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Comparative study of the neurotrophic effects elicited by VEGF-B and GDNF in preclinical *in vivo* models of Parkinson's disease

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Abstract

Vascular endothelial growth factor B (VEGF-B) has recently been shown to be a promising novel neuroprotective agent for several neurodegenerative conditions. In the current study we extended previous work on neuroprotective potential for Parkinson's disease (PD) by testing an expanded dose range of VEGF-B (1 μ g and 10 μ g) and directly comparing both neuroprotective and neurorestorative effects of VEGF-B in progressive unilateral 6-hydroxydopamine (6-OHDA) PD models to a single dose of glial cell line-derived neurotrophic factor (GDNF, 10 μ g), that has been established by several groups as a standard in both preclinical PD models. In the amphetamine-induced rotational tests the treatment with 1 and 10 μ g VEGF-B resulted in significantly improved motor function of 6-OHDA-lesioned rats compared to vehicle-treated 6-OHDA-lesioned rats in the neuroprotection paradigm. Both doses of VEGF-B caused an increase in tyrosine hydroxylase (TH)-positive cell and fiber count in the substantia nigra (SN) and striatum in the neuroprotective experiment. The effect size was comparable to the effects seen with GDNF. In the neurorestoration paradigm, VEGF-B injection had no significant effect in either the behavioral or the immunohistochemical analyses, whereas GDNF injection significantly improved the amphetamine-induced rotational behavior and reduced TH-positive neuronal cell loss in the SN. We also present a strong positive correlation ($p = 1.9 \times 10^{-50}$) of the expression of *VEGF-B* with nuclear-encoded mitochondrial genes involved in fatty acid metabolism in rat midbrain, pointing to the mitochondria as a site of action of VEGF-B. *GDNF* showed a positive correlation with nuclear-encoded mitochondrial genes that was not nearly as strong ($p = 0.018$). VEGF-B counteracted rotenone-induced reduction of (a) fatty acid transport protein 1 and 4 levels and (b) both Akt protein and phosphorylation levels in SH-SY5Y cells. We further verified VEGF-B expression in the human SN pars compacta of healthy controls and Parkinson's disease patients, in neuronal cells that show co-expression with neuromelanin. These results have demonstrated that VEGF-B has potential as a neuroprotective agent for PD therapy and should be further investigated.

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Keywords

neuroprotective factors; rodent 6-OHDA model; human substantia nigra pars compacta; mitochondria; FATP1; FATP4

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disease characterized by the cardinal movement symptoms bradykinesia, resting tremor, muscle rigidity, and postural abnormalities (Savitt et al., 2006; Olanow et al., 2009). Non motor symptoms of PD also include autonomic dysfunction, pain and sensory disorder, sleep impairment, and dementia (Olanow et al., 2009). The motor symptoms largely result from degeneration of dopaminergic (DA) neurons projecting from the substantia nigra (SN) to the caudate putamen. PD symptoms appear after a loss of 70–80% of DA neurons has occurred and approximately 1–2% of the population over the age of 65 is afflicted with PD (Olanow et al., 2009).

The majority of PD patients are idiopathic with approximately 10% being familial (Farrer, 2006). Some environmental factors have been linked to an increased risk of PD, for example rural well water use, pesticide use, and occupations such as mining or welding (Farrer, 2006). Although many genes have been identified, the exact mechanism in which the variation in PD-linked genes leads to neurodegeneration is not fully understood, however past research has pointed to mitochondrial dysfunction, oxidative damage, aberrant protein aggregation, and deficits in ubiquitin-mediated proteolysis (Olanow et al., 2009).

Current therapies are able to provide symptomatic relief but are unable to halt the progression of the disease (Olanow et al., 2009). A variety of neurotrophic factors, particularly those in the glial cell line-derived neurotrophic factor (GDNF)-family (GDNF and neurturin), have shown much promise in this regard in preclinical studies, demonstrating robust effects in rodent and primate models. For example, fibroblast growth factor (FGF; Timmer et al., 2007) has been shown to be neuroprotective. Others have been shown to be neuroprotective and neurorestorative in preclinical models of PD; for example GDNF (Hoffer et al., 1994; Kearns and Gash, 1995; Tomac et al., 1995a; Gash et al., 1996; Kirik et al., 2004), neurturin (Horger et al., 1998; Rosenblad et al., 1999; Gasmi et al., 2007), as well as cerebral dopamine neurotrophic factor (CDNF; Lindholm et al., 2007; Voutilainen et al., 2011) and mesencephalic astrocyte-derived neurotrophic factor (MANF; Voutilainen et al., 2009).

Initial clinical trials of neurotrophic factors of the GDNF family, however, have not been successful likely because of a lack of information regarding the optimum dosing, delivery methods and choice of individual factors (Nutt et al., 2003; Lang et al., 2006). There is still reason to hope that trophic factor therapy may become a reality for patients with PD (Sherer et al., 2006) and experimental and clinical investigations to help clarify the potential role of GDNF and other neurotrophic factors to PD have been underway since. Several trials of viral gene therapy vectors in PD patients while not successful yet in slowing disease progression have shown the safety and tolerability of adeno-associated virus (AAV-2) gene delivery (Kapli et al., 2007; Marks et al., 2008 and 2010; Christine et al., 2009; LeWitt et al., 2011), and Cere-120 (AAV2-neurturin) is currently being reevaluated in a second phase II clinical study with different injection sites and increased viral titer. It is therefore important to continue to evaluate novel growth factors in standard animal models concurrently with clinical trials now optimizing the delivery of traditional growth factors in the GDNF-family.

VEGF-B is a substantially different growth factor with neuroprotective capabilities (Poesen et al., 2008; Dhondt et al., 2011), a member of the vascular endothelial growth factor (VEGF) family (Rosenstein et al., 2004) rather than the GDNF-family. VEGF-A is the most studied VEGF family member but due to its angiogenic capabilities but is less suitable for use as a neurotrophic agent (Olsson et al., 2006). VEGF-B has very little angiogenic activity but it has the potential to inhibit apoptosis (Li et al., 2008) and increase stimulation in proliferation of neuronal cultures *in vitro* (Sun et al., 2004). VEGF-B was up-regulated after exposing rat midbrain cultures to the pesticide rotenone (Falk et al., 2009a), a candidate environmental risk factor for PD (Tanner et al., 2011). Furthermore, exogenous supplementation of VEGF-B levels in this model system acted as a neuroprotective agent facilitating neuronal survival (Falk et al., 2009a). Based on those results VEGF-B was further evaluated as a putative neuroprotective agent *in vivo* (Falk et al., 2011) demonstrating that a 3 μ g VEGF-B injection into the rat striatum after a mild progressive 6-OHDA was neuroprotective. The dose had been determined based on the lowest dose of other neurotrophic factors shown to be effective.

These findings have led us to further investigate effects of VEGF-B in both a neuroprotective and a neurorestorative preclinical PD model. In the present study we have tested an expanded dose range of VEGF-B (1 and 10 μ g) and directly compared it to 10 μ g GDNF, a dose that has been established by several groups and can be considered as a standard to use in the mild progressive 6-OHDA lesion rat PD model. Intra-striatal growth factor injection was conducted in rats either 6 hours prior to 6-OHDA-lesioning to test neuroprotective effects, or four weeks after the lesion to test neurorestorative effects. After injection, behavioral tests and immunohistochemical analyses were conducted to measure any improvement in disease state. In order to further investigate the therapeutic potential for PD and understand the mechanism of action of VEGF-B, we also investigated VEGF-B expression in the human SN of PD patients, and effects of VEGF-B on fatty acid transporter proteins (FATPs), sometimes also referred to as Acyl-coenzyme A synthetase very long (ACSVLs), and Akt signaling in rotenone-treated SH-SY5Y cells.

EXPERIMENTAL PROCEDURES

Animals

Male Sprague-Dawley (Charles River, Wilmington, MA) rats were used in this experiment, weighing 250–280 grams at the start. Rats were housed in groups of three on a 12 hour light-dark cycles at room temperature (RT). Food pellets and water were available to them at all times. The experimental design was approved by the Institutional Animal Care and Use Committee at the University of Arizona and conformed to the guidelines of the National Institutes of Health.

Administration of 6-OHDA and the neurotrophic factors in the rat model

(a) Neuroprotection paradigm—The unilateral 6-OHDA lesion was administered by injecting freshly made 20 μ g 6-OHDA in one 4 μ l deposit (Sigma, St Louis, MO; 5.0 μ g/ μ l in 0.9% sterilized saline with 0.02% ascorbic acid) into the ventral lateral striatum at the following coordinates: A/P +0.8; L/P –2.5 and D/V –5.2 according to the atlas of Paxinos and Watson (1997). The growth factors (10 μ g GDNF, 1 and 10 μ g VEGF-B) were injected 6 hours prior to the 6-OHDA lesion into the same location. GDNF (Preprotech, Rocky Hill, NJ) was dissolved in Na-Citrate and VEGF-B₁₈₆ (R&D Systems, Minneapolis, MN) was dissolved in phosphate buffered saline (PBS; Sigma, St Louis, MO). The vehicle control groups were not significantly different and combined. The rate of the injection was 0.5 μ l per min using a Stoelting microinjector (Stoelting Co., Wood Dale, IL), and the Hamilton syringe was left in place for 5 additional minutes to prevent back flow of solution. Rats were

pretreated (30 min prior to the infusion of 6-OHDA) with 12.5 mg/kg desipramine (Sigma, St Louis, MO) given intraperitoneal (*i.p.*) to prevent damage to noradrenergic neurons. Ketamine: xylazine 80 mg/kg; 12 mg/kg, *i.p.* was utilized as anesthetic.

(b) Neurorestoration paradigm—In this experiment we have injected 6-OHDA, total 20 µg in the striatum in two deposits (10 µg/4 µl each) at the following coordinates: 1.) A/P +1.6; L/M +2.2; D/V -5; and 2.) A/P -0.4; L/M +4; D/V -5. The coordinates of the GDNF/VEGF-B injections (10 µg GDNF, 3 µg VEGF-B) at 4 weeks post-6-OHDA lesion were: A/P +1; L/M +2.7; D/V -5 (Paxinos and Watson, 1997). At 13 weeks post-6-OHDA lesion the tissue analysis described below was conducted.

Behavioral analysis in the rat model

For the neuroprotection paradigm (Figure 1 A) amphetamine-induced rotation tests were conducted at 2, 4 and 6 weeks post-injection of 6-OHDA and for the neurorestoration paradigm (Figure 1 B) behavioral tests were done 3, 6, 8, 10 and 12 weeks after injection of 6-OHDA. Cylinder tests were conducted on the day prior to amphetamine-induced rotation testing. Amphetamine-induced rotation tests were executed by injection of D-amphetamine (Sigma, St Louis, MO; 5 mg/kg, *i.p.*). Full clockwise and counterclockwise turns were recorded for a time period of 100 min in plexiglass cylinders (38.0 cm diameter; 38.0 cm height) by a blinded observer counting the rotations from a video recording at 2 x playback speed. Cylinder behavioral tests were conducted in plexiglass cylinders (14.5 cm diameter; 30.0 cm height) and with an additional baseline test conducted 1 week prior to 6-OHDA injection in the neuroprotection experiment. Animals were grouped to have a mean 50% forepaw preference in each group. Forepaw touches on cylinder walls were counted by a blinded observer on a video for a minimum of 20 touches during a 5 – 10 min recording period and calculated by a formula given in Schallert, 2006: $[(\text{ipsi} + 1/2 \text{ both})/(\text{ipsi} + \text{contra} + \text{both})] \times 100$, which sets non-bias at 50%, and plotted as % contralateral forepaw contacts. The data were graphed using Graph Pad Prism 5 (GraphPad Software, San Diego, CA).

Perfusion and Tissue preparation

At the end of the studies rats were anesthetized with an overdose of 500 µl ketamine:xylazine (80 mg/ml:12 mg/ml), then perfused intracardially with phosphate buffered saline (PBS) and 4% paraformaldehyde at pH 7.4. The brains were harvested, fixated in 4% paraformaldehyde over night, and then placed in PBS. With a Pelco 101 vibrotome (Pelco 101 Series-1000, Pelco, Clovis, CA) 40 µm sections were cut and mounted on Surgipath glass slides.

Tyrosine hydroxylase immunohistochemistry in rodents

Staining was conducted as outlined prior (Falk et al., 2011). We used a 1:10,000 dilution of primary antibody (rabbit anti-tyrosine hydroxylase; Millipore, Temecula, CA) and an anti-rabbit (Rbt) goat biotin (1:1,000) antibody (Millipore, Temecula, CA). Sections were then washed and placed in ABC reagent (VECTASTAIN Elite ABC Kit, PK-6100) and visualized using 3,3'-diaminobenzidine (DAB; Vector-Laboratories, Burlingame, CA) as a chromogen. For TH staining photomicrographs were taken using an Olympus IX07 inverted microscope and digital CCD camera using Olympus MagnaFire software, with a constant illumination table. Images were acquired after calibration of the system to ensure adequate exposure and avoid saturation of gray levels on a high magnification (20x Objective). From each animal, three sections from the SN at levels A/P: -4.8, -5.3 and -5.8 according to the atlas of Paxinos and Watson, 1997 were selected for quantitative analysis of neuronal count. AP -5.3 was identified by the presence of the medial terminal nucleus. Then 3 coronal sections at A/P +1.6, A/P +1.0 and A/P +0.2 according to the atlas of Paxinos and

Watson, 1997 were analyzed for the striatal measurements. The Image-J program (Wayne Rasband, Bethesda, MD) was used to count cells only in the SN and presented as the percentage of TH-positive neurons in the lesioned side relative to the intact side. Optical density (OD) of images was also analyzed using Image-J and non specific background staining as determined by measuring the OD in the adjacent cortex was subtracted from the OD in the striatum. Measurements were taken over the entire striatum. Measurements are presented as percentage of the intact side defined as 100%.

SH-SY5Y cell culture

The human neuroblastoma cell line, SH-SY5Y (ATCC, Manassas, VA), was cultured in Roswell Park Memorial Institute (RPMI) medium 1640 (Life Technologies, Carlsbad, CA) containing 10% FBS (fetal bovine serum) and kept at 37° C in a humidified 5% CO₂ Incubator. 50 nM rotenone (Sigma-Aldrich, St. Louis, MO) was used to induce neurodegeneration, leading in our hands to 45% reduction of viability after 48 hours as measured with XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; Sigma-Aldrich, St Louis, MO); data not shown.

VEGFR-1 Staining

Cultures were fixed on day 4 after splitting them into 35 mm² dishes in an MEM/HEPES (pH 7.0) solution containing 4% sucrose and 4% paraformaldehyde. Details on the procedure have been published (Falk et al., 2009). The fixed cells were incubated in primary Rbt anti-human VEGFR-1 antibody solution (1:100; R&D Systems, Minneapolis, MN) in permeation buffer supplemented with 3% donkey serum for 24 hr at RT. To allow visualization under fluorescent light, cultures were incubated in a biotinylated secondary antibody solution (1:1,000; donkey (Dky) anti-Rbt IgG) for 1 hr at RT. The cells were washed as previously described and incubated in a solution of fluor-conjugated streptavidin (1:100,000; AlexaFluor 555; Molecular Probes, Eugene OR) for 1 hr at RT. The cells were washed, and PBS solution (pH 7.4) was applied for microscopic visualization and storage. To determine the optimal conditions for immunocytochemistry, initial studies were carried out with a range of dilutions of the primary antibody from 1:5,000 to 1:50. Fluorescent images were acquired digitally on an Olympus IX70 inverted microscope and camera using Olympus MagnaFire software with a 20x objective. Phase contrast images were also acquired at the same magnification.

Protein Extraction and Bradford Protein Assay

Material: 1% protease inhibitor (PI) in Lysis Buffer; 10% BME (β-Mercaptoethanol) and 1% Bromophenol Blue added to 2X Laemmli Buffer. Media was suctioned from each 35 mm² cell culture dish, dishes were rinsed 2 x with 1 mL ice cold 1X PBS. 100 µL of Lysis Buffer + PI was added and swirled around the bottom of each dish, left on ice for 5–10 min. Cells were then scraped with plastic cell scrapers and harvested. Lysate was sonicated (Sonic Dismembrator, Fisher Scientific) 10 x at Level 3. 10 µL cell lysate was removed for a standard Bradford protein assay, using a plate reader (Molecular Devices, Sunnyvale, CA, USA) and Softmax Pro Software (Molecular Devices, LLC, Sunnyvale, CA). Remaining volume of cell lysate was used to make a 1:1 solution with 2X Laemmli Buffer, boiled for 5 min, placed on ice and aliquots were stored at –80°C.

Western analysis

Samples were thawed at RT followed by 3 min at 100° C. NuPAGE 10% Bis-Tris (Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane) gels were used (Novex by Life Technologies, Carlsbad, CA) with 1X Running Buffer (NuPAGE MOPS SDS Running Buffer, Life Technologies, Carlsbad, CA) + Antioxidant (Invitrogen Technologies,

Carlsbad, CA). 5 μ L of Bio-Rad Kaleidoscope Prestained Standards (Bio-Rad Laboratories, Hercules, CA) and 10 μ g of protein samples were loaded, and gels were run using the XCell II Blot Module (Invitrogen Technologies, Carlsbad, CA) with PowerEase500 power supply (Invitrogen Technologies, Carlsbad, CA) at 125 V, 50 mA and 50 W for approximately 1 hr 40 min, according to Invitrogen protocols. Proteins were transferred onto pure nitrocellulose transfer membranes (Bio-Rad Laboratories, Hercules, CA) using the XCell II Blot Module with PowerEase 500 at 19 V, 50 mA and 50 W, for 14 hr. Transfer membranes were removed and rinsed with 1X TBST (Tris-Buffered Saline + Tween 20) 2×10 min and were blocked with 10% nonfat milk in 1X TBST for 30 min at RT on a shaker, followed by rinsing with 1X TBST for 15 min and a second 2 hr blocking cycle. After blocking, they were washed 4×10 with 1X TBST. Transfer membranes were incubated in primary antibody with 5% BSA (Santa Cruz Biotechnology, Inc., Dallas, TX) with 0.02% Sodium Azide in 1X TBST overnight at -4° C on shaker, washed 4×10 min with 1X TBST, followed by incubation in secondary antibody in 1% BSA in 1X TBST for 1 hour at RT on shaker. Following incubation, transfer membranes were washed 4×10 min with 1X TBST. For protein detection, ECL (enhanced chemiluminescence; GE Healthcare, Piscataway, NJ) was used according to manufacturer's instructions. Western blots were imaged using Bio-Rad Quantity One 4.6.3 Imaging Software (Bio-Rad Laboratories, Hercules, CA) and quantified with Image J. After imaging, blots were rinsed with 1X TBST 2×10 min. If necessary, blots were stripped using a moderately stringent stripping buffer (50 mM Tris (pH=6.8), 1% SDS, 1% Sodium Azide) twice for 15 min each. Blots were washed 4×10 min with 1X TBST and probed again. Primary antibodies: mouse (Ms) monoclonal anti- β -Actin (1:10,000; Sigma-Aldrich, St Louis, MO); anti-Akt (pan) (C67E7) Rbt mAb (1:1,000), anti-phospho-Akt (Thr308) (244F9) Rbt mAb (1:1,000), anti-phospho-Akt (Ser473) (D9E) XP Rbt mAb (1:1,000), Cell Signaling Technology, Inc., Danvers, MA; FATP4: anti-ACSVL4 (H-100): sc-25670 Rbt pAb (1:500) and FATP1: anti-ACSVL5 (M-100): sc-25541 Rbt pAb (1:500), Santa Cruz Biotechnology Inc., Dallas, TX. Secondary antibodies: Dky X Rbt IgG HRP (1:5,000), Dky X Ms IgG (H+L) HRP (1:5,000), EMD Millipore, Billerica, MA.

VEGF-B immunohistochemistry in the human substantia nigra pars compacta (SNpc)

Brains of Parkinson's disease patients and healthy individuals had a postmortem interval of 20–38 hr. Information about the cause of death was not available. Samples were selected at the Department of Psychiatry and Department of Pathology, University of Würzburg, Germany. Search string for selection was age > 70 years for control and PD group. Neurologist's Diagnosis of Parkinson's disease (PD) was based on UK Brain Bank criteria (Litvan et al., 2003) including idiopathic Parkinson's disease, excluding atypical Parkinson syndromes like multiple system atrophy (MSA), progressive supranuclear palsy (PSP), drug-induced Parkinson syndromes, or other diseases presenting with PD like symptoms (Wilson's disease, Huntington's disease). Neuropathologic assessment by a consultant neuropathologist confirmed clinical diagnosis of PD. Controls were confirmed to be neuropathologically normal using histology from paraffin-embedded brain tissue blocks.

Processing: After death the brain was removed including cerebellum and brainstem. Section was done into right and left halves by cutting midsagittally through the corpus callosum, midbrain, cerebellum and brainstem. One half was processed using fixation in 10% formalin, the other half was frozen. Formalin fixed tissues were then blocked, paraffin embedded and used for immunohistology. The midbrain sections were cut on a microtome into 6 μ m coronal sections. After rehydrating the sections in xylene and a graded alcohol series, slides were microwaved for 15 min in 10 mM sodium citrate, pH 6.0. Slides were washed with PBS; endogenous peroxidases were blocked using 0.3% hydrogen peroxide in 40% methanol for 10 min and blocked using 5% normal goat serum in PBS supplemented

with 0.3% Triton and washed again with PBS (three times for 10 min). The polyclonal rabbit anti-human VEGF-B antibody diluted 1:50 (Santa Cruz Biotechnology, CA; sc-13083) mapping at the N-terminus of VEGF-B; diluted in PBS plus 3% goat serum) was added and incubated at 4°C overnight in a humid chamber. The secondary antibody, coupled with biotin (Vector Laboratories, Burlingame, CA), was diluted the same way in PBS plus 1.5% goat serum and added after washing the slides with PBS. After incubation for 30 min at room temperature and a brief wash with PBS, an ABC enhancer complex coupled with peroxidase (Vector Laboratories) was added and incubated for 30 min at room temperature. After washing with PBS, the substrate (ABC-Kit, Vector Laboratories) was added, and the reaction was stopped in distilled water after the desired degree of staining was reached. Finally, slides were dehydrated again and mounted using CV mount (Leica, Bensheim, Germany). Staining was visualized using an Axioplan 2 imaging microscope (Carl Zeiss Microimaging, Oberkochen, Germany) equipped with an Axio-Cam MR color digital camera (Carl Zeiss Microimaging) using a 40x Plan Neofluar and a 63xPlan/Apochromat objective and the AxioVision 4.6 software package (Carl Zeiss Microimaging).

Gene expression analysis of *VEGF-B*, *GDNF* and nuclear-encoded mitochondrial genes in rat midbrain cultures

The Pearson correlation analysis of the *VEGF-B* and *GDNF* genes with a list of nuclear-encoded mitochondrial was performed using R-project and BioConductor data analysis tools (<http://www.r-project.org>, <http://www.bioconductor.org>), based on the analysis of Hagberg et al., 2010, who established a correlation of *VEGF-B* with those mitochondrial genes in endothelial cells. To evaluate the *VEGF-B* and *GDNF* expression we used data collected from RNA extracts of rat midbrain cultures from prior experiments (Falk et al., 2009a). Since we wanted to investigate if *VEGF-B* expression was indeed also correlated with mitochondrial gene expression in the midbrain irrespective of treatment condition in those prior experiments the data were pooled from all 11 Affymetrix RAT ST 1.0 expression arrays to increase the statistical power (either untreated, treated with 20 nM rotenone, the unrelated pigment epithelium-derived factor at 1 ng/ml, or a combination of these agents). Data from the arrays were normalized using the RMA function of the BioConductor OLIGO library and annotated using BioConductor tools. Probe sets for each gene on the RAT ST 1.0 array were then averaged. The methods for preparation of and maintenance of the rat midbrain cultures have been reported in detail previously (Falk et al., 2009b). The methods of RNA isolation, the gene array procedure and analysis have been reported in detail in Falk et al., 2009a.

Data Analysis

Statistical analysis was performed using SPSS software, 16.0 (SPSS, Chicago, IL) and GraphPad Prism, software 5.0 (GraphPad Software, Inc., La Jolla, CA). Differences between groups in the behavioral analysis were assessed using Student's *t*-test or parametric analysis of variance (mixed repeated measure ANOVA) followed by appropriate post hoc testing. Differences between groups in the immunohistochemical and western analysis were assessed with one-way ANOVA, followed by the appropriate post hoc test. The null hypothesis was rejected when $p < 0.05$. In the neuroprotection experiment a total of 55 Sprague-Dawley rats were used, 5 cases were excluded due to the injection point being out of the striatum; 4 outliers in the amphetamine-induced rotation experiment (3 in the vehicle group and 1 in the V10 group) were excluded since they were more than 2 Standard Deviations greater than the mean and presented with Cook's distance above $Di = 4/n$ (they rotated between 1890 and 1990 times/100 min). In the neurorestoration experiment 30 Sprague-Dawley rats were used. The concordance of the behavioral and immunohistochemical measures was evaluated by calculating the Pearson's correlation using SPSS.

RESULTS

Behavioral improvement after VEGF-B and GDNF in the neuroprotection experiment

The neuroprotective effects of VEGF-B were compared with GDNF in a unilateral progressive 6-OHDA lesion rat PD model. The experimental paradigm is depicted in Figure 1A. Rats were injected with vehicle, VEGF-B (1 and 10 μ g) or GDNF (10 μ g), 6 hours prior to 6-OHDA injections and then evaluated with amphetamine-induced rotational behavioral tests (Figure 2A). The vehicle group showed a high number of rotations at 2, 4 and 6 week, indicating a significant destruction of DA neurons in the lesioned side. The VEGF-B-groups and the GDNF group showed a significant decrease in ipsiversive turning in amphetamine-induced rotation behavior tests at 2, 4 and 6 weeks after injection compared to vehicle (Figure 2A). The improvements seen in the GDNF and VEGF-B groups were not significantly different, even though a trend of a slight superiority of GDNF is evident from the graph. The non-drug-induced cylinder test was used as another behavioral measure to quantify the lesion caused by 6-OHDA. Lesioned animals use their contralateral forepaw less frequently in exploring the cylinder. Vehicle-treated rats showed a significant reduction from baseline values (Figure 2B) in contralateral forepaw preference after the 6-OHDA-lesion, whereas in all 3 treatment groups the repeated measures ANOVAs did not reveal any significant change over time. This indicates that a significant forepaw deficit was induced by the 6-OHDA lesion in the vehicle group that was not seen in either treatment group. However, VEGF-B and GDNF treatments resulted in no significant between group effects to restore forepaw preference in this test when comparing all groups.

VEGF-B and GDNF rescued DA neurons and fibers in the neuroprotection experiment

To verify the extent of the lesion immunohistochemical analysis of the SN and striatum stained with an anti-TH antibody was performed after the end of the neuroprotection experiment. Intrastriatal injection of 6-OHDA caused a ~46% reduction in the number of TH-positive neurons in the SN in the lesioned side compared to the non-lesioned hemisphere (Figure 3). Pretreatment with VEGF-B (1 μ g and 10 μ g) and GDNF (10 μ g) caused an increase in surviving TH-positive cell bodies in the SN (~30%, ~34%, and ~20% percent loss respectively) against the 6-OHDA induced degeneration (Figure 3). There was a significant difference between 10 μ g VEGF-B and 10 μ g GDNF ($p < 0.05$). 1 μ g VEGF-B and 10 μ g GDNF were not significantly different. To further investigate the lesioned state, we measured the optical density (OD) of TH-positive fibers in the striatum. Both VEGF-B groups and the GDNF group showed an increase in TH-positive fiber density survival compared to vehicle (Figure 4). There was no significant difference between the VEGF-B (1 μ g and 10 μ g) groups and the GDNF (10 μ g) groups (Figure 4).

GDNF but not VEGF-B significantly reduced behavioral deficits in the neurorestoration experiment

Neurorestorative effects of VEGF-B were tested using the 6-OHDA model and the experimental paradigm is depicted in Figure 1B. Since our data indicate that the neuroprotective effect of VEGF-B (Figures 2 and 3; Falk et al., 2011) is already maximal at the 1 μ g and 3 μ g doses we chose a dose of 3 μ g for the neurorestoration experiment. Rats were injected with vehicle (n=10), VEGF-B (3 μ g; n=10) and GDNF (10 μ g; n=10). At the baseline (BL-3) vehicle-treated rats showed a higher ipsiversive amphetamine-induced rotational count since they were lesioned in two locations in the striatum compared to being lesioned in one location for the neuroprotective studies. The rationale behind the 2 lesion paradigm was to generate a lesion that was stable over the course of 14 weeks post-6-OHDA. The data from Figure 2 indicate that in our hands a single 6-OHDA lesion did show a slightly reduced amphetamine-induced rotation count in the vehicle controls at the end of the experiment at 6 weeks post-lesion. This could potentially lead to a significant reduction

of the amphetamine-induced rotations in an experiment extending to 14 week post-6-OHDA-lesion, and therefore the 2 lesion approach that has been shown to improve the long-term stability of the lesion (Kirik et al., 1998; Voutilainen et al., 2011) was chosen. VEGF-B-treated rats in the neurorestoration study showed no significant decrease in ipsiversive amphetamine-induced rotations over the 12 week period post-6-OHDA injection (Figure 5A). The GDNF-treated group on the other hand did show a significant decrease at 10 and 12 weeks (Figure 5A). For the cylinder test a baseline (BL-3) was established three weeks after injection of 6-OHDA and further tests occurred at 6, 8, 10 and 12 weeks post-6-OHDA. VEGF-B and GDNF injection resulted in no significant improvement in this test when compared to vehicle (Figure 5B). When only comparing the GDNF-treated group with the vehicle group at 12 weeks post-6-OHDA a significant improvement was apparent (two tailed *t*-test, $p < 0.05$).

GDNF and not VEGF-B significantly increased the number of surviving DA neurons in the SN in the neurorestoration experiment

TH immunohistochemical analysis of the SN was conducted at the end of the experiment. Intrastriatal injection of 6-OHDA in the neurorestoration paradigm caused a ~74% reduction of TH-positive cell bodies in the SN in vehicle-treated rats ($n=10$). VEGF-B (3 μ g; $n=10$) and GDNF 10 μ g; $n=10$) in this experiment were injected four weeks after injection of 6-OHDA. The VEGF-B group showed a 6% increase in DA neuronal neuron count in the SN (~68% reduction) compared to the vehicle group, whereas the GDNF group showed a significant 13% increase in DA neuron count (~61% reduction) over the vehicle group (Figure 6A). To further investigate the lesioned state, we measured the OD of TH-positive fibers in the striatum. Neither the VEGF-B group nor the GDNF group showed an increase in TH-positive fiber density survival compared to vehicle (Figure 6B).

To investigate if there was concordance between the behavioral and morphological results, we calculated the Pearson's correlation of the behavioral and immunohistochemical analyzes. There was a significant negative correlation between the amphetamine-induced rotation count at the last test of each experiment with both the SN TH cell count ($r = -0.57$; $p < 0.001$; $n = 76$) and the striatal TH OD measurement ($r = -0.41$; $p < 0.001$; $n = 76$). The SN TH cell count and the striatal TH OD measurement showed a positive correlation ($r = 0.64$; $p < 0.001$; $n = 76$).

The expression of the VEGF-B gene correlates with nuclear-encoded mitochondrial genes in the rat midbrain

Since mitochondrial dysfunction is central to PD and since there has been prior data showing a correlation of *VEGF-B* with nuclear-encoded mitochondrial genes in endothelial cells (Hagberg et al., 2010), we next conducted a Pearson correlation analysis of the expression of the *VEGF-B* gene in rat midbrain cultures (Falk et al., 2009a) with a list of nuclear-encoded mitochondrial genes previously shown to be tightly correlated with expression of the *VEGF-B* gene in endothelial cells (Hagberg et al., 2010), utilizing gene array data from prior experiments (Falk et al., 2009a). A plot of the correlation of *VEGF-B* with those mitochondrial genes is shown in Figure 7A (grey bars) and revealed 23 genes with a correlation coefficient of > 0.5 (8 of those had a correlation coefficient of > 0.8). To compare the results of the correlation analysis between the *VEGF-B* gene and the target gene set, the distribution of correlation coefficients between the *VEGF-B* gene and all genes on the array was determined and the result is shown in Figure 7A (black bars). The proportion of genes with a high positive or negative correlation with *VEGF-B* expected by chance was then evaluated with the Fisher exact test. This analysis revealed a highly significant positive correlation of *VEGF-B* ($p = 1.9e-50$) with the set of 23 nuclear-encoded mitochondrial genes, identified below, in rat midbrain cultures. Of those 23 genes 22 are involved in fatty

acid metabolism. *Hadhb* (hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase) encodes the beta subunit of the mitochondrial trifunctional protein, which catalyzes the last three steps of mitochondrial beta-oxidation of long chain fatty acids. *Mcee* (methylmalonyl CoA epimerase) catalyzes the interconversion of D- and L-methylmalonyl-CoA during the degradation of odd chain-length fatty acids. *Cs* (citrate synthase), *Ogdh* (oxoglutarate (alpha-ketoglutarate) dehydrogenase lipoamide), *Sdhb* (succinate dehydrogenase complex, subunit B, iron sulfur), *Sdhc* (succinate dehydrogenase complex, subunit C), *Idh3g* (isocitrate dehydrogenase [NAD] subunit gamma) and *Fh1* (fumarate hydratase 1) are involved in the Krebs cycle (Stryer, 1995). *Pdha1* (pyruvate dehydrogenase (lipoamide) alpha 1) catalyzes the overall conversion of pyruvate to acetyl-CoA and CO₂. *Chchd10* (coiled-coil-helix-coiled-coil-helix domain containing 10) has a functional role in complex IV activity (Martherus et al., 2010). *Coq9* (coenzyme Q9 homolog, ubiquinone biosynthesis protein), *Ndufv1*, *Ndufa9*, *Ndufb2*, *Ndufb8*, *Ndufb9*, *Ndufs1*, *Ndufs2* (parts of the NADH dehydrogenase [ubiquinone] flavoprotein complex), *Etfb* (electron-transfer-flavoprotein, beta polypeptide), *Uqcrc1* (ubiquinol-cytochrome c reductase core protein I) and *Etfdh* (electron transfer flavoprotein-ubiquinone oxidoreductase) are involved in the mitochondrial membrane respiratory electron transport chain (Duncan et al. 2009; Stryer, 1995). *Hsdl2* (hydroxysteroid dehydrogenase-like protein 2) is of unknown function, has apparently no steroid dehydrogenase activity but has been implicated in fatty acid metabolism (Kowalik et al., 2009). *Chchd3* (coiled-coil-helix-coiled-coil-helix domain containing 3) is required for maintenance of mitochondrial cristae integrity (Darshi et al., 2012).

The same correlation analysis was conducted with *GDNF* (Figure 7B). *GDNF* also showed a significant correlation ($p = 0.018$) with the nuclear-encoded mitochondrial genes, but the correlation was not nearly as strong as for *VEGF-B*. The following 15 genes, *Chchd10*, *Coq9*, *Cs*, *Etfb*, *Fh1*, *Hadhb*, *Hsdl2*, *Mcee*, *Ndufb2*, *Ndufb8*, *Ndufb9*, *Ndufv1*, *Ogdh*, *Sdhb* and *Sdhc* (succinate dehydrogenase complex, subunit D) showed a correlation coefficient of greater than 0.5. The two lists have 14 genes in common, with *Sdhc* seen only in the *GDNF* correlation.

VEGF-B normalized rotenone-induced reduction of FATP level and Akt signaling in SH-SY5Y cells

We further evaluated potential down-stream targets of VEGF-B with western analysis in a commonly used cell line model that displays hallmark characteristics of DA neurons, the SH-SY5Y neuroblastoma cell line treated with rotenone (for example, Wang et al., 2002; Ruan et al., 2010; Yu et al., 2013). We first established that the VEGF-B receptor VEGFR-1 indeed is expressed in the cells with immunocytochemistry (Figure 8). We then evaluated the effect of VEGF-B in these cells in 4 experimental groups (control; + 20 ng/mL VEGF-B; + 50 nM rotenone; 50 nM rotenone + 20 ng/mL VEGF-B) at 30 min, 1 hr, 6 hr, 12 hr, 24 hr and 48 hr time points post-treatment. VEGF-B was administered 1 hr prior to rotenone. (1) We evaluated fatty acid transport protein (FATP) expression, since FATPs had been shown to be up-regulated by VEGF-B in endothelial cells (Hagberg et al., 2010) and since *VEGF-B* correlated in rat midbrain with nuclear-encoded mitochondrial genes involved in fatty acid metabolism. Since FATP1 (ACSVL5) and FATP4 (ACSVL4) are the only FATPs that have been reported to be expressed in the brain these two isoforms were chosen (Schaap et al., 1997; Utsunomiya et al., 1997; Hirsch et al., 1998). VEGF-B did not have a significant effect on the FATP1 and 4 levels when given in the control situation, but it counteracted the loss of FATP1 (Figure 9A,B) and FATP4 (Figure 9C,D) after rotenone exposure, increasing the protein levels toward control levels. (2) Akt protein level and phosphorylation were investigated since Akt signaling has been implicated downstream from VEGFR-1 in other cellular systems (Olsson et al., 2006; Falk et al., 2010). There was a significant 25%

reduction in Akt total protein level at 24 and 48 hr post-rotenone that was significantly reversed in the VEGF-B co-treatment group (Figure 10A,B). There was also a significant reduction in the phosphorylation level at both Akt phosphorylation sites serine 473 (Figure 10C,D) and threonine 308 (Figure 10E,F) 1hr after rotenone exposure that was significantly reversed by VEGF-B pre-treatment.

VEGF-B is expressed in the human SNpc

We then evaluated expression of VEGF-B in the human SNpc. We stained coronal paraffin embedded SNpc slices from healthy controls and PD patients for VEGF-B and evaluated staining with light microscopy. All PD patients were in advanced (end) stage of the disease (Hoehn & Yahr stage 4). The samples were age matched with healthy controls mean age: 78.7 ± 8 years and PD patients: mean age 80.4 ± 6 years. From the sample photomicrographs (Figure 11) it is apparent that there is clear expression of VEGF-B (arrows) in neuronal shaped cells that show co-expression of neuromelanin (arrowheads) in both the healthy control (left panel) and the PD patient (right panel). A semi-quantitative analysis from postmortem SNpc tissue of 4 PD patients did not show any overt changes in the VEGF-B protein level compared to 3 healthy controls (Table 1).

DISCUSSION

VEGF-B was first studied in a unilateral progressive 6-OHDA-lesion model of dopaminergic nigrostriatal pathways (Falk et al., 2011). This standard model (Dauer and Przedborski, 2003; Meredith et al., 2008; Cannon and Greenamyre, 2010) facilitates progressive axonal degeneration of the midbrain DA neurons, and allows for the study of not only neuroprotective but also neurorestorative effects by either injecting the neurotrophic factors before or after the 6-OHDA-injection. In this PD model GDNF has been shown to protect DA neurons when injected into the SN or the striatum (Kearns and Gash 1995; Shults et al., 1996; Kirik et al., 2000).

The injection of GDNF in our experiments resulted in similar effects when compared to the reported results in the literature in both the neuroprotection and the neurorestoration study. Using GDNF as our positive control we have shown that VEGF-B-treatment as well leads to a significant improvement in the amphetamine-induced rotational behavior in the neuroprotection study. GDNF can be considered as the gold standard growth factor in preclinical models of PD and our data showed VEGF-B was only slightly inferior in terms of its protective effect (Figures 2, 3 and 4). In the non-drug-induced cylinder test in the neuroprotection study the GDNF and both VEGF-B treated groups showed no significant difference from the vehicle control group (Figure 2B). Since this experimental paradigm isn't as sensitive to the extent of the lesion as the amphetamine-induced rotation paradigm this was not unexpected. The cylinder data did confirm, however, that the vehicle-treated animals showed a significant loss of motor function compared to baseline, indicating that the mild lesion was detectable in vehicle-treated rats, whereas this was not the case in the groups treated with either neurotrophic factor. The immunohistochemical analysis correlated with the behavioral study. VEGF-B's neuroprotective action led to an increased number of DA cell bodies remaining in the SN and fiber density in the striatum (Figure 3 and 4). Our results showed that VEGF-B protects DA neurons from degeneration to a similar degree when compared to GDNF at the same $10 \mu\text{g}$ dose. Interestingly there was a similar degree of protection seen even with the lowest VEGF-B dose tested, $1 \mu\text{g}$. In the neurorestoration study only the GDNF group showed a significant restoration (Figure 5 and 6). The mechanism of neurorestoration effects is incompletely understood, but may include restoration of the TH-phenotype, from damaged but surviving, DA neurons, as shown here. Our data reproduced well published findings of a neurorestorative effect of GDNF and showed a slow development of reduced behavioral deficit over time that reached the

maximum benefit at 8 weeks post-growth factor injection (for example Lindholm et al., 2007, Voutilainen, et al., 2009 and Cohen et al., 2011 reported a similar slow time course of the GDNF effect). We cannot rule out that the lack of effect by VEGF-B may be due in part to the low 3 μg dose or due to suboptimal diffusion properties. Due to the fact that maximal neuroprotection is already achieved at 1 μg and since the VEGF-B₁₈₆ isoform that we have used in our study has been shown prior to be highly diffusible due to the lack of matrix binding that restricts the diffusion of VEGF-B₁₆₇ (Olofsson, et al., 1996; Poesen et al., 2008), we would hypothesize that a neurorestorative effect might require more long-term supplementation with VEGF-B which would require expression from a gene therapy vector. To summarize VEGF-B protected DA neurons in the SN and the DA terminals in the striatum in the same range as GDNF, albeit with a slightly reduced magnitude while not providing significant neurorestoration. We can also compare our data with the published results from other neurotrophic factors that are currently investigated. VEGF-B protected DA neurons equally to MANF (Voutilainen et al., 2009) and is somewhat more effective in protecting striatal DA fibers when compared to MANF. CDNF (Lindholm et al., 2007) is slightly more effective than VEGF-B in protecting DA neurons, when comparing the 3 μg (Falk et al., 2009a) and 10 μg doses. None of these other factors besides VEGF-B has been tested at a 1 μg dose. These differences could be due to the factors themselves or the relative place along their dose-response curves that was examined. While there is a clear neuroprotective potential for VEGF-B, it does not show the neurorestorative potential that GDNF, MANF and CDNF display in this particular preclinical rodent model (Lindholm et al., 2007, Voutilainen, et al., 2009 and Cohen et al., 2011). Nevertheless VEGF-B appears worthy of further study, since it likely has a significantly different mechanism of action (*vide infra*). Neurorestoration in the toxin rodent model may not be the best predictor of efficacy in a slowly progressing human disease where continuous neurodegeneration occurs. A modest acute neuroprotective effect may well translate into a therapeutic meaningful effect, if a constant long-term supply of VEGF-B can be achieved. Neurotrophic factors are still considered a hopeful prospect for PD therapy (Sherer et al., 2006), but given the lack of success in the initial clinical trials of the GDNF-family of growth factors and since GDNF has recently been shown to not be neuroprotective in an α -synuclein rat model of PD (Decressac et al., 2011), we would argue novel protective growth factors using different intracellular cascades still need to be examined. VEGF-B should therefore in the future be studied after long-term expression with a gene therapy vector, including testing of VEGF-B in a rat α -synuclein PD model.

Our data from the rat midbrain show that *VEGF-B* co-expressed with nuclear-encoded mitochondrial genes (Figure 7). This indicates the action of VEGF-B might involve mitochondrial function. This novel finding from rat midbrain is further substantiated by recent publications proving (1) the linkage of *VEGF-B* gene expression with various mitochondrial genes across other cell types (Hagberg et al., 2010), (2) down-regulating of pro-apoptotic genes recently shown to be regulated by the VEGFR-1 receptor in other cell types (Li et al., 2008), including the “BH-3 only” family and (3) the up-regulation of fatty acid transport into the mitochondria by VEGF-B in endothelial cells (Hagberg et al., 2010). (4) VEGF-B normalized the mitochondrial membrane potential in toxin-treated sensory neurons (Dhondt et al., 2011). This is especially interesting since the mitochondria are thought to be at the center of both genetic and potential environmental causes for PD, and DA neurons in the SN are known to have a high mitochondrial oxidant stress, due to their high metabolic demands (Olanow et al., 2009; Surmeier et al., 2011).

Although neuroprotective effects of VEGF-A overexpression in models of PD have been reported (Yasuhara et al., 2004), detrimental vascular effects such as edema and disruption of the blood-brain barrier occurred (Harrigan et al., 2003; Rite et al., 2007). These negative side effects have not been observed after sustained VEGF-B delivery into a rodent brain in

an amyotrophic lateral sclerosis (ALS) model (Poesen et al., 2008) or genetic VEGF-B overexpression in a model of retrograde degeneration of sensory neurons (Dhondt et al., 2011), which is not surprising given that VEGF-B only binds to the protective VEGFR-1, whereas VEGF-A binds to both VEGFR-1 and VEGFR-2, the receptor thought to mediate the angiogenic effects (Olsson et al., 2006; Falk et al., 2010). VEGFR-1 on the other hand is tied to protective effects in many organs, including the brain. In DA neurons the pathways for VEGF-B have not yet been elucidated. In general VEGF-B's mammalian receptor VEGFR-1 activates downstream pathways that are used to help with protective processes during cellular stress (Olsson et al., 2006; Falk et al., 2010), including regulation of apoptotic pathways (Li et al., 2008). Recently regulation of fatty acid transport by VEGF-B has also been implicated in endothelial cells (Hagberg et al., 2010) which could aid a cell during metabolic stress. FATPs are a large evolutionarily conserved family of proteins that mediate transport of long chain fatty acids into cells, with FATP1 and 4 being expressed in brain (Hirsch et al., 1998). Indeed, using the SH-SY5Y cell line, a commonly used human cell line model exhibiting hallmark characteristics of DA cells, we could show that VEGF-B does counteract mitochondrial stress-induced (rotenone) reduction of FATP1 and 4 levels by bringing the levels back up toward control levels (Figure 9). We could hypothesize that in the context of the mitochondrial stress-induced reduction of FATP1 and 4 levels the up-regulation of these FATPs toward normal control levels via VEGF-B allows for increased ATP production by the remaining intact mitochondria, thereby helping the cells with compromised mitochondrial function to survive. In mitochondrial complex I low ATP production and consequent high proton-motive force and a reduced coenzyme Q pool contributes to the production of superoxide radicals, while on the other hand, mitochondria with normal ATP production and subsequent low proton-motive force in the mitochondrial matrix produce much lesser superoxide radicals (Murphy, 2009). In this context it is important to point out that 22 of 23 nuclear-encoded mitochondrial genes shown to strongly correlate with *VEGF-B* in rat midbrain (Figure 7) are involved in fatty acid metabolism (beta-oxidation, Krebs cycle and electron transport chain). The only gene not directly involved in fatty acid metabolism, *Chchd3*, is required for mitochondrial cristae stability and a recent study indicates that cristae shape determines respiratory efficiency (Cogliati et al., 2013). Signaling via the anti-apoptotic regulator Akt is also implemented in the VEGF-B response, since VEGF-B normalized the reduced Akt levels and the reduced phosphorylation levels of Akt, at both sites required for maximal Akt activation, after rotenone-treatment (Figure 10). The fact that there was only an effect of VEGF-B evident in the rotenone-treated cells and not the controls is commensurate with the role of a stress-induced response similar to what has been demonstrated in the SOD1 mouse model of amyotrophic lateral sclerosis (ALS), where knock-out of VEGF-B did lead to a worsening of the disease state, while in otherwise healthy control VEGF-B knock-out mice there was no obvious deficit (Poesen et al., 2008). And primary dorsal root ganglion cultures lacking VEGF-B or VEGFR-1 exhibited increased neuronal stress and were more susceptible to paclitaxel induced cell death, while addition of VEGF-B antagonized neuronal stress, maintained the mitochondrial membrane potential and stimulated neuronal survival (Dhondt et al., 2011).

On the other hand, the correlation of *GDNF* ($p = 0.018$) with nuclear-encoded mitochondrial genes was not nearly as strong as shown for *VEGF-B* ($p = 1.9 \times 10^{-50}$), indicating that GDNF does not play a very important role in regulation of fatty acid metabolism, in keeping with the established role of GDNF in the brain. Homodimeric GDNF-family ligands activate RET tyrosine kinase (TK) by first binding their cognate GDNF-family receptor- α (GFR α) receptors, and these pathways are well studied in DA neurons (Airaksinen and Saarma, 2002; Lindgren et al., 2008). Since VEGF-B works using a different receptor than GDNF, the two could potentially be additive or even synergistic with each other to produce more profound effects.

The facts that VEGF-B indeed is expressed in the human SNpc of both healthy controls and PD patients (Figure 11) in neurons co-expressing with neuromelanin and that the presence of VEGF-B's receptor VEGFR-1 (Flt 1) in neuromelanin containing neurons in the SN of PD patients has been established by Wada et al. 2006, allow us to speculate that the intracellular pathways necessary for the neuroprotective action of VEGF-B may be present in dopaminergic neurons in PD patients. An analysis from postmortem SNpc tissue of 4 PD patients did not show any overt changes in the VEGF-B protein level compared to healthy controls. Therefore a lack of VEGF-B in DA neurons may not be directly causative of PD, but this is not necessary to make VEGF-B a promising therapeutic candidate for PD. We hypothesize that VEGF-B up-regulation is a protective response to cellular oxidative stress that leads to increased mitochondrial load and activation of apoptotic cascades, and that therefore enhancing this endogenous protective response might have a protective effect in neurodegenerative disease such as PD.

CONCLUSION

Our results have shown that VEGF-B is a neuroprotective factor for DA neurons, regulates fatty acid transporters and Akt signaling, and is expressed in the human SNpc of PD patients. Future studies should aim to test the effects and safety of long-term expression of VEGF-B and evaluate potential synergistic effects of VEGF-B and GDNF.

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- Neuroprotective effects of VEGF-B in rat 6-OHDA model are comparable to GDNF
- VEGF-B is not neurorestorative in rat 6-OHDA model, while GDNF is
- *VEGF-B* expression has strong correlation with mitochondrial genes in rat midbrain
- VEGF-B restored reduced levels of FATP1+4 and Phospho-Akt post-rotenone in SH-SY5Y
- VEGF-B is expressed in the human substantia nigra pars compacta of PD patients

Figure 1.

Scheme depicting the experimental paradigms and timelines for (A) the neuroprotection study (BL = baseline before lesion) and (B) the neurorestoration study (BL-3 = baseline 3 weeks after 6-OHDA-lesion).

Figure 2.

Neuroprotection: (A) Amphetamine-induced rotations. Intra-striatal infusion of either 1 μ g (V1) or 10 μ g (V10) VEGF-B, or 10 μ g GDNF (G) 6 hrs prior to the 6-OHDA injection reduced amphetamine-mediated ipsiversive rotations, as compared to vehicle-injected controls, at 2, 4 and 6 week (mean rotations $\pm$ SEM; $n=8-19$ /group; Fisher LSD post-hoc test compared to the vehicle control group after one-way ANOVAs; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $\#p = 0.08$). **(B) Cylinder test.** Lesion-induced forepaw preference measured by the cylinder test (mean % contralateral forepaw contacts; $n=9-22$ /group). Repeated-measures ANOVAs confirmed a significant reduction in contralateral forepaw contacts post 6-OHDA-lesion when compared to baseline only in the vehicle control group ($*p < 0.05$) but not in either GDNF (G) or VEGF-B-treated groups (V1 and V10). No statistical significant differences between the vehicle and the treatment groups were detected at the different time points (Fisher LSD post-hoc test of raw data after one-way ANOVAs).

Figure 3. Neuropreservation: Dopaminergic cell number

1 μ g (V1) and 10 μ g (V10) VEGF-B and 10 μ g GDNF (G) partially protected TH-positive neurons in the SN. The example photomicrographs taken at lower magnification in the left panel (A, B, C, D) depicts the intact side and in the right panel (E, F, G, H) depict the lesioned side at level A/P + 1.0 mm. Both 1 μ g (F) and 10 μ g (G) doses of VEGF-B protected neurons in the SN as well as GDNF (H) when compared to vehicle (E). (I) The cell number of TH-positive cells in the ipsilateral SN (circled in A) of 6-OHDA lesioned animals was compared to the intact contralateral side (mean TH-positive cells $\pm$ SEM; n=9–22/group, one-tail Fisher LSD post-hoc test compared to the vehicle control group after one-

way ANOVA; * $p < 0.05$, ** $p < 0.02$, *** $p < 0.001$). 1 μg (V1) and 10 μg (V10) VEGF-B and 10 μg GDNF (G) partially protected TH-positive neurons in the SN.

Figure 4. Neuroprotection: Dopaminergic terminal density

The example photomicrographs examples depicted (A–D) were taken at level A/P –5.8 mm and show the non-lesioned control side on the left. All 3 treatments showed a partial protective effect when compared to the vehicle group (A). There was no clearly visible difference between either of the 1 μ g VEGF-B (C), or the 10 μ g VEGF-B (D) groups and the 10 μ g GDNF (B) group. (E) The optic density of TH-IR fibers in the ipsilateral striatum of 6-OHDA-lesioned animals was compared to the intact contralateral side (mean OD $\pm$ SEM; n=9–22/group; one-tail Fisher LSD post-hoc test compared to the vehicle control group after one-way ANOVA; * p < 0.05, ** p < 0.01). 1 μ g (V1) and 10 μ g (V10) VEGF-B and 10 μ g GDNF (G) partially protected TH-positive fibers in the striatum.

Figure 5.

Neurorestoration: (A) Amphetamine-induced rotation BL-3 = baseline count at 3 weeks post 6-OHDA injection, 1 week before growth factor injection. Intra-striatal infusion of 3 μ g VEGF-B did not reduce amphetamine-mediated ipsiversive rotations, while 10 μ g GDNF did reduce amphetamine-induced ipsiversive rotation as compared to BL-3, at both 10 weeks (* $p < 0.05$) and 12 weeks (** $p < 0.01$) post-6-OHDA injection (mean rotations $\pm$ SEM; $n=10$ /group; Bonferroni post-hoc tests after repeated-measures ANOVAs). A linear mixed model was fit to the whole data and revealed a significant main effect of group ($p < 0.05$). Specifically, the GDNF group had significantly lower rotations than the vehicle ($p < 0.05$) and VEGF-B ($p < 0.01$). No significant difference was found between vehicle and VEGF-B group. **(B) Cylinder test.** Lesion-induced forepaw preference measured by the cylinder test (mean % contralateral forepaw contacts; $n=7-10$ /group). No statistical significant differences between the vehicle and the treatment groups were detected at the different time points (Fisher LSD post-hoc tests of the raw data after one-way ANOVAs).

Figure 6.

Neurorestoration: (A) Dopaminergic cell number. The cell number of TH-positive cells in the ipsilateral SN of 6-OHDA lesioned animals was compared to the intact contralateral side and presented as % of control side (mean TH-positive cells $\pm$ SEM; $n=10$ /group, Bonferroni post-hoc test compared to the vehicle control group after one-way ANOVA). 10 μ g GDNF did significantly increase TH-positive neuron number in the SN ($*p < 0.05$) when compared to vehicle while 3 μ g VEGF-B did not. **(B) Dopaminergic terminal density.** The optic density of TH-IR fibers in the ipsilateral striatum of 6-OHDA-lesioned animals was compared to the intact contralateral side (mean OD $\pm$ SEM; $n=10$ /group; one-tail Fisher LSD post-hoc test compared to the vehicle control group after one-way ANOVA). There was no significant difference between vehicle control vs. either treatment group.

Figure 7. Pearson correlation analysis of the *VEGF-B* and *GDNF* genes with lists of nuclear-encoded mitochondrial genes

To compare results of the correlation analysis between *VEGF-B* (A) or *GDNF* (B) and the target gene set, the distribution of correlation coefficients between that same gene and all genes on the array was determined and the result are shown. The correlation with *VEGF-B* or *GDNF* is plotted in 0.1 steps from -1.0 to 1.0 on the X axis. The gray bars depict the correlation of *VEGF-B* (A) or *GDNF* (B) with a set of nuclear-encoded mitochondrial genes and the black bars depict the correlation of *VEGF-B* (A) or *GDNF* (B) with all genes. The sample plot of the correlation of *VEGF-B* (A) with mitochondrial genes revealed 23 genes with a correlation coefficient (cc) of > 0.5 . The proportion of genes with a high positive or negative correlation with the given gene expected by chance was then evaluated with the Fisher exact test. This analysis revealed a highly significant positive correlation of *VEGF-B* with the set of mitochondrial genes ($p = 1.9\text{e-}50$), while there was the expected Gaussian distribution of correlations when compared with all genes. The sample plot of the correlation of *GDNF* (B) with mitochondrial genes revealed 15 genes with a cc of > 0.5 . In this case the analysis revealed a significant positive correlation of *GDNF* with the set of mitochondrial genes ($p = 0.018$), while there was the expected Gaussian distribution of correlations when compared with all genes.

Figure 8. Expression of VEGFR-1 in SH-SY5Y cells

(A) Phase contrast and (B) Fluorescence photomicrographs showing expression of VEGFR-1 in all SH-SY5Y cells in the same field of view. Immunocytochemical staining was done using an anti-human VEGFR-1 antibody and visualization with AlexaFluor 555. Scale bar corresponds to 10 μ m.

Figure 9. Semi-quantitative western analysis of FATP1 and 4 expression in SH-SY5Y cells
 Example western blots for FATP1 (A) and FATP4 (C) and the internal control β -actin at 30 min, 1 and 6 hr (upper panels), and 12, 24 and 48 hr (lower panels) time points. The summary graphs depict the mean relative density of the bands normalized to percent of control level ($\pm$ S.E.M) for FATP1 (B) and FATP4 (D). Rotenone (50 nM) treatment significantly decreased FATP1 level at the 1, 6, 24 and 48 hr time points and decreased FATP4 levels at the 1, 6, 12, 24 and 48 hr time points. Treatment with VEGF-B (20 ng/mL) one hour prior to rotenone (50 nM) administration significantly reversed this rotenone-induced reduction of FATP1 levels at the 6, 24 and 48 hr time points and significantly reversed this rotenone-induced reduction of FATP4 levels at the 12 and 24 hr time points. The statistical differences among experimental conditions within each time point are

indicated by asterisks (* $p < 0.05$, ** $p < 0.01$; one-way ANOVA before normalization to control; Tukey post hoc test; $n=3$).

Figure 10. Semi-quantitative western analysis of total Akt and phospho-Akt protein expression
 Example western blots for total Akt and the internal control β -actin (A), phospho-Akt (Ser473) (C) and phospho-Akt (Thr308) (E) at 30 min, 1 and 6 hr (upper panels), and 12, 24 and 48 hr (lower panels) time points post-rottenone. The summary graphs for total Akt (B), phospho-Akt (Ser473) (D) and phospho-Akt (Thr308) (F) depict the mean relative density of the bands normalized to percent of control level ($\pm$ S.E.M). Rotenone (50 nM) treatment decreased total Akt protein level at the later time points. Treatment with VEGF-B (20 ng/mL) one hour prior to rotenone (50 nM) administration significantly reversed rotenone-induced reduction of total Akt protein at the 24 and 48 hr time points. Rotenone (50 nM) treatment also significantly decreased both phospho-Akt (Ser473) and phospho-Akt (Thr308) protein level at the 1 hr time point. Treatment with VEGF-B (20 ng/mL) one hour prior to rotenone (50 nM) administration significantly reversed this rotenone-induced reduction of both phospho-Akt (Ser473) and phospho-Akt (Thr308) protein levels at the 1 hr time point. The statistical differences among experimental conditions within each time point are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$; one-way ANOVA before normalization to control; Tukey post hoc test; $n=3$).

Figure 11. VEGF-B is expressed in the human SNpc

Representative photomicrographs of immunohistochemical detection of VEGF-B in postmortem human brain slices from a Parkinson's disease patient (right panel) and an age matched healthy individual (left panel) verifying expression of VEGF-B (black precipitate; arrows) in neuronal shaped cells also showing neuromelanin expression (brown; arrow heads) in the human SNpc. Scale bars correspond to 20 μ m.

Table 1
Semi-quantitative analysis of neuronal VEGF-B staining in the human SNpc:

<i>probe-ID</i>	<i>control neuronal</i>	<i>PD neuronal</i>	<i>probe-ID</i>	<i>antibody signal</i>
C3	+++	+++	P1	+
C4	+++	+++	P3	++
C5	++	+++	P4	+++
		++	P5	