Published in final edited form as:

Pharmacol Biochem Behav. 2013 March ; 104: 10–19. doi:10.1016/j.pbb.2012.12.022.

Glial cell-line derived neurotrophic factor (GDNF) replacement attenuates motor impairments and nigrostriatal dopamine deficits in 12-month-old mice with a partial deletion of *GDNF*

Ofelia M. Littrell^a, Ann-Charlotte Granholm^b, Greg A. Gerhardt^a, and Heather A. Boger^{b,*}

^aDepartment of Anatomy and Neurobiology, Parkinson's Disease Translational Research Center of Excellence, University of Kentucky Medical Center, 306 Davis Mills Bldg., 800 Rose St., Lexington, KY 40536, USA

^bDepartment of Neurosciences and Center on Aging, Medical University of South Carolina, 173 Ashley Ave., BSB Suite 403, Charleston, SC 29425, USA

Abstract

Glial cell-line derived neurotrophic factor (GDNF) has been established as a growth factor for the survival and maintenance of dopamine (DA) neurons. In phase I clinical trials, GDNF treatment in Parkinson's disease patients led to improved motor function and GDNF has been found to be down regulated in Parkinson's disease patients. Studies using GDNF heterozygous (*Gdnf*^{+/−}) mice have demonstrated that a partial reduction of GDNF leads to an age-related accelerated decline in nigrostriatal DA system- and motor-function and increased neuro-inflammation and oxidative stress in the substantia nigra (SN). Therefore, the purpose of the current studies was to determine if GDNF replacement restores motor function and functional markers within the nigrostriatal DA system in middle-aged *Gdnf*^{+/−} mice. At 11 months of age, male *Gdnf*^{+/−} and wildtype (WT) mice underwent bilateral intra-striatal injections of GDNF (10 µg) or vehicle. Locomotor activity was assessed weekly 1–4 weeks after treatment. Four weeks after treatment, their brains were processed for analysis of GDNF levels and various DAergic and oxidative stress markers. An intrastriatal injection of GDNF increased motor activity in *Gdnf*^{+/−} mice to levels comparable to WT mice (1 week after injection) and this effect was maintained through the 4-week time point. This increase in locomotion was accompanied by a 40% increase in striatal GDNF protein levels and SN GDNF expression in *Gdnf*^{+/−} mice. Additionally, GDNF treatment significantly increased the number of tyrosine hydroxylase (TH)-positive neurons in the SN of middle-aged *Gdnf*^{+/−} mice, but not WT mice, which was coupled with reduced oxidative stress in the SN. These studies further support that long-term changes related to the dysfunction of the nigrostriatal pathway are influenced by GDNF expression and add that this dysfunction appears to be responsive to GDNF treatment. Additionally, these studies suggest that long-term GDNF depletion alters the biological and behavioral responses to GDNF treatment.

Keywords

Dopamine; Glial cell-line derived neurotrophic factor; Parkinson's disease; Neurodegeneration; Striatum; Substantia nigra

*Corresponding author at: Department of Neurosciences, Medical University of South Carolina, 173 Ashley Ave., BSB Suite 403, Charleston, SC 29425, USA. Tel.: +1 843 876 2230; fax: +1 843 792 4423. boger@musc.edu..

1. Introduction

The use of neurotrophic factor supplementation, particularly in the context of Parkinson's disease (PD), has been widely investigated with promising findings of enhanced neuron function and behavioral measures (Peterson and Nutt, 2008). In particular, GDNF has shown restorative effects in numerous animal models exhibiting dopamine (DA)-neuron dysfunction including the aged and 6-hydroxydopamine (6-OHDA) lesioned rat (Hebert and Gerhardt, 1997; Hoffer et al., 1994) and the aged and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-lesioned non-human primate (Grondin et al., 2002, 2003). GDNF has also demonstrated protective effects from 6-OHDA- and MPTP-induced cytotoxicity in rodents (Kearns et al., 1997; Tomac et al., 1995). Furthermore, in phase I clinical trials GDNF showed significant therapeutic potential in Parkinson's disease patients (Gill et al., 2003; Slevin et al., 2005) with a 25% improvement on the Unified Parkinson Disease Rating Scale motor score.

GDNF is a target derived neurotrophic factor that is expressed at highest levels in the developing striatum with a decline in expression in adulthood (Stromberg et al., 1993). There is also evidence for decreased GDNF expression in the brains of Parkinson's disease patients (Chauhan et al., 2001; Jenner and Olanow, 1998) and dysregulation in aged rats with 6-OHDA lesions (Yurek and Fletcher-Turner, 2001). In light of the restorative- and protective-effects of GDNF on DA neurons and the prominent role of GDNF in development (Granhölm et al., 2000), the effects of a chronic GDNF depletion have been investigated using GDNF heterozygous mice (*Gdnf*^{+/-}), which have decreased GDNF protein expression in the brain (Boger et al., 2006; Pichel et al., 1996). *Gdnf*^{+/-} mice display a unique aging phenotype — exhibiting locomotor deficiencies, decreases in tyrosine hydroxylase (TH) staining in the SN, and functional changes in DA-release and -uptake in the striatum (Boger et al., 2006; Littrell et al., 2012). Typically, motor and DA-neuron functional measures decline around 12 months of age in *Gdnf*^{+/-} mice.

It has been suggested that inflammation contributes to nigrostriatal dysfunction in *Gdnf*^{+/-} mice because of increased microglial cell activation and exacerbated microglial responses in methamphetamine-induced toxicity models (Boger et al., 2007). Indeed, neuro-inflammation is strongly implicated in the degeneration and dysfunction of the nigrostriatal pathway related to PD and parkinsonism (He et al., 2001; Hunter et al., 2007; Zecca et al., 2008). The inflammatory response is thought to be related to oxidative stress (Hald and Lotharius, 2005) — both processes having been associated with neurodegeneration (Aubin et al., 1998; Chan et al., 2012; Jenner and Olanow, 1996; Sugama et al., 2003). In addition to being linked to PD pathogenesis (Jenner and Olanow, 2006), oxidative stress is implicated in age-associated neurodegeneration (Chakrabarti et al., 2011). Since GDNF treatment reduces neurotoxicity related to oxidative stress (Ortiz-Ortiz et al., 2011; Sawada et al., 2000), oxidative stress markers in *Gdnf*^{+/-} mice were investigated. Preliminary data from our laboratory have shown that markers of oxidative stress are altered in the SN of *Gdnf*^{+/-} mice. Cyclooxygenase-2 (COX-2) is a known cytokine that can be released from glial cells and is involved in neuro-inflammatory and oxidative stress pathways (Gupta et al., 2011).

To further investigate results from preliminary studies, the current studies assess oxidative stress markers (COX-2) as well as levels of an antioxidant (superoxide dismutase-2 (SOD-2)) in this model of GDNF depletion and examine if GDNF treatment in middle-aged *Gdnf*^{+/-} mice affects these oxidative stress markers. Motor behavior (locomotor activity) and DA-neuron functional measures are enhanced in studies using GDNF treatment in animal models (Grondin et al., 2003; Hebert and Gerhardt, 1997; Kordower et al., 2000). Thus, similar locomotor measures and DA-neuron functional measures were investigated after GDNF treatment in *Gdnf*^{+/-} mice. The primary aim of these studies was to test the

hypothesis that age-related DA-neuron dysfunction, potential causes of dysfunction, and concomitant motor impairments are reduced by GDNF treatment in *Gdnf*^{+/-} mice. In particular, the following questions were investigated in *Gdnf*^{+/-} and age-matched WT mice: 1) Does GDNF treatment affect spontaneous or stimulated locomotor activity? 2) Does GDNF treatment restore the number of DA neurons in the SN? 3) Does GDNF treatment attenuate oxidative stress markers in the SN?

2. Material and methods

2.1. Animals

A nonfunctional GDNF allele was generated by replacing part of exon 3, which encodes the GDNF protein with a selectable marker neomycin phosphotransferase expressing cassette. Generation and genotyping of *Gdnf*^{+/-} mice is described in detail in previous work (Pichel et al., 1996). Mice were obtained from a colony established at the Medical University of South Carolina. Mice were bred on a C57Bl/6J background consistent with NIH approved protocols. After transfer to the University of Kentucky, mice were acclimated for a minimum of 1 week before experimentation. Male *Gdnf*^{+/-} mice (12 months of age) were compared with age-matched WT mice in all experiments. Mice were housed 3–4 per cage with food and water provided *ad libitum*. Mice were maintained under 12:12 h light/dark cycle at an ambient temperature of 20–22 °C. Protocols for animal care were in agreement with NIH approved guidelines and compliant with local institutional protocols at the University of Kentucky Medical Center and Medical University of South Carolina. Procedures were in strict agreement with the *Guide for the Care and Use of Laboratory Animals*.

2.2. Reagents

Recombinant methionyl human GDNF (Amgen, Thousand Oaks, CA, USA) expressed in *Escherichia coli* as previously described (Lin et al., 1993) was used.

2.3. Delivery of GDNF

GDNF was dissolved (5 µg/µL) in sterile (0.22 µm filtered) citrate buffer (10 mM sodium citrate, 150 mM NaCl, pH=5) as previously described (Hebert et al., 1996).

Animals used for survival surgical procedures were anesthetized with isoflurane gas (1.5–2.5% in O₂) and positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). A craniotomy was performed for access to the targeted structures and GDNF or vehicle was delivered using a 26-gauge needle (cannula only) (22026-01, point style-3; Hamilton Company, Reno, NV) attached to a 25-µL Hamilton syringe (80408, point style 3; Hamilton Company, Reno, NV) using plastic tubing (Zeus Inc., Orangeburg, SC). *Gdnf*^{+/-} mice (12-month-old) (*n*=14) and age-matched WT littermate mice (*n*=8) were treated with 10 µg of GDNF (bilaterally) or equivalent volume of citrate buffer (vehicle) to the striata. The dose was selected based on previous studies in rodents (Hebert et al., 1996; Hudson et al., 1995). Stereotaxic coordinates were (from *bregma* (mm) (bilaterally)): anterior–posterior: +1.0, medial–lateral: +/-1.5, dorsal–ventral: -3.0 (Franklin and Paxinos, 2001; Kirik et al., 2004). Solution delivery was controlled using a KD Scientific model infusion pump (model 100, KD Scientific Inc., Holliston, MA). Solution delivery began 5 min after lowering to the appropriate depth. GDNF and vehicle treatments were administered bilaterally (10 µg per hemisphere or equivalent volume (2 µL) of vehicle) at a rate of 0.2 µL/min for 10 min. The needle remained in the brain after completing solution delivery and was slowly retracted after 10 min. This procedure was repeated bilaterally and the overlying burr holes were covered with bone wax before closing the incision with dissolvable sutures (4-0 Caprosyn™, Covidien; Norwalk, CT). Topical analgesic ointment (Neosporin® with

pramoxine HCl; Rite Aid Corp.) was applied to the incision site following surgical procedures and daily out to 3 days post-operatively. During surgical procedures and the immediate recovery period following surgery, animals rested on a heating pad connected to a re-circulating water bath (Gaymar Industries, Inc., Orchard Park, NY) maintained at 37 °C. Animals recovered in their home cage under observation in the laboratory (~2 h) before transport to the animal housing facility. Animal health was assessed daily for a minimum of 1 week for signs of postoperative distress. There was significant attrition due to anesthetic intolerance in all treatment groups and genotypes. Thus, some of the treated animals were not viable for use in brain tissue analysis. The resulting sample size is indicated in the Results sections.

2.4. Locomotor activity

Locomotor activity (total distance traveled) was assessed using a Digiscan Animal Activity Monitor system (Omnitech Electronics Model RXYZCM (8); TAO, Columbus, OH, USA), details of which have been previously described (Halberda et al., 1997). Animals were tested for spontaneous motor activity prior to treatment with vehicle or GDNF and randomly assigned to treatment groups. Spontaneous motor activity was determined weekly (1–4 weeks after treatment) using the total distance traveled over a 1-hour period. At the 4-week time point, animals were injected with saline (0.9% NaCl, 0.01 mL/g body weight, i.p.) before measuring spontaneous locomotor activity. The saline injection served as a negative control for stimulated motor activity. Previous studies from our laboratory have demonstrated that *Gdnf*^{+/−} mice have greater DAT activity (Boger et al., 2007). Since it has been established in the literature that blocking the DAT increases locomotion (Garris et al., 2003), the DAT inhibitor, nomifensine (Altar and Marshall, 1988; Littrell et al., 2012; Ross, 1979), was administered systemically (7.5 mg/kg i.p.) to measure stimulated motor activity (4 weeks after treatment only).

2.5. Euthanasia and brain preparation

Procedures related to immunohistochemical measures and protein quantification (ELISA and Western blot) were carried out 4 weeks after GDNF treatment (~24 h after nomifensine administration) for behavioral measures to allow drug washout (Lindberg and Syvalahti, 1986; Lindberg et al., 1986). Mice were euthanized by decapitation while under deep urethane anesthesia (1.25 g/kg, 0.9% NaCl i.p.) and their brains were dissected. Brains were post-fixed in 4% paraformaldehyde at 4 °C for 48 h to allow for full penetration of fixative to deep structures and then transferred to 30% sucrose until cryo-sectioning for immunohistochemical studies (45 µm; Microm, Zeiss, Thornwood, NY). Remaining tissues for protein quantification (ELISA and Western blot) were stored at −80 °C until processing for analysis described below.

2.6. Substantia nigra immunohistochemistry (TH-ir, GDNF-ir, and COX-2-ir)

Immunohistochemistry (immunoreactivity (ir)) was performed as previously described (Boger et al., 2006). A polyclonal TH antibody (TH; 1:5000; Pel-Freeze Inc., Roger, AZ), a polyclonal GDNF antibody (1:100; Santa Cruz Biotechnology, Inc.), and a polyclonal COX-2 antibody (1:250; EMD Millipore, San Diego, CA) were used for free-floating immunohistochemistry on the SN — using every 3rd section for TH and every 6th section for GDNF. Peroxidase activity was quenched by treating sections with H₂O₂, methanol and 0.01 M tris buffered saline (TBS) (ratio of 1:2:7; pH=7.6) for 15 min followed by permeabilization in TBS with 0.25% TritonX-100 (TBST) and treatment with sodium-m-iodate (0.1 M, in TBS; 20 min). Non-specific binding was controlled by incubation at room temperature in 10% normal goat serum for 30 min. Sections were incubated with the primary antibody for 24 h (TBST with 3% NGS), washed with TBST and then incubated with the secondary antibody (1:200, Vector Labs, Burlingame, CA) and avidin–biotin

complex (ABC kit, Vector Labs). The reaction was developed using 3'3'-diaminobenzidine (0.05% of 3% H₂O₂) using 2.5% nickel ammonium sulfate to enhance the reaction. Sections were mounted on glass slides and cover-slipped.

2.7. Unbiased stereological cell counts

Quantitative estimates of total numbers of TH-ir neurons in the SN were determined 4 weeks after a single treatment. Unbiased stereological cell counts from serial sections through the midbrain were used and methods previously described were followed (Gundersen et al., 1988; West, 1993). The optical fractionator system used in the present study was Stereo Investigator stereological software (MicroBrightfield, Colchester, VT) coupled to a Prior H128 computer-controlled *x-y-z* motorized stage. Outline contours were drawn at low magnification (10×) and the outlined region measured with a systematic random design of dissector-counting frames. The counting frame area was 2500 μm² and the sampling grid area was 100×100 μm (Boger et al., 2006). The counting brick was approximately 35 μm thick after excluding the upper and lower guard zones (2.5 μm each). The slides were coded and TH-ir cells were counted using a 40× objective lens with a 1.4 numerical aperture. The selection of the first section from the SN in the rostral position was random and every 3rd section was counted — rendering a systematic random design. The total number of TH-ir neurons was calculated. Outline contours drawn for SN cell counts included areas containing predominately TH-labeled dopaminergic neurons (substantia nigra pars compacta (SNc) and lateralis). The substantia nigra pars reticulata (SNr) — containing mostly TH fibers — was excluded. The *cell number was counted* on every 3rd section through the SN in all 24 groups on the same sections used for stereological assessment (randomly measuring at least 150 neurons per brain across all stereological sections) according to previous protocols (Boger et al., 2006, 2007; Zaman et al., 2008).

2.8. Immunohistochemical image analysis

Analysis of SN GDNF-ir and COX-2-ir was performed using the NIH Image program as described previously (Choe and McGinty, 2000; Boger et al., 2007, 2009). Briefly, slide background was subtracted and the LUT scale was adjusted using density slicing. This approach captures all labeled profiles above a threshold density and interactively discriminates them from density values below the threshold. The software then automatically measures the mean optical density and # of pixels per area of the extracted profiles in the selected medial or lateral striatal regions. Two parameters were obtained from this procedure: the area covered by the specific profile population (field area), and the mean density of the specific profiles. Total immunoreactivity (integrated density) is obtained by multiplying the field area times the mean density value.

2.9. GDNF protein quantification (ELISA)

GDNF protein levels were assessed 4 weeks after a single treatment using a commercially available assay kit from Promega (Madison, WI) and a standard protocol (Albeck et al., 2003). The overlying cortex was peeled away from coronal sections and the entire striatum was dissected including the medial and lateral striatum but excluding the nucleus accumbens. Flat-bottom plates were coated with the corresponding capture antibody, which binds the soluble captured neurotrophin. The captured neurotrophin was bound by a second specific antibody, which was detected using a species-specific antibody conjugated to horseradish peroxidase as a tertiary reactant. Unbound conjugates were removed by subsequent wash steps according to Promega protocols. After an incubation period with chromagenic substrate, color change was measured in an ELISA plate reader at 450 nm. According to the assay kit the quantification range for GDNF was 7.8–500 pg/mL and cross reactivity with other trophic proteins ranged <2–3%. Protein levels of GDNF were expressed as pg/mg wet weight of tissue.

2.10. SOD-2 Western blot

Striatal samples (20 μ g) from 12-month-old WT and *Gdnf*^{+/-} mice were loaded in duplicate and separated on 4–12% NuPAGE Bis Tris gels (Invitrogen, Carlsbad, CA) at 150 V for 45 min. After separation onto the gel, the gel was transferred via wet transfer onto a nitrocellulose membrane for 1 h at 30 V. The membrane was then removed from the cassette and blocked for 1 h in 5% non-fat milk in PBS at room temperature and subsequently incubated overnight at room temperature in a SOD-2 primary antibody (1:5000; rabbit polyclonal; Abcam, Cambridge, MA). After 24 h, blots were washed in PBS-T (0.1% Tween-20) and incubated in secondary antibody (1:200; Peroxidase-conjugated goat anti-rabbit IgG, Jackson Immunoresearch, West Grove, PA) in 5% non-fat milk in PBS for 1 h at room temperature. Blots were then washed in PBS-T and imaged on a Kodak Image Station 4000 (4 exposures, 15 s each) using Immobilon chemiluminescent reagent (Millipore, Bellerica, MA). For loading control, blots were incubated with anti-actin antibody for 1 h at room temperature, followed by incubation with secondary antibody and were re-imaged under the same settings. The samples were normalized to actin. Integrated density of the bands was analyzed using AlphaEaseFC (FluorChem 9900) and results were reported as percent of WT.

2.11. Data analysis and statistics

Pre-treatment locomotor activity was analyzed using a two-tailed unpaired *t* test. Spontaneous locomotor activity was analyzed using a 2 (Genotype) $\times$ 2 (Treatment) $\times$ 4 (intervals) mixed-factor ANOVA with repeated measures on the interval factor. Stimulated motor activity was measured (4 weeks after treatment only) and was analyzed using a genotype $\times$ treatment 1 (GDNF/vehicle) $\times$ treatment 2 (nomifensine/saline) three-way ANOVA.

Immunohistochemical data (TH-ir, GDNF-ir, and COX-2), GDNF protein quantification (ELISA), and SOD-2 Western Blot data were analyzed using a 2 (Genotype) $\times$ 2 (Treatment) ANOVA design. Additional analysis included a one-way ANOVA followed by Scheffe's post-hoc analysis when a significant F-value existed ($p < 0.05$) to assess differences across groups.

3. Results

3.1. GDNF administration attenuates hypoactivity in 12-month-old *Gdnf*^{+/-} mice

As we have previously shown (Littrell et al., 2012), spontaneous locomotor activity in *Gdnf*^{+/-} mice (pre-treatment) is decreased ($p < 0.001$; $t(20) = 3.515$, $n = Gdnf^{+/-}$: 14, WT: 8) (Fig. 1). Spontaneous locomotor activity was measured in mice treated with GDNF or vehicle and analyzed using a 2 (Genotype) $\times$ 2 (Treatment) $\times$ 4 (Time) mixed-factor ANOVA with repeated measures on the interval. Analysis showed a significant Genotype $\times$ Treatment interaction ($F(1,79) = 5.114$, $p = 0.0265$) as well as a significant Treatment $\times$ Time interaction ($F(3,79) = 9.255$, $p < 0.0001$) (Fig. 1). Additionally, there was a significant main effect of GDNF Treatment ($F(1,79) = 71.096$, $p < 0.0001$) with a general increase in locomotor activity in GDNF-treated groups of both genotypes (weeks 1–4 in *Gdnf*^{+/-} mice and weeks 1–2 in WT mice). There was also a significant main effect of Time ($F(3,79) = 32.315$, $p < 0.0001$) with both genotypes (GDNF-treated) demonstrating an initial increase in locomotor activity that declined over the 4-week testing period. These data taken together demonstrate that *Gdnf*^{+/-} mice had a greater increase in locomotor activity response to a single GDNF intracranial administration and this effect was longer lasting compared to GDNF-treated WT mice.

Mice treated with GDNF (n =WT: 5 *Gdnf*^{+/−}: 7) or vehicle (n =WT: 4–5 *Gdnf*^{+/−}: 7) were also tested for stimulated motor activity following nomifensine (4-weeks after GDNF treatment). We have previously shown that mice with a partial deletion of *Gdnf* have greater DAT activity with no difference in DAT protein expression at 3- and 12-months of age (Boger et al., 2007). Stimulated motor activity was investigated to determine if GDNF-treatment normalizes the exacerbated nomifensine-induced motor response in *Gdnf*^{+/−} mice as shown previously (Littrell et al., 2012). Data were analyzed using a Genotype×Treatment 1 (GDNF/vehicle)×Treatment 2 (nomifensine/saline) three-way ANOVA. Consistent with our previous work (Littrell et al., 2012), the administration of nomifensine, compared to saline, increased locomotor activity in both genotypes, regardless of treatment with GDNF or vehicle ($F(1,38)=158.057$, $p<0.0001$). There was a significant interaction between Treatment 1 (GDNF/vehicle) and Treatment 2 (nomifensine/saline) ($F(1,38)=4.263$, $p=0.0458$) with GDNF-treated groups showing a greater response to nomifensine in both genotypes (Fig. 1). These data suggest a synergistic effect of combining GDNF treatment with nomifensine to increase locomotor activity.

3.2. GDNF protein levels are increased 4 weeks after GDNF administration

To quantify effective increases in GDNF protein after striatal treatment, ELISA was used to determine protein levels in the striatum of GDNF- and vehicle-treated *Gdnf*^{+/−} ($n=6$) and WT mice ($n=4$) (Fig. 2). Striatal GDNF protein level analysis revealed significant main effects of Genotype [$F(1,16)=36.353$, $p<0.001$] and Treatment [$F(1,16)=14.997$, $p<0.01$], but no significant interaction of the two factors existed (Fig. 2). An additional one-way ANOVA analysis revealed significant group differences [$F(3,15)=17.457$; $p<0.001$]. Scheffe's post-hoc tests showed vehicle-treated *Gdnf*^{+/−} mice had lower GDNF protein levels than vehicle-treated WT mice ($p<0.01$). There was also a significant difference in protein levels after treatment in *Gdnf*^{+/−} mice ($p<0.05$). Wild-type (WT) mice treated with GDNF did not show a significant difference in protein (versus vehicle) ($p>0.05$). GDNF treatment in *Gdnf*^{+/−} mice restored striatal GDNF levels to those detected in WT mice ($p>0.05$ versus WT (vehicle)).

Immunohistochemistry was used to determine GDNF-ir density in the SN after striatal GDNF treatment (Fig. 3). Integrated density analysis of the results showed an increase in staining intensity after treatment with GDNF. Vehicle-treated *Gdnf*^{+/−} mice demonstrated reduced SN GDNF-ir compared to vehicle-treated WT mice (Fig. 3A, B). Four weeks after bilateral intrastratial injections of GDNF, GDNF-ir density in the SN was increased in both genotypes (Fig. 3C, D, E). A 2×2 ANOVA revealed significant main effects of Genotype [$F(1,16)=68.646$; $p<0.001$, n =*Gdnf*^{+/−}: 6 WT:4] and Treatment [$F(1,16)=208.524$, $p<0.001$], however a significant interaction did not exist (Fig. 3E). A one-way ANOVA analysis revealed significant group differences [$F(3,15)=85.064$, $p<0.001$]. Scheffe's post-hoc analysis revealed that vehicle-treated *Gdnf*^{+/−} mice had lower GDNF-ir compared to vehicle-treated WT mice ($p<0.001$; Fig. 3E). Treatment with GDNF significantly increased GDNF-ir 4 weeks after treatment in both genotypes compared to vehicle-treated groups ($p<0.001$). These data suggest that striatal GDNF administration increased the supply of GDNF in the SN.

3.3. Intrastratial GDNF administration increases SN tyrosine hydroxylase (TH)-immunoreactivity

The number of DA neurons, detected as TH-positive neurons, was determined in *Gdnf*^{+/−} and WT mice 4 weeks after GDNF treatment (Fig. 4). As previously reported (Boger et al., 2006), middle-aged *Gdnf*^{+/−} mice had fewer SNc TH-positive neurons compared to WT mice, as well as less TH-ir in the SNr (Fig. 4A, B, E). As shown in Fig. 4C and D, GDNF administration increased SN TH-ir 4 weeks post-administration (both in the SNc and SNr,

regardless of genotype). A significant Genotype×Treatment interaction [$F(1,16)=5.008$, $p<0.05$] existed in regards to the number of TH-positive neurons in the SN (Fig. 4E). An additional one-way ANOVA analysis was performed and showed significant group-wise differences ($F(3,16)=31.700$, $p<0.001$). Scheffe's post-hoc analysis revealed *Gdnf*^{+/−} mice had significantly fewer TH-positive neurons than WT mice ($p<0.001$, vehicle treated). Additionally, GDNF treatment increased the number of TH-positive neurons in *Gdnf*^{+/−} mice ($p<0.001$, Fig. 4E). However, GDNF treatment did not affect the number of TH-positive neurons in WT mice ($p>0.05$; Fig. 4E). Treatment with GDNF in *Gdnf*^{+/−} mice normalized the number of TH-positive neurons to values comparable to control (WT (vehicle-treated); $p>0.05$).

3.4. Oxidative stress in the SN is reduced 4 weeks after intrastriatal GDNF administration

Initial microarray analysis demonstrated that mRNA for the pro-inflammatory cytokine, COX-2, is upregulated in the SN of *Gdnf*^{+/−} mice compared to WT mice (preliminary results, unpublished). In the current study, COX-2 was assessed via immunohistochemistry. As shown in Fig. 5A and B, COX-2-ir is higher in the SN of middle-aged *Gdnf*^{+/−} mice ($n=6$) compared to WT ($n=4$) mice (vehicle treated). Treatment with GDNF ($n=WT$: 4 *Gdnf*^{+/−}: 6) reduced COX-2-ir in the SN of mice, regardless of genotype (Fig. 5C, D). Nigral COX-2-ir was influenced by significant main effects of Genotype [$F(1,16)=9.169$, $p<0.01$] and Treatment [$F(1,16)=15.703$, $p<0.01$], however, no significant interaction existed (Fig. 5E). Analysis also revealed a significant one-way ANOVA [$F(3,16)=9.889$, $p<0.001$]. Vehicle-treated *Gdnf*^{+/−} mice had significantly higher COX-2-ir compared to vehicle-treated WT mice ($p<0.05$). GDNF administration significantly reduced COX-2-ir in the SN of *Gdnf*^{+/−} mice compared to vehicle-treated *Gdnf*^{+/−} mice ($p<0.01$), however, no difference existed between vehicle-treated and GDNF-treated WT mice ($p>0.05$). There was no significant difference ($p>0.05$) in COX-2-ir in GDNF-treated *Gdnf*^{+/−} mice compared to WT (vehicle-treated) mice.

To follow up preliminary studies, protein levels of the endogenous antioxidant (SOD-2) were assessed in the SN (Fig. 6). There was a significant Genotype×Treatment interaction [$F(1,16)=9.831$, $p<0.01$, $n=WT$: 4 *Gdnf*^{+/−}: 6] of SOD-2 protein levels (Fig. 6). Scheffe's post-hoc analysis showed that vehicle-treated *Gdnf*^{+/−} mice had significantly lower protein levels of SOD-2 compared to vehicle-treated WT mice ($p<0.01$) as detected by western blots. Four weeks after intra-striatal administration of GDNF, *Gdnf*^{+/−} mice treated with GDNF had a significant increase in SOD-2 protein levels compared to vehicle-treated *Gdnf*^{+/−} mice ($p<0.01$), however no significant difference existed between WT vehicle-treated and GDNF-treated WT mice ($p>0.01$). In addition, a positive correlation exists between the level of SOD-2 and locomotor activity performance ($r=0.542$, $p<0.05$), as evident by significantly lower locomotor activity and SOD-2 levels of saline-treated *Gdnf*^{+/−} mice.

4. Discussion

As previously reported (Boger et al., 2006), mice with a 40% reduction of GDNF demonstrate reduced locomotor activity and fewer TH-positive nigral neurons at 12 months of age. These studies add that *Gdnf*^{+/−} mice demonstrate increased nigral oxidative stress with higher levels of COX-2 and reduced levels of an endogenous antioxidant (SOD-2) compared to age-matched WT mice. Four weeks after bilateral intra-striatal administration of GDNF (10 µg) in *Gdnf*^{+/−} mice, several parameters were normalized to levels comparable to WT mice including: locomotor activity, GDNF protein levels within the striatum and SN, TH-ir and the number of TH-positive neurons in the SN, and oxidative stress markers. Collectively these data further support the important role GDNF plays in the maintenance and survival of DA neurons and highlight specific consequences of long-term GDNF

depletion related to oxidative stress — a potential mechanism of DA-neuron degeneration in aging and Parkinson's disease.

4.1. GDNF levels are increased by striatal GDNF treatment

A significant 36% reduction in GDNF protein in the vehicle-treated *Gdnf*^{+/-} mice compared to vehicle-treated WT mice corroborates previous reports of protein reductions in naïve *Gdnf*^{+/-} mice (Boger et al., 2006). Delivery of GDNF to the striatum of *Gdnf*^{+/-} mice led to a 40% increase in striatal GDNF protein levels compared to vehicle-treated *Gdnf*^{+/-} mice. A comparable increase in GDNF protein in the striatum was not seen in GDNF-treated WT mice, although there was an increase (statistically insignificant) in GDNF protein. It is unknown how processing or clearance of exogenous GDNF may be altered in *Gdnf*^{+/-} mice. It is possible that a higher dose of GDNF is necessary to result in significant changes in WT mice, which do not demonstrate protein reductions (Boger et al., 2006). Thus, treatment with GDNF led to an increase in striatal protein only in *Gdnf*^{+/-} mice. The effective striatal protein levels were comparable to WT (vehicle-treated) animals but did not reach levels equivalent to that seen in GDNF-treated WT mice. Thus, protein levels in GDNF-treated *Gdnf*^{+/-} mice were restored to levels comparable to WT counterparts ($p>0.05$).

Qualitative TH-ir histological studies showed that striatal GDNF treatment increased staining intensity of GDNF in the SN of both WT and *Gdnf*^{+/-} mice. Although GDNF expression is highest in the developing striatum (Stromberg et al., 1993), retrograde transport to the SN (Barroso-Chinea et al., 2005) is suggested to play a critical role in activation of the nigral localized GDNF receptor — GDNF family receptor- $\alpha 1$ (GFR $\alpha 1$) (Quartu et al., 2007). Retrograde transport of GDNF by striatal DA terminals has been previously demonstrated in animal models — occurring within 24 h after striatal delivery (Ai et al., 2003; Lapchak et al., 1997). In the current studies, retrograde transport processes are supported by the increase in GDNF-staining intensity in the SN. This finding makes increased GFR $\alpha 1$ activation in the SN after GDNF treatment likely, although this possibility requires further investigation. In the current study, it is not known if the exogenously applied GDNF protein is being detected in either striatal or nigral measures or if the detected GDNF is from an endogenous source. It is possible that endogenous GDNF expression is upregulated by an acute GDNF treatment through a positive feedback mechanism (Barak et al., 2011; He and Ron, 2006). This possibility is promising considering the long-term (4-week) effect after a single treatment, as seen in the current studies. Alterations in endogenous GDNF expression by GDNF treatment are worth further investigation in *Gdnf*^{+/-} mice.

4.2. Locomotor behavior enhanced by GDNF treatment

As shown previously, *Gdnf*^{+/-} mice demonstrated reduced spontaneous locomotor activity and enhanced sensitivity to nomifensine-stimulated locomotor activity compared to WT counterparts (Boger et al., 2006; Littrell et al., 2012). Treatment with GDNF increased spontaneous locomotor activity in both genotypes, an effect that appears to diminish over time. *Gdnf*^{+/-} mice displayed a more sustained increase in activity with greater activity than GDNF-treated WT mice at 3- and 4-weeks after treatment. Importantly, these studies showed that GDNF treatment can restore behavioral deficits in middle-aged *Gdnf*^{+/-} mice to comparable activity levels as WT counterparts. It is hypothesized that *Gdnf*^{+/-} mice demonstrate increased sensitivity to GDNF treatment due to chronic GDNF depletion and compensatory changes in GFR $\alpha 1$ expression — a finding demonstrated in rats (Kozlowski et al., 2004; Pruett and Salvatore, 2010). Because changes in GFR $\alpha 1$ expression have been correlated with TH-ir (Pruett and Salvatore, 2010), GFR $\alpha 1$ expression has been suggested to regulate nigral DA tissue content. Thus, changes in DA availability may also contribute to the long-term enhancing effects of GDNF — sustained in *Gdnf*^{+/-} mice. The enhanced

activity in GDNF-treated *Gdnf*^{+/-} mice versus GDNF-treated WT mice may also be due to increased DA sensitivity in *Gdnf*^{+/-} mice — supported by increased DA receptor expression (Kelly et al., 1998; Littrell et al., 2012).

The nomifensine-induced increase in locomotor activity was seen in all genotypes and treatment groups and is consistent with the established pharmacological and behavioral effects of the DAT inhibitor (Altar and Marshall, 1988; Littrell et al., 2012). Sensitivity to nomifensine was increased in GDNF-treated groups of both genotypes compared to vehicle-treated groups of the same genotype. This enhanced effect may be attributed to increased DA-neuron function in GDNF-treated groups as stated above. Future studies will be conducted to assess DA neuron function following GDNF administration in *Gdnf*^{+/-} mice.

Gdnf^{+/-} mice demonstrate an interesting, and paradoxical, aging phenotype with increased DAT function (Hebert and Gerhardt, 1999; Littrell et al., 2012). Although the mechanism and function of DAT-related changes in *Gdnf*^{+/-} mice are not fully understood, one of the proposed consequences of increased transporter activity is increased vulnerability to toxins like MPTP, 6-OHDA and methamphetamine, which utilize the DAT for cellular entry and neurotoxic effects (Boger et al., 2007; Gainetdinov et al., 1997; Storch et al., 2004). Due to the neuroprotective effects from DAT-utilizing neurotoxins provided by GDNF treatment (Cass et al., 2006; Cohen et al., 2011; Tomac et al., 1995), the effect of GDNF-replacement on a DAT-functional measure (stimulated motor activity) was investigated with the prediction that it would be normalized (i.e. decreased DAT activity, decreased sensitivity to nomifensine). GDNF treatment potentially normalizes the nomifensine locomotor response in GDNF-treated *Gdnf*^{+/-} mice to levels comparable to GDNF-treated WT mice.

Increased DAT activation by nomifensine (increased nomifensine-induced locomotor activity) is indicated in GDNF-treated animals of both genotypes. Although direct measures of DA-uptake *in vivo* confirms increased DAT-activity (Littrell et al., 2012) in naïve *Gdnf*^{+/-} mice, the stimulatory actions of nomifensine in these studies could involve multiple aspects of neurotransmission that could be influenced by the DA-enhancing effects of GDNF. The enhanced motor response to nomifensine in *Gdnf*^{+/-} mice may be due to increased receptor activation (increased DA-receptor expression (Littrell et al., 2012)), increased DA content/synapse functionality (increased TH-positive neurons), and/or increased number of transporters (increased number of DA-synapses). Although largely speculative, if GDNF-treated *Gdnf*^{+/-} mice show enhanced DA-neuron functionality we would predict the presumed compensatory mechanisms (e.g. increased DA-receptors) would normalize and, eventually, *Gdnf*^{+/-} mice would display a comparable stimulated motor response to WT (vehicle-treated) mice. The functional and durational threshold for these changes to occur is not known in this model and should be investigated in future studies.

4.3. GDNF treatment increased tyrosine hydroxylase immunoreactivity

Consistent with prior studies establishing histological differences between genotypes (Boger et al., 2006), there was a decrease (48%) in number of TH-positive neurons in vehicle-treated *Gdnf*^{+/-} mice versus vehicle-treated WT mice. Treatment with GDNF increased (36%) the number of TH-positive neurons in the SN of *Gdnf*^{+/-} mice compared to vehicle-treated *Gdnf*^{+/-} mice. While the number of TH-positive neurons is substantially lower than GDNF-treated WT mice, GDNF treatment in *Gdnf*^{+/-} mice restored the number of TH-positive neurons to comparable levels to vehicle-treated WT counterparts (*p*>0.05). The increase in TH-positive neurons supports increased nigrostriatal DA-neuron function, which we would expect to be accompanied with enhanced neurochemical levels and accompanying behavioral improvements. Although an increase in staining of TH – the rate-limiting enzyme in DA synthesis – is encouraging, investigation of other aspects of TH activity and DA-neuron function are necessary to further characterize the functional effect(s) of GDNF

treatment in *Gdnf*^{+/-} mice. It is unknown if the TH-ir increase after GDNF treatment is a result of increased numbers of TH-positive neurons or upregulation of TH expression in existing neurons. Both possibilities have been associated with the effects of GDNF and/or GDNF-receptor presence/activation (Cao et al., 2010; Pruett and Salvatore, 2010).

Interestingly, GDNF-treated WT mice did not display an increase in number of TH-positive neurons versus vehicle-treated WT counterparts ($p>0.05$). Although enhancement of DA neurochemical levels has been reported in the intact nigrostriatal pathway of young (Hebert et al., 1996) and aged rodents (Hebert and Gerhardt, 1997), prior animal studies investigating TH-ir after GDNF treatment have yielded inconsistent results. A decrease in TH-ir after GDNF delivery to the intact nigrostriatal pathway of the rat (Rosenblad et al., 2003) has been reported, but another study in the aged primate reported an increase in number of TH-positive neurons after GDNF delivery (Palfi et al., 2002). It has been proposed that GDNF treatment causes a transient phenotypic shift — evident by an initial downregulation of TH protein but an increase in phosphorylation of TH (Salvatore et al., 2004). In Parkinsonian rats (6-OHDA striatal lesioned) treated with GDNF, an initial decline in TH-ir has been demonstrated with a delayed increase in TH-positive neurons (8 weeks after GDNF treatment (Cohen et al., 2011)). Although the current studies were modeled after *in vivo* studies that addressed similar neurochemical and behavioral parameters in the aged rat (Hebert and Gerhardt, 1997), it is possible that the effect of GDNF on TH may require a longer time course (8+ weeks) than examined here (4 weeks). We postulate that *Gdnf*^{+/-} mice, demonstrating a chronic GDNF depletion, may be more sensitive to GDNF supplementation due to a compensatory increase in surface expression of GDNF receptors as demonstrated in aging and lesion models (Kozlowski et al., 2004; Pruett and Salvatore, 2010).

4.4. GDNF treatment reduced markers of oxidative stress

Oxidative stress has been implicated in the pathology of neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Federico et al., 2012; Gandhi and Abramov, 2012). In particular, the selective degeneration of subsets of DA-neurons of the SNc (Hirsch et al., 1988) highlight the predisposition of DA neurons to oxidative stress and damage — a characteristic sometimes attributed to the auto-oxidation of DA, generation of reactive oxygen species and mitochondrial dysfunction (Gandhi and Abramov, 2012). Preliminary microarray work from our laboratory indicate that *Gdnf*^{+/-} mice as young as 3 months of age demonstrate heightened markers of oxidative stress in the SN including: higher mRNA expression of cytokines (COX-2 and iNOS) and reduced mRNA expression of endogenous antioxidants (SOD-2, catalase, and glutathione peroxidases) (data not published). Indeed, protein analysis of COX-2 and SOD-2 in the current studies support increased oxidative stress in 12-month-old *Gdnf*^{+/-} mice as *Gdnf*^{+/-} mice have greater COX-2 and lower SOD-2 protein levels in the SN compared to WT mice. Furthermore, just 4 weeks following a single administration of GDNF these responses were attenuated in the SN of *Gdnf*^{+/-} mice to levels comparable to WT mice. The favorable effects of GDNF on oxidative stress markers confer with previous studies that demonstrate GDNF protection against neurotoxins, such as paraquat, that act primarily through oxidative stress mechanisms (Dong et al., 2007; Ortiz-Ortiz et al., 2011). Attenuating oxidative stress provides a potential mechanism by which GDNF treatment may preserve DA-neuron function in *Gdnf*^{+/-} mice as seen by the normalization of TH-positive neurons. It has been demonstrated that *Gdnf*^{+/-} mice experience exacerbated microglial responses (Boger et al., 2007) in methamphetamine-induced toxicity models. This inflammatory response may be mediated by COX-2 and negatively affect DA-neuron viability as demonstrated in MPTP models (Vijitruth et al., 2006). Indeed, blocking COX-2 signaling protects mice from MPTP-induced insults (Feng et al., 2002; Teismann and Ferger, 2001) and mice with reduced GDNF signaling capacity

experience differential DA-neuron vulnerability to MPTP-induced insults (Boger et al., 2008).

Together these data support a potential role of GDNF in the regulation of endogenous antioxidant molecules (e.g. SOD-2) and apparent suppression of oxidative stress markers such as COX-2. These data continue to support an important role of GDNF in the regulation of oxidative stress (Chao and Lee, 1999; Dong et al., 2007). Additional studies are needed to further elucidate the role of GDNF in oxidative stress and to determine if chronic administration of GDNF aids in the long-term suppression of oxidative stress — a potential therapeutic strategy for neurodegenerative disease (Chan et al., 2012; Choi et al., 2006; Federico et al., 2012).

5. Conclusions

Long-term changes in nigrostriatal DA-neuron function related to GDNF depletion are suspected due to the critical role of GDNF in developmental processes (Granholtm et al., 2000; Stromberg et al., 1993). These studies further investigated and confirmed functional abnormalities of DA neurons and behavioral deficits accompanying a chronic, partial GDNF depletion. The therapeutic benefit of GDNF treatment is strongly supported by motor improvement seen in PD patients (Gill et al., 2003; Slevin et al., 2005). However, these studies emphasize that functional deficiencies in a GDNF depletion model also appear to be responsive to GDNF replacement. Furthermore, sensitivity to GDNF treatment appears to be influenced by a chronic and partial GDNF depletion with WT and *Gdnf*^{+/-} mice demonstrating altered responses to equivalent doses of GDNF. Taken together, these studies suggest that regulation of endogenous GDNF likely causes behavioral and histopathological abnormalities within the nigrostriatal pathway in middle-aged *Gdnf*^{+/-} mice. The identified abnormalities, oxidative stress and loss of DA-neuron phenotypic markers (TH-ir), may underlie the known behavioral (locomotor) impairments and are potential targets for motor restoration using neurotrophic factor replacement.

Acknowledgments

This work was supported by grants from the following sources: NIA AG033687 (HAB); AG023630 (ACh); NIA NIH Training Grant 1T32 DA022738 (GAG/OML); NIH Training Grant 5T32AG000242-14 (GAG/OML); USPHS NS39787 (GAG).

Role of funding source The funding agencies did not have any role in determining the study design, or collection, analysis and interpretation of data. Additionally, funding sources did not have a role in the writing of this manuscript or decision to submit for publication.

References

- Ai Y, Markesbery W, Zhang Z, Grondin R, Elseberry D, Gerhardt GA, et al. Intraputamenal infusion of GDNF in aged rhesus monkeys: distribution and dopaminergic effects. *J Comp Neurol*. 2003; 461:250–61. [PubMed: 12724841]
- Albeck D, Mesches MH, Juthberg S, Browning M, Bickford PC, Rose GM, et al. Exogenous NGF restores endogenous NGF distribution in the brain of the cognitively impaired aged rat. *Brain Res*. 2003; 967:306–10. [PubMed: 12650994]
- Altar CA, Marshall JF. Neostriatal dopamine uptake and reversal of age-related movement disorders with dopamine-uptake inhibitors. *Ann N Y Acad Sci*. 1988; 515:343–54. [PubMed: 3364894]
- Aubin N, Curet O, Deffois A, Carter C. Aspirin and salicylate protect against MPTP-induced dopamine depletion in mice. *J Neurochem*. 1998; 71:1635–42. [PubMed: 9751197]
- Barak S, Ahmadianehrani S, Kharazia V, Ron D. Positive autoregulation of GDNF levels in the ventral tegmental area mediates long-lasting inhibition of excessive alcohol consumption. *Transl Psychiatry*. 2011; 1

- Barroso-Chinea P, Cruz-Muros I, Aymerich MS, Rodriguez-Diaz M, Afonso-Oramas D, Lanciego JL, et al. Striatal expression of GDNF and differential vulnerability of midbrain dopaminergic cells. *Eur J Neurosci*. 2005; 21:1815–27. [PubMed: 15869477]
- Boger HA, Middaugh LD, Huang P, Zaman V, Smith AC, Hoffer BJ, et al. A partial GDNF depletion leads to earlier age-related deterioration of motor function and tyrosine hydroxylase expression in the substantia nigra. *Exp Neurol*. 2006; 202:336–47. [PubMed: 16889771]
- Boger HA, Middaugh LD, Patrick KS, Ramamoorthy S, Denehy ED, Zhu H, et al. Long-term consequences of methamphetamine exposure in young adults are exacerbated in glial cell line-derived neurotrophic factor heterozygous mice. *J Neurosci*. 2007; 27:8816–25. [PubMed: 17699663]
- Boger HA, Middaugh LD, Zaman V, Hoffer B, Granholm AC. Differential effects of the dopamine neurotoxin MPTP in animals with a partial deletion of the GDNF receptor, *GFRalpha1*, gene. *Brain Res*. 2008; 1241:18–28. [PubMed: 18822276]
- Boger HA, Middaugh LD, Granholm AC, McGinty JF. Minocycline restores striatal tyrosine hydroxylase in GDNF heterozygous mice but not in methamphetamine-treated mice. *Neurobiol Dis*. 2009; 33:459–66. [PubMed: 19110059]
- Cao JP, Li FZ, Zhu YY, Yuan HH, Yu ZQ, Gao DS. Expressions and possible roles of GDNF receptors in the developing dopaminergic neurons. *Brain Res Bull*. 2010; 83:321–30. [PubMed: 20884338]
- Cass WA, Peters LE, Harned ME, Seroogy KB. Protection by GDNF and other trophic factors against the dopamine-depleting effects of neurotoxic doses of methamphetamine. *Ann N Y Acad Sci*. 2006; 1074:272–81. [PubMed: 17105923]
- Chakrabarti S, Munshi S, Banerjee K, Thakurta IG, Sinha M, Bagh MB. Mitochondrial dysfunction during brain aging: role of oxidative stress and modulation by antioxidant supplementation. *Aging Dis*. 2011; 2:242–56. [PubMed: 22396876]
- Chan T, Chow AM, Cheng XR, Tang DW, Brown IR, Kerman K. Oxidative stress effect of dopamine on alpha-synuclein: electroanalysis of solvent interactions. *ACS Chem Neurosci*. 2012; 3:569–74. [PubMed: 22860226]
- Chao CC, Lee EH. Neuroprotective mechanism of glial cell line-derived neurotrophic factor on dopamine neurons: role of antioxidation. *Neuropharmacology*. 1999; 38:913–6. [PubMed: 10465695]
- Chauhan NB, Siegel GJ, Lee JM. Depletion of glial cell line-derived neurotrophic factor in substantia nigra neurons of Parkinson's disease brain. *J Chem Neuroanat*. 2001; 21:277–88. [PubMed: 11429269]
- Choe ES, McGinty JF. N-Methyl-D-aspartate receptors and p38 mitogen-activated protein kinase are required for cAMP-dependent cyclase response element binding protein and Elk-1 phosphorylation in the striatum. *Neuroscience*. 2000; 101:607–17. [PubMed: 11113310]
- Choi J, Sullards MC, Olzmann JA, Rees HD, Weintraub ST, Bostwick DE, et al. Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. *J Biol Chem*. 2006; 281:10816–24. [PubMed: 16517609]
- Cohen AD, Zigmond MJ, Smith AD. Effects of intrastriatal GDNF on the response of dopamine neurons to 6-hydroxydopamine: time course of protection and neurorestoration. *Brain Res*. 2011; 1370:80–8. [PubMed: 21062624]
- Dong A, Shen J, Krause M, Hackett SF, Campochiaro PA. Increased expression of glial cell line-derived neurotrophic factor protects against oxidative damage-induced retinal degeneration. *J Neurochem*. 2007; 103:1041–52. [PubMed: 17935603]
- Federico A, Cardaioli E, Da Pozzo P, Formichi P, Gallus GN, Radi E. Mitochondria, oxidative stress and neurodegeneration. *J Neurol Sci*. 2012; 322:254–62. [PubMed: 22669122]
- Feng ZH, Wang TG, Li DD, Fung P, Wilson BC, Liu B, et al. Cyclooxygenase-2-deficient mice are resistant to 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced damage of dopaminergic neurons in the substantia nigra. *Neurosci Lett*. 2002; 329:354–8. [PubMed: 12183047]
- Franklin, K.; Paxinos, G. The mouse brain in stereotaxic coordinates. Academic Press; New York: 2001.

- Gainetdinov RR, Fumagalli F, Jones SR, Caron MG. Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *J Neurochem.* 1997; 69:1322–5. [PubMed: 9282960]
- Gandhi S, Abramov AY. Mechanism of oxidative stress in neurodegeneration. *Oxid Med Cell Longev.* 2012; 2012:428010. [PubMed: 22685618]
- Garris PA, Budygin EA, Phillips PE, Venton BJ, Robinson DL, Bergstrom BP, et al. A role for presynaptic mechanisms in the actions of nomifensine and haloperidol. *Neuroscience.* 2003; 118:819–29. [PubMed: 12710989]
- Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med.* 2003; 9:589–95. [PubMed: 12669033]
- Granhölm AC, Reyland M, Albeck D, Sanders L, Gerhardt G, Hoernig G, et al. Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J Neurosci.* 2000; 20:3182–90. [PubMed: 10777782]
- Grondin R, Zhang Z, Yi A, Cass WA, Maswood N, Andersen AH, et al. Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced Parkinsonian monkeys. *Brain.* 2002; 125:2191–201. [PubMed: 12244077]
- Grondin R, Cass WA, Zhang Z, Stanford JA, Gash DM, Gerhardt GA. Glial cell line-derived neurotrophic factor increases stimulus-evoked dopamine release and motor speed in aged rhesus monkeys. *J Neurosci.* 2003; 23:1974–80. [PubMed: 12629203]
- Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, et al. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *Apmis.* 1988; 96:857–81. [PubMed: 3056461]
- Gupta A, Kumar A, Kulkarni SK. Targeting oxidative stress, mitochondrial dysfunction and neuroinflammatory signaling by selective cyclooxygenase (COX)-2 inhibitors mitigates MPTP-induced neurotoxicity in mice. *Prog Neuropsychopharmacol Biol Psychiatry.* 2011; 35:974–81. [PubMed: 21291942]
- Halberda JP, Middaugh LD, Gard BE, Jackson BP. DAD1- and DAD2-like agonist effects on motor activity of C57 mice: differences compared to rats. *Synapse.* 1997; 26:81–92. [PubMed: 9097408]
- Hald A, Lotharius J. Oxidative stress and inflammation in Parkinson's disease: is there a causal link? *Exp Neurol.* 2005; 193:279–90. [PubMed: 15869932]
- He DY, Ron D. Autoregulation of glial cell line-derived neurotrophic factor expression: implications for the long-lasting actions of the anti-addiction drug, Ibogaine. *FASEB J.* 2006; 20:2420–2. [PubMed: 17023388]
- He Y, Appel S, Le W. Minocycline inhibits microglial activation and protects nigral cells after 6-hydroxydopamine injection into mouse striatum. *Brain Res.* 2001; 909:187–93. [PubMed: 11478935]
- Hebert MA, Gerhardt GA. Behavioral and neurochemical effects of intranigral administration of glial cell line-derived neurotrophic factor on aged Fischer 344 rats. *J Pharmacol Exp Ther.* 1997; 282:760–8. [PubMed: 9262339]
- Hebert MA, Gerhardt GA. Age-related changes in the capacity, rate, and modulation of dopamine uptake within the striatum and nucleus accumbens of Fischer 344 rats: an in vivo electrochemical study. *J Pharmacol Exp Ther.* 1999; 288:879–87. [PubMed: 9918602]
- Hebert MA, Vanhorne CG, Hoffer BJ, Gerhardt GA. Functional effects of GDNF in normal rat striatum: presynaptic studies using in vivo electrochemistry and microdialysis. *J Pharmacol Exp Ther.* 1996; 279:1181–90. [PubMed: 8968339]
- Hirsch E, Graybiel AM, Agid YA. Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature.* 1988; 334:345–8. [PubMed: 2899295]
- Hoffer BJ, Hoffman A, Bowenkamp K, Huettl P, Hudson J, Martin D, et al. Glial-cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic-neurons in-vivo. *Neurosci Lett.* 1994; 182:107–11. [PubMed: 7891873]
- Hudson J, Granhölm AC, Gerhardt GA, Henry MA, Hoffman A, Biddle P, et al. Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits in vivo. *Brain Res Bull.* 1995; 36:425–32. [PubMed: 7712205]

- Hunter RL, Dragicevic N, Seifert K, Choi DY, Liu M, Kim HC, et al. Inflammation induces mitochondrial dysfunction and dopaminergic neurodegeneration in the nigrostriatal system. *J Neurochem.* 2007; 100:1375–86. [PubMed: 17254027]
- Jenner P, Olanow CW. Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology.* 1996; 47:S161–70. [PubMed: 8959985]
- Jenner P, Olanow CW. Understanding cell death in Parkinson's disease. *Ann Neurol.* 1998; 44:S72–84. [PubMed: 9749577]
- Jenner P, Olanow CW. The pathogenesis of cell death in Parkinson's disease. *Neurology.* 2006; 66:S24–36. [PubMed: 16717250]
- Kearns CM, Cass WA, Smoot K, Kryscio R, Gash DM. GDNF protection against 6-OHDA: time dependence and requirement for protein synthesis. *J Neurosci.* 1997; 17:7111–8. [PubMed: 9278545]
- Kelly MA, Rubinstein M, Phillips TJ, Lessov CN, Burkhart-Kasch S, Zhang G, et al. Locomotor activity in D2 dopamine receptor-deficient mice is determined by gene dosage, genetic background, and developmental adaptations. *J Neurosci.* 1998; 18:3470–9. [PubMed: 9547254]
- Kirik D, Georgievska B, Bjorklund A. Localized striatal delivery of GDNF as a treatment for Parkinson disease. *Nat Neurosci.* 2004; 7:105–10. [PubMed: 14747832]
- Kordower JH, Emborg ME, Bloch J, Ma SY, Chu Y, Leventhal L, et al. Neurodegeneration prevented by lentiviral vector delivery of GDNF in primate models of Parkinson's disease. *Science.* 2000; 290:767–73. [PubMed: 11052933]
- Kozlowski DA, Miljan EA, Bremer EG, Harrod CG, Gerin C, Connor B, et al. Quantitative analyses of GFRalpha-1 and GFRalpha-2 mRNAs and tyrosine hydroxylase protein in the nigrostriatal system reveal bilateral compensatory changes following unilateral 6-OHDA lesions in the rat. *Brain Res.* 2004; 1016:170–81. [PubMed: 15246853]
- Lapchak PA, Jiao S, Collins F, Miller PJ. Glial cell line-derived neurotrophic factor: distribution and pharmacology in the rat following a bolus intraventricular injection. *Brain Res.* 1997; 747:92–102. [PubMed: 9042532]
- Lin LFH, Doherty DH, Lile JD, Bektesh S, Collins F. Gdnf — a glial-cell line derived neurotrophic factor for midbrain dopaminergic-neurons. *Science.* 1993; 260:1130–2. [PubMed: 8493557]
- Lindberg RL, Syvalahti EK. Metabolism of nomifensine after oral and intravenous administration. *Clin Pharmacol Ther.* 1986; 39:378–83. [PubMed: 3956054]
- Lindberg RL, Syvalahti EK, Pihlajamaki KK. Disposition of nomifensine after acute and prolonged dosing. *Clin Pharmacol Ther.* 1986; 39:384–8. [PubMed: 3956055]
- Littrell OM, Pomerleau F, Huettl P, Surgener S, McGinty JF, Middaugh LD, et al. Enhanced dopamine transporter activity in middle-aged Gdnf heterozygous mice. *Neurobiol Aging.* 2012; 33(427):e1–14. [PubMed: 21144620]
- Ortiz-Ortiz MA, Moran JM, Ruiz-Mesa LM, Bonmatty RG, Fuentes JM. Protective effect of the glial cell line-derived neurotrophic factor (GDNF) on human mesencephalic neuron-derived cells against neurotoxicity induced by paraquat. *Environ Toxicol Pharmacol.* 2011; 31:129–36. [PubMed: 21787677]
- Palfi S, Leventhal L, Chu Y, Ma SY, Emborg M, Bakay R, et al. Lentivirally delivered glial cell line-derived neurotrophic factor increases the number of striatal dopaminergic neurons in primate models of nigrostriatal degeneration. *J Neurosci.* 2002; 22:4942–54. [PubMed: 12077191]
- Peterson AL, Nutt JG. Treatment of Parkinson's disease with trophic factors. *Neurotherapeutics.* 2008; 5:270–80. [PubMed: 18394569]
- Pichel JG, Shen L, Sheng HZ, Granholm AC, Drago J, Grinberg A, et al. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature.* 1996; 382:73–6. [PubMed: 8657307]
- Pruett BS, Salvatore MF. GFR alpha-1 receptor expression in the aging nigrostriatal and mesoaccumbens pathways. *J Neurochem.* 2010; 115:707–15. [PubMed: 20731758]
- Quartu M, Serra MP, Boi M, Ferretti MT, Lai ML, Del Fiacco M. Tissue distribution of Ret, GFRalpha-1, GFRalpha-2 and GFRalpha-3 receptors in the human brainstem at fetal, neonatal and adult age. *Brain Res.* 2007; 1173:36–52. [PubMed: 17825269]

- Rosenblad C, Georgievska B, Kirik D. Long-term striatal overexpression of GDNF selectively downregulates tyrosine hydroxylase in the intact nigrostriatal dopamine system. *Eur J Neurosci*. 2003; 17:260–70. [PubMed: 12542662]
- Ross SB. The central stimulatory action of inhibitors of the dopamine uptake. *Life Sci*. 1979; 24:159–67. [PubMed: 33315]
- Salvatore MF, Zhang JL, Large DM, Wilson PE, Gash CR, Thomas TC, et al. Striatal GDNF administration increases tyrosine hydroxylase phosphorylation in the rat striatum and substantia nigra. *J Neurochem*. 2004; 90:245–54. [PubMed: 15198683]
- Sawada H, Ibi M, Kihara T, Urushitani M, Nakanishi M, Akaike A, et al. Neuroprotective mechanism of glial cell line-derived neurotrophic factor in mesencephalic neurons. *J Neurochem*. 2000; 74:1175–84. [PubMed: 10693950]
- Slevin JT, Gerhardt GA, Smith CD, Gash DM, Kryscio R, Young B. Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputamenal infusion of glial cell line-derived neurotrophic factor. *J Neurosurg*. 2005; 102:216–22. [PubMed: 15739547]
- Storch A, Ludolph AC, Schwarz J. Dopamine transporter: involvement in selective dopaminergic neurotoxicity and degeneration. *J Neural Transm*. 2004; 111:1267–86. [PubMed: 15480838]
- Stromberg I, Bjorklund L, Johansson M, Tomac A, Collins F, Olson L, et al. Glial-cell line-derived neurotrophic factor is expressed in the developing but not adult striatum and stimulates developing dopamine neurons in-vivo. *Exp Neurol*. 1993; 124:401–12. [PubMed: 7904571]
- Sugama S, Yang L, Cho BP, DeGiorgio LA, Lorenzl S, Albers DS, et al. Age-related microglial activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurodegeneration in C57BL/6 mice. *Brain Res*. 2003; 964:288–94. [PubMed: 12576189]
- Teismann P, Ferger B. Inhibition of the cyclooxygenase isoenzymes COX-1 and COX-2 provide neuroprotection in the MPTP-mouse model of Parkinson's disease. *Synapse*. 2001; 39:167–74. [PubMed: 11180504]
- Tomac A, Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, et al. Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. *Nature*. 1995; 373:335–9. [PubMed: 7830766]
- Vijitruth R, Liu M, Choi DY, Nguyen XV, Hunter RL, Bing G. Cyclooxygenase-2 mediates microglial activation and secondary dopaminergic cell death in the mouse MPTP model of Parkinson's disease. *J Neuroinflammation*. 2006; 3:6. [PubMed: 16566823]
- West MJ. New stereological methods for counting neurons. *Neurobiol Aging*. 1993; 14:275–85. [PubMed: 8367009]
- Yurek DM, Fletcher-Turner A. Differential expression of GDNF, BDNF, and NT-3 in the aging nigrostriatal system following a neurotoxic lesion. *Brain Res*. 2001; 891:228–35. [PubMed: 11164827]
- Zaman V, Boger HA, Granholm AC, Rohrer B, Moore A, Buhusi M, et al. The nigrostriatal dopamine system of aging GFRalpha-1 heterozygous mice: neurochemistry, morphology and behavior. *Eur J Neurosci*. 2008; 28:1557–68. [PubMed: 18973577]
- Zecca L, Wilms H, Geick S, Claasen JH, Brandenburg LO, Holzknecht C, et al. Human neuromelanin induces neuroinflammation and neurodegeneration in the rat substantia nigra: implications for Parkinson's disease. *Acta Neuropathol*. 2008; 116:47–55. [PubMed: 18343932]

Fig. 1.

Spontaneous and nomifensine-induced locomotor activity in GDNF-treated *Gdnf*^{+/-} and WT mice. Total distance traveled was measured prior to treatment and weekly after striatal treatment with GDNF or vehicle. Spontaneous motor activity was measured at all time points. Stimulated motor activity following nomifensine (7.5 mg/kg i.p.) was measured 4-weeks after treatment. *Gdnf*^{+/-} mice displayed a significant decrease (** $p=0.0022$; $t_{20}=3.515$) in locomotor activity versus WT mice. Spontaneous locomotor activity following treatment was analyzed using a 2 (Genotype)×2 (Treatment)×4 (intervals) mixed-factor ANOVA with repeated measures. There was a significant effect of GDNF treatment ($F_{1,79}=71.096$, $p<0.0001$) with greater locomotor activity in GDNF-treated groups. There was also a significant effect of time ($F_{3,79}=32.315$, $p<0.0001$) with both GDNF-treated genotypes exhibiting an increase in locomotor activity after treatment that diminished over the 4-week testing period. There were also significant interactions: Genotype×Treatment ($F_{1,79}=5.114$, $p=0.0265$) and Treatment×Time ($F_{3,79}=9.255$, $p<0.0001$) (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).

Fig. 2.

GDNF treatment increased striatal GDNF tissue content. At 12-months of age, *Gdnf*^{+/-} mice had significantly lower striatal GDNF tissue concentration compared to age-matched WT mice ($p < 0.001$, vehicle). Administration of GDNF increased striatal tissue concentrations of GDNF in *Gdnf*^{+/-} mice ($p < 0.01$) and GDNF levels were increased to levels comparable ($p > 0.05$) to vehicle-treated WT mice (** $p < 0.01$; *** $p < 0.001$).

Fig. 3.

Nigral GDNF-ir is increased after intrastriatal GDNF Administration. Photomicrographs of GDNF-ir in the SN of (A) vehicle-treated WT mice, (B) vehicle-treated *Gdnf*^{-/-} mice, (C) GDNF-treated WT mice, and (D) GDNF-treated *Gdnf*^{-/-} mice. (E) Quantification of the average integrated density of GDNF-ir in the SN. Vehicle-treated *Gdnf*^{-/-} mice had lower SN GDNF-ir compared to vehicle-treated WT mice ($p < 0.001$). GDNF administration resulted in an increase in SN GDNF-ir, regardless of genotype ($p < 0.001$), compared to vehicle-treated mice. Magnification=10 $\times$.

Fig. 4.

Increased number of TH-positive neurons after GDNF administration. Dopamine-producing neurons in the SN are shown using TH-ir: (A) vehicle-treated WT mice, (B) vehicle-treated *Gdnf*^{+/-} mice, (C) GDNF-treated WT mice, and (D) GDNF-treated *Gdnf*^{+/-} mice. (E) Quantification of the estimated number of TH-positive neurons in the SN. Vehicle-treated *Gdnf*^{+/-} mice have fewer TH-positive neurons than vehicle-treated WT mice ($p < 0.001$). GDNF administration gave rise to a significant increase in the number of TH-positive neurons in *Gdnf*^{+/-} mice compared to vehicle-treated *Gdnf*^{+/-} mice ($p < 0.001$). No differences existed in the number of TH-positive neurons between vehicle-treated and GDNF-treated WT mice ($p > 0.05$). Magnification=10 $\times$.

Fig. 5.

GDNF treatment decreased the expression of COX-2 in the SN of *Gdnf*^{+/-} mice. COX-2-ir measured in the SN of (A) vehicle-treated WT mice, (B) vehicle-treated *Gdnf*^{+/-} mice, (C) GDNF-treated WT mice, and (D) GDNF-treated *Gdnf*^{+/-} mice. (E) Quantification of the integrated density of COX-2-ir in the SN. COX-2-ir was significantly greater in the SN of vehicle-treated *Gdnf*^{+/-} mice compared to vehicle-treated WT mice ($p < 0.001$). GDNF administration resulted in a significant decrease in COX-2-ir in the SN of *Gdnf*^{+/-} mice compared to vehicle-treated *Gdnf*^{+/-} mice ($p < 0.001$), to levels comparable to vehicle-treated WT mice ($p > 0.05$). Magnification=60 $\times$.

Fig. 6. GDNF treatment increased levels of the endogenous antioxidant, SOD-2 in *Gdnf*^{+/-} mice. (A) Immunoblot of SOD-2 protein in the SN of vehicle-treated WT mice, vehicle-treated *Gdnf*^{+/-} mice, GDNF-treated WT mice, and GDNF-treated *Gdnf*^{+/-} mice. (B) Quantification of the integrated density of SOD-2 in the SN. Vehicle-treated *Gdnf*^{+/-} mice have significantly lower protein levels of SOD-2 compared to vehicle-treated WT mice ($p < 0.01$). GDNF treatment significantly increased protein levels of SOD-2 in the SN of *Gdnf*^{+/-} mice compared to vehicle-treated *Gdnf*^{+/-} mice ($p < 0.01$), however, there was no change in SOD-2 in the SN of WT mice after GDNF treatment ($p > 0.05$).