Published in final edited form as:

Neuropeptides. 2013 February ; 47(1): 43–49. doi:10.1016/j.npep.2012.08.004.

A synthetic five amino acid propeptide increases dopamine neuron differentiation and neurochemical function

OM Littrell, Ph.D.¹, JL Fuqua, Ph.D.¹, AD Richardson, Ph.D.¹, J. Turchan-Cholewo, Ph.D.¹, ER Hascup, Ph.D.¹, P Huettl, M.S.¹, F Pomerleau, M.Sc.¹, LH Bradley, Ph.D.^{1,2}, DM Gash, Ph.D.¹, and GA Gerhardt, Ph.D.^{1,*}

¹Department of Anatomy & Neurobiology and the Morris K. Udall Parkinson's Disease Research Center of Excellence, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY, 40536

²Department of Molecular & Cellular Biochemistry and the Center of Structural Biology, University of Kentucky College of Medicine, 800 Rose Street, Lexington, KY, 40536

Abstract

A major consequence of Parkinson's disease (PD) involves the loss of dopaminergic neurons in the substantia nigra (SN) and a subsequent loss of dopamine (DA) in the striatum. We have shown that glial cell line-derived neurotrophic factor (GDNF) shows robust restorative and protective effects for DA neurons in rats, non-human primates and possibly in humans. Despite GDNF's therapeutic potential, its clinical value has been questioned due to its limited diffusion to target areas from its large size and chemical structure. Several comparatively smaller peptides are thought to be generated from the prosequence. A five amino-acid peptide, dopamine neuron stimulating peptide-5 (DNSP-5), has been proposed to demonstrate biological activity relevant to neurodegenerative disease. We tested the *in vitro* effects of DNSP-5 in primary dopaminergic neurons dissected from the ventral mesencephalon of E14 Sprague Dawley rat fetuses. Cells were treated with several doses (0.03, 0.1, 1.0, 10.0 ng/mL) of GDNF, DNSP-5, or an equivalent volume of citrate buffer (vehicle). Morphological features of tyrosine hydroxylase positive neurons were quantified for each dose. DNSP-5 significantly increased ($p < 0.001$) all differentiation parameters compared to citrate vehicle (at one or more dose). For *in vivo* studies, a unilateral DNSP-5 treatment (30 μ g) was administered directly to the SN. Microdialysis in the ipsilateral striatum was performed 28 days after treatment to determine extracellular levels of DA and its primary metabolites (3,4-dihydroxyphenylacetic acid and homovanillic acid). A single treatment significantly increased (~66%) extracellular DA levels compared to vehicle, while DA metabolites were unchanged. Finally, the protective effects of DNSP-5 against staurosporine-induced cytotoxicity were investigated in a neuronal cell line showing substantial protection by DNSP-5. Altogether, these studies strongly indicate biological activity of DNSP-5 and suggest that DNSP-5 has neurotrophic-like properties that may be relevant to the treatment of neurodegenerative diseases like PD.

© 2012 Elsevier Ltd. All rights reserved.

*Corresponding author contact: Dr. Greg A. Gerhardt, 306 Davis Mills Bldg., 800 Rose Street, Lexington, KY 40536-0098, (gregg@uky.edu), Phone: 1+ 859.323.4531, Fax: 1+ 859.257.5310.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict of interest statement

DMG, GAG, and LHB declare that 3 patents are pending regarding the reported findings.

Keywords

dopamine; glial cell line-derived neurotrophic factor; Parkinson's disease; striatum

Introduction

Parkinson's disease (PD) is characterized by the loss of dopamine (DA) neurons in the substantia nigra (SN) and concomitant decreases in synaptic DA in the striatum (Bernheimer, et al., 1973). Current therapies are designed to increase DA levels by enhancing DA synthesis and/or decreasing DA metabolism (Goudreau, 2006; Salawu, et al., 2010) and can restore motor deficits associated with nigrostriatal dysfunction (Salawu, et al., 2010). However, current therapies remain only symptomatic treatment regimens that fail to slow the progression of the disease and may cause unfavorable side effects (Muller and Russ, 2006; Salawu, et al., 2010). One promising PD therapeutic agent, glial cell line-derived neurotrophic factor (GDNF), has shown robust restorative and protective effects on DA neurons (Grondin and Gash, 1998; Hebert and Gerhardt, 1997; Kearns, et al., 1997; Lin, et al., 1993). Despite evidence for therapeutic potential demonstrated in phase I clinical trials (Gill, et al., 2003; Slevin, et al., 2005), the clinical utility of GDNF has been questioned due to a failed phase II trial, evidence of potential toxicity (Lang, et al., 2005), and limited diffusion of GDNF to target areas (Salvatore, et al., 2006; Sherer, et al., 2006).

The proregion of GDNF is predicted to yield small amidated peptides that have been investigated by our group and others (Bradley, et al., 2010; Immonen, et al., 2008; Kelps, et al., 2011). Recently, an 11 amino acid sequence derived from the rat (Immonen, et al., 2008) and human (Bradley, et al., 2010; Kelps, et al., 2011) prosequences of GDNF was associated with biological effects including: 1) enhanced survival and morphological complexity of primary DA neuron cell cultures 2) protection from cytotoxicity of 6-hydroxydopamine, staurosporine, and gramicidin 3) increased resting extracellular levels of DA *in vivo* (Bradley, et al., 2010) and 4) increased excitability of rat CA1 pyramidal neurons (Immonen, et al., 2008). Here we investigated the neurotrophic-like effects of a different propeptide, distinct from the DNSP-11 sequence, dopamine neuron stimulating peptide-5 (DNSP-5). The 5 amino acid sequence is highly conserved among related species [Phe-Pro-Leu-Pro-Ala (human, mouse, rat)] and is predicted to be generated from the proteolytic processing of GDNF (Bradley, et al., 2010; Immonen, et al., 2008; Kelps, et al., 2011). The effects of DNSP-5 in dopaminergic systems were investigated *in vitro* using primary DA neurons dissected from the ventral mesencephalon on E14 rat embryos. Differentiation parameters were quantified and compared to GDNF and vehicle treated cultures. Functional neurochemical enhancement of the nigrostriatal pathway was also assessed in animals treated with a single dose of DNSP-5 to the SN. After treatment, extracellular DA and DA metabolites [(3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)], were quantified in the striatum using *in vivo* microdialysis and high performance liquid chromatography with electrochemical detection (HPLC-EC). Finally, the protective effects of DNSP-5 from staurosporine were evaluated in the PC12 neuronal cell line. These studies support biological activity of DNSP-5 that we believe is relevant to the treatment of neurodegenerative diseases like PD.

Materials and Methods

Ethics Statement

Animal procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee and were in strict agreement with AAALACI guidelines.

Materials and Reagents

Cell culture materials and reagents were obtained from Invitrogen (Carlsbad, CA) and Sigma-Aldrich Co. (St. Louis, MO). Reagents for microdialysis and HPLC-EC were obtained from Fisher Scientific (Fisher Chemical Fairlawn, NJ) and Sigma-Aldrich (St. Louis, MO), respectively.

All animals used were obtained from Harlan Laboratories Inc. (Indianapolis, IN).

Reagents

DNSP-5 (sequence: FPLPA-amide) was synthesized and purified to 98% purity by AC Scientific (Duluth, GA) and W.M. Keck Foundation Biotechnology Resource Laboratory at Yale University New Haven, CT. GDNF was obtained from Peprotech Inc (Rocky Hill, NJ).

DNSP-5 treatment of Mesencephalic Cells

Mesencephalic cells were dissected from the ventral mesencephalon of E14 fetuses of timed pregnant SD rats as previously described (Bradley, et al., 2010). Briefly, mesencephalic tissues were collected in cold Neurobasal™ medium and rinsed twice with cold PBS. The cells were chemically dissociated with TrypLE® (~20 minutes, 37°C) and fetal bovine serum and Hank's balanced salt solution (HBSS, Ca²⁺ or Mg²⁺ to inactivate the trypsin) was added. Mechanical dissociation was accomplished by trituration using a series of pipette tips of decreasing diameter to yield a single cell suspension. The cell suspension was centrifuged at 169 g and the pellet was re-suspended in Dulbecco's Modified Eagle Medium (DMEM) with F12 supplement (DMEM/F12). Cells were plated (4000 cells/μL) on poly-D-lysine coated plates. Neurobasal™ media (2 mM glutamine, 100 units penicillin/streptomycin) was added to the wells after adhesion. Each media change (on the initial plating day and day 2) contained GDNF or DNSP-5 (0.03, 0.1, 1.0 and 10 ng/mL) in citrate buffer, or vehicle (citrate buffer only; added following media additions). Cell cultures were stained for tyrosine hydroxylase (TH) immunoreactivity using methods modified from those previously published (Xing, et al., 2007). For morphology, five fields (minimum 15 cells/field; 2–6 independent experiments) were photographed at 20× magnification using an IX71 Olympus inverted microscope (Olympus America, Center Valley, PA) and Image-pro plus v6.2 software (Media Cybernetics, Bethesda, MD). Primary branches and total branches (number of primary branches and collateral branches) of TH-positive neurons were counted by hand and the combined neurite length was quantified using a Bioquant Image Analysis System (R&M Biometrics).

Infusion Delivery of DNSP-5 or Vehicle

Fischer 344 rats were treated, unilaterally, with 5 μL of DNSP-5 (6 μg/μL) or vehicle (5 μL) under isoflurane anesthesia as previously described (Bradley, et al., 2010). Briefly, DNSP-5 (30 μg) or vehicle (equivalent volume) was delivered to the right SN via two injection sites (0.25 μL/min over 10 minutes per site) using a KD Scientific model 100 infusion pump (KD Scientific Inc., Holliston, MA) (Hebert, et al., 1996). Stereotaxic coordinates were (relative to bregma, mm): Point 1: (AP): –5.6; (ML): –1.7; (DV): –7.3 and Point 2: AP: –5.6; ML: –2.5; DV: –6.8 (Paxinos and Watson, 2005).

Reverse Microdialysis

CMA 11 microdialysis probes (4 mm, membrane length) were used for the reverse microdialysis studies as described (Bradley, et al., 2010). Before *in vivo* use, probes were submerged in a standard solution of analytes of interest (DA, DOPAC, HVA) and one sample was collected using the same collection interval and flow rate as *in vivo* collection methods (1 μL/min, 20-minute intervals). The sample analyte concentration was determined

and divided by the standard concentration to calculate the percent recovery for each probe. Probes were stereotactically placed in the right striatum ((mm) from bregma: AP: +1.5; ML: -2.3; DV: -8.0) (Paxinos and Watson, 2005). Three microdialysis aCSF solutions were used for reverse microdialysis: (1) aCSF (124 mM NaCl, 3 mM KCl, 1 mM $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 1 mM $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 1 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 25 mM NaHCO_3 , 5.9 mM d-glucose) (2) high-potassium aCSF (27 mM NaCl, 100 mM KCl, 1 mM $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 1 mM $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 1 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 25 mM NaHCO_3 , 5.9 mM d-glucose) and (3) d-amphetamine aCSF (250 μM d-amp, 124 mM NaCl, 3 mM KCl, 1 mM $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 1 mM $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, 1 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 25 mM NaHCO_3 , 5.9 mM d-glucose) (adjusted to pH range 7.2–7.4). Dialysate samples were analyzed by HPLC-EC for DA and its primary metabolites, DOPAC and HVA. Basal levels were determined after reaching a stable baseline during perfusion of isotonic (1) aCSF. Three samples were used to calculate basal levels of DA and metabolites ($n=5$ animals/group). Evoked DA levels using high-potassium aCSF or d-amphetamine aCSF were determined from the appropriate sample during each stimulation. Due to probe variability, concentration values obtained from *in vivo* samples were normalized using the percent recovery for individual microdialysis probes.

Analysis of Dialysates (HPLC-EC)

HPLC-EC was used for the analysis of DA and its primary metabolites, DOPAC and HVA in dialysate samples. The protocol used was modified from previously published methods (Hall, et al., 1989). A citrate-acetate buffer mobile phase (4% methanol, 0.34 mM 1-octanesulfonic acid, pH = 4.1) was used and analyte separation was accomplished using a C18 column (4.6 mm $\times$ 75 mm, 3 μm particle size, Shiseido CapCell Pak UG120, Shiseido Co., LTD., Tokyo, Japan). Electrochemical detection was carried out using dual-channel coulometric detectors (ESA model 5014B dual analytical cell; E1 = +300 mV; E2 = -250 mV; pre-conditioning cell: +100 mV). Analytes of interest, DA, DOPAC, and HVA, were identified based on retention times of standards and dialysate concentrations were quantified by peak area based on standard concentrations.

JC-1 Mitochondrial Membrane Potential Assay

After treatment with DNSP-5 and staurosporine, PC12 cells were incubated for 30 minutes at 37°C in a 5% CO_2 incubator in the presence of 10 μM of the green fluorescent JC-1 [5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodine, T-3168 (Invitrogen)] and washed in Locke's solution. Optical measurements were acquired with excitation at 485 nm and emission at 527 nm and 590 nm. The levels of fluorescence at each emission wavelengths were quantified and ratio of measurements was assessed. Pertinent data were expressed as percent of the mean control values $\pm$ S.E.M. for mean optical measurements.

Statistics

Mesencephalic cell culture data were analyzed by a one-way ANOVA with Bonferroni's post-hoc tests to compare each treatment dose (GDNF or DNSP-5) to vehicle (citrate buffer vehicle) treated cultures. In addition, individual t-tests (unpaired) were performed for all differentiation parameters and all doses to compare GDNF and DNSP-5. Three samples from baseline microdialysis measurements were averaged for each subject to determine basal neurochemical levels. One value for each stimulation per subject was used for the DA release studies (potassium and d-amphetamine). Because the direction of change (positive) after DNSP-5 treatment was predicted (based on prior studies with GDNF and propeptide treatment (Bradley, et al., 2010; Hebert, et al., 1996)), a one-tailed unpaired t test was used to compare treatment groups. A simple linear correlation (Pearson r) was performed to determine the relationship between basal and evoked DA levels. Turnover ratios were calculated for baseline microdialysis levels of DA and DA-metabolites and treatment groups

were compared using a two-tailed unpaired t test. In the JC-1 assay, pertinent data were analyzed using a one-way ANOVA.

Significance was defined as $p < 0.05$ for all analyses.

Results

DNSP-5 is one of the predicted peptide products generated from post-translational processing of GDNF (Bradley, et al., 2010; Immonen, et al., 2008). The high conservation of the DNSP-5 sequences among related species suggests biological importance. Indeed, biological activity of DNSP-5 is indicated by these studies. Several differentiation parameters of both GDNF and DNSP-5 treated cultures were compared to citrate buffer vehicle (mean values $\pm$ SEM; Figure 1 A–C) and showed significant increases in the number of primary branches (Figure 1A). The lowest tested dose (0.03 ng/mL) was sufficient for GDNF to increase the number of primary branches (26% increase, $^{**}p < 0.01$), while a higher (1.0 ng/mL) dose was required of DNSP-5 to produce a significant increase in the number of primary branches (27% increase, $^{***}p < 0.001$). At the 1.0 ng/mL dose, there was a significant difference between GDNF and DNSP-5 treatment ($^{*}p = 0.0478$, $t_{176} = 1.993$). Both total number of branches and combined neurite length were increased at nearly all doses of both GDNF and DNSP-5 (Figure 1B, C, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). GDNF treatment increased the number of total branches when compared to citrate vehicle (45% increase, mean increase of all doses). DNSP-5 also increased total number of branches (42% increase, mean increase of all doses). Combined neurite length was increased in both GDNF (38% increase, mean increase of all doses) and DNSP-5 (32%, mean increase of all doses) treated cell cultures. At the 0.03 ng/mL dose, there was a significant difference between GDNF and DNSP-5 treatment ($p = 0.0049$, $t_{189} = 2.609$). Photographs of vehicle, GDNF and DNSP-5 treated cell cultures depict increased cell differentiation by GDNF and DNSP-5 (Figure 1D). Other indicators of neurotrophic effects, cell survival and soma size, were unchanged following DNSP-5 treatment ($p > 0.05$, data not shown).

Both GDNF and a previously studied GDNF propeptide, DNSP-11, have demonstrated robust enhancing effects on nigrostriatal DA neuron function in numerous animal models after only one injection (Bradley, et al., 2010; Grondin, et al., 2003; Hebert and Gerhardt, 1997; Hebert, et al., 1996). The optimal dose for DNSP-5 was chosen based on previous *in vivo* studies using the propeptide DNSP-11 (Bradley, et al., 2010). Functional changes in DA neurochemical levels in the nigrostriatal pathway of the rat were investigated after a single unilateral dose (30 μ g) of DNSP-5 was administered to the right SN. *In vivo* microdialysis studies were performed 28 days later in the striatum. Microdialysis samples were collected at 20-minute intervals to determine extracellular levels of DA and its metabolites (Figure 2A). Baseline DA (Figure 2B) and metabolite (DOPAC and HVA, Figures 2C and 2D, respectively) levels were quantified (shown as mean $\pm$ SEM; $n = 5$). Dopamine levels were significantly increased ($^{*}p < 0.05$) (66% increase) compared to vehicle treated animals. Both baseline DOPAC and HVA metabolite levels were unchanged compared to vehicle ($p > 0.05$). Because baseline DA levels were significantly changed by DNSP-5 and metabolite levels were unchanged, turnover ratios were calculated and analyzed. Dopamine turnover ratios for combined metabolites [DA:(DOPAC+HVA)] were increased by DNSP-5 treatment ($^{*}p = 0.0352$, $t_8 = 2.087$) [Vehicle: 4.6 ± 0.31 , DNSP-5: 6.5 ± 0.85 (ratio $\times 10^3$)]. In addition, turnover calculated with respect to DOPAC (DA:DOPAC) was significantly different ($^{**}p = 0.0048$, $t_8 = 3.383$) [Vehicle: 7.9 ± 0.48 , DNSP-5: 10.1 ± 0.45 (ratio $\times 10^3$)]. Turnover ratios for HVA (DA:HVA) were not different ($p > 0.05$) between treatment groups. Evoked release of DA was determined by reverse microdialysis with high potassium or d-amphetamine aCSF (Figure 2A). Neither evoked release of DA by potassium (Figure 2E) nor d-amphetamine (Figure 2F) was significantly different between DNSP-5 and

vehicle treatments ($p > 0.05$, $n = 5$). Neither evoked release of DA by potassium nor d-amphetamine stimulation was correlated with basal DA levels ($p > 0.05$, all).

Previously, DNSP-11 was shown to provide significant protection from staurosporine – a non-selective protein kinase inhibitor that induces apoptosis and loss of mitochondrial potential (Bradley, et al., 2010). To extend this line of investigation using DNSP-5, we examined the protective effects from staurosporine-induced loss of mitochondrial potential at an equal molar concentration in PC-12 neuronal cells. Following 6 hours of 1 μ M staurosporine exposure, the mitochondrial potential was significantly decreased by approximately 30% as measured by JC-1 assay (Figure 3), consistent with a staurosporine-induced increase of mitochondrial permeability and resultant loss of cytochrome c observed previously (Bradley, et al., 2010). At 10 nM, DNSP-5 provided significant protection of mitochondrial potential from staurosporine in PC12 neuronal cells, with the determined mitochondrial potentials nearly 90% of cells treated with citrate buffer alone (Figure 3).

Discussion

The present studies show that a proGDNF peptide, DNSP-5, enhances differentiation of primary DA neurons at several doses. Although there are indications of a dose-response with DNSP-5 treatment within certain morphological parameters, there is not a clear upper or lower dose limit for cell morphological enhancement in general. Only one DNSP-5 treatment dose resulted in significant increases in primary number of branches and the increase in combined neurite length with DNSP-5 treatment was nearly uniform at all doses – suggesting a larger dose range may be necessary. The effect of DNSP-5 on total branches is convincing and suggests a dose-response relationship. Collectively, the differential effects of equal doses of DNSP-5 on the three morphological parameters are interesting and worth further examination. By definition, neurotrophic factors regulate cell differentiation and proliferation – likely reflecting their prominent role in development (Granhölm, et al., 2000; Stromberg, et al., 1993). Enhanced morphological complexity of primary dopaminergic neurons is apparent in previous studies using GDNF (Schaller, et al., 2005; Widmer, et al., 2000). An important finding of the current studies is that DNSP-5 also enhanced differentiation parameters in dopaminergic neurons, supporting transforming growth factor-like properties. DNSP-5 may increase the number of axonal varicosities and functional DA terminals, producing increased sites for DA synthesis and neurotransmission. Thus, the increased morphological complexity seen *in vitro* is a possible explanation for the observed increase in extracellular basal DA *in vivo* 24 days after a single injection. Additionally, TH activity is enhanced by GDNF (Lin, et al., 1993; Salvatore, et al., 2004), a possible mechanistic explanation for the *in vivo* neurochemical findings seen after DNSP-5 treatment. Ongoing studies are focused on these possibilities and determination of the source of extracellular DA i.e. are there changes in DA synthesis and storage or evidence for an independent presynaptic mechanism.

The effects of DNSP-5 on DA neurons could be of particular importance in neurodegenerative diseases like PD where neuronal plasticity phenomena are implicated (Stephens, et al., 2005). Compensatory changes in PD are supported by evidence that striatal DA loss must be severe (>80% loss) before behavioral deficits are apparent (Hornykiewicz, 1993) and the nigrostriatal pathway has shown significant compensatory ability following injury in animal models (Castaneda, et al., 1990). Thus, utilizing the compensatory ability of dopaminergic neurons with neurotrophic factors is a promising alternative or adjunct therapeutic strategy to current treatments. Current PD therapies function mainly by: increasing DA levels through synthesis/metabolism modulation or receptor activation with selective agonists (Djaldetti and Melamed, 2002). Improved therapies are needed (Ravina, et

al., 2003) because current drug regimens provide only temporary symptomatic relief (Goudreau, 2006) and do not slow disease progression (Salawu, et al., 2010).

The increase in extracellular DA is a substantial finding that supports *in vivo* functional changes in DA neurons. We believe this finding is particularly relevant to PD because DA levels are markedly reduced in PD (Bernheimer, et al., 1973). Conceivably an increase in basal extracellular DA levels may result in decreased DA-release through negative feedback pathways e.g. DA-autoreceptor and or DA-transporter modulation (Cass and Gerhardt, 1994). This does not appear to be the case in these studies. Dopamine release was not correlated with basal DA levels in any treatment group (both d-amphetamine and potassium). Increased extracellular DA seen after DNSP-5 treatment contrasts with the effect of GDNF, which does not affect resting DA levels (Hebert, et al., 1996). Furthermore, the effects of GDNF treatment include increased metabolite levels (DOPAC and HVA) and evoked DA release (Hebert, et al., 1996) - effects that are not reproduced in the current DNSP-5 treatment studies. Indeed, additional calculations reflect increased turnover ratios [DA:DOPAC and DA:(DOPAC +HVA)] in DNSP-5 treated animals. Although some interpretations may propose this supports decreased DA turnover (decreased metabolism), this finding is likely due to the increase in basal DA levels without a corresponding increase in metabolites in DNSP-5 treated animals. Turnover ratios using HVA (DA:HVA) were unchanged between treatment groups. It is relevant to clarify that although HVA is the primary metabolite in primates (Sharman, et al., 1967; Sternberg, et al., 1983), DOPAC is the primary DA metabolite in the rodent CNS (Dedek, et al., 1979; Kopin, 1985; Kopin, 1994).

These functional differences between DNSP-5 and GDNF suggest different underlying cellular mechanisms. Cytosolic binding partners of the related propeptide, DNSP-11, indicate metabolic and/or apoptotic cellular pathways are possible players in the apparent functional changes in DA neurons (Bradley, et al., 2010). DNSP-11 does not appear to interact with the GDNF receptor - GDNF family receptor alpha-1 (GFR α 1) (Bradley, et al., 2010; Sariola and Saarma, 2003). We predict that DNSP-5 may participate in similar pathways as DNSP-11, which are also independent of GFR α 1. Both DNSP-5 and DNSP-11 provided preservation of mitochondrial potentials and increased caspase-3 activation in dopaminergic cell lines. These cellular effects support a non-GDNF neuroprotective signaling mechanism (Figure 3) (Bradley, et al., 2010). Although DNSP-11 provided protection from staurosporine and 3-nitropropionate in non-neuronal HEK-293 cells (Kelps, et al., 2011), DNSP-5 did not provide comparable protection. Thus, the protective effects of DNSP-5 may be more selective for neurons.

Conclusions

Collectively, these preliminary studies support biological activity of DNSP-5 relevant to neurodegenerative diseases. Neurotrophic factors have long been regarded as promising therapeutic agents for the treatment of PD (Peterson and Nutt, 2008). In particular, GDNF has shown substantial promise in numerous animal models (Ai, et al., 2003; Gash, et al., 1998; Hebert and Gerhardt, 1997; Kirik, et al., 2001; Lapchak, et al., 1997; Winkler, et al., 1996). The current work corroborates previous work with DNSP-11 – demonstrating neurotrophic-like qualities of propeptides (Bradley, et al., 2010; Kelps, et al., 2011). Although propeptides were previously thought to function only to assist in the processing and secretion of the mature secreted neurotrophin protein (Rattenholl, et al., 2001), the presented studies support specific trophic effects on DA systems. Because the post-translational processing model of GDNF predicts DNSP-11 and DNSP-5 are generated simultaneously, DNSP-11 and DNSP-5 may act in concert — exhibiting synergistic effects along with GDNF signaling. This mechanistic speculation is currently being investigated.

Although the mechanism of action is poorly defined at this time and, certainly, additional studies are necessary to establish physiological effects of DNSP-5, these studies provide support for distinct biological activity of propeptides – an unexpected and relatively unexplored source. These studies are encouraging considering some notable advantages of small peptides as treatment strategies. The simple structure, lack of heparin binding (Kelps, et al., 2011), intrinsic *in vitro* stability, and comparatively small size of DNSP-5 may circumvent some delivery limitations associated with larger trophic factors like GDNF (Kelps, et al., 2011; Lang, et al., 2005; Salvatore, et al., 2006). In addition, the small size of DNSP-5 makes it an appropriate candidate for modification to enhance bioavailability and as well as, potentially, administration by oral or nasal delivery (Born, et al., 2002). These delivery routes are marked improvements over the invasive intracranial delivery approaches required for GDNF delivery to the brain (Bjorklund, et al., 2000; Gill, et al., 2003; Grondin, et al., 2002; Patel and Gill, 2007).

In conclusion, DNSP-5 exhibits neurotrophic-like effects on dopamine-producing neurons in primary cell cultures and function-enhancing effects *in vivo*. With favorable physical properties and physiological activities, further studies are warranted to evaluate DNSP-5 as a therapeutic agent for the treatment of neurodegenerative disorders like PD.

Acknowledgments

The authors would like to thank Lloyd Greene (Columbia University) for providing PC12 cells.

Funding Sources

GAG: NIH Training Grants: 5T32 AG000242-14, T32 DA022738 (OML/JLF); USPHS NS39787; DA017186; AG13494; NSF EEC-0310723.

DMG: T32-AG00242; PO1-13494; NINDS P50-NS039787; endowed funds

LHB: NIH COBRE Pilot P20RR20171; NINDS P50-NS039787; NINDS NS075694; PhRMA Foundation; Columbus Foundation; and University of Kentucky College of Medicine Startup Funds.

References

1. Ai Y, Markesbery W, Zhang Z, Grondin R, Elseberry D, Gerhardt GA, Gash DM. Intraputamenal infusion of GDNF in aged rhesus monkeys: distribution and dopaminergic effects. *J.Comp Neurol.* 2003; 461:250–261. [PubMed: 12724841]
2. Bernheimer H, Birkmayer W, Hornykiewicz O, Jellinger K, Seitelberger F. Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J.Neurol.Sci.* 1973; 20:415–455. [PubMed: 4272516]
3. Bjorklund A, Kirik D, Rosenblad C, Georgievska B, Lundberg C, Mandel RJ. Towards a neuroprotective gene therapy for Parkinson's disease: use of adenovirus, AAV and lentivirus vectors for gene transfer of GDNF to the nigrostriatal system in the rat Parkinson model. *Brain Res.* 2000; 886:82–98. [PubMed: 11119690]
4. Born J, Lange T, Kern W, McGregor GP, Bickel U, Fehm HL. Sniffing neuropeptides: a transnasal approach to the human brain. *Nature neuroscience.* 2002; 5:514–516.
5. Bradley LH, Fuqua J, Richardson A, Turchan-Cholewo J, Ai Y, Kelps KA, Glass JD, He X, Zhang Z, Grondin R, Littrell OM, Huettl P, Pomerleau F, Gash DM, Gerhardt GA. Dopamine neuron stimulating actions of a GDNF propeptide. *PLoS One.* 2010; 5:e9752. [PubMed: 20305789]
6. Cass WA, Gerhardt GA. Direct in vivo evidence that D2 dopamine receptors can modulate dopamine uptake. *Neurosci.Lett.* 1994; 176:259–263. [PubMed: 7830960]
7. Castaneda E, Whishaw IQ, Robinson TE. Changes in striatal dopamine neurotransmission assessed with microdialysis following recovery from a bilateral 6-OHDA lesion: variation as a function of lesion size. *J.Neurosci.* 1990; 10:1847–1854. [PubMed: 1693953]

8. Dedek J, Baumes R, Tien-Duc N, Gomeni R, Korf J. Turnover of free and conjugated (sulphonyloxy) dihydroxyphenylacetic acid and homovanillic acid in rat striatum. *Journal of neurochemistry*. 1979; 33:687–695. [PubMed: 479883]
9. Djaldetti R, Melamed E. New drugs in the future treatment of Parkinson's disease. *J.Neurol*. 2002; 249(Suppl 2):II30–II35. [PubMed: 12375061]
10. Gash DM, Gerhardt GA, Hoffer BJ. Effects of glial cell line-derived neurotrophic factor on the nigrostriatal dopamine system in rodents and nonhuman primates. *Adv.Pharmacol*. 1998; 42:911–915. [PubMed: 9328046]
11. Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson disease. *Nat Med*. 2003; 9:589–595. [PubMed: 12669033]
12. Goudreau JL. Medical management of advanced Parkinson's disease. *Clin.Geriatr.Med*. 2006; 22:753–772. v. [PubMed: 17000334]
13. Granholm AC, Reyland M, Albeck D, Sanders L, Gerhardt G, Hoernig G, Shen L, Westphal H, Hoffer B. Glial cell line-derived neurotrophic factor is essential for postnatal survival of midbrain dopamine neurons. *J.Neurosci*. 2000; 20:3182–3190. [PubMed: 10777782]
14. 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; 23:1974–1980. [PubMed: 12629203]
15. Grondin R, Gash DM. Glial cell line-derived neurotrophic factor (GDNF): a drug candidate for the treatment of Parkinson's disease. *J.Neurol*. 1998; 245:35–42.
16. Grondin R, Zhang Z, Yi A, Cass WA, Maswood N, Andersen AH, Elsberry DD, Klein MC, Gerhardt GA, Gash DM. Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys. *Brain*. 2002; 125:2191–2201. [PubMed: 12244077]
17. Hall ME, Hoffer BJ, Gerhardt GA. Rapid and Sensitive Determination of Catecholamines in Small Tissue Samples by High-Performance Liquid-Chromatography Coupled with Dual-Electrode Coulometric Electrochemical Detection. *Lc Gc-Magazine of Separation Science*. 1989; 7:258–265.
18. 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; 282:760–768. [PubMed: 9262339]
19. Hebert MA, Vanhorne CG, Hoffer BJ, Gerhardt GA. Functional effects of GDNF in normal rat striatum: Presynaptic studies using in vivo electrochemistry and microdialysis. *Journal of Pharmacology and Experimental Therapeutics*. 1996; 279:1181–1190. [PubMed: 8968339]
20. Hornykiewicz O. Parkinson's disease and the adaptive capacity of the nigrostriatal dopamine system: possible neurochemical mechanisms. *Adv Neurol*. 1993; 60:140–147. [PubMed: 8420131]
21. Immonen T, Alakuijala A, Hytonen M, Sainio K, Poteryaev D, Saarma M, Pasternack M, Sariola H. A proGDNF-related peptide BEP increases synaptic excitation in rat hippocampus. *Exp.Neurol*. 2008; 210:793–796. [PubMed: 18280470]
22. 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; 17:7111–7118. [PubMed: 9278545]
23. Kelps KA, Turchan-Cholewo J, Hascup ER, Taylor TL, Gash DM, Gerhardt GA, Bradley LH. Evaluation of the physical and in vitro protective activity of three synthetic peptides derived from the pro- and mature GDNF sequence. *Neuropeptides*. 2011; 45:213–218. [PubMed: 21507484]
24. Kirik D, Georgievskia B, Rosenblad C, Bjorklund A. Delayed infusion of GDNF promotes recovery of motor function in the partial lesion model of Parkinson's disease. *Eur.J.Neurosci*. 2001; 13:1589–1599. [PubMed: 11328352]
25. Kopin IJ. Catecholamine metabolism: basic aspects and clinical significance. *Pharmacological reviews*. 1985; 37:333–364. [PubMed: 3915090]
26. Kopin IJ. Monoamine oxidase and catecholamine metabolism. *Journal of neural transmission*. 1994; (Supplementum 41):57–67. [PubMed: 7931266]
27. Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, Brooks DJ, Hotton G, Moro E, Heywood P, Brodsky MA, Burchiel K, Kelly P, Dalvi A, Scott B, Stacy M, Turner D, Wooten VGF, Elias WJ, Laws ER, Dhawan V, Stoessl AJ, Matcham J, Coffey RJ, Traub M. Randomized controlled

- trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. *Annals of Neurology*. 2005; 59:459–466. [PubMed: 16429411]
28. Lapchak PA, Gash DM, Jiao S, Miller PJ, Hilt D. Glial cell line-derived neurotrophic factor: a novel therapeutic approach to treat motor dysfunction in Parkinson's disease. *Exp.Neurol*. 1997; 144:29–34. [PubMed: 9126148]
 29. Lin LFH, Doherty DH, Lile JD, Bektesh S, Collins F. Gdnf - A Glial-Cell Line Derived Neurotrophic Factor for Midbrain Dopaminergic-Neurons. *Science*. 1993; 260:1130–1132. [PubMed: 8493557]
 30. Muller T, Russ H. Levodopa, motor fluctuations and dyskinesia in Parkinson's disease. *Expert.Opin.Pharmacother*. 2006; 7:1715–1730. [PubMed: 16925499]
 31. Patel NK, Gill SS. GDNF delivery for Parkinson's disease. *Acta Neurochir*. 2007; (Suppl 97):135–154.
 32. Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press; 2005.
 33. Peterson AL, Nutt JG. Treatment of Parkinson's disease with trophic factors. *Neurotherapeutics*. 2008; 5:270–280. [PubMed: 18394569]
 34. Rattenholl A, Ruoppolo M, Flagiello A, Monti M, Vinci F, Marino G, Lilie H, Schwarz E, Rudolph R. Pro-sequence assisted folding and disulfide bond formation of human nerve growth factor. *J Mol Biol*. 2001; 305:523–533. [PubMed: 11152610]
 35. Ravina BM, Fagan SC, Hart RG, Hovinga CA, Murphy DD, Dawson TM, Marler JR. Neuroprotective agents for clinical trials in Parkinson's disease: a systematic assessment. *Neurology*. 2003; 60:1234–1240. [PubMed: 12707423]
 36. Salawu F, Olokoba A, Danburam A. Current management of Parkinson's disease. *Ann Afr Med*. 2010; 9:55–61. [PubMed: 20587924]
 37. Salvatore MF, Ai Y, Fischer B, Zhang AM, Grondin RC, Zhang Z, Gerhardt GA, Gash DM. Point source concentration of GDNF may explain failure of phase II clinical trial. *Exp.Neurol*. 2006; 202:497–505. [PubMed: 16962582]
 38. Salvatore MF, Zhang JL, Large DM, Wilson PE, Gash CR, Thomas TC, Haycock JW, Bing G, Stanford JA, Gash DM, Gerhardt GA. Striatal GDNF administration increases tyrosine hydroxylase phosphorylation in the rat striatum and substantia nigra. *J.Neurochem*. 2004; 90:245–254. [PubMed: 15198683]
 39. Sariola H, Saarma M. Novel functions and signalling pathways for GDNF. *J Cell Sci*. 2003; 116:3855–3862. [PubMed: 12953054]
 40. Schaller B, Andres RH, Huber AW, Meyer M, Perez-Bouza A, Ducray AD, Seiler RW, Widmer HR. Effect of GDNF on differentiation of cultured ventral mesencephalic dopaminergic and non-dopaminergic calretinin-expressing neurons. *Brain Res*. 2005; 1036:163–172. [PubMed: 15725414]
 41. Sharman DF, Poirier LJ, Murphy GF, Sourkes TL. Homovanillic acid and dihydroxyphenylacetic acid in the striatum of monkeys with brain lesions. *Canadian journal of physiology and pharmacology*. 1967; 45:57–62. [PubMed: 4961824]
 42. Sherer TB, Fiske BK, Svendsen CN, Lang AE, Langston JW. Crossroads in GDNF therapy for Parkinson's disease. *Mov Disord*. 2006; 21:136–141. [PubMed: 16470786]
 43. 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. *Journal of Neurosurgery*. 2005; 102:216–222. [PubMed: 15739547]
 44. Stephens B, Mueller AJ, Shering AF, Hood SH, Taggart P, Arbuthnott GW, Bell JE, Kilford L, Kingsbury AE, Daniel SE, Ingham CA. Evidence of a breakdown of corticostriatal connections in Parkinson's disease. *Neuroscience*. 2005; 132:741–754. [PubMed: 15837135]
 45. Sternberg DE, Heninger GR, Roth RH. Plasma homovanillic acid as an index of brain dopamine metabolism: enhancement with debrisoquin. *Life sciences*. 1983; 32:2447–2452. [PubMed: 6855448]
 46. Stromberg I, Bjorklund L, Johansson M, Tomac A, Collins F, Olson L, Hoffer B, Humpel C. Glial-Cell Line-Derived Neurotrophic Factor Is Expressed in the Developing But Not Adult Striatum

- and Stimulates Developing Dopamine Neurons In- Vivo. *Experimental Neurology*. 1993; 124:401–412. [PubMed: 7904571]
47. Widmer HR, Schaller B, Meyer M, Seiler RW. Glial cell line-derived neurotrophic factor stimulates the morphological differentiation of cultured ventral mesencephalic calbindin- and calretinin-expressing neurons. *Exp Neurol*. 2000; 164:71–81. [PubMed: 10877917]
48. Winkler C, Sauer H, Lee CS, Bjorklund A. Short-term GDNF treatment provides long-term rescue of lesioned nigral dopaminergic neurons in a rat model of Parkinson's disease. *J.Neurosci*. 1996; 16:7206–7215. [PubMed: 8929429]
49. Xing B, Liu M, Bing G. Neuroprotection with pioglitazone against LPS insult on dopaminergic neurons may be associated with its inhibition of NF-kappaB and JNK activation and suppression of COX-2 activity. *J Neuroimmunol*. 2007; 192:89–98. [PubMed: 17976742]

Figure 1. Enhanced differentiation of primary dopaminergic neurons

Mesencephalic cells were dissected from the ventral mesencephalon and treated with a range of concentrations of GDNF (gray), DNSP-5 (black), or vehicle. The mean ($\pm$ SEM) values for differentiation parameters are shown for several doses of GDNF and DNSP-5. Both GDNF and DNSP-5 showed neurotrophic or neurotrophic-like effects when compared to vehicle (citrate buffer) cultures by Bonferroni's *post-hoc* tests (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). A) The number of primary branches were increased significantly with GDNF treatment ($F_{4,452} = 4.41$, $p = 0.0017$, vehicle: $n=135$; GDNF: $n=89$ (0.03 ng/mL dose), $n=79$ (1.0 ng/mL)). DNSP-5 also increased the number of primary branches ($F_{4,515} = 4.83$, $p = 0.0008$, vehicle: $n=135$; DNSP-5: $n=99$ (1 ng/mL dose)). B) Combined neurite length was

significantly increased by all doses of GDNF ($F_{4,452} = 9.36$, $p < 0.0001$, vehicle: $n = 135$, GDNF: $n = 89$ (0.03 ng/mL), $n = 106$ (0.1 ng/mL), $n = 79$ (1.0 ng/mL), $n = 48$ (10 ng/mL) and most doses of DNSP-5 ($F_{4,515} = 6.71$, $p < 0.0001$, vehicle: $n = 135$ DNSP-5: $n = 102$ (0.03 ng/mL), $n = 111$ (0.1 ng/mL), $n = 99$ (1.0 ng/mL), $n = 73$ (10 ng/mL)). C) The total number of branches was also increased at most doses of GDNF ($F_{4,452} = 12.41$, $p < 0.0001$, vehicle: $n = 135$, GDNF: $n = 89$ (0.03 ng/mL), $n = 79$ (1.0 ng/mL), $n = 48$ (10 ng/mL)) and all doses of DNSP-5 ($F_{4,515} = 9.53$, $p < 0.0001$, vehicle: $n = 135$; DNSP-5: $n = 102$ (0.03 ng/mL), $n = 111$ (0.1 ng/mL), $n = 99$ (1.0 ng/mL), $n = 75$ (10 ng/mL)). D) Photographs demonstrate the enhancing effects of DNSP-5 that compared with GDNF.

Figure 2. Increased basal DA with DNSP-5 treatment

A) 28 days after intranigral DNSP-5 (30 μg) the ipsilateral striatum was sampled via *in vivo* microdialysis for extracellular levels of DA (shown in trace) and the primary metabolites of DA (DOPAC, HVA, not shown). Microdialysis samples were collected at 20-minute intervals with 3 samples used to calculate baseline levels (shown for DA) (A), and similarly for metabolites. High potassium (100 mM) aCSF or d-amphetamine (250 μM) aCSF was used to evoke DA release. The quantified data analyzed are shown (mean ± SEM (B–F)). B) DNSP-5 significantly increased ($t_8 = 1.974$, $*p = 0.0419$, $n = 5$) baseline DA levels compared to citrate buffer vehicle: mean (nM) ± SEM, vehicle: 26.0 ± 4.9 ; DNSP-5: $43.1 \pm$

7.1,) while metabolite levels were unchanged ($p > 0.05$). DNSP-5 treatment did not affect evoked DA release by E) potassium nor F) d-amphetamine ($p > 0.05$, both, $n = 5$)

Figure 3. Protective effect of DNSP-5 from staurosporine in the PC12 neuronal cell line DNSP-5 protection (blue bar) from 1 μ M staurosporine-induced cytotoxicity (red bar) was determined by measuring mitochondrial potentials 6 h after treatment by 10 nM DNSP-5 using a JC-1 assay. The control was citrate buffer alone. One-way ANOVA was used to test for significance among groups, followed by Tukey's post hoc analysis (*** $p < 0.001$ vs. control; # $p < 0.05$ vs. staurosporine).