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MOLECULAR AND CELLULAR CHARACTERIZATION OF THE DOPAMINE
NEURON STIMULATING PEPTIDES

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the Doctor of Philosophy in the
College of Medicine at the
University of Kentucky

By

Kristen Alice Kelps

Lexington, Kentucky

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Lexington, Kentucky

ABSTRACT OF DISSERTATION

MOLECULAR AND CELLULAR CHARACTERIZATION OF THE DOPAMINE
NEURON STIMULATING PEPTIDES

Parkinson's disease, the second most common neurodegenerative disease, is characterized by the loss of dopaminergic neurons within the substantia nigra. Currently, the treatments available for PD are symptomatic treatments that do not stop the progression of the disease. Trophic molecules, such as glial cell-line derived neurotrophic factor (GDNF), have been evaluated as potential therapeutic molecules that could stop the loss of neurons and potentially restore some of the neurons that have already been lost. However, these trophic molecules are large making them difficult to produce and delivery. Here we characterize three peptides (DNSP-5, DNSP-11, and DNSP-17) to determine it they are stable and offer protective effects similar to GNDF allowing them to be potential therapeutic molecules.

The data presented here involves the evaluation of the molecular and cellular mechanism of DNSP-5, DNSP-11, and DNSP-17, which are derived from prosequence of GDNF. Initial studies were carried out to evaluate the physical characteristics of these three peptides to determine their viability as potential therapeutic molecules. The structure and stability of these peptides were evaluated. Based on the data it was determined that the three peptides do not interact in vitro, allowing for further individual evaluations of the peptides. It was also determined that the peptides were stable when stored at both -80°C and 37°C for one month, allowing them to both potentially be stored during treatment.

Cell culture assays and proteomic profiling were utilized to determine binding partners and potential mechanisms through which DNSP-11 may be able to mediate apoptosis. It was determined that DNSP-11 was able to interact with a variety of binding partners that are involved in metabolism. These studies have aided in the understanding of neurotrophic factor prosequence function, but will also serve as a starting point for the development of novel trophic factors for PD treatment.

Finally, the interaction between DNSP-11 and GAPDH was evaluated as a potential anti-apoptotic mechanism. GAPDH has previously shown to play a role in mediating apoptotic pathways. It was hypothesized that the observed interaction between DNSP-11 and GAPDH could mediate that role of GAPDH in apoptosis and afford DNSP-11 its observed anti-apoptotic effects. It was observed that while DNSP-11's interaction with GAPDH may play a role in its anti-apoptotic effects, it does not appear to be the only mechanism involved. Based on this data, it is likely that the other metabolic binding partners play a role in DNSP-11's anti-apoptotic mechanisms and therefore, these interactions should be further evaluated.

KEYWORDS: GDNF, DNSP-11, GAPDH, Anti-apoptotic, Peptides

Kristen Kelps

Student's Signature

December 17, 2013

Date

MOLECULAR AND CELLULAR CHARACTERIZATION OF THE DOPAMINE
NEURON STIMULATING PEPTIDES

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The dissertation is dedicated to my granddad, Donald Lambert, for constantly

stressing the importance of education.

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Chapter One: Introduction

I. Overview

This chapter provides background information on Parkinson's disease (PD) including its history, pathophysiology, and etiology. This includes information about the genetic and environment influences on PD. Details about the current treatment for PD are also presented here. These current therapies function to treat the symptoms of PD, but do not stop the loss of dopaminergic neurons associated with PD. Investigations of neurotrophic factors, such as glial cell-line derived neurotrophic factor (GDNF), for the treatment of PD are also discussed here including the observed challenges associated with large molecule therapies. Due to these challenges, we have introduced a group of three small peptides derived from the pro- and mature sequence of GDNF as potential therapeutic molecules. The chemical characteristics of these peptides, as well as their protective effects, are evaluated and discussed here.

II. Introduction to Parkinson's disease

a. Background/History

Though symptoms of PD have been observed and recorded in writings and artwork since ancient times, such as in AD 175 when Galan described what he referred to as shaking palsy in his writings, it wasn't until 1817 when James Parkinson published "An Essay on the Shaking Palsy" (Parkinson, 2002) that Parkinson's Disease was first recognized as a medical condition (Figure 1.1).

The essay was based on six patients that Parkinson had observed in his practice and on the streets of the surrounding area. Sixty years after the publication of Parkinson's essay, Jean Martin Charcot began studying PD, recognized the importance of James Parkinson's work and named the condition after him, but it was not until the 1960s that the pathology of PD became well understood.

PD is associated with a characteristic set of motor symptoms. In order for PD to be diagnosed, the patient must have bradykinesia coupled with at least one of four other symptoms that include resting tremor, gait difficulty, postural instability, and rigidity (Hardy *et al.*, 2006). PD was initially classified as a motor disorder; however, it is becoming clear that a number of non-motor symptoms accompany PD and that there are a variety of autonomic symptoms associated with the disease, the most common of which is constipation. As the disease progresses, it is common for symptoms such as anxiety, depression, and eventually dementia to develop.

Currently affecting approximately one and a half million Americans with sixty thousand new cases diagnosed each year (Parkinson's Disease Foundation, 2013), Parkinson's disease is the second most common neurodegenerative disease. These numbers represent a significant financial impact on the patients and their families as well as on society as a whole. Currently, PD costs America twenty-five billion dollars each year (Parkinson's Disease Foundation, 2013). This accounts for costs associated with treatment and medical care and social security payments, as well as the lost productivity, lost wages of the patients suffering from PD, and the cost to caregivers. The

individual cost of treatment for PD is also significant. Medications for treatment

of the disease cost approximately twenty-five hundred dollars each year with the

additional costs of up to one hundred thousand dollars for surgical therapeutic

options (Parkinson's Disease Foundation, 2013).

b. Pathophysiology

i. Loss of dopaminergic neurons

PD pathology is characterized by a loss of dopaminergic neurons in the

basal ganglia, specifically the substantia nigra pars compacta region of the

midbrain. The neurons within the substantia nigra pars compacta communicate

with the striatum also found in the basal ganglia. The loss of dopaminergic

neurons in the substantia nigra results in an overall reduction in the amount of

dopamine that is released in the striatum (Figure 1.2). As a result, a lack of

communication is caused between the basal ganglia and the motor cortex, which

leads to a loss of motor control.

ii. Lewy body formation

Another characteristic of PD is the presence of Lewy bodies in the brain.

Lewy bodies are intracellular inclusions containing α-synuclein. While α-

synuclein's physiological function is currently unknown, in pathological

conditions, α-synuclein has been observed as a major component of intracellular

inclusion bodies common in several neurodegenerative diseases (Alim *et al.*,

2004). They are toxic to dopaminergic neurons and have been widely associated

with the death of dopaminergic neurons that is observed in PD. Though there is a loss of dopaminergic neurons in the substantia nigra, Lewy body formation does not appear initially in this area of the brain. Braak staging of PD describes how Lewy bodies first appear in regions of the brain other than the substantia nigra, such as the olfactory bulb, and then progress through the brain and eventually reach the substantia nigra at which point motor symptoms become apparent (Braak *et al., 2002; 2003*).

Lewy body formation is a hallmark of all PD cases. However, in cases of parkinsonism, a condition characterized by motor symptoms similar to PD, Lewy body formation is not necessarily observed but, when present, they are primarily found only within the substantia nigra rather than throughout the brain as they are in PD. Similarly to PD, however, parkinsonism symptoms can also be treated by the replacement of dopamine in the brain (Hardy *et al*., 2006).

iii. Other deficits

While the loss of dopaminergic neurons is primarily responsible for the symptoms of PD, there is widespread neuron loss in the central nervous system, including the locus ceruleus (LC) (Greenfield and Bosanquet, 1953; Chan-Palay and Asan, 1989; Forno, 1996) and the nucleus basalis of Meynert (NBM) (Arendt *et al.,* 1983; Gaspar and Gray, 1984; Nakano and Hirano, 1984). Data from recent studies looking at genetic and environmental causes of PD have revealed the role of mitochondrial dysfunction in the disease (Greenamyre et al., 2001; Dawson and Dawson, 2003; Castello et al., 2007; Mortiboys et al., 2007; Gash *et*

al., 2008; Bueler, 2009). Additionally, it has been observed that sporadic cases of PD are associated with a decrease in mitochondrial complex I activity.

c. Etiology

      PD currently affects one and a half million people in the United States with 50-60,000 new cases diagnosed each year (parkinson.org). Age is the largest risk factor for PD with the onset of idiopathic PD occurring between 55 and 80 years old (Tanner, 1992). PD is most commonly thought to be the result of a combination of factors, including genetic and environmental influences (Thomas, 2009). It currently affects 1% of the over 65 population (Tanner, 1992). While familial forms of PD are less common and only account for 5-10% of all cases, the onset of disease with familial PD is earlier, usually less than 45 years of age (Dawson and Dawson, 2003; Bueler, 2009). Despite all of the work that has been done in the field, the etiology is still not well understood.

i. Genetics

      There is evidence from epidemiologic studies that PD has a genetic component. One study showed that residents in the general population in New Jersey had a 2% chance of having PD at the age of eighty (Lazzarini *et al.,* 1994). However, if the patient had a parent or sibling with PD, their chance of getting PD increased to 5-6%, and if the patient had a parent and sibling suffering from PD then their probability of having PD increased to between 20-40% (Lazzarini *et al.,* 1994). Even though PD has been shown to have a

genetic link, the majority of PD cases are sporadic with less than 10% of the cases resulting from strictly genetic causes (Thomas and Beal, 2007; Bueler, 2009). One distinction between the two forms of PD is that familial PD cases typically have an earlier onset of symptoms than sporadic cases.

Familial PD has been shown to result from mutations in six genes, including α-synuclein *(SNCA), leucine-rich repeat kinase 2 (LRRK2), parkin (PRKN), DJ1, PTEN-induced putative kinas (PINK1),* and *ATPase type 13A2 (ATP13A2)* (Table 1.1) (Bekris *et al.*, 2010). The genes associated with PD exhibit both autosomal dominant inheritance (*SCNA, LRRK2)* and autosomal recessive inheritance (*PARK2, DJ1, PINK1, ATP13A2*). Variations in additional genes, such as *MAPT, LRRK2,* and *GBA*, have been shown to be risk factors and increase susceptibility for PD (Bekris *et al.*, 2010). While all of these mutations result in some form of PD, there are differences between the resulting disease characteristics depending on the gene mutation that is responsible.

Increased levels of expression of *SNCA* (PARK$_1$/PARK$_4$) have been observed in presynaptic nerve terminals all through the mammalian brain (George, 2002). The protein is typically found in an unfolded form that can take on both monomeric and oligomeric conformations (Uversky, 2003) and is associated with Lewy bodies (Spillantini *et al.,* 1997). Mutations in *SNCA* (autosomal dominant) are often associated with an increase in self-aggregation and oligomerization of the protein (Conway *et al.,* 1998; Pandey *et al.,* 2006) and the resulting form of PD that occurs typically has an early onset and progresses

quickly (Bekris *et al.*, 2010). This form of PD is also accompanied by a few

atypical symptoms, including myclonus and hypoventilation (Bekris *et al.*, 2010).

  LRRK2 is expressed in numerous body regions, including the central

nervous system (CNS), heart, kidney, lung, liver, and peripheral leukocytes

(Zimpich *et* al*., 2004;* Paisan-Ruiz *et al.,* 2004). Within these regions, the protein

can be located in a variety of different cellular areas, such as the cytosol,

mitochondrial outer membrane, plasma membrane, lysosomes, endosomes,

transport vesicles, Golgi apparatus, synaptic vesicles, and lipid rafts (West *et al.,*

2005; Biskup *et al.,* 2006; Hatano *et al.,* 2007). While it is known that mutations

in *LRRK2* result in PD, the normal function of the protein is not well understood;

although, the protein has been identified as a tyrosine kinase-like protein (Mata

et al., 2006) and has been shown to be involved in protein-protein interactions

(Zimprich *et al.,* 2004). Mutations in *LRRK2* (autosomal dominant) are different

from mutations in SNCA in that these mutations result in a PD that typically has a

later onset and does not result in dementia (Zimprich *et al.,* 2004); this form of

PD has been shown to result in cell loss and Lewy body formation in the

substantia nigra, along with limited Lewy body formation in the cortex (Khan *et*

al., 2005).

  PRKN is a member of the "ring between ring finger" family of E3 ubiquitin

ligases that is found within the cytosol, synaptic vesicles, Golgi complex,

endoplasmic reticulum, and the mitochondrial outer membrane (Shimura *et al.,*

2000; Kubo *et al.,* 2001; Darios *et al.,* 2003: von Coelln *et al.,* 2004; Mouatt-

Prigent *et al.,* 2004). Mutations in *PRKN* result in alterations in the protein's

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solubility, localization, and its ability to form aggregations (Cookson *et al.,* 2003; Gu *et al.,* 2003; Wang *et al.,* 2005). These mutations (autosomal recessive) result in early onset PD, with the typical onset being between childhood and 40 years of age (Lucking *et al.,* 2000), and are accompanied by dystonia. Characteristics of this form of PD include gliosis and cell loss in the substantia nigra; however, there are typically no Lewy bodies formed and less involvement of the locus coeruleus (Takahashi *et al.,* 1994; Mori *et al.,* 1998). Additionally, this form of PD is responsive to levodopa.

DJ1 is a member of the peptidase C56 family of proteins and it forms a homodimer (Moore *et al.,* 2003). It is expressed throughout the body, including the brain, (Bandopadhyay *et al.,* 2004) but is primarily found within the cytosol, and can also be located within the mitochondria (Zhang *et al.,* 2005). PD that results from mutations in *DJ1* (autosomal recessive) is rare and, therefore, there is little clinical information about the resulting disease. The onset of this form of PD is typically between the ages of 20 and 40 (Bonifati *et al.,* 2003). However, it has been reported that these patients also suffer from psychiatric symptoms, short stature, and brachydactyly (Dekker *et al.,* 2003; 2004).

PINK1's function is unknown; however, it is known that it contains a serine/ threonine protein kinase domain (Valente *et al.,* 2001) and there is evidence that it is involved in neuroprotection against mitochondrial dysfunction and proteasome-induced apoptosis (Valente *et al.,* 2001; Valente *et al.,* 2004; Akundi *et al.*, 2012). It is expressed in the brain and systemic organs, and locates to the mitochondrial matrix and the intermembrane space (Silvestri *et al.,* 2005). *PINK1*

(autosomal recessive) mutations result in an early onset form of PD (fourth to fifth decade of life); however, this form of PD progresses more like late onset PD in that it progresses slowly. This form of PD responds well to levodopa and is often associated with dementia (Valente *et al.,* 2001; Hatano *et al.,* 2004; Bonifati *et al.,* 2005; Tan *et al.,* 2006).

ATP13A2 is a lysosomal membrane protein that is a member of the P5 subfamily of ATPases (Ramirez *et al.,* 2006). It is expressed in the brain, mostly in the ventral midbrain (Ramirez *et al.,* 2006). Mutations in *ATP13A2I* (autosomal recessive) results in an atypical form of PD known as Kufor-Rakeb syndrome, a disease characterized by early onset symptoms (11-16 years old). It is responsive to levedopa and its symptoms include dementia and supranuclear gaze palsy (Najim al-Din *et al.,* 1994; Williams *et al.,* 2005).

Identification of the genetic mutations that are linked to familial forms of PD helps to better understand the mechanisms that are involved in the development of the disease. Some of the genes that have been identified have shown evidence that damage to the mitochondria is a contributing factor to PD progression (Valente *et al.,* 2001; Valente *et al.,* 2004; Silvestri *et al.,* 2005; Zhang *et al.,* 2005). However, even with this evidence, the exact mechanism that is responsible for the development and progression of PD is still unclear.

ii. Environmental

While there has been a genetic component identified in the development of PD, there are still only approximately 20% of patients that have a family history

of the disease. This indicates that a large number of cases result from some other cause, such as environmental factors (Bonifati *et al.,* 1995). Recent studies have shown that there are links between environmental toxins and incidents of PD. Studies that have looked at regional differences in PD rates demonstrate that environmental toxins may be a contributing factor in the development of the disease. Increases in PD rates in rural communities suggest that toxins such as pesticides and herbicides could increase the risk of developing PD (Koller *et al.,* 1990; Granieri *et al.,* 1991; Svenson *et al.,* 1993; Hubble *et al.,* 1993), while higher incidences of PD in industrialized, urban areas imply that byproducts of industrialization may be linked to PD (Aquilonius and Hartvig, 1986; Schoenberg *et al.,* 1988; Rybicki *et al.,* 1993). However, not all studies agree on the observed increase in PD in rural and industrial, urban areas.

PD has been linked to exposure to pesticides (Costello *et al.*, 2009; Dhillon *et al.*, 2008; Gorell *et al.*, 1998; Menegon *et al.*, 1998), heavy metals, and organic pollutants. One example of a solvent that has been linked to the development of PD is trichloroethylene (TCE). Patients that have undergone long term TCE exposure have been linked to cases of PD and Parkinsonism (Gash *et al.,* 2008). A study evaluating twins who were exposed to several different solvents has also shown that exposure to TCE significantly increased the risk for developing PD. Other solvents such as perchloroethylene (PERC) and carbon tetrachloride (CCl_4) were associated with an increased risk of developing PD (Goldman *et al.*, 2012). Because environmental toxins have been shown to contribute to the causes of PD, toxins such as 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat are all commonly used to induce animal models of PD. These toxins provide valuable information about the mechanisms that underlie the neuropathology of PD. Currently, it is thought that these toxins, like TCE, induce mitochondrial dysfunction and cause oxidative stress, damaging dopaminergic neurons (Greenamyre *et al.*, 2001; Castello *et al.*, 2007; Mortiboys *et al.*, 2007; Gash *et al.,* 2008).

MPTP has been shown to induce PD-like symptoms in rodents, primates, and humans through its selective neurotoxic effects on dopaminergic neurons (Langston *et al.,* 1984; Ballard *et al.,* 1985). MPTP-induced toxicity is primarily an outcome of impairment in mitochondrial respiration (Nicklas *et al.,* 1985; Przedborski and Jackson-Lewis, 1998; Przedborski *et al.,* 2000) as a result of MPP+, the toxic metabolite, being taken up by nigrostriatal neurons (Chiba *et al.,* 1984). The observed impairment in mitochondrial respiration has been shown to be a result of inhibition of mitochondrial complexes I, III, and IV (Desai *et al.,* 1996).

Rotenone, a commonly used pesticide and piscicide, has been shown to inhibit mitochondrial complex I in nigrostriatal dopaminergic neurons (Betaret *et al.,* 2000; Amberg *et al.*, 2012). The primary cause of damage to mitochondrial complexes I and II by rotenone is an increase in reactive oxygen species (ROS) (Panov *et al.,* 2005). It has also been shown that the result of complex I inhibition as a result of chronic rotenone exposure results in an increase in

aggregation of α-synuclein and ubiquitin, which results in oxidative damage and caspase dependent death (Sherer *et al.,* 2002).

Paraquat is a commonly used herbicide that has structural similarities to MPTP, although it does not appear to work through the same mechanism. The mechanism by which paraquat induces parkinsonism is not well understood. There is, however, evidence that paraquat causes some weak inhibition of mitochondrial complex I, in contrast to both MPTP and rotenone which have strong mitochondrial complex I inhibitory properties (Richardson *et al.,* 2005). In addition, there is evidence that exposure to paraquat can cause lipid peroxidation that results in damage to dopaminergic neurons (Dinis-Oliveira *et al.,* 2006).

Data about regional differences in the frequency of PD have provided evidence that suggests exposure to environmental toxins likely result in an increased risk of developing PD (Racette, 2012). While there are studies showing these regional differences correlating with increased incidences in both rural (Koller *et al.,* 1990; Granieri *et al.,* 1991; Svenson *et al.,* 1993; Hubble *et al.,* 1993) and industrial, urban areas (Aquilonius *et al.,* 1986; Schoenberg *et al.,* 1988; Rybicki *et al.,* 1993), there are also studies that refute these differences. Many of the inconsistencies in these studies result from limited sample sizes being available (Racette, 2012). Therefore, methods to improve sample size and evaluate the dose response data are necessary to better understand the role of environmental toxins in the development of PD.

iii. Excitotoxicity

Excitotoxicity is suspected as one of the causative factors resulting in the death of dopaminergic neurons observed in PD (Levy *et al.,* 2009). Evidence indicates that changes in glutamate, the major excitatory neurotransmitter in the central nervous system, regulation can have significant impact on neurons (Olney, 1990). It is believed that excitotoxicity can damage neurons directly via overstimulation of NMDA receptors; however, in PD it is indicated that damage as a result of excitotoxicity follows an indirect route (Albin and Greenamyre, 1992; Greene and Greenamyre, 1996).

iv. Inflammation

Inflammation plays a role in many neurodegenerative diseases, including PD. The immune response most commonly associated with neurodegenerative disease is the activation of microglia (Kreutzberg, 1996). It has been shown that levels of proinflammatory cytokines, such as tumor necrosis factor-α and interferon-γ, are up regulated in the brain after exposure to toxins that have been linked to PD (Litteljohn *et al.*, 2010). This increase in cytokines then results in a loss of dopaminergic neurons.

In addition to inflammation in the CNS, there is also evidence that systemic inflammation can exacerbate the neuronal loss in PD. It has been observed that cytokine mRNA are increased in the microglia during animal models of PD while protein levels such as IL-1β do not increase (Depino *et al.*, 2003). This has given rise to the theory of "primed microglia," that can be

"activated" after a secondary peripheral proinflammatory stimulus. Once these primed microglia become activated, they continue to contribute to neurotoxicity by producing free radicals and cytokines.

d. Treatments

Current PD treatments (Table 1.2) function by treating the symptoms but do not actually reverse the cell loss that is responsible for causing the symptoms. During the initial treatment (less than five years), PD symptoms can be greatly reduced and quality of life improved. However, as time passes, all of the treatments become less effective and the symptoms return (Mayo Clinic, 2012).

i. Symptomatic treatments

Levodopa is the current gold standard of treatment for PD symptoms. It is a naturally occurring precursor for dopamine that can be taken orally because, unlike dopamine, levodopa can cross the blood brain barrier. After levodopa is taken, it is transported across the blood brain barrier to the brain where it is converted into dopamine by DOPA decarboxylase, or L-amino acid decarboxylase in striatal dopaminergic neuron terminals (Figure 1.3) (Lopez *et al.*, 2001). In the drug Sinement, levodopa is combined with carbidopa, which helps to prevent the conversion of levodopa to dopamine outside of the brain. One of the most noticeable side effects of levodopa treatment is the appearance of dyskinesia, which affects the patient's voluntary movement. Lowering the dose of levodopa can reduce dyskinesia, but will also reduce the level of

effectiveness in treating the PD symptoms.   Further, as levodopa treatment continues, it tends to exhibit a "wearing off" effect and loses its effectiveness as the disease progresses.

Another class of PD treatments includes dopamine receptor agonists. These therapies do not increase the amount of dopamine present in the brain, but instead, act to mimic the presence of dopamine, by interacting with the dopamine receptors present in the brain (Figure 1.3). Most of the currently available dopamine agonists on the market interact with the D_2 receptor, although there is cross talk with other dopamine receptors (Table 1.3). Dopamine agonists were introduced for the treatment of PD in the late 1970s (Factor, 1999) and were initially used as a combination therapy with levodopa. However, it has been observed that dopamine agonists offer beneficial effects at all stages of PD both when given in conjunction with levodopa and as a monotherapy. When given with levodopa, dopamine agonists can delay levodopa-induced motor complications (Jankovic and Stacy, 2007).

MAO-B is an enzyme found in the outer mitochondrial membrane that is responsible for metabolizing dopamine in the brain. Therefore, if the levels of MAO-B in the brain are reduced or MAO-B is rendered inactive, dopamine levels in the brain would remain elevated. MAO-B inhibitors, such as Selegiline (Deprenyl), function by inhibiting the breakdown of endogenous dopamine, as well as that produced as a result of levodopa treatment, resulting in an overall increase in the total amount of dopamine available in the brain (Figure 1.3).

There are a few other treatments that can be used but that are less common because they are not as effective and the side effects associated with the treatment are more severe. Catechol O-methyltransferase (COMT) inhibitors function by inhibiting the breakdown of levodopa (Figure 1.3). They can be used in combination with levodopa therapy. However, they have been associated with liver damage and are therefore only used in patients that are not responding to other treatment options.

Anticholinergics can be used to reduce PD associated tremors. However, these drugs offer modest effects and are often associated with significant side effects. NMDA blockers can be prescribed to treat the early symptoms of PD. They are often used later to supplement levodopa treatment to help alleviate dyskinesia side effects.

ii. Physical therapy and surgical treatments

Physical therapy can be used to help combat the symptoms of PD. While these exercises cannot stop the disease from progressing, the muscle maintenance can help to counteract some of the effects of the disease.

Deep brain stimulation is a surgery that can be performed on patients with advanced PD. During this surgery, electrodes are implanted in the brain. These electrodes can deliver current to the brain to help reduce the symptoms of PD. This treatment option is primarily used for patients who are no longer responding to other therapies, such as levodopa. It can also be used to eliminate some of the dyskinesia that is associated with levodopa.

iii. Growth factors

Attempts to target the source of PD and not just treat the symptoms have been undertaken. Based on their endogenous role, neurotrophic factors are one class of molecules that have been evaluated for their potential to treat PD. Neurotrophic factors are a class of functionally related proteins that play a key role in neurite formation and growth during development and after injury (Ibáñez, 1998). Because of their native cellular function, neurotrophic factors have received considerable attention as potential therapeutic agents for neurodegenerative disorders, including PD.

1. Role of growth factors in the treatment of neurodegenerative diseases

The first neurotrophic factor described was nerve growth factor (NGF) (Levi-Montalcini and Hamburger, 1951). Studies have shown that NGF levels are decreased in PD patients and animal models (Lorigados *et al.,* 1992, 1996). Therefore, it is a reasonable choice for use as a potential treatment of PD. Studies have shown that in rodent models of PD, NGF can increase the survival of grafted adrenal chromaffin cells which results in an increase in the functionality of the treatment (Date *et al.,* 1997; Silani *et al.,* 1990). However, this work has not translated with consistent results into primate models and clinical trials (Hurtig et al., 1989; Peterson et al., 1989; Olson et al, 1990).

The function of brain-derived neurotrophic factor (BDNF) is to promote the survival and differentiation of cholinergic neurons (Alterson *et a*l., 1990; Knusel *et al.*, 1991) and has been shown to play a role in neurogenesis (Benraiss *et al.*,

2001; Zigova *et al.*, 1998). Similar to NGF, levels of BDNF have been shown to be reduced in PD patient's brains (Howells *et al.*, 2000, Mogi *et al.*, 1999). BDNF has been shown to effect dopaminergic neurons in culture (Feng *et al.*, 1999; Hyman *et al.*, 1991; Yoshimoto *et al.*, 1995) and in whole tissue after both 6-OHDA and MPTP exposure (Hung and Lee, 1996; Levivier et al., 1995). BDNF has also been shown to reverse behavioral and neurochemical deficits when administered to the substantia nigra before and after nigrostriatal injury (Altar et al., 1992).

Neurotrophin-3 (NT-3), also known as neurotensin, and neurotrophin-4/5 (NT-4/5) are also members of the neurotrophin family. Like the other neurotrophins, NT-3 promotes survival and differentiation of neurons. It primarily functions through the TrkC receptor (Lamballe *et al.*, 1991; Tessarollo *et al.*, 1993); however, it interacts with the TrkB receptor to a lesser extent. NT-3 has been shown to protect dopaminergic neurons (Gu *et al.*, 2009). Additionally, NT-4/5 has been shown to have protective effects on a variety of different types of neurons, including dopaminergic neurons (Hyman *et al.*, 1991, 1994).

The glial cell-line derived neurotrophic factor (GDNF) family of proteins, which includes GDNF, neurturin, artemin and persephin, is a small subfamily of the transforming growth factor beta (TGF-β) superfamily (Krieglstein *et al.,* 1995; Baloh *et al.,* 1998; Milbrandt *et al.,* 1998). The GDNF family of proteins have been shown to play key roles in numerous cell processes, including cell survival, neurite outgrowth, cell differentiation, and cell migration. These functions are carried out through their ability to specifically activate RET tyrosine kinase

pathways. Due to their unique role, this family of trophic factors has been evaluated as potential candidates for neurodegenerative disorder treatment.

GDNF is a 33-45 kDa protein that interacts with a multi-subunit receptor system that consists of GDNF family receptors alpha-1 (GFRα1) and RET (Figure 1.4). Interaction with these receptors can initiate a variety of intracellular pathways, including Ras-MAPK (Worby *et al.,* 1996), PLCγ-dependent pathway (Borello *et al.,* 1996), phosphoinositol 3-kinase (PI3-K), and Jun N-terminal kinase (JNK) (Van Weering and Bos, 1998). The second member of the GDNF family of proteins is neurturin (NTN). NTN is 12 kDa that has been shown to support the survival of sympathetic neurons (Kotzbauer *et al.,* 1996). Like GDNF, NTN interacts with the RET receptor, but it can interact with GFRα1 and GFRα2 receptors (Figure 1.4) to induce activation of the MAP-K and PI3K pathways and promote survival of dopaminergic neurons (Creedon *et al.,* 1997). It has been observed that both GDNF and NTN can interact with GFRα1 and GFRα2 (Figure 1.4); however, GDNF preferentially interacts with GFRα1, while NTN is more likely to interact with GFRα2 (Figure 1.4) (Sanicola *et al.,* 1997; Klein *et al.,* 1997). Artemin (ARTN) interacts primarily with GFRα3, although there is some crosstalk with GFRα1 (Airaksinen *et al.,* 1999). Persephin (PSPN) only interacts with GFRα4 and RET and does not crosstalk with any of the other GDNF family receptors (Figure 1.4) (Lindahl *et al.,* 2000). GFRα4 is unique in that, when compared to the other GDNF family receptors, it does not contain the first N-terminal Cys-rich domain.

NTN, a GDNF family member, has a 40% sequence similarity to GDNF and has shown promise as a PD therapeutic. Ceregene Inc. developed CERE-120, a recombinant AAV2- based vector that encodes NTN. CERE-120 has been shown to result in rapid (within four weeks) and long lasting (up to one year) expression of NTN (Gasmi *et al.*, 2007b). AAV2-NTN has been shown to protect neurons from 6-OHDA in rats (Gasmi *et al.*, 2007a). Additionally, AAV2-NTN was shown to increase TH+ neurons in aged monkeys (Herzog *et a*l., 2007) and protect in MPTP- lesion monkeys (Kordower *et al.*, 2006). Based on the success of CERE-120 in animal models, it was moved into phase I clinical trials. During the phase I clinical trials, beneficial effects, such as a 40% reduction in UPDRS-motor "off" scores, a substantial increase in "on" time without dyskinesias, a reduction in "total off time" and improvement on timed motor tasks, were observed without any significant adverse reactions being reported (Marks *et al.*, 2008). CERE-120 then moved into phase II clinical trials. However, this trial did not show any significant beneficial results (Bartus, 2012). This was likely due to the difficulties associated with the delivery of the molecule to the brain.

2. Glial cell line-derived neurotrophic factor

GDNF, the most promising neurotrophic factor for the treatment of PD to date, has been shown to increase the survival of cultured dopaminergic neurons, which lead to further investigation in connection with PD (Lin *et al.*, 1993). In addition, *in vivo* GDNF has been shown to protect and restore neurons after cytotoxic insults (Hoffer *et al.,* 1994; Tomac *et al.,* 1995; Gash *et al.,* 1996;

Herbert and Gerhardt, 1997; Cass *et al.,* 1999). GDNF has also shown promise

extensively as a therapeutic by increasing dopamine levels in the substantia

nigra, improving motor deficits and exhibiting long-lasting effects in aged

primates and PD patients (Salvatore *et al.*, 2004). Unfortunately, the therapeutic

development of GDNF has been discontinued because of the reported presence

of GDNF antibodies in clinical trials, unpublished data indicating cerebellar

toxicity at high concentrations in primates, and patent protection issues (Gill *et

al.,* 2006; Slevin *et al.,* 2005; Lang *et al.*, 2006).

Like all neurotrophic factors, GDNF is initially produced as a 221 amino

acid inactive precursor protein that is cleaved to produce a 134 amino acid

mature protein (Lin *et al.,* 1993). The mature form of GDNF is a glycosylated

homodimer that is approximately 33-45 kDa, with the monomer being

approximately 16 kDa (Lin *et al.,* 1994). While the cellular function of the mature,

processed GDNF protein is known, the role of the highly conserved proprotein

sequence is not fully understood.

Numerous studies have shown GDNF to have promise as a potential

therapeutic for PD. In addition to being able to enhance the growth and survival

of unstressed neurons (Lin *et al.* 1993), GDNF has also been shown to protect

against toxins *in vitro,* such as 1-methyl-4-phenylpyridinium ion (MPP+) (Hou *et

al.,*1996). Further benefits of GDNF have been observed in rodent models; the

administration of GDNF to the substantia nigra prior to treatment with the toxin 6-

hydroxydopamine (6-OHDA) results in a significant protection of dopaminergic

neurons (Kearns and Gash, 1995; Kearns *et al.,* 1997; Fox *et al.,* 2001). This

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neuronal protection is also associated with an observed protection from the

reduction in evoked dopamine release that is typically associated with 6-OHDA

treatment (Cass and Manning, 1999).   Based on the data from the rodent

models, studies in which GDNF was administered to non-human primates were

carried out. Again, increases in dopamine levels (Gash *et al.,* 1995; Grondin *et al.,* 2002) and improvement in PD related behaviors were observed in MPTP

primate models (Gash *et al.,* 1996; Zhang *et al.,* 1997).

3. Clinical trials of GDNF

Based on the data from rodent and non-human primate studies, GDNF

was moved into phase I clinical trials. The initial trials were open-label trials

carried out in Bristol, UK and at the University of Kentucky, Lexington, KY. These

studies used a programmable pump (Medtronic) and catheters system for the

delivery of GDNF (Gill *et al.,* 2003; Slevin *et al.,* 2005). In both of these clinical

trials a significant improvement in the Unified Parkinson's Disease Rating Scale

(UPDRS) was observed (Gill *et al.,* 2003; Slevin *et al.,* 2005).

However, despite the initial promise of the GDNF studies, it has failed to

advance beyond phase II clinical trials. The phase II clinical trials failed to meet

the criteria set out for significant improvement in the patients that was required

for the studies to continue (Lang *et al.,* 2006; Sherer *et al.,* 2006). Complications

associated with the delivery of a large protein to the brain are believed to have

contributed to the failure of GDNF in clinical trials (Gill *et al.,* 2003; Slevin *et al.,*

2005; Lang *et al.,* 2006). The delivery catheter and the distribution of GDNF

varied during the clinical trials and may have contributed to the appearance of auto-antibodies, as well as the lack of significant improvements in the patients that were treated (Gill *et al.,* 2003; Slevin *et al.,* 2005; Lang *et al.,* 2006).

III. Challenges of growth factors

The development of growth factors as PD therapeutics faces several difficulties that must be overcome. The major obstacle is delivering a large molecule, such as GDNF (33-45 kDa), to the brain because they cannot cross the blood brain barrier. This has resulted in the use of invasive delivery techniques, such as CED through a surgically implanted catheter, to target administration of growth factors. These treatments require surgical procedures, which are limited by several factors including training qualified surgeons and having adequate space to perform the procedure. In addition, growth factors can be challenging to synthesis for use in a therapeutic agent. One possible solution to address and help eliminate these problems is to develop small molecules, which possess neurotrophic-like properties and can be modified for systemic deliver, as an alternative therapeutic.

a. Large size-difficult to deliver

In order to treat PD, it is necessary to deliver the drug to the affected area of the brain. However, it is difficult to deliver large molecules to the brain through systemic methods because these molecules cannot cross the blood brain barrier. In order to circumvent this problem, direct delivery of the molecule to the affected

brain region was required. In the case of the GDNF clinical trials, this was achieved by implanting a pump in the abdomen that could hold a one-month supply of the drug. This pump was connected via tubing to the brain so that GDNF could be directly delivered by convection enhanced delivery (CED) (Yin *et al.*, 2011; Taylor *et al.*, 2013). However, this requires an intracranial surgical procedure for implantation and monitoring of the device.

b. Delivery methods

When considering delivering GDNF to the CNS, it is important to note that GDNF receptors are found throughout the nervous system meaning that delivery to specific regions could be essential for reducing any off target effects. It has been shown that GDNF distribution to the target tissues correlates with the observed effects on midbrain dopamine neurons and improvement in motor function (Gash *et al.*, 2005). In order to overcome the problems associated with delivering large molecules to the brain, a variety of different techniques have been developed. These include delivering the growth factors by convection enhanced delivery (CED), which can increase the distribution of the drug within the brain. Also, other delivery methods are being tested; these include using viral vectors to deliver the growth factors to the brain.

i. Convection enhanced delivery

Convection enhanced delivery (CED) is a technique used to deliver molecules to solid tissue by using a bulk flow infusion system. CED allows

clinically relevant volumes of molecules to be distributed to clinically relevant

tissues (Bobo *et al.,* 1994; Morrison *et al.,* 1994). The use of bulk flow in CED

offers several advantages, such as allowing for a greater distribution volume than

simple diffusion (Bobo *et al.*, 1994). Further, a more uniform distribution of the

molecule that is being delivered within the target region is made possible with

CED, which also allows the entire molecule is able to be delivered to the target

region (Morrison *et al.,* 1994). This helps to cut down on the dose required for

the maximum effect (Morrison *et al.,* 1994).

This method has become widely accepted as a method for delivering

therapeutic agents to targeted brain regions (Lonser *et al.*, 1998; Bankiewicz *et

al.*, 2000; Krauze *et al.*, 2005). While CED has been shown to be an effective

delivery method for growth factors, including GDNF, to the brain, it is essential to

carefully control this delivery to prevent the material from being deposited into

undesirable locations, which can lead to adverse effects, such as the induction of

auto-antibodies (Lang *et al.*, 2006). In an attempt to avoid these complications,

studies looking at co-infusion of an MRI tracer with GDNF have been conducted

to determine the distribution of GDNF (Gimenez *et al.*, 2011). These studies

would allow neurosurgeons to more accurately tract the distribution of GDNF in

the brain and monitor the delivery in real time. This helps to reduce unwanted

side effects associated with GDNF being delivered outside of the desired location

in the brain as well as allowing for surgeons to know that delivery was successful

prior to symptomatic relief.

ii. AAV, Adenovirus, Lentivirus

Delivery of growth factors using adenoviral (Ad) vectors, adeno-associated viral (AAV) vectors, and lentiviral (LV) vectors have all been shown to provide efficient transduction in rodents, with LV also exhibiting transduction in non-human primates (Kordower JH et al., 1999; Kordower JH et al,).

Ad vectors are capable of holding large pieces of DNA (8 kb) and can infect both dividing and non-dividing cells. The DNA carried by Ad vectors does not integrate into the host DNA so it is best used for transient expression in non-dividing cells. While intracerebral delivery of GDNF by Ad vectors (Ad-GDNF) has been shown to offer protection in rats with 6-OHDA lesions (Choi-Lundberg *et al.*, 1997; Choi-Lundberg *et al.* 1998), there has also been evidence that the transduced cells also express adenoviral proteins that can induce a host immune reaction (Wood *et al.*, 1996; Kajiwara *et al.*, 1997).

AAV vectors are lacking 96% of their viral genome but have the genes required for integrating the transgene into the host genes, thereby allowing for stable expression in non-dividing cells. Because the majority of the viral genome is absent in AAV vectors, the risk of causing a host immune response is diminished. However, their DNA capacity (<5 kb) is less than that of Ad vectors. Additionally, transgenes carried by AAV vectors exhibit a delayed expression of several days with full expression at about 2-3 weeks after delivery (Monahan and Samulski, 2000; Peel and Klein, 2000). While AAV vectors have been shown to be efficient for transducing neurons, they do not affect all types of neurons and brain regions equally because they require binding to heparin sulfate

proteoglycans. Treatment of 6-OHDA lesion rats with AAV-GDNF has been shown to rescue dopamine neuron cell bodies; however, the denervation of TH-positive fibers in the striatum was not affected (Mandel *et al.*, 1997; Mandel *et al.*, 1999).

LV vectors are developed from retroviruses, which are highly pathogenic. They are capable of carrying large pieces of DNA (9 kb) and can stably integrate into their host and exhibit long-term expression. LV-GDNF has been shown to induce GDNF expression in rats, mice, and monkeys (Bensadoun *et al.,* 2000; Kordower and Bjorklund, 2013). In rats, this expression is similar to that seen with AAV-GDNF (Mandel RJ *et al.,* 1997; Kirik *et al.,* 2000). LV-GDNF was shown to increase the percentage of TH-positive neurons after lesions (Deglon *et al.,* 2000; Bensadoun *et al.,* 2000).

iii. Other neurotrophic factors

Despite the evidence that BDNF can protect dopaminergic neurons, its large size presents difficulty when trying to deliver the molecule to the brain. Due to this challenge, other delivery methods have been investigated, including *in vivo* gene transfer by adeno-associated viral vector (AAV) (Klein *et a*l., 1999). Even though BDNF does show potential for protecting dopaminergic neurons, there has not been enough evidence to move it to clinical trials. This is due, in part, to the fact that while it shows neuroprotective effects, it does not exhibit neurorestorative effects.

Additionally, mesencephalic astrocyte-derived neuorotrophic factor (MANF), is expressed at high levels in the hippocampus, cortex, and midbrain (Lindholm *et al.*, 2008). It has been shown to promote the survival of ventral midbrain dopaminergic neurons (Petrova *et al.*, 2003, 2004). It has also been shown to be neuroprotective and neurorestorative in 6-OHDA treated rats (Voutilainen *et al.*, 2009). Conserved dopamine neurotrophic factor (CDNF) has also been shown to provide effects similar to MANF in 6-OHDA treated rats (Lindholm *et al.*, 2007). It is thought that the activity of MANF and CDNF is through their protection from endoplasmic reticulum stress (Apostolou *et al.*, 2008; Mizobunchi *et al.*, 2007).

Other neurotrophic factors are unlikely to overcome the problems that have plagued GDNF. These alternative treatments still possess the qualities that have made it difficult for GDNF to move out of phase II clinical trials. Other neurotrophic factors are still large and because of this, they can be complex to produce and face the same difficulties of delivery that affected GDNF. Ultimately, the development of a smaller molecule with protective effects similar to that of GDNF and other neurotrophic factors would overcome these obstacles.

IV. Biotherapeutic strategies

Neurotrophic factors, such as GDNF, possess many qualities that make them desirable molecules for use in treating neurodegenerative diseases, such as PD. Endogenously neurotrophic factors function to support cell survival and growth. This function makes them good potential therapeutic candidates for

treating neurodegenerative disease because they are likely to be neuroprotective and neurorestorative. There is evidence that GDNF provides protection by offering mitochondrial protection and therefore mediating apoptotic pathways (Bradley *et al.*, 2010; Turchan-Cholewo *et al.*, unpublished).

Neurotrophic molecules have been attractive as drug candidates because of their endogenous roles of promoting cell survival. Current PD therapies, such as levodopa, dopamine agonists, and MAO-B inhibitors, work to alleviate the symptoms of PD, but do not prevent or reverse the cell loss that is the cause of PD. Furthermore, these treatments have side effects that commonly develop after repeated, long-term use. Some of these side effects can be as debilitating as the symptoms of PD. Therefore, newly developed therapies need to be neuroprotective (stopping cell death) and neurorestorative (promoting the growth of new neurons). These characteristics will allow biotherapeutic strategies to do more than treat the symptoms of PD; they will actually be able to stop and reverse the pathology.

Damage to the mitochondria, particularly complex I of the electron transport chain, has been shown to play a key role in the pathology of PD. Novel and more effective therapies can be developed by evaluating biotherapeutics that are able to provide mitochondrial protection. There is evidence that many of the neurotrophic molecules that have been evaluated as PD therapeutics function through mitochondrial protective mechanisms.

Programmed cell death, or apoptosis, is the primary form of cell death observed in PD. The observed apoptosis associated with PD is primarily a result

of deficits in cellular respiration. The use of therapeutics, such as neurotrophic molecules, that can prevent apoptosis is essential for the success of the treatment.

One of the major problems associated with current biotherapeutic strategies for neurodegenerative diseases is the delivery of large molecules to the brain. Thus, a viable approach towards the development of a potential PD therapeutic is to construct and evaluate novel, smaller molecules with GDNF-like activity. These smaller molecules offer the advantage of being easier to synthesize and purify in addition to ultimately being simpler to modify and deliver to the brain by less invasive methods.

V. Peptides offer attractive molecular advantage

Small molecules, such as peptides, offer advantages over larger biotherapeutic strategies. A significant advantage centers on their small size. The small size makes the use of peptides more realistic for non-invasive delivery to the brain. In addition, peptides are simpler to synthesize, modify, and purify than larger molecules. These peptides can then be chemically modified to allow for easier delivery and more effective action of the peptide. In addition, peptides are already used in nature as signaling peptides with a wide range of sequence diversity available.

a. Chemical synthesis

  Chemical synthesis of peptides originated in work done by Emil Fischer (Kent, 1988). Following Fischer's work, interest in peptide synthesis rose along with interest in the research of biologically active peptides, such as neuropeptide hormones and neurotransmitters. Currently, peptides are produced by solid phase peptide synthesis, a process that involves the stepwise elongation of a peptide (Merrifield, 1963; Kent, 1988). This procedure is almost exclusively used to produce peptides for research purposes (Kent, 1988). Due to their small size, peptides can be more easily produced by chemical synthesis than larger, more complex recombinantly-produced proteins like GDNF. For example, mature GDNF is a dimer that takes on a cystiene knot formation, which is more complicated to produce recombinantly. In order for GDNF to be functional after it is recombinantly produced, it must be able to refold correctly to form its active confirmation. In addition, it is necessary to be able to purify a therapeutic agent before it can be used. Small peptides can be purified by relatively simple methods, such as high-performance liquid chromatography (HPLC). The relative ease with which peptides can be produced and purified makes them attractive alternatives to large molecules, such as proteins, as potential therapeutics.

b. Modification

  Modifying peptides either through changing the amino acid sequence or by chemically modifying the peptide to change their properties can help to improve not only their therapeutic function, but it can also allow them to be

delivered more easily and improve their biodistribution. For the treatment of

neurodegenerative diseases, such as PD, it is necessary that the molecule get

into the affected brain region. By modifying peptides, they can be given

systemically or perhaps intranasally and still access the brain. Further, chemically

modifying the peptides can alter the biodistribution of a molecule as well as alter

the *in vivo* stability of the peptides. In order to allow the peptides to have their

desired effect after treatment, it is necessary for the peptides to be relatively

stable once they have been delivered into the body. A lack of *in vivo* stability can

result in the peptides being quickly broken down and having a limited effect.

Some peptides are inherently unstable *in vivo* and, therefore, being able to

increase the *in vivo* stability of these peptides can increase the amount of time

that they are present within the body and in doing so, they can improve the

observed therapeutic effect.

c.   *Peptide signaling molecules*

Peptides are used as biological signaling molecules. Peptides are

involved in numerous important roles within the body, including roles as

neurohormones and neurotransmitters. One example of peptides that are

involved in a biological signaling pathway is the neuropeptide Y family, which

includes three thirty-six amino acid peptides (neuropeptide Y, peptide YY, and

pancreatic polypeptides). This family of peptides interacts with G-protein-coupled

receptors to help regulate energy balance (Nguyen *et al.,* 2011).

VI. Growth factor prosequences

　　As with many excreted proteins, growth factors are first translated as a
preproprotein. Processing of the growth factors during maturation results in the
removal of the pre- and prosequences to produce the mature growth factor.
Growth factor prosequences have long been believed to have very little, if any,
function other than guiding the processing and folding of the mature protein.
However, recent studies have shown that the prosequences of some growth
factors, including NGF and BDNF, possess additional anti-apoptotic activities.
This emerging evidence of anti-apoptotic activity suggests that growth factor
prosequences may play a role in cell survival and maintenance that complements
the activity of the mature growth factors that they are derived from.

a. Roles in apoptosis

　　Mature NGF promotes cell survival by binding to the TrkA and p75[NTR]
receptors. The p75[NTR] is also involved in the binding of proNGF to a heterodimer
that consist of both this and the sortilin receptors (Figure 1.5) (Nykjaer *et al.*,
2004). The recruitment of the sortilin receptor is necessary for proNGF's ability
to induce apoptosis (Nykjaer *et al.*, 2004). Brain-derived Neurotrophic factor
(BDNF), which is also a member of the TGF-β family, is able to promote the
survival and differentiation of neurons through the TrkB receptor tyrosine kinase.
However, BDNF also causes low levels of cell death, which appears to be
mediated by the p75[NTR] receptor (Teng *et al.*, 2005).

It has been observed that proBDNF is able to cause apoptosis at a much higher level than mature BDNF. ProBDNF seems to function through the same mechanism as proNGF. It is able to bind to the heterodimer of p75^{NTR} and sortilin receptors, both of which are required for its apoptotic activity (Teng *et al.*, 2005).

Studies looking at the function of proNGF have shown that the isolated prosequence of NGF can be used to block the induction of apoptosis (Nykjaer *et al.*, 2004). When cells expressing the sortilin and p75^{NTR} receptors are treated with only the prosequence of NGF, it appears that it is able to competitively inhibit proNGF binding and keep sortilin from being recruited to the p75^{NTR} receptor to form an active heterodimer and therefore reduce the levels of apoptosis (Nykjaer *et al.*, 2004).

VII. DNSP-11

Similar to NGF and BDNF, GDNF is also produced as a proprotein. It is hypothesized that the GDNF prosequence functions in much the same way. This would mean that some proGDNF is able to escape its normal processing and remain as proGDNF in the body. This unprocessed proGDNF could be causing an increase in cell death. However, when GDNF is normally processed, the cleaved prosequence can work to block the activity of proGDNF and increase cell survival by inhibiting apoptosis that would be induced by proGDNF.

a. Processing

The GDNF presequence contains a signal sequence that targets the protein to the rough endoplasmic reticulum (rER), where it is processed and folded within the rER/Golgi complex secretory pathway. It has long been believed that these prosequences were solely responsible for guiding the processing and folding of the mature growth factor; however, it has recently been shown that the prosequences of other trophic factors possess alternate apoptosis mediating functions compared to the mature proteins (Chao and Bothwell, 2002; Nykjaer *et al.*, 2004). This has led to the investigation of the activity of the prosequence of GDNF. Evaluation of the GDNF proprotein for potential protease sites found internal endopeptidase sites were predicted to yield three individual amidated peptides of 5, 11, and 17 amino acids in length (Figure 1.6) (Immonen *et al.,* 2008; Bradley *et al.*, 2010; Kelps *et al.,* 2011). The rat homolog of 11, named BEP, amino acid sequence (DNSP-11) from GDNF's prosequence has been shown to effect hippocampal neurons in a neurotropic-like manner (Immonen *et al.*, 2008).

b. Potential as a therapeutic

Preliminary evaluations of these peptides show similar functional effects as mature GDNF, thus making them viable candidates for further development as a PD therapeutic. The peptides have been shown to increase neurite outgrowth in primary mesocephalic cell culture models, as well as positively affect the behavior in a young Fisher 344 rat model of dopaminergic dysfunction (Bradley

et al., 2010). Treatment with DNSP-11 has been shown to increase basal dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in rats (Bradley *et al.*, 2010). While the cellular mechanism of these peptide sequences is unknown, these results coupled with the functional data from other neurotrophic factor prosequences, strongly suggest that the GDNF family of prosequences exhibit cellular functions. In addition, peptides offer advantages of a smaller size, being easier to deliver and synthesize, and less potential for causing an immune response than the larger mature protein. Thus, the propeptides require further evaluation and characterization to determine their functional role and potential for PD therapeutic development. Furthermore, a combination of *in vitro* and *in vivo* data from a collaborative research group has aided in focusing our attention on DNSP-11.

By furthering our understanding of the mechanism by which DNSP-11 is able to provide these anti-apoptotic effects, DNSP-11's potential as a therapeutic can be maximized. This understanding also provides a foundation on which further studies to evaluate the functionality of DNSP-11 can be based. Presented here is an evaluation of a potential mechanism that may contribute to some of the observed beneficial effects of DNSP-11. This mechanism involves DNSP-11's interaction with GAPDH, which is known to mediate an apoptotic pathway.

c. DNSP-11 and GAPDH

The mechanism by which DNSP-11 is able to provide its observed anti-apoptotic effects is currently not known. One potential binding partner for DNSP-11 that has been identified is glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which has previously been shown to be involved in mediating an apoptotic pathway (Bradley *et al.*, 2010). Under conditions of cell stress, GAPDH is nitrosylated. Once GAPDH is nitrosylated, it is no longer metabolically active, resulting in two distinct pools of GAPDH existing in stressed cells. The nitrosylated GAPDH can now interact with and bind to Siah-1, an E3 ubiqutin ligase (Hara *et al.*, 2005). When Siah-1 is not bound to GAPDH, it is unstable and so it is quickly degraded within the cell. However, binding to nitrosylated GAPDH stabilizes Siah-1 and prevents it from being quickly degraded and allows it to have prolonged effects (Hara *et al.*, 2005).

GAPDH lacks a nuclear localization signal, so it normally is not located within the nucleus. However, Siah-1 does possess a nuclear localization signal and can, therefore, move into the nucleus. Due to the presence of a nuclear localization signal on Siah-1, when nitrosylated GAPDH binds to Siah-1, the complex is able to move into the nucleus. Since the binding of nitrosylated GAPDH functions to stabilize Siah-1, it is able to degrade its nuclear targets for an extended period of time (Hara *et al.*, 2005). This leads to activation of an apoptotic pathway (Figure 1.7).

This pathway is of particular interest because deprenyl, a current therapeutic used to treat early stages of PD, has been shown to mediate the

GAPDH-Siah-1 apoptotic pathway (Hara *et al.*, 2006). Deprenyl was shown to reduce the amount of GAPDH nitrosylation (Hara *et al.*, 2006). The reduction in GAPDH nitrosylation is associated with a reduction in nuclear GAPDH because there is less GAPDH that is capable of interacting with Siah-1 (Hara *et al.*, 2006). Here we hypothesize that DNSP-11 is also able to reduce the amount of nuclear GAPDH by interacting with the GAPDH pathway. Additionally, it is hypothesized that through mediating the GAPDH apoptotic pathway, DNSP-11 is able to reduce the observed amount of apoptosis.

VIII. Thesis outline

The data presented here involves the evaluation of the molecular and cellular mechanism of DNSP-5, DNSP-11, and DNSP-17, which are derived from prosequence of GDNF. Initial studies were carried out to evaluate the physical characteristics of these three peptides. Cell culture assays and proteomic profiling were utilized to determine binding partners and potential mechanisms through which DNSP-11 may be able to mediate apoptosis. These studies will not only contribute to the understanding of neurotrophic factor prosequence function, but will also serve as a starting point for the development of novel trophic factors for PD treatment. Finally, the interaction between DNSP-11 and GAPDH was evaluated as a potential anti-apoptotic mechanism.
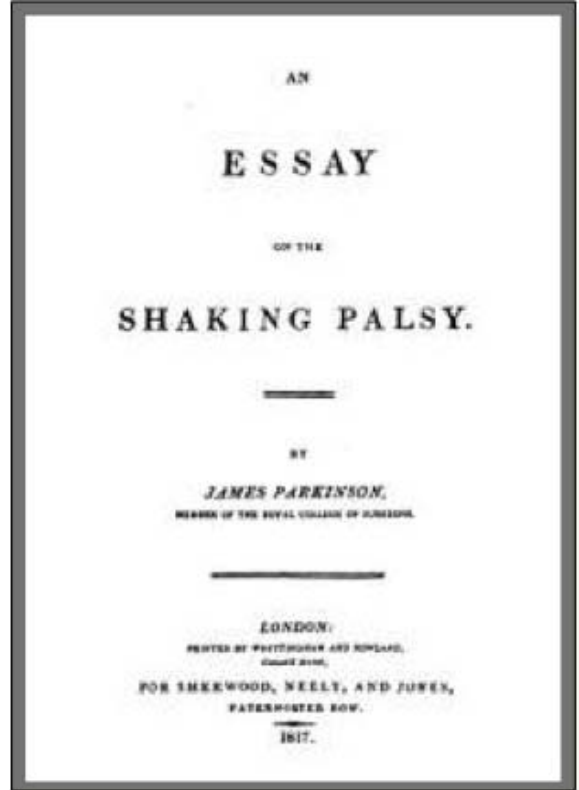
Figure 1.1: The history of Parkinson's disease. (A) shows a statue of Wilhelm von Humboldt (1767-1835) by Friedrich Drake. This statue shows the stooped posture of von Humboldt which is characteristic of PD. von Humboldt also described his other neurological symptoms, including tremor and bradykinesia, in letters (Horowski *et al.,* 1995). (B) James Parkinson wrote "An Essay on the Shaking Palsy" in 1817 (Parkinson, 2002). It was the first description of PD and described six cases that Parkinson observed as patients or on the streets.
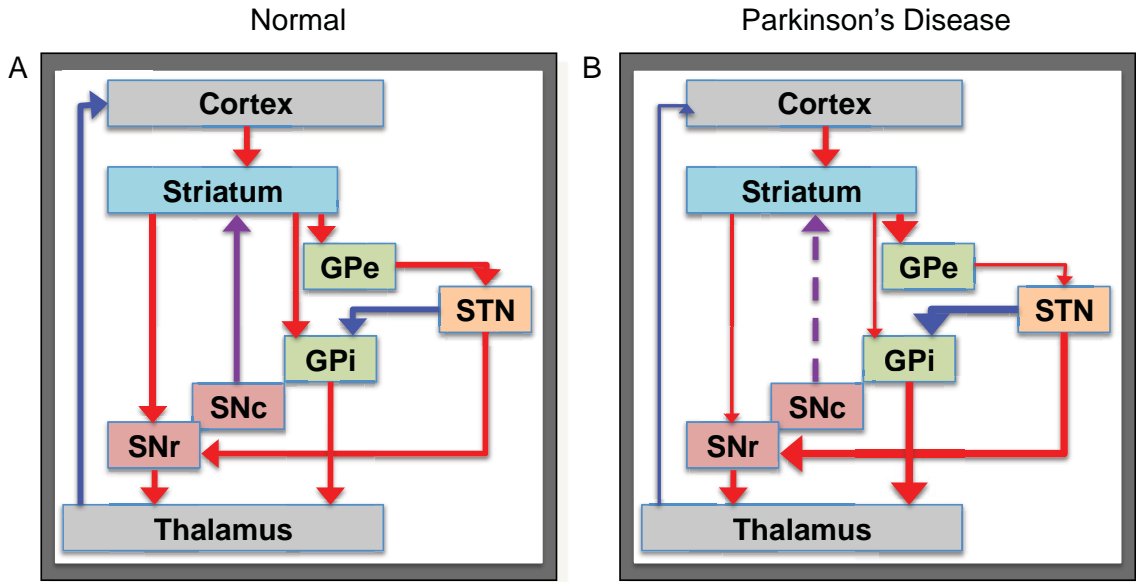
Figure 1.2: Changes in signaling in the basal ganglia in Parkinson's disease. (A) shows the signaling pathway between the neurons of the basal ganglia in normal brain. In Parkinson's disease, the loss of neurons within the substantia nigra result in changes within the signaling pathway (shown in (B)) that result in the observed loss of motor control. Key: Red arrows indicate inhibitory input, blue arrows indicate excitatory input, purple indicates both inhibitory and excitatory input. The thickness of the arrow indicates the relative amount of input. Modified from Wichmann and Dostrovsky, 2011 (SNr - substantia nigra pars reticulata; SNc - substantia nigra pars compacta; GPi - globus pallidus: internal segment; GPe - globus pallidus: external segment; STN -subthalamic nucleus).

Table 1.1: Genetics of Parkinson's disease. The genes that have been linked to familial Parkinson's disease are displayed here along with their position, inheritance pattern, and the type of Parkinsonism that results from each mutation. Modified from Bekris *et al.*, 2010.

PARK Locus	Gene	Map Position	Inheritance	Type of Parkinsonism
PARK1/ PARK4	*SNCA*	4q21	AD	EOPD
PARK2	*Parkin*	6q25.2-q27	AR	Juvenile and EOPD
PARK6	*PINK1*	1p35-p36	AR	EOPD
PARK7	*DJ-1*	1p36	AR	EOPD
PARK8	*LRRK2*	12q12	AD	EOPD
PARK9	*IATP13A2*	1p36	AR	Kufor-Rakeb syndrome

Table 1.2: Current Parkinson's disease treatments. The current therapeutics for PD are displayed here along with the mechanism of each of the treatments (Mayo Clinic, 2012).

Drug Name	Drug Class
Selegiline	MAO-B Inhibitor
Rasagiline	MAO-B Inhibitor
Pramipexole	Dopamine Receptor Agonist
Ropinirole	Dopamine Receptor Agonist
Levodopa	Dopamine Replacement
Entacapone	COMT Inhibitor
Carbidopa	Dopa-decarboxylase Inhibitor
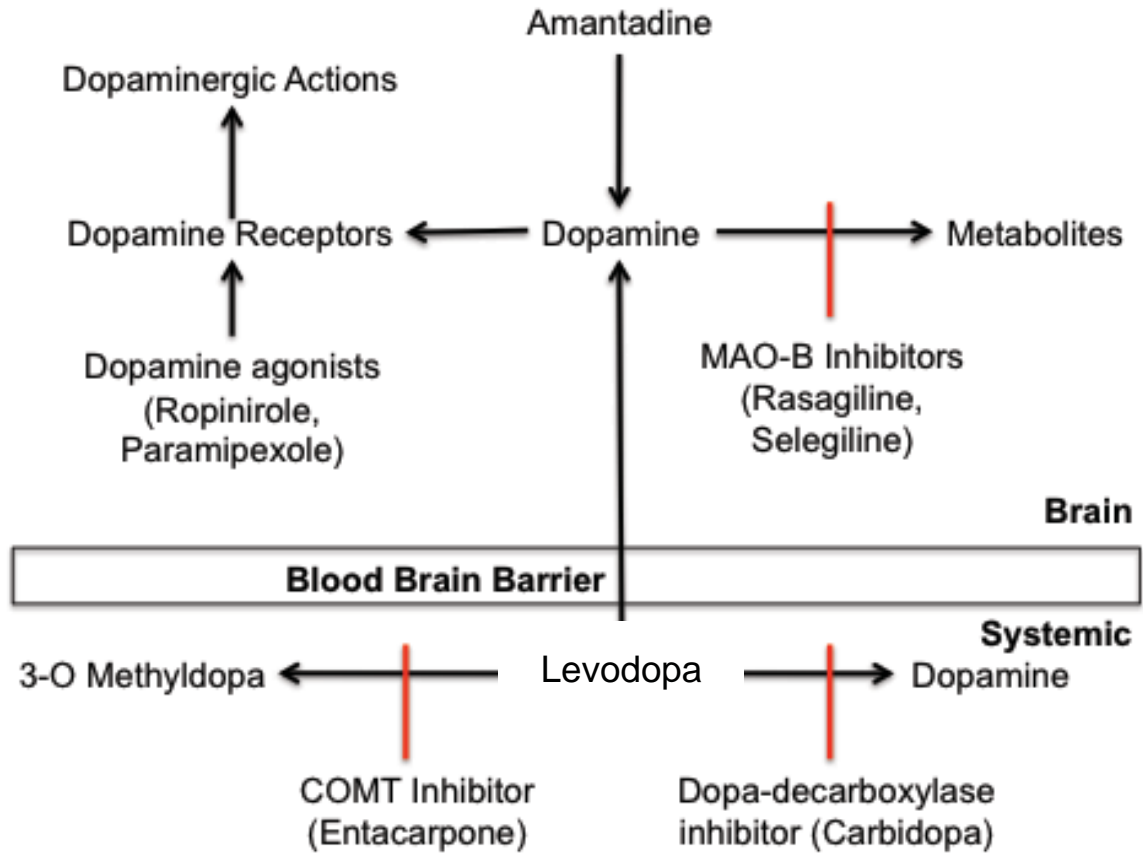
Figure 1.3: Mechanisms of current Parkinson's disease treatments. The current standard treatment for PD is levodopa. Levodopa can be delivered systemically and cross the blood brain barrier. Once it reaches the brain it can be converted to dopamine. In order to prevent levodopa from being converted to dopamine outside of the brain, it is often given in combination with carbidopa, a dopa-decarboxylase inhibitor. Additionally, MAO-B inhibitors, which prevent dopamine from being broken down in the brain, can be used to treat PD. As a replacement for the lost dopamine, dopamine agonists can be used to activate dopamine receptors (Lopex *et al.*, 2001; Factor, 1999).

Table 1.3: Interaction of dopamine agonists with dopamine receptors. The interaction between common dopamine agonists and the dopamine receptors is summarized here. Most of the currently available dopamine agonists interact with the D_2 receptor; however, there is cross talk with other dopamine receptors. Modified from Factor, 1999.

Dopamine Agonist	D1	D2	D3	D4	D5
Dopamine	+	++	+++	++	+++
Bromocriptine	-	++	+	+	+
Pergolide	+	+++	+++	+++	+
Pramipexole	-	+++	+++	++	-
Ropinirole	-	+++	+++	-	-
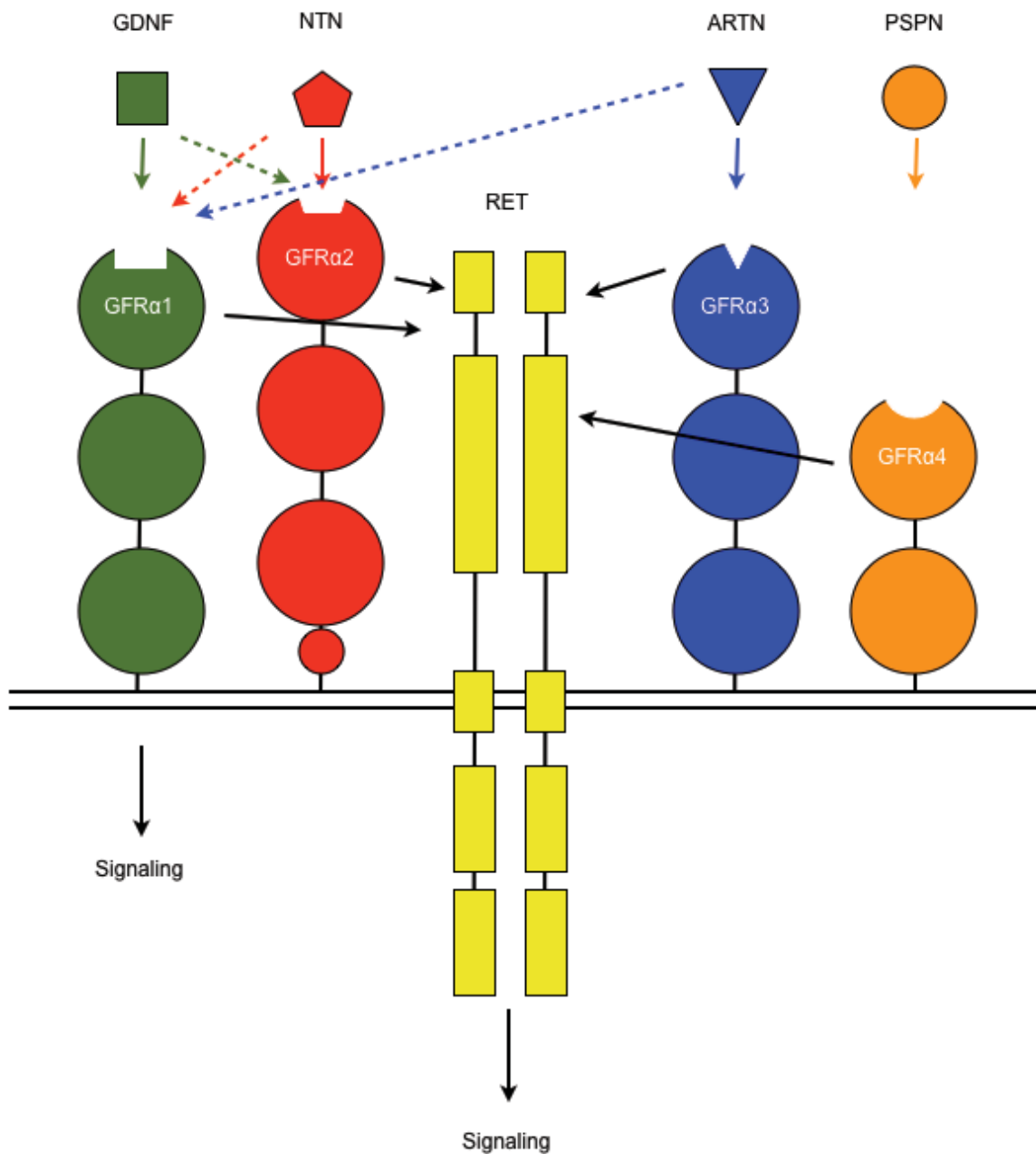
Figure 1.4: GDNF family interactions with GFRα receptors. The GDNF family of proteins interact with the GFRα family of receptors, in addition to the RET receptor, to initiate a variety of intracellular pathways. The interaction of the GDNF proteins is shown here. GDNF has been shown to primarily interact with GFRα1, while interacting with GFRα2 to a lesser extent. NTN preferentially interacts with GFRα2 with some cross talk with GFRα1. ARTN may bind to GFRα1, but predominantly interacts with GFRα3. Finally, PSPN has been shown to only interact with GFRα4 (Saarma, 2000).
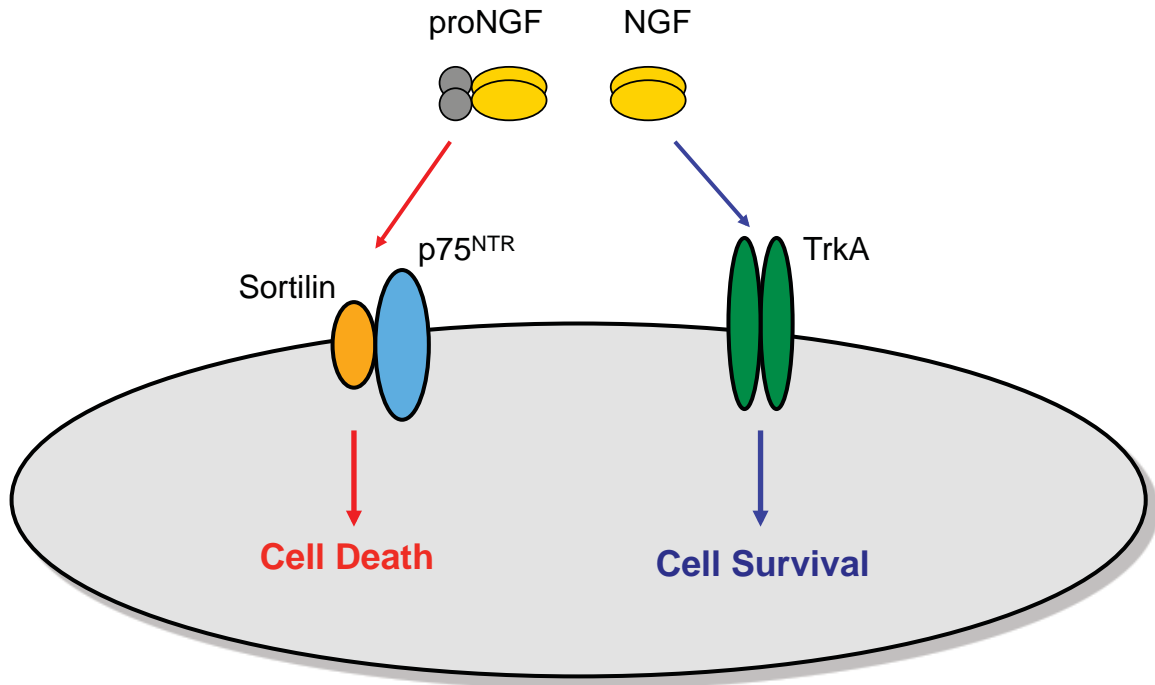
Figure 1.5: NGF and proNGF's roles in cell survival. Mature NGF has been shown to promote cell survival by interacting with the TrkA receptors. However, the proNGF interacts with the a heterodimer consisting of p75^{NTR} and sortilin. When proNGF interacts with this receptor it initiates cell death instead of cell survival. Modified from Nykjaer *et al.*, 2004.
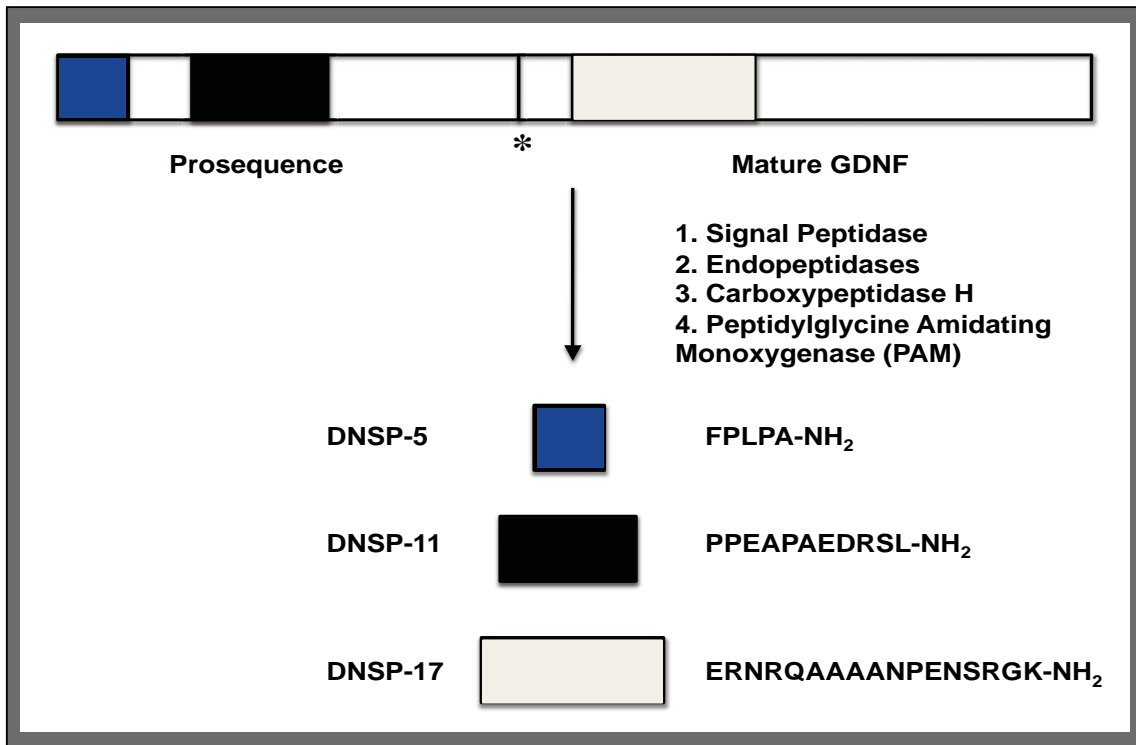
Figure 1.6: Processing of the DNSPs. GDNF is originally produced as a preproprotein. This protein is processed in the rough endoplasmic reticulum and golgi complex for secretion. It is hypothesized that during this processing three amidated peptides of 5, 11, and 17 amino acids in length are produced as a result of the presence of internal endopeptidase sites identified with in the proGDNF sequence (Bradley *et al*, 2010).
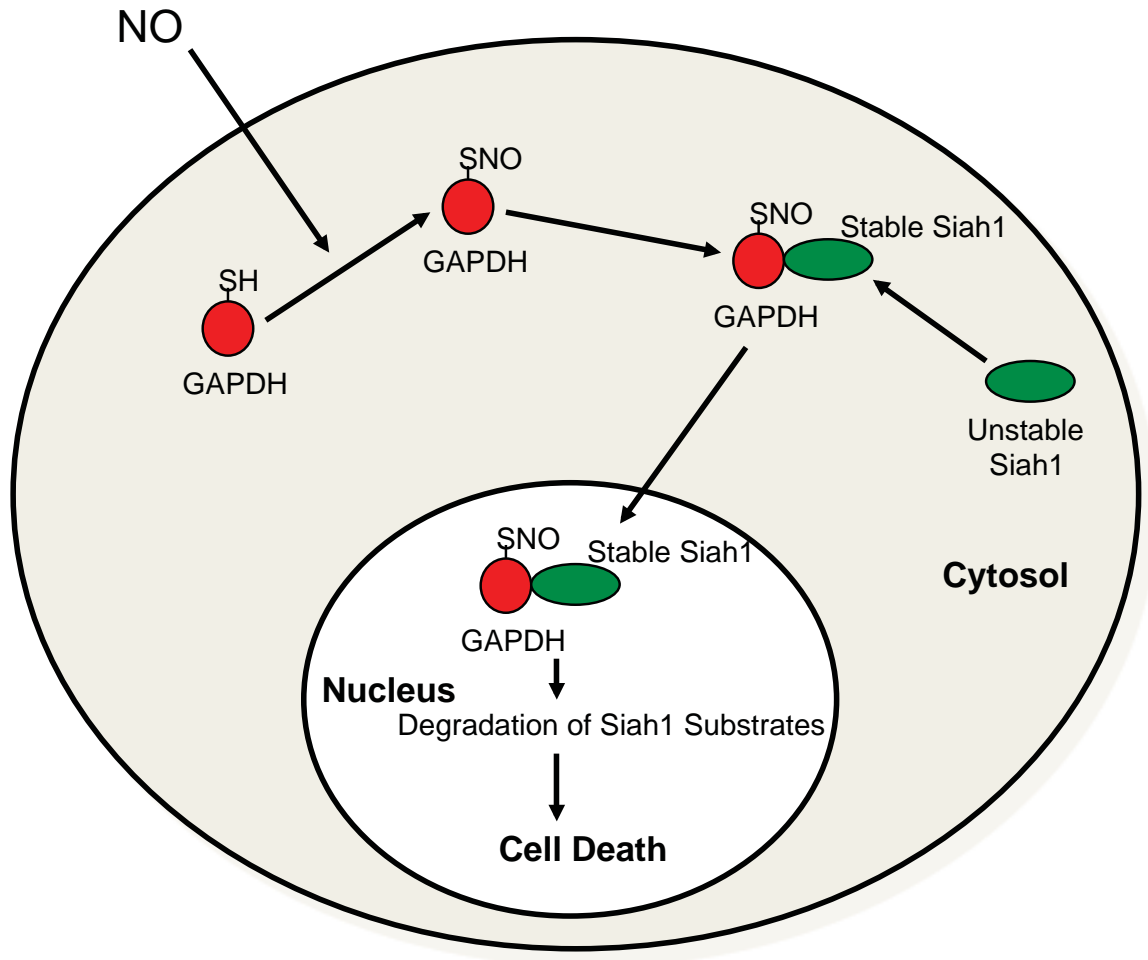
Figure 1.7: Role of GAPDH in mediating apoptosis. It has been observed that GAPDH is involved in mediating an apoptotic pathway. As part of this pathway, GAPDH becomes nitrosylated after which the nitrosylated GAPDH is capable of binding to Siah1, an E3 ubiquitin ligase. The binding of GAPDH to Siah1 stabilizes the normally unstable Siah1. The GAPDH-Siah1 complex then moves into the nucleus, where Siah1 degrades its nuclear targets and initiates apoptosis. Modified from Hara et al., 2005

Chapter Two: Methods

I. Stability study

Individual (0.3 and 1.0 mg/mL) and combination solutions of DNSP-5,

DNSP-11, and DNSP-17 were made in sterile citrate buffer (10 mM Citrate + 150

mM NaCl, pH 5.0). Samples were then stored at -80°C and 37°C for 0, 3, 7, 10,

14, 17, 21, 25, 28, or 31 days. At these intervals, aliquots were analyzed for

degradation using RP-HPLC (Waters Breeze System) with dH2O (HPLC grade)

+ 0.1% trifluoroacetic acid (TFA) as the aqueous mobile phase. Samples were

loaded to a C4 column (4.6 mm 75 mm, 300 Å pore size, GRACE/Vydac

214TP54, Deerfield, IL) at a flow rate of 1 mL/min and the column flow through

was monitored at 214 nm with a Waters 2486 dual-wavelength UV/VIS detector.

Samples were eluted with a linear gradient of the organic mobile phase

(acetonitrile + 0.1% TFA), to a final aqueous:organic phase ratio of 75:25 after 30

min. All solvents were HPLC grade, degassed and filtered prior to use. At 31

days, aliquots were subjected to LC-MS analysis.

II. Far-UV circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed for each purified

peptide sample (DNSP-5, 130 μM; DNSP-11, 21 μM; DNSP-17, 13 μM) in 50 mM

sodium phosphate buffer, pH 7.0. Measurements were made in a 1 mm quartz

cuvette using a Jasco J-810 spectrophotometer. Spectra were recorded as the

average of four far-UV wavelength scans from 250 to 190 nm with 0.5 nm steps and 8 s averaging time.

III. Heparin affinity chromatography

Peptide and GDNF samples (10 μM) in 10 mM sodium citrate, pH 5.6 were loaded to a 1 mL HiTrapTM Heparin HP Column (GE Healthcare) at 1mL/min. Column elutant was simultaneously monitored for peptide/protein (k = 215 nm) and salt concentration using an AKTA Explorer 100 equipped with UV/Vis detector and conductivity monitor. Following column loading and washing, heparin-binding samples were eluted with a high-salt linear gradient (10 mM sodium citrate + 2 M NaCl, pH 5.6). All buffers were freshly prepared, filtered and degassed prior to use.

IV. Caspase-3 activity assay

HEK-293 cells were plated to 100,000 cells/well. Cell cultures were exposed to defined dosages of DNSP-5, DNSP-11, or DNSP- 17 and either 1 μM staurosporine or 8 mM 3-nitropropionate exposure. The Enz Chek (Invitrogen) caspase-3 kit was used to monitor caspase-3 activity. Fluorescence measurements were made after 12 h of treatment (excitation/emission 496/520 nm) using a Molecular Devices Spectramax M5 plate reader. Protein levels of lysed cells were measured by BCA assay (BioRad) and normalized for every experiment. Data are expressed as percent of control and were repeated a minimum of 3 times.

V. Terminal dUTP nick-end labeling (TUNEL)

After treatment with DNSP-11, cells are fixed and labeled to assess degenerative nuclear changes as indicated by the extent of high-molecular weight DNA strand breaks. This was performed by the Apo-BrdU TUNEL Assay (Invitrogen).

VI. Pull down analysis with isolated substantia nigra from young Fischer 344 rats

Young F344 substantia nigra tissue samples are homogenized in Buffer A (20 mM HEPES, pH 7.5, 10% Glycerol, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, Complete™ Mini EDTA Free tablet (for 10 mL; Roche)). Samples are then centrifuged for 30 minutes at 100,000 x g at 4°C, and the supernatant (cytosolic fraction) is collected. Buffer B (Buffer A plus 0.5% Brig 58) is then added to the pellet, mixed thoroughly, and incubated on ice for 15 minutes. The samples are then centrifuged for 30 minutes at 100,000 x g at 4°C. The supernatant (membrane bound fraction) is then collected (pellet discarded). 50 µg of the bDNSP-11 is added to both the cytosolic and membrane bound fractions and incubated for 15 minutes on ice. The samples are then added to 125 µL aliquot of streptavidin magnetic beads (New England Biolabs, Beverly MA) to isolate bound proteins. The beads are extensively washed (three times) in 100 µL Buffer A (cytosolic) or 100 µL Buffer B (membrane bound). Bound proteins will be eluted from the beads with 2x50 µL of Solubilization/Rehydration Solution (7 M Urea, 2 M Thiourea, 50 mM DTT, 4% CHAPS, 1% NP-40, 0.2% Carrier ampholytes, 0.0002% Bromophenol blue) and then once with 100 µL of Solubilzation/

Rehydration Solution. Aliquots were pooled and the proteins are analyzed by 2D-PAGE and identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

VII. *In vitro* pull down of GAPDH

Binding studies were performed using purified samples of GAPDH (Sigma) and bDNSP-11. The two were incubated together in binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA, 50 µM NAD$^+$, 10 mM sodium phosphate, 50 µM Glyceraldehyde-3-phosphate) at 4°C for 1 hour. The sample were then mixed with streptavidin magnetic beads and incubated at 4°C for 1 hour and washed three times with binding buffer. The protein was then removed from the beads with SDS sample running buffer. The samples were separated by SDS-PAGE.

VIII. *In vitro* S-nitrosylation of GAPDH

Based on the procedure described by Jaffrey et al., 2001. Pure GAPDH (Sigma) is pretreated with either S-nitrosoglutathione (GSNO, active NO donor) or glutathione (inactive control). Following incubation at RT in the dark, unreacted GSNO/glutathione is removed by four buffer exchanges into binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA, 50 µM NAD$^+$, 10 mM sodium phosphate, and 50 µM glyceraldehyde-3-phosphate) using a Amicon Ultra 5,000 MWCO spin column.

IX. *In vitro* pull down assay

A solution of 25 µL GFRα1 (1 mg/mL) was incubated with 50 µL of Dynabeads® (Invitrogen) in wash and bind buffer (0.1 M sodium phosphate, pH 8.2, 0.01% Tween® 20) for 10 minutes at room temperature. The beads were then washed three times in 100 µL of wash and bind buffer. 2 µg of GDNF was added and incubated for 1 hour at 4°C. 25 µL GFRα1 (1 mg/mL) was incubated with 40 µg of biotinylated DNSP-11 (bDNSP-11) for 1 hour at 4°C. They were then added to 50 µL of hydrophilic streptavidin magnetic beads (New England Biolabs) and incubated for an hour at 4°C.

X. Western blots

Protein concentrations were determined by performing a Bradford. Samples were then normalized to 30 µg of total protein. The samples were run on a 10% acrylamide gel for 1.5 hours at 200 volts. The gels were then transferred onto PVDF membrane at 200 mA for 45 minutes. Membranes were then blocked with 5% milk for 2 hours at room temperature. After being washed three times for five minutes each with 1X Tris buffered saline with Tween (TSB-T), the membranes were incubated overnight at 4°C with primary antibody. The membranes were then washed three times for five minutes each with 1X TBS-T and incubated with the corresponding secondary antibody for 2 hours at room temperature. Staining was imaged on the Odyssey and the Odyssey software package (LI-COR) was used to calculate the amount of protein present for analysis.

Chapter 3: Evaluation of the physical and *in vitro* protective activity of three

synthetic peptides derived from the pro- and mature GDNF sequence

I. Introduction

Glial cell line-derived neurotrophic factor (GDNF), due to its potent and

specific neurotrophic effects on dopaminergic neurons (Lin *et al.*, 1993), has

been extensively examined as a therapeutic agent for the treatment of age-

related neurodegenerative diseases, such as Parkinson's disease (PD) (Gash *et

al.*, 1996; Gill *et al.*, 2003; Lang *et al.*, 2006; Slevin *et a*l., 2005). However,

GDNF has not advanced beyond phase II clinical trials, primarily due to

challenges attributed to the direct intracranial delivery of large proteins (Gash *et

al.*, 2005; Lang *et al.*, 2006; Patel and Gill, 2007; Salvatore *et al.*, 2006).

Furthermore, GDNF binds heparin with high affinity (Lin *et al.*, 1993, 1994), and

likely other heparin-related molecules abundant in the brain matrix (Rickard *et a*l.,

2003; Sariola and Saarma, 2003), which hinders its predictable biodistribution

following a direct injection (Gash *et al.*, 2005; Lapchak *et al.*, 1998; Patel and Gill,

2007; Piltonen *et al.*, 2009; Salvatore *et al.*, 2006).

While additional delivery strategies have been examined to improve GDNF

delivery and distribution *in vivo*, including convection enhanced delivery (CED)

(Fiandaca *et al.*, 2008; Hamilton *et al.*, 2001; Morrison *et al.*, 2007), co-infusion

with heparin during CED (Hamilton *et al.*, 2001), removal of the GDNF N-terminal

heparin binding domain (Piltonen *et al.*, 2009), viral vector delivery (Kordower *et

al., 2000; Ramaswamy *et al.*, 2009; Wang *et al.*, 2002) and encapsulated GDNF-producing cells (Lindner *et al.*, 1995; Lindvall and Wahlberg, 2008), they have not advanced to clinical applications. The challenges associated with the delivery and distribution of large molecules to the central nervous system (CNS) have led to exploration of alternative approaches. One such approach to circumvent these delivery and distribution challenges would be to utilize small, neurotrophic-like functional molecules.

Recently, it has been hypothesized that functional, carboxy-terminally amidated peptides of 5, 11, and 17 amino acids are processed from the rodent and human GDNF precursor and mature sequences upon proteolytic cleavage by furin-like endopeptidases (Bradley *et al.*, 2010; Immonen *et al.*, 2008). Based on initial studies showing all three peptides possessed some dopaminergic activities, they were named dopamine neuron stimulating peptides (Bradley *et al.*, 2009). While these peptides have not been isolated endogenously to date, initial studies in rat hippocampal CA1 pyramidal neurons showed that the rat 11-mer sequence (named brain excitatory peptide, BEP) significantly induced synaptic excitability, while the 5- and 17-mer sequences failed to show statistical significance (Immonen *et al.*, 2008). Furthermore, we have shown the human 11-mer sequence (named dopamine neuron stimulating peptide-11, DNSP-11) exhibits neurotrophic-like properties including (i) promotion of the survival of primary fetal mesencephalic neurons; (ii) in vitro protection from 6-hydroxydopamine (6-OHDA) in primary mesencephalic and MN9D dopaminergic cell culture; (iii) improving the neurochemical resting levels of dopamine and its

metabolites for up to 28 days following a single injection into the rat substantia nigra; and (iv) significantly improving apomorphine-induced rotational behavior in a severe, PD rat model (Bradley *et al.*, 2010). Collectively, these data support the further characterization and translational evaluation of these peptides as lead therapeutic candidates.

Here data is presented to show the initial *in vitro* physical characterization of DNSP-5, DNSP-11, and DNSP-17. We show that all three peptides are soluble and stable under a variety of conditions in vitro. In addition, DNSP-5 and DNSP-11 are shown to not interact with heparin, which would enhance their *in vivo* biodistribution following delivery to the brain. Finally, it is shown that DNSP-11 offers significant protection, from both staurosporine- and 3-nitropropionate (3-NP)-induced cytotoxicity in HEK-293 cells, supporting the potential for broad beneficial effects on other, non-neuronal cell types. These data provide the basis for future evaluation and development of the dopamine neuron stimulating peptides as a disease modifying therapeutic.

II. Methods

a. *Stability study*

Individual (0.3 and 1.0 mg/mL) and combination solutions of DNSP-5, DNSP-11, and DNSP-17 were made in sterile citrate buffer (10 mM Citrate + 150 mM NaCl, pH 5.0). Samples were then stored at -80°C to 37°C for 0, 3, 7, 10, 14, 17, 21, 25, 28, or 31 days. At these intervals, aliquots were analyzed for degradation using RP-HPLC (Waters Breeze System) with dH2O (HPLC grade)

+ 0.1% trifluoroacetic acid (TFA) as the aqueous mobile phase. Samples were loaded to a C4 column (4.6 mm 75 mm, 300 Å pore size, GRACE/Vydac 214TP54, Deerfield, IL) at a flow rate of 1 mL/min and the column flow through was monitored at 214 nm with a Waters 2486 dual-wavelength UV/VIS detector. Samples were eluted with a linear gradient of the organic mobile phase (acetonitrile + 0.1% TFA), to a final aqueous:organic phase ratio of 75:25 after 30 min. All solvents were HPLC grade, degassed and filtered prior to use. At 31 days, aliquots were subjected to LC-MS analysis.

b. Far-UV circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed for each purified peptide sample (DNSP-5, 130 μM; DNSP-11, 21 μM; DNSP-17, 13 μM) in 50 mM sodium phosphate buffer, pH 7.0. Measurements were made in a 1 mm quartz cuvette using a Jasco J-810 spectrophotometer. Spectra were recorded as the average of four far-UV wavelength scans from 250 to 190 nm with 0.5 nm steps and 8 s averaging time.

c. Heparin affinity chromatography

Peptide and GDNF samples (10 μM) in 10 mM sodium citrate, pH 5.6 were loaded to a 1 mL HiTrapTM Heparin HP Column (GE Healthcare) at 1mL/min. Column elutant was simultaneously monitored for peptide/protein (λ = 215 nm) and salt concentration using an AKTA Explorer 100 equipped with UV/Vis detector and conductivity monitor. Following column loading and washing,

heparin-binding samples were eluted with a high-salt linear gradient (10 mM

sodium citrate + 2 M NaCl, pH 5.6). All buffers were freshly prepared, filtered

and degassed prior to use.

d. Caspase-3 activity assay

HEK-293 cells were plated to 100,000 cells/well. Cell cultures were

exposed to defined dosages of DNSP-5, DNSP-11, or DNSP- 17 and either 1 μM

staurosporine or 8 mM 3-nitropropionate exposure. The Enz Chek (Invitrogen)

caspase-3 kit was used to monitor caspase-3 activity. Fluorescence

measurements were made after 12 h of treatment (λ excitation/λ emission

496/520 nm) using a Molecular Devices Spectramax M5 plate reader. Protein

levels of lysed cells were measured by BCA assay (BioRad) and normalized for

every experiment. Data expressed as percent of control and were repeated a

minimum of 3 times.

III. Results

a. RP-HPLC analysis and long-term stability of the DNSPs

Reverse phase HPLC (RP-HPLC) was used to isolate and purify DNSP-5,

DNSP-11, and DNSP-17 from an aqueous tripeptide mixture solution (Figure

3.1A). The individual DNSPs were separated from an aqueous tripeptide mixture

solution on a C4 reverse phase column with increasing concentrations of

acetonitrile, in an inverse relationship to their size (Table 3.1). The identification

of each well-resolved peak was confirmed by liquid chromatography mass spectrometry (LC-MS) (Table 3.1).

RP-HPLC and LC-MS were used to monitor the stability of DNSP- 5, DNSP-11, and DNSP-17. The peptides were stored in citrate buffer (10 mM Citrate + 150 mM NaCl, pH 5.0) at -80°C and 37°C for 31 days and 4°C for one week. These conditions were chosen based on their relevance to long-term storage and use in future *in vivo* translational studies. The peptides were stable at all temperatures tested, thus allowing for the confident use of the individual peptides for further investigation when stored *in vitro* at these temperatures (Table 3.1, Figure 3.2). Furthermore, LC-MS and amino acid sequencing data confirmed there was no intrinsic degradation within these sequences (i.e. deamidation) upon storage; each peptide sequence was as originally synthesized (Figure 3.2). Additional studies performed at 4°C found no degradation at one week as determined by RP-HPLC.

b. Far-UV circular dichroism structural analysis

The peptide backbone structure of DNSP-5, DNSP-11, and DNSP-17 were examined using circular dichroism (CD) spectroscopy in the far-UV region. All three peptides exhibited a minimum ellipticity value between 196–200 nm, with small spectral signatures observed (shoulders between 208–230 nm), typical of small, soluble peptides of similar length sampling multiple backbone conformations including random coil, turn, polyproline II and α- helix (Figure 3.1B). Additionally, CD was utilized to determine if there are intermolecular

interactions between DNSP-5, DNSP-11, and DNSP-17 *in vitro*. The tripeptide

mixture has similar far-UV CD spectra as the additive spectra of the individual

peptides, with minor differences at 200 nm that are within experimental error

(Figure 3.1B), thus demonstrating that the three peptides do not interact *in vitro*.

c. Heparin binding analysis

We investigated the heparin-binding properties of the DNSPs using heparin

affinity chromatography. Human GDNF binds tightly to the heparin Sepharose

column, eluting at high salt concentrations [0.8 M NaCl] in 10 mM sodium citrate

buffer (pH 5.6), consistent with earlier data (Lin *et al.*, 1994). DNSP-17, the

sequence of which is present within the heparin binding N-terminus of mature

GDNF, binds strongly to the heparin column with an identical elution profile to

mature GDNF (Figure 3.3). However, the peptides derived from the GDNF

prosequence, DNSP-5 and DNSP-11, have no affinity for the heparin column,

eluting in the column flow through (Figure 3.3).

d. Protection from staurosporine and 3-nitropropionate in HEK-293 cells

While DNSP-11 has analogous *in vivo* neurotrophic-like properties, it likely

functions differently than GDNF. We showed that DNSP-11 does not directly

interact with the physiological receptor of mature GDNF, GFRa1 (Bradley *et al.*,

2010). Furthermore, treatment with 10 ng/mL (10 nM) DNSP-11 was shown to

block 1 μM staurosporine-induced cytotoxicity in nutrient-deprived dopaminergic

B65 cells, and its neuroprotective effects included preventing the release of

cytochrome c from mitochondria (Bradley *et al.*, 2010). DNSP-11 proteomic pull down studies identified 16 proteins by MALDI-TOF mass spectrometry, 11 of which possess metabolic functions (Bradley *et al.*, 2010). Collectively, these data support our hypothesis that DNSP-11's neurotrophic effects are mediated through the mitochondria.

To extend this line of investigation, we examined the protective roles of the DNSPs from the activation of caspase-3, a pro-apoptotic protein, in cultured non-neuronal HEK-293 cells. Following 12 h of staurosporine (1 μM) exposure, caspase-3 activity was significantly increased by approximately 80%, consistent with initiation of apoptosis (Figure 3.4A). Between 10 to 100 nM, DNSP-11 provided significant protection (return to control values) from staurosporine-induced activation of caspase-3 (Figure 3.4A). At dosages lower than 10 nM, the protective effects of DNSP-11 were not significant. Both DNSP-5 and DNSP-17 did not provide any significant protection from staurosporine-induced activation of caspase-3 at the tested dosages in the non-neuronal cell line, with the exception of the lowest (1 nM) DNSP-17 dosage (Figure 3.4A).

To determine if the protective effects were specific for mitochondria, we examined the protection afforded by the DNSPs against 3-nitropropionic acid (3-NP) activation of caspase-3 activity in HEK-293 cells. 3-NP is an apoptosis-inducing, mitochondrial specific toxin that irreversibly inhibits succinate dehydrogenase of the Kreb's cycle and complex II of respiration, resulting in mitochondria membrane permeabilization and caspase-3 activation (Beal, 1994; Ludolph *et al.*, 1991; Nasr *et al.*, 2009; Palfi *et al.*, 1996). Exposure of 8 mM 3-

NP to HEK-293 cells for 12 h significantly increased caspase-3 activity approximately 100% over control (Figure 3.4B). At a same concentration (10 nM) that provided staurosporine protection, DNSP-11 provided significant protection from 3-NP induced activation of caspase-3 activity in HEK-293 cells, whereas equimolar concentrations of DNSP-5 and DNSP-17 provided no significant protection (Figure 3.4B).

IV. Discussion

Neurotrophic factors have received considerable attention as potential therapeutic agents for neurodegenerative disorders, including PD. However, because of unsuccessful clinical trials as well as prolonged patent protection/ litigation, the clinical applications of these native molecules have yet to be realized. Large trophic factors, such as GDNF, have inherent pharmacological disadvantages and challenges: they must be delivered to the CNS by invasive procedures. Thus, smaller molecules like the DNSPs, which are relatively easy to synthesize and modify to improve bioavailability, have the potential for more widespread use in the clinic (Thorne and Frey, 2001).

Because of the stringent requirements of biotherapeutics for *in vitro* stability and solubility, the DNSPs were evaluated for these properties (Bell, 1997; Powell, 1994). RP-HPLC and LC-MS data showed that each of the DNSPs were stable at -80°C and 37°C for one month without any appreciable loss or intrinsic modification of the peptides. Other common storage and experimental temperatures tested had similar results, supporting that the DNSPs remain stable

for long-term storage and delivery, such as an internal implanted pump stored in the abdominal cavity holding one month's supply of peptide. Additional evidence supports long term storage of the peptide at -80°C and shows that peptides still retain their potency and protection after storage for up to 2 years (Bradley *et al.*, unpublished data). Thus, the DNSPs are stable *in vitro*, which is essential if they are to be used in clinical applications.

It was hypothesized that the processed DNSPs might have endogenous/physiological function. It is possible that the DNSPs form an intermolecular complex for bioactivity. We show that the far-UV CD spectra of the individual DNSPs are an additive for the tripeptide mixture, thereby suggesting that these peptides do not interact *in vitro*. These spectra show dynamic structural characteristics that would be expected of small, soluble peptides of 5, 11, and 17 amino acids. Furthermore, this data shows no signs of aggregation of the peptides, which could lead to complications with delivery and potentially activate an immune response.

To gain insight into the biodistribution properties of the DNSPs, we examined their heparin binding properties by affinity chromatography. Heparin binding has been shown to limit the biodistribution of GDNF, thereby affecting its therapeutic targeting following an intracranial injection (Gash et al., 2005; Lapchak *et al.*, 1998). Unlike mature GDNF, both DNSP-5 and DNSP-11 do not bind heparin (Figure 3.3), thus suggesting that the GDNF prosequence-derived peptides would have enhanced volume of distribution properties when delivered intracranially. Although the apparent lack of heparin binding might make it

difficult to control the diffusion of these peptides into non-targeted/undesired regions of the brain, our previous immunohistochemical staining data with DNSP-11 showed rapid uptake into neurons found both in the substantia nigra, pars reticulata and substantia nigra, pars compacta within 30 min following a direct injection (Bradley *et al.*, 2010). Furthermore, increased resting levels of dopamine and its metabolites were observed for up to 28 days in normal and 6-OHDA-lesioned Fischer 344 rats following a single injection of DNSP-11 into the substantia nigra, with no adverse effects observed under these conditions (Bradley *et al.*, 2010). *In vivo* studies with DNSP-5 are currently ongoing.

Heparin affinity chromatography of DNSP-17, an amidated 17 amino acid sequence putatively derived from amino acids 13–29 within the N-terminal region of mature GDNF, exhibited an equal binding profile as GDNF (Figure 3.3). These data are consistent with earlier studies, which have demonstrated that heparin binding activity is located within the GDNF N-terminal domain, however, the residues primarily responsible for heparin binding are unresolved (Alfano *et al.*, 2007; Parkash *et al.*, 2008; Piltonen *et al.*, 2009). Complete removal of the highly-basic, 38 amino acid residue N- terminal domain resulted in an elimination of heparin binding and increased GDNF biodistribution *in vivo* (Piltonen *et al.*, 2009). Recent data from the crystal structure of the GDNF-GFRa1 complex, show basic GDNF residues Arg35, Lys37, and Arg39 to be interacting with sucrose octasulfate, a heparin mimic (Parkash *et al.*, 2008). However, GDNF residues 1–34 were unresolved in the receptor-complex crystal structure (Parkash *et al.*, 2008) to rule out additional binding sites. In an earlier study,

ELISA and affinity chromatography data showed that removal of the N-terminal domain residues 24–39 (ND2) resulted in a weakening of heparin binding, whereas deletion of residues 4–23 (ND1) had little change relative to full-length GDNF (Alfano *et al.*, 2007). Given DNSP-17's strong heparin-binding properties, this suggests that a significant portion of the N-terminal GDNF heparin binding domain is provided by this sequence, likely the two dibasic BXB clusters at the N- and C-terminal ends of the peptide sequence (corresponding to mature GDNF residues Arg14, Arg16, Arg27, and Lys29). Thus, DNSP-17 could provide a further tool towards understanding of the unresolved role of heparin binding and GDNF signaling (Alfano *et al.*, 2007; Piltonen *et al.*, 2009). Additionally, DNSP-17 may serve as a co-infusate with GDNF to improve its distribution following a direct injection or CED.

DNSP-11 appears to be a functional proGDNF-derived peptide. Initial studies of the rat homolog of DNSP-11, BEP, showed a significant increase in synaptic excitability of rat CA1 pyramidal neurons, as well as broad-binding within the adult rat brain (Immonen *et al.*, 2008). Additional studies demonstrated that DNSP-11 exhibits neurotrophic-like effects *in vivo*, including long-lived increases in rat resting dopamine levels following a single nigral injection (Bradley *et al*., 2010). Furthermore, DNSP-11 was shown to be protective at a single dose from 6-OHDA, staurosporine and gramicidin cytotoxins in dopaminergic cell lines (Bradley *et al.*, 2010). Consistent with these prior observations, DNSP-11 provides significant dose-dependent protection from staurosporine and 3-NP in the non-neuronal, HEK-293 cell line (Figure 3.4A, B). While staurosporine is a

broad-based cytotoxin, 3-NP targets succinate dehydrogenase of the respiratory complex II of the mitochondrial electron transport chain, supporting that DNSP-11's protective mechanism of action involves mitochondria. These data are further supported by recent findings showing protection from specific mitochondrial toxins (Turchan-Cholewo *et al.*, submitted).

Unlike DNSP-11, DNSP-5 did not provide significant protection from staurosporine and 3-NP in HEK-293 cells. In addition, DNSP-17 showed limited protection from staurosporine (only at 1 nM) and failed to produce 3-NP protection. Previous work further demonstrated that the DNSP-5 and the DNSP-17 peptide sequences failed to produce significant neuronal excitability (Immonen *et al.*, 2008). Collectively, these early findings support a limited mitochondrial protective role for DNSP-5 and DNSP-17. However, given the findings with DNSP-11, further studies are warranted to establish the possible functional roles of these relatively unexplored sequences.

V. Conclusion

The emergence of naturally occurring, physiologically functional propeptides from the neurotrophic factor family provides a wealth of untapped sequences for exploration and evaluation. As these newly characterized peptides undergo further therapeutic evaluation, it is necessary to conduct studies with molecules characterized under a variety of experimental and storage conditions for reproducibility and translation. Here we show that the DNSPs are inherently stable and soluble under these conditions. Of the three peptides, DNSP-5 and

66

DNSP-11 do not bind heparin, which would facilitate their biodistribution

properties when delivered in the brain. Finally, we show that DNSP-11 exhibits

protection from the cytotoxins staurosporine and 3-NP, in HEK-293 cells,

supporting a potentially broad role as a disease altering therapeutic.
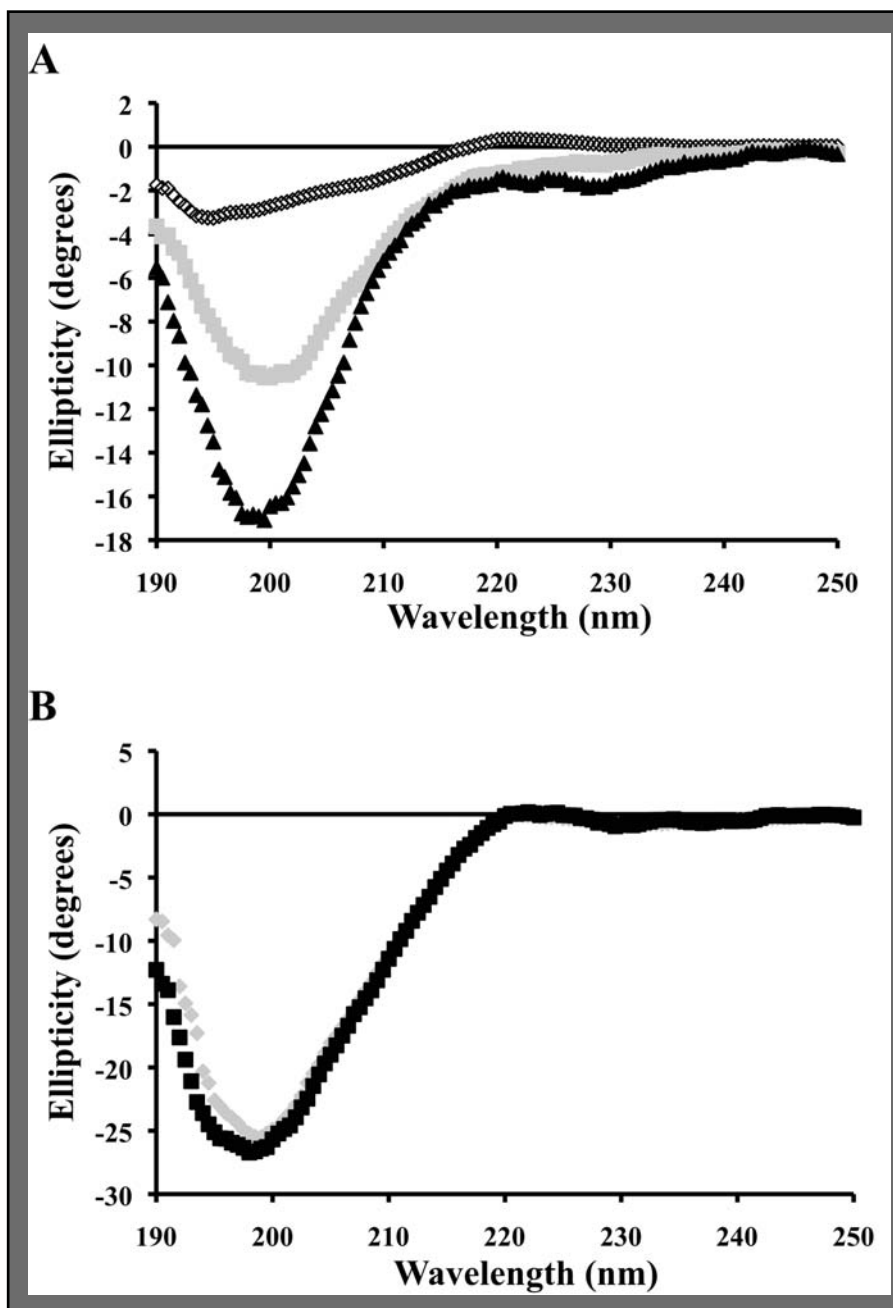
Figure 3.1: Physical characterization of the DNSPs. (A) A tripeptide mixture of DNSP-5, DNSP-11, and DNSP-17 was loaded to a C4 column in dH2O and 0.1% TFA. After ten minutes of wash (1 mL/min), samples were eluted with a linear gradient (red) of the organic mobile phase (acetonitrile + 0.1% TFA), to a final aqueous:organic phase ratio of 75:25 after 30 minutes. Elution of the peptides was monitored at 214 nm. (B) Far-UV CD analysis of DNSP-5 (open circles), DNSP-11 (gray squares), and DNSP-17 (back triangles) shows that the peptides have backbone characteristics of small, soluble peptides (Kelps *et al.*, 2011).

Table 3.1: Characterization of the DNSPs *in vitro* stability after incubation at 37°C for 31 days. Integration of the RP-HPLC elution peaks for each of the DNSPs were used to calculate the percent of peptide remaining after incubation at 37 °C for 31 days. These samples were then submitted to mass spectrometry, in which the determined molecular weight following extended incubation was in agreement with the calculated sequence molecular weight (Modified from Kelps *et al.*, 2011).

Propeptide	Retention Time	% Acetonitrile	Calculated Molecular Weight	Molecular Weight After 30 Days (37°C)	% Remaining After 30 Days (37°C)
DNSP-5	24.4	19.4%	542	542.1	93.6%
DNSP-11	20.2	15.2%	1180	1180.3	97.6%
DNSP-17	15.5	10.5%	1868	1868.1	94.6%
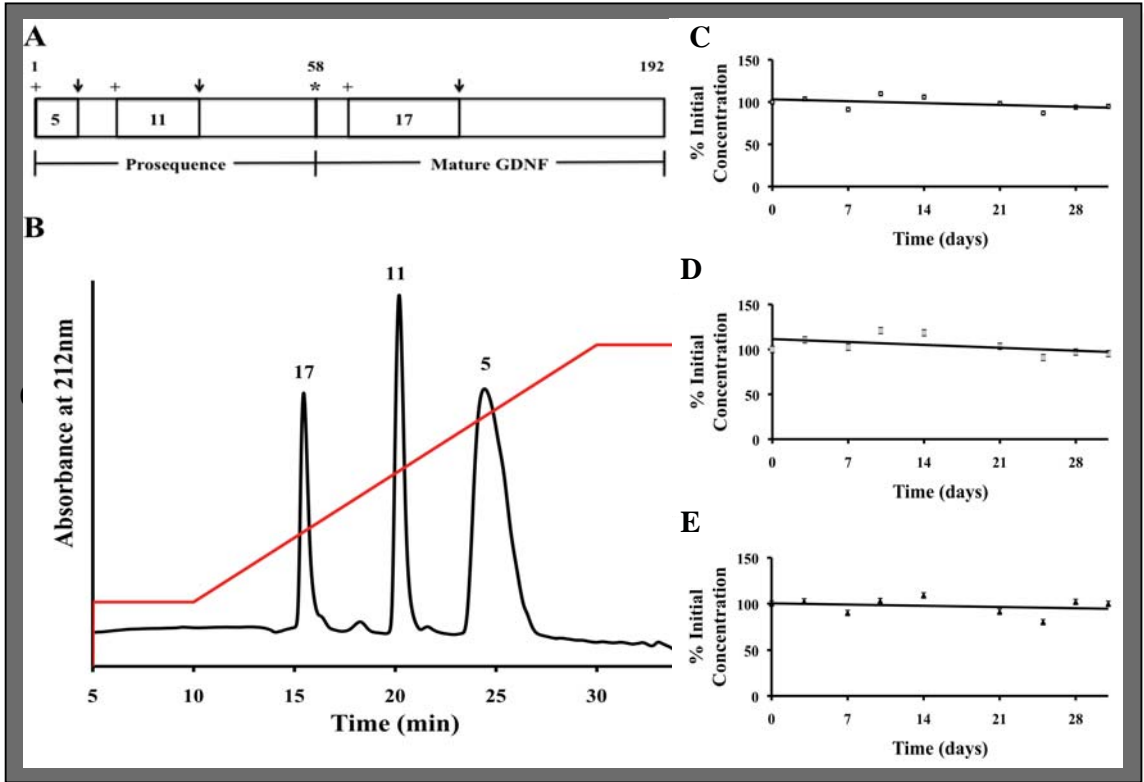
Figure 3.2:The CD analysis of the stability of the DNSPs *in vitro*. (A) shows the locations of the sequences of the proposed DNSPs. (B) shows an HPLC purification of DNSP-5, 11, and 17. The DNSP-5 was shown to be stable (C) for up to one month at 37°C by HPLC analysis. The DNSP-11 was shown to be stable (D) for up to one month at 37°C by HPLC analysis. The DNSP-17 was shown to be stable (E) for up to one month at 37°C by HPLC analysis (Modified from Kelps *et al.*, 2011).
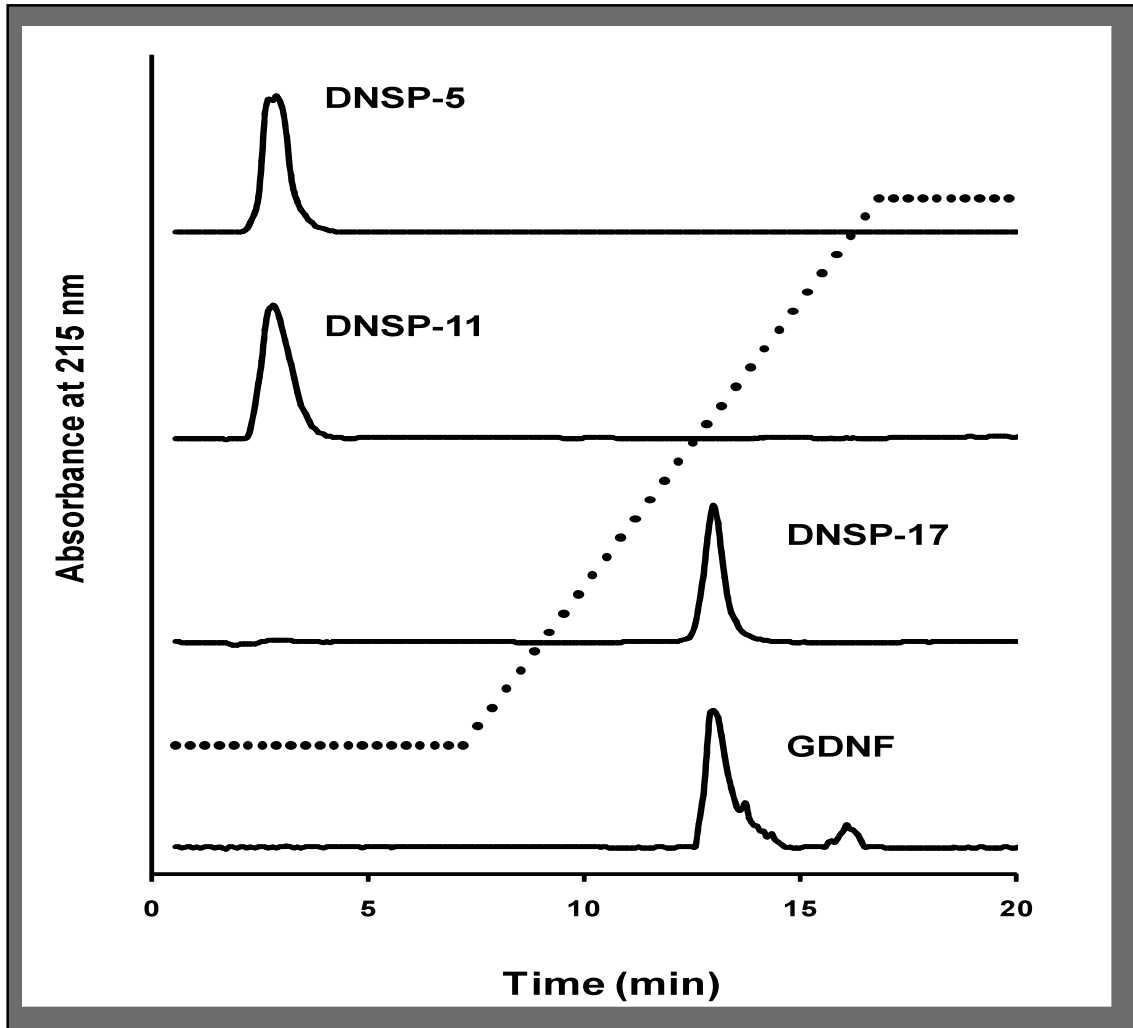
Figure 3.3: Heparin affinity chromatography of the DNSPs and GDNF. 10 μM samples of the synthetic DNSPs and GDNF (in 10 mM sodium citrate, pH 5.6) were applied to a HiTrap™ Heparin HP affinity column and elutant was monitored at 215 nm. After sample loading and wash, a high salt (10 mM sodium citrate + 2 M NaCl) linear gradient was applied (dashed line) to elute heparin-binding samples (Modified from Kelps *et al.*, 2011).
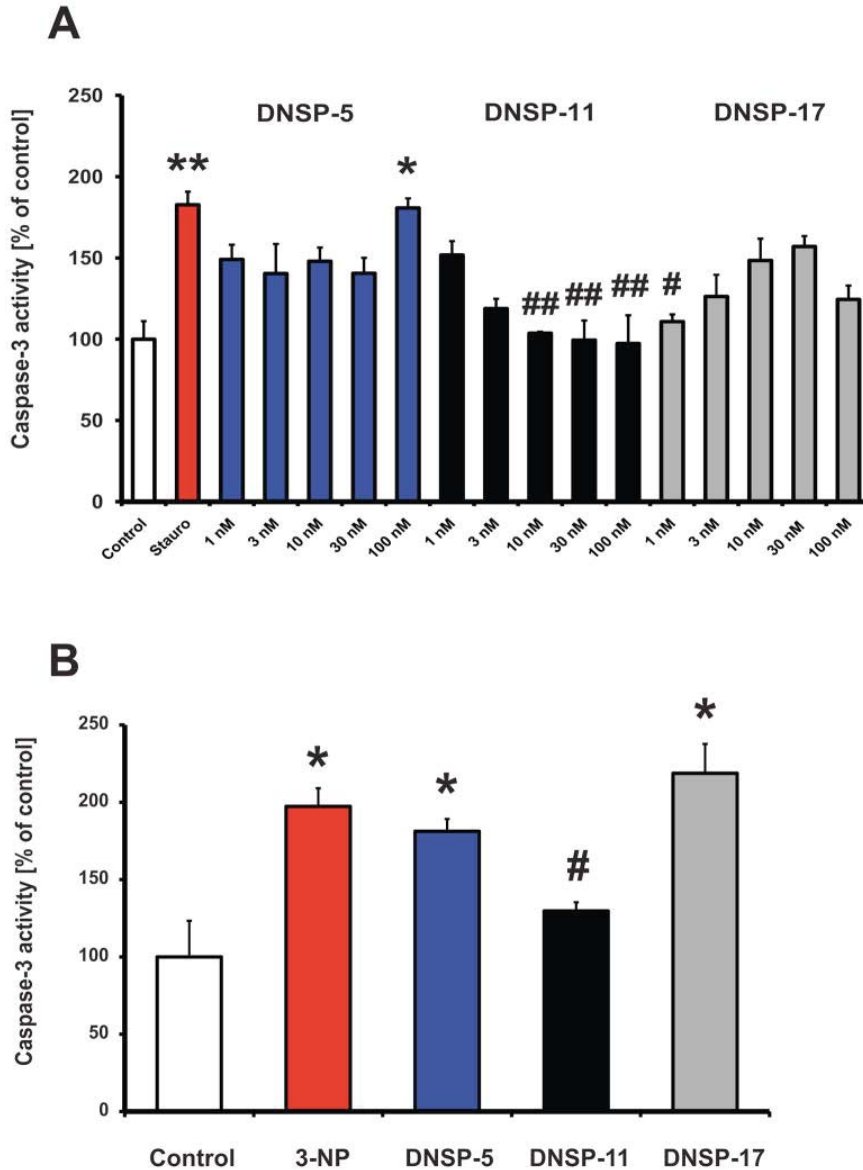
Figure 3.4: Protective effects of the DNSPs. (A) Dose responses (1 nM – 100 nM) for DNSP-5 (blue bars), DNSP-11 (black bars), and DNSP-17 (gray bars) protection from 1 µM staurosporine-induced cytotoxicity (red bar), were measured by caspase-3 activity 12 hours after treatment in HEK-293 cells. Stauro-staurosporine. (B) Protection from 8 mM 3-nitropropionate (3-NP) was measured by caspase-3 activity assay 12 hours after treatment with 10 nM treatment with the DNSPs. For both experiments, the control (open bar) was citrate buffer alone. One-way ANOVA was used to test for significance amongst groups, followed by Tukey's post-hoc analysis (*p<0.05, **p<0.01 vs control; #p<0.05, ##<0.0172 vs toxin) (Modified from Kelps *et al.*, 2011).

Chapter 4: Role of GAPDH in DNSP-11 protection

I. Introduction

Mature glial cell line-derived neurotrophic factor (GDNF) has been shown to provide neuroprotective and neurorestorative effects in cellular and animal models of Parkinson's disease (PD). Based on the observed protective effects of GDNF, it advanced to clinical trials, but was not able to advance beyond that point, partially because of problems associated with using large molecule biotherapeutics, including production and delivery of these molecules to the brain.

GDNF, which was originally identified in 1993 (Lin *et al.*, 1993), is a member of the transforming growth factor-β (TGF-β) superfamily. Mature GDNF has been shown to exhibit trophic effects on embryonic dopaminergic neurons (Tomac *et al.*, 1995), spinal motor neurons (Henderson *et al.*, 1994), and central noradrenergic neurons (Arenas *et al.*, 1995). GDNF has been shown to provide its observed protective effects primarily through its interaction with the receptor GDNF family receptor α1 (GFRα1) (Jing *et al.*, 1996; Treanor *et al.*, 1996). After GDNF binds to GFRα1, it activates the receptor tyrosine kinase (RTK), Ret (Jing *et al.*, 1996; Treanor *et al.*, 1996; Trupp *et al.*, 1996). Through the interactions of GDNF with GFRα1 and Ret, GDNF is able to support cell survival by reducing the observed pro-apoptotic activity of Ret when it is not in the presence of GDNF (Bordeaux *et al.*, 2000).

While GDNF has been shown to provide neuroprotective and neurorestorative effects in some models of PD, these protective effects have not been universally observed in all models of the disease. Studies have shown that preadministering GDNF is not able to reduce α–synuclein-induced death of dopaminergic neurons (Decressac et al., 2011). Treatment with GDNF was also unable to improve behavior in this PD model (Decressac et al., 2011).

Thus, alternative therapies that provide neuroprotection, while working via a nonGFRα1/RET pathway might be needed to treat certain forms of PD. Small molecules with similar proposed therapeutic potential have been evaluated as possible alternatives to GDNF. Three peptides of 5, 11, and 17 amino acids in length were identified as potential endogenous processing products (Bradley et al., 2010). Preliminary evaluations of these three peptides show similar functional effects as mature GDNF, thus making them viable candidates for further development as a potential PD therapeutic. The peptides have been shown to increase neurite outgrowth in primary mesocephalic cell culture models, as well as positively affect the behavior in a young Fisher 344 rat model of dopaminergic dysfunction (Bradley et al., 2010). Treatment with DNSP-11 has been shown to increase basal dopamine (DA), dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) in Fisher 344 rats (Fuqua, 2010). While the cellular mechanism of these peptide sequences is unknown, these results coupled with the functional data from other neurotrophic factor prosequences, strongly suggest that the GDNF family of prosequences exhibit in vivo functions. Based on the observed anti-apoptotic effects in cellular models of DNSP-11, it

was hypothesized that DNSP-11 functioned through a similar mechanism to mature GDNF (Bradley *et al.,* 2010).

The mechanism by which DNSP-11 is able to provide its observed protective effects and antiparkinsonian properties is not known. Here, that mechanism is investigated. Since DNSP-11 was derived from the sequence of GDNF and had been observed to support cell survival similar to mature GDNF (Bradley *et al.,* 2010), initial studies looking at DNSP-11's potential interactions with GFRα1 were conducted. These initial binding studies did not indicate an interaction between DNSP-11 and GFRα1 (Bradley *et al.,* 2010). Based on the lack of evidence to support DNSP-11 functioning through the same mechanism as mature GDNF, proteomic studies were carried out in order to identify other potential binding partners. In so doing, several potential binding partners were identified. One of these binding partners was glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

GAPDH is an enzyme that catalyzes the phosphorylation of glyceraldehyde-3-phosphate – a process requiring inorganic phosphate and nicotinamide adenine dinucleotide (NAD) - during carbohydrate metabolism. Because of GAPDH's role in carbohydrate metabolism, it is abundant in the cytoplasm of all cells. The abundance of GAPDH makes it suitable for many cellular assays as a control for protein levels within the cell.

GAPDH has previously been shown to be involved in mediating an apoptotic pathway (Hara, M.R. *et al.,* 2005). Under conditions of cellular stress, GAPDH has been shown to become nitrosylated. This observed nitrosylation

occurs on cysteine 150 (Padgett, C.M. and Whorton A.R., 1995). Once this

nitrosylation occurs, GAPDH is no longer metabolically active, leaving two

distinct pools of GAPDH in stressed cells. The nitrosylated GAPDH can then

interact with and bind to Siah-1, an E3 ubiqutin ligase. When Siah-1 is not bound

to GAPDH, Siah-1 is normally unstable and so it is quickly degraded within the

cell (Hara, M.R. *et al.,* 2005). However, binding to nitrosylated GAPDH stabilizes

Siah-1 and prevents it from being quickly degraded, allowing it to have prolonged

effects. GAPDH lacks a nuclear localization signal, so it normally is not found

within the nucleus. Conversely, Siah-1 possesses a nuclear localization signal

and can therefore move into the nucleus. Therefore, when nitrosylated GAPDH

binds to Siah-1, it is able to move into the nucleus. Since the binding of

nitrosylated GAPDH helps to stabilize Siah-1, it is able to degrade its nuclear

targets for an extended period of time (Hara, M.R. *et al.,* 2005). This leads to

activation of an apoptotic pathway.

Based on the observed interaction between DNSP-11 and GAPDH and

GAPDH's role in apoptosis, it was hypothesized that DNSP-11's protective

mechanism are through a non-GFRα1 mediated mechanism and further

hypothesized that GAPDH plays a role in this protection. The effect of DNSP-11

on GAPDH nitrosylation was determined by looking at levels of nuclear GAPDH,

which is elevated after nitrosylation has occurred. In addition to looking at

nuclear GAPDH levels, GAPDH activity assays were used to determine if

changes in nitrosylation levels occurred after treatment of GAPDH with DNSP-11.

Presented here is the initial investigation regarding the cellular mechanism of DNSP-11's anti-apoptotic effects. Also, data evaluating the possible interactions between DNSP-11 and GFRα1 as a potential pathway through which DNSP-11 offers anti-apoptotic activity is presented. DNSP-11's role in a feedback mechanism resulting in an upregulation of GDNF mRNA and protein is also investigated. Finally, a proteomic screen of potential binding partners in which GAPDH, a protein known to play a role in apoptosis, is presented. Interactions between DNSP-11 and the GAPDH apoptotic pathway were further evaluated to determine if this is a mechanism by which DNSP-11 may be providing some anti-apoptotic effects.

II. Methods

a. Caspase-3 activity assay

HEK-293 cells were plated to 100,000 cells/well. Cell cultures were exposed to defined dosage of DNSP-11 and either 1 μM staurosporine or 8 mM 3-nitropropionate exposure. The Enz Chek (Invitrogen) Caspase-3 kit was used to monitor levels of caspase-3 activity. Fluorescence measurements were made after 3 hours of treatment (excitation/emission 496/520 nm) using a Molecular Devices Spectramax M5 plate reader. Protein levels of lysed cells were measured by BCA assay (BioRad) and normalized for every experiment. Data are expressed as percent of control and were repeated a minimum of 3 times.

b. Terminal dUTP nick-end labeling (TUNEL)

After treatment with DNSP-11, cells are fixed and labeled to assess degenerative nuclear changes as indicated by the extent of high-molecular weight DNA strand breaks. This was performed by the Apo-BrdU TUNEL Assay (Invitrogen).

c. Pull down analysis with isolated substantia nigra from young Fischer 344 rats

Young F344 substantia nigra tissue samples are homogenized in Buffer A (20 mM HEPES, pH 7.5, 10% Glycerol, 2 mM EGTA, 2 mM EDTA, 1 mM DTT, Complete™ Mini EDTA Free tablet (for 10 mL; Roche)). Samples are then centrifuged for 30 minutes at 100,000 x g at 4°C, and the supernatant (cytosolic fraction) is collected. Buffer B (Buffer A plus 0.5% Brig 58) is then added to the pellet, mixed thoroughly, and incubated on ice for 15 minutes. The samples are then centrifuged for 30 minutes at 100,000 x g at 4°C. The supernatant (membrane bound fraction) is then collected (pellet discarded). 50 μg of the bDNSP-11 is added to both the cytosolic and membrane bound fractions and incubated for 15 minutes on ice. The samples are then added to 125 μL aliquot of streptavidin magnetic beads (New England Biolabs, Beverly MA) to isolate bound proteins. The beads are extensively washed (three times) in 100 μL Buffer A (cytosolic) or 100 μL Buffer B (membrane bound). Bound proteins will be eluted from the beads with 2x50 μL of Solubilization/Rehydration Solution (7 M Urea, 2 M Thiourea, 50 mM DTT, 4% CHAPS, 1% NP-40, 0.2% Carrier ampholytes, 0.0002% Bromophenol blue) and then once with 100 μL of Solubilzation/

Rehydration Solution. Aliquots were pooled and the proteins are analyzed by 2D-PAGE and identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

d. In Vitro Pull Down of GAPDH

Binding studies were performed using purified samples of GAPDH (Sigma) and bDNSP-11. The two were incubated together in binding buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA, 50 µM NAD^+, 10 mM sodium phosphate, 50 µM Glyceraldehyde-3-phosphate) at 4°C for 1 hour. The sample were then mixed with streptavidin magnetic beads and incubated at 4°C for 1 hour and washed three times with binding buffer. The protein was then removed from the beads with SDS sample running buffer. The samples were separated by SDS-PAGE.

e. In vitro S-Nitrosylation of GAPDH

Based on the procedure described by Jaffrey et al., 2001. Pure GAPDH (Sigma) is pretreated with either S-nitrosoglutathione (GSNO, active NO donor) or glutathione (inactive control). Following incubation at RT in the dark, unreacted GSNO/glutathione is removed by four buffer exchanges into binding buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1 mg/ml BSA, 50 µM NAD^+, 10 mM sodium phosphate, and 50 µM glyceraldehyde-3-phosphate) using a Amicon Ultra 5,000 MWCO spin column.

f. In vitro pull down assay

A solution of 25 µL GFRα1 (1 mg/mL) was incubated with 50 µL of Dynabeads® (Invitrogen) in wash and bind buffer (0.1 M sodium phosphate, pH 8.2, 0.01% Tween® 20) for 10 minutes at room temperature. The beads were then washed three times in 100 µL of wash and bind buffer. 2 µg of GDNF was added and incubated for 1 hour at 4°C. 25 µL GFRα1 (1 mg/mL) was incubated with 40 µg of biotinylated DNSP-11 (bDNSP-11) for 1 hour at 4°C. They were then added to 50 µL of hydrophilic streptavidin magnetic beads (New England Biolabs) and incubated for an hour at 4°C.

g. Western blots

Protein concentrations were determined by performing a Bradford. Samples were then normalized to 30 ug of total protein. The samples were run on a 10% acrylamide gel for 1.5 hours at 200 volts. The gels were then transferred onto PVDF membrane at 200 mA for 45 minutes. Membranes were then blocked with 5% milk for 2 hours at room temperature. After being washed three times for five minutes each with 1X Tris buffered saline with Tween (TSB-T), the membranes were incubated overnight at 4°C with primary antibody. The membranes were then washed three times for five minutes each with 1X TBS-T and incubated with the corresponding secondary antibody for 2 hours at room temperature. Staining was imaged on the Odyssey and the Odyssey software package (LI-COR) was used to calculate the amount of protein present for analysis.

III. Results

a. DNSP-11 reduces apoptosis

DNSP-11 has been shown to promote the survival of primary fetal mesencephalic neurons, protect from 6-hydroxydopamine (6-OHDA) toxicity in primary mesencephalic and MN9D dopaminergic cell culture, improve the neurochemical resting levels of dopamine and its metabolites for up to 28 days following a single injection into the rat substantia nigra, and significantly improve apomorphine-induced rotational behavior in a severe, PD rat model (Bradley *et al.*, 2010). Based on these data, the cellular protective effects of DNSP-11 were compared to that of GDNF through the use of two different measures of apoptosis. Levels of apoptosis after treatment with 100 uM 6-OHDA and either DNSP-11 and GDNF were measured by both a TUNEL assay and a caspase-3 activity assay.

The TUNEL assay showed that treatment with 100 uM 6-OHDA significantly increased the amount of TUNEL staining that was observed. This increase in TUNEL staining indicates an increase in apoptosis as measured by an increase in DNA fragmentation that results from apoptosis. However, both 1 ng/mL of DNSP-11 and GDNF were able to significantly reduce the quantity of TUNEL staining after a 24 hour treatment, indicating that DNSP-11 is able to protect the cells from apoptosis at similar levels to GDNF (Figure 4.1A). These results, along with data from *in vivo* studies, support the hypothesis that DNSP-11 has a similar effect to mature GDNF. This data was confirmed by caspase-3 activity assay data. The caspase-3 activity assay also showed a

significant increase in the caspase-3 activity after treatment with 100 uM 6-OHDA indicating an increase in apoptosis. After treatment with both 1 ng/mL DNSP-11 and GDNF for 3 hours, levels of caspase-3 activity were significantly reduced (Figure 4.1B). However, DNSP-11 did not reduce apoptosis as measured by caspase-3 activity at the same level as GDNF.

b. DNSP-11 does not bind GFRα1

Based on the similarities that were observed between DNSP-11's ability to protect against 6-OHDA induced apoptosis and the protection offered by mature GDNF, the interaction between DNSP-11 and GFRα1 was evaluated in order to determine if DNSP-11 is functioning through the same mechanism as mature GDNF, which is known to function through interactions with the GFRα1 receptor. In order to determine if DNSP-11 interacted with GFRα1, a pull down assay was performed with biotinylated DNSP-11. No interaction was observed between DNSP-11 and GFRα1 as indicated by the absence of GFRα1 in the elution lanes when DNSP-11 and GFRα1 were incubated together. Incubation of GDNF with GFRα1 resulted in the expected interaction between GDNF and GFRα1, indicating binding of GDNF to GFRα1 by the presence of both GDNF and GFRα1 in the elution lane (Figure 4.2). This data indicates that DNSP-11 does not interact with GFRα1 *in vitro* and therefore, may not be mediating apoptotic pathways through the same mechanism as mature GDNF.

c. DNSP-11 does not upregulate GDNF mRNA levels

After it was observed that DNSP-11 does not bind GFRα1 *in vitro,* investigation continued in order to determine if DNSP-11's anti-apoptotic effects were mediated through other mechanisms affecting overall GDNF levels. Since evidence indicates that DNSP-11 did not function through the same mechanism as mature GDNF, it was hypothesized that DNSP-11 may provide its anti-apoptotic effects by increasing the levels of GDNF via a positive feedback mechanism. In order to further evaluate the potential mechanisms of DNSP-11's action, it was evaluated whether DNSP-11 was able to influence GDNF mRNA levels through a feedback mechanism. GDNF mRNA levels were determined by real time PCR after treatment of HEK293 cells with 1 or 10 nM DNSP-11. There was no observed increase in GDNF mRNA levels during treatments with DNSP-11 at 6, 12, 18, and 24 hours (Figure 4.3).

d. DNSP-11 does not upregulate GDNF protein levels

Although data suggests that DNSP-11 does not upregulate levels of GDNF mRNA after treatment for 6, 12, 18, and 24 hours, it is important to determine if DNSP-11 has any influence over the protein levels of mature GDNF after treatment. In order to determine if mature GDNF protein levels were upregulated after DNSP-11 treatment, Western blots were performed. MN9D cells were treated with either 1 or 10 nM DNSP-11 for 3, 6, 12, 18 and 24 hours. After treatment, the total protein was collected from the lysed cells and analyzed by Western blot.

It was observed that there was no significant upregulation of GDNF protein levels under any of the tested conditions (Figure 4.4). This indicates that GDNF protein levels are not influenced by the presence of DNSP-11. Therefore, it is unlikely that DNSP-11 functions by a feedback mechanism that results in the upregulation of GDNF. Thus, the observed *in vivo* and *in vitro* protective effects against 6-OHDA are likely working through a different mechanism from mature GDNF.

e. Proteomic analysis

Since DNSP-11 does not appear to work through the same receptor pathway as GDNF, nor does DNSP-11 seem to function by increasing the levels of GDNF mRNA or protein, it is important to determine the mechanism by which DNSP-11 is providing cellular protection. In order to determine a potential mechanism of action, a proteomic pull down screen was performed (Figure 4.5). Two dimensional gel analysis following a pull down assay was used to determine potential binding partners. Homogenate from rat substantia nigra were utilized for these studies. Binding partners were isolated by incubation of the homogenate with biotinylated DNSP-11. A pull down of the biotinylated DNSP-11 along with its binding partners was then performed. The binding partners were observed by 2D-PAGE gel analysis. The 2D-PAGE analysis allows for proteins to be separated based on two properties. The 2D-PAGE analysis used to evaluate the binding partners of DNSP-11 separated the proteins initially by differences in their molecular weights and then by the differences based on their

isoelectric point. After the potential binding partners were observed on a 2D-PAGE gel, they were then identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry in the University of Kentucky Center of Structural Biology.

The membrane and cytosolic protein fraction were analyzed separately from one another. There was a potential binding partner observed on the 2D gel of the membrane fraction. However, that potential binding partner could not be identified by MALDI-TOF mass spectroscopy.

Numerous spots were observed within the cytosolic fraction of the 2D gel analysis (Figure 4.6; Table 4.1). Of these sixteen observed proteins eleven of the potential binding partners, including aconitate hydralase, alpha enolase, aspartate aminotransferase, creatine kinase, fructose bisphosphate aldolase, glutamate dehydrogenase, glutamine synthetase, glyceraldehyde 3 phosphate dehydrogenase, L-lactate dehydrogenase B chain, malate dehydrogenase, and pyruvate kinase M1/M2, were known to have metabolic functions, while other binding partners played a role in the cytoskeleton, neuronal development, and translation. Of the identified potential binding partners, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was previously known to play a role in apoptosis (Hara, M.R. *et al.,* 2005). Due to the known role of GAPDH in apoptosis and as a target for the current PD therapeutic, deprenyl, its interactions with DNSP-11 were pursued further.

f. Confirmation of GAPDH-DNSP-11 binding

Before further analysis of GAPDH role in DNSP-11 activity was performed, it was important to validate the observed interaction with DNSP-11. In order to confirm GAPDH-DNSP-11 binding, an *in vitro* pull down was performed using biotinylated DNSP-11 and purified GAPDH protein. Rabbit GAPDH was incubated in the presence and absence of DNSP-11. After the incubation, the samples were incubated with streptavidin beads to pull down the biotinylated DNSP-11 and anything that is bound to the DNSP-11.

It was observed that there was binding of GAPDH to the biotinylated DNSP-11 as indicated by the presence of GAPDH in the elution lane on the gels (Figure 4.7B). However, in the absence of DNSP-11 in the incubation, there was no band observed in the elution lanes of the gel (Figure 4.7A). This indicates that GAPDH is not binding to the streptavidin beads nonspecifically and is in fact binding to DNSP-11 and therefore, would be able to play a potential role in the activity of DNSP-11.

g. Nitrosylation of GAPDH decreases DNSP-11 binding

In order to further evaluate the role of DNSP-11 in potentially mediating the GAPDH apoptotic pathway, the involvement of DNSP-11 at various steps of the apoptotic pathway was observed. It is known that nitrosylation of GAPDH allows it to bind to Siah-1 and be transported to the nucleus. Therefore, it is important to determine if DNSP-11 is able to bind to nitrosylated GAPDH and potentially prevent it from binding to Siah-1. Based on the observed anti-

apoptotic properties of DNSP-11, it was hypothesized that DNSP-11 was mediating this pathway by preventing the nitrosylated GAPDH from binding to Siah-1. In order to determine if nitrosylation of GAPDH is able to alter it's binding to DNSP-11, GAPDH was nitrosylated *in vitro* and then binding to biotinylated DNSP-11 was observed by pull down analysis.

After nitrosylation of GAPDH with GSNO for 30 minutes at 37°C, reduced binding of DNSP-11 to GAPDH was observed (Figure 4.8). This was indicated by the reduction in the amount of GAPDH observed in the elution lanes of the gel of the nitrosylated GAPDH (Figure 4.8). This observation implies that DNSP-11 binding to GAPDH is reduced by the nitrosylation of GAPDH. Because of this, it seems unlikely that DNSP-11 is mediating the GAPDH apoptotic pathway by interacting with nitrosylated GAPDH and preventing it from binding to Siah-1. If DNSP-11 is able to mediate that GAPDH apoptotic pathway, it is likely doing so at another step of the pathway.

h. DNSP-11 reduces nitrosylation of GAPDH

To further evaluate DNSP-11's role in mediating GAPDH's apoptotic pathway, DNSP-11's role in influencing the nitrosylation of GAPDH was observed. In order to determine if DNSP-11 incubation influenced the nitrosylation of GAPDH, a GAPDH activity assay was used. When GAPDH is not nitrosylated, it is able to carry out its metabolic activities. GAPDH is an enzyme that plays a role in glycolysis by breaking down glucose. This metabolic activity can be measured in a spectrophotometer by measuring the oxidation of NADH to

NAD that is a result of GAPDH activity. Alternatively, nitrosylated GAPDH is no longer active and able to carry out these metabolic activities. Therefore, an increase in the amount of GAPDH nitrosylation should be accompanied by a decrease in GAPDH activity. If DNSP-11 does inhibit nitrosylation of GAPDH, then GAPDH should be able to maintain a higher level of activity in the presence of GSNO, which can nitrosylate GAPDH.

When GAPDH was incubated only, enzymatic activity was observed. However, when GAPDH was pre-incubated with 1 mM of GSNO, no GAPDH activity was observed. When GAPDH was incubated with 5 mM DNSP-11 only for one hour prior to measuring GAPDH activity, activity was still observed at similar levels as GAPDH that was not incubated with DNSP-11. DNSP-11 was able to increase the amount of GAPDH activity that was observed in the presence of 1 mM GSNO to similar levels as what was observed in the absence of GSNO. DNSP-11 was able to partially restore GAPDH activity in the presence of 10 mM GSNO, but at the levels of 100 mM GSNO little GAPDH activity was observed when 5 mM DNSP-11 was present. This data indicates that DNSP-11 seems to be able to inhibit the nitrosylation of GAPDH (Figure 4.9). By blocking the nitrosylation of GAPDH, it is possible for DNSP-11 to modify the GAPDH apoptotic pathway and afford some protection to the cells from apoptosis.

i. Alteration of nuclear GAPDH levels

Previous studies have shown that during apoptosis, GAPDH levels are elevated in the nucleus of the cell (Hara, M.R. *et al.,* 2005). This elevation occurs

as a result of GAPDH being nitrosylated. The nitrosylation of GAPDH allows it to bind to Siah-1, an E3 ubiquitin ligase. Siah-1 has a nuclear localization signal that allows the GAPDH-Siah-1 complex to move into the nucleus. GAPDH helps to stabilize Siah-1, which is normally quickly degraded and, therefore, not normally present in the nucleus for extended periods of time. However, when it is bound to GAPDH, Siah-1 is stabilized and can then degrade its nuclear targets, leading to apoptosis. In order to determine if DNSP-11 is mediating the pathway, nuclear GAPDH levels were observed by Western blot analysis of samples after treatment with toxin either with or without DNSP-11.

In MN9D cells it was observed that DNSP-11 decreased the amount of nuclear GAPDH that was observed after treatment with 20 µM 6-OHDA for 18 hours (Figure 4.10). However, due to the fact that the MN9D cells showed high levels of cell death as a result of treatment with 6-OHDA, studies were moved into HEK293 cells, a more stable cell line. Treatment of HEK293 cells 100 µM etoposide resulted in an increase in nuclear GAPDH after 24 hours of treatment (Figure 4.11, Figure 4.12). However, western blots showed no significant decrease in nuclear GAPDH when these cells were treated with either 1 or 10 nM of DNSP-11 (Figure 4.11, Figure 4.12).

Recent studies have indicated that the effects of DNSP-11 occur more quickly than originally hypothesized with the peptide being internalized within minutes of treatment. Therefore, the studies performed in MN9D cells at 18 hours post treatment may have been to late to see any observed changes in nuclear GAPDH levels. Based on this observation, studies have been carried out

to evaluated nuclear GAPDH levels at shorter time periods. It has been noted

that nuclear GAPDH is elevated after treatment for 5 minutes with both 1 μM

staurosporine and 20 μM 6-OHDA. However, no changes in nuclear GAPDH

were observed in cells treated with 8 mM 3-NP at this time point. Treatment with

100 nM DNSP-11 was able to reduce nuclear GAPDH levels after staurosporine

treatment only (Figure 4.13, Figure 4.14). At this time point, changes in cytosolic

GAPDH were not observed (Figure 4.13, Figure 4.14). These studies indicate a

need to evaluate the actions of DNSP-11 at shorter time periods.

IV. Discussion

It has been observed that DNSP-11 is able to provide protection from

apoptosis. Here data has been presented to show that DNSP-11 is able to

protect from 6-OHDA induced apoptosis. DNSP-11 has been shown to reduce

the levels of apoptosis similarly to the reduction in apoptosis that has been

observed after treatment with GDNF. Two different apoptotic screens were used

to evaluate this. Measurements of both TUNEL staining and caspase-3 activity

levels showed a significant reduction of apoptosis in 6-OHDA treated MN9D

cells. Since DNSP-11 is able to reduce apoptosis to similar levels as GDNF, it is

reasonable to hypothesis that DNSP-11 may be functioning through the same

pathway as GDNF.

Mature GDNF is known to promote cell survival through its binding to the

receptor GFRα1. To test if DNSP-11 was providing protection from apoptosis

through the same mechanism as mature GDNF, a pull down assay with

biotinylated DNSP-11 and GFRα1 was performed. This assay will allow for determination of DNSP-11's ability to bind with GFRα1, potentially allowing for DNSP-11 to function by signaling through the GFRα1 receptor. DNSP-11 was not observed to bind to GFRα1, while the mature GDNF was shown to bind to GFRα1 as expected. Since DNSP-11 does not interact with GFRα1, it suggests the DNSP-11 does not function through the binding to GFRα1 in a similar mechanism as mature GDNF, meaning that while DNSP-11 is able to protect from apoptosis similarly to mature GDNF, it likely does so through the mechanism unique from that of mature GDNF.

Since DNSP-11 has been shown to not interact with GFRα1 *in vitro*, it is hypothesized that it functions through a different mechanism than mature GDNF to offer protective effects. Based on this hypothesis, the role of DNSP-11 in a potential feedback mechanism with GDNF was evaluated. Because the cellular protection that was observed with both GDNF and DNSP-11 were similar, it is reasonable to hypothesize that DNSP-11 may provide this protection by upregulating the levels of mature GDNF. Therefore, the observed protection is a result of the increase in GDNF levels. In order to test this hypothesis, studies were performed to determine if treatment with DNSP-11 resulted in changes in GDNF at both the mRNA and protein levels. After treating MN9D cells for 6-24 hours with either 1 or 10 nM DNSP-11, no significant increase in GDNF mRNA levels were observed. DNSP-11 does not appear to be initiating a feedback mechanism that influences the levels of GDNF mRNA. In addition, there was no significant increase in GDNF protein levels after treatment with 1 or 10 nM

DNSP-11 for between 3-24 hours. Since neither GDNF mRNA nor protein levels were increased by DNSP-11 treatment, it can be concluded that DNSP-11 does not function by initiating a feedback mechanism resulting in an upregulation in GDNF mRNA or protein. This feedback pathway would result in an increase in GDNF levels, which could lead to an increased activation of the GFRα1 pathway, resulting in the observed reduction in apoptosis.

Collectively these data support the hypothesis that DNSP-11 may not function through the same mechanism as mature GDNF by interacting with GFRα1. It has also been shown that DNSP-11 does not function to upregulate GDNF, either at the mRNA or protein level. These data indicate that DNSP-11 is functioning by a mechanism that is distinct from that of mature GDNF.

In order to determine by what mechanism DNSP-11 is functioning, a proteomic screen of potential binding partners was performed. A two-dimensional gel analysis was performed with young Fischer 344 rat substantia nigra homogenate. The observed binding partners on the gels were then identified by MALDI-TOF mass spectroscopy. Both the membrane and cytosolic fractions were evaluated during this process. None of the observed binding partners in the membrane fraction could be identified. However, within the cytocolic fraction, sixteen proteins were identified. Of these sixteen proteins eleven of the potential binding partners, including aconitate hydralase, alpha enolase, aspartate aminotransferase, creatine kinase, fructose bisphosphate aldolase, glutamate dehydrogenase, glutamine synthetase, glyceraldehyde 3 phosphate dehydrogenase, L-lactate dehydrogenase B chain, malate dehydrogenase, and

pyruvate kinase M1/M2, were known to have metabolic functions. Further,

proteins with additional functions such as chaperones (heat shock cognate 71

kDa protein), cytoskeletal proteins (actin and tubulin), neuronal development

(dihydropyrimidinase-related protein 2), and translation (elongation factor-1 alpha

2) were identified. Of the potential binding partners that were identified, one,

GAPDH, was known to play a role in apoptosis, in addition to being a key

metabolic protein. Because of GAPDH's known role in apoptosis, its interaction

with DNSP-11 was further evaluated for a potential role in offering cellular

protection that has been observed with DNSP-11 treatment.

In order to confirm binding between DNSP-11 and GAPDH, an *in vitro* pull

down with biotinylated DNSP-11 and purified GAPDH was performed. This pull

down showed that biotinylated DNSP-11 was able to bind to the purified GAPDH

further supporting the idea that DNSP-11 and GAPDH interact with one another

and that this interaction may be able to mediate apoptotic pathways. Verifying

GAPDH as a DNSP-11 binding partner allows for the further investigation of

GAPDH in DNSP-11's anti-apoptotic activities.

GAPDH is known to play a role in an apoptotic pathway that involves

GAPDH interacting with Siah-1, an E3 ubiquitin ligase. This pathway allows for

an increase in nuclear GAPDH and Siah-1 because of the binding of Siah-1 to

GAPDH after nitrosylation of GAPDH has occurred. Siah-1's nuclear localization

signal allows the complex to be transported to the nucleus, where it is stabilized

and longer lasting degradation of Siah-1's nuclear targets leads to apoptosis.

Therefore, one of the markers that can be used to evaluate this pathway is the

nuclear level of GAPDH. If this apoptotic pathway is being activated, the amount of GAPDH in the nucleus should be elevated (Hara, H.R. *et al.,* 2005). In order to determine if DNSP-11 was interacting with the GAPDH apoptotic pathway, Western blots of the nuclear fraction of GAPDH were evaluated after treatment with toxins which elevated the activation of the GAPDH apoptotic pathway. In order to determine if the interaction of DNSP-11 and GAPDH plays a role in mediating apoptosis, the interaction of DNSP-11 with GAPDH at different stages of the GAPDH-Siah-1 pathway were investigated. Since nitrosylation of GAPDH is an initiating factor in this pathway, it was important to determine if DNSP-11 was able to influence the nitrosylation of GAPDH. Also, interaction of DNSP-11 with GAPDH after nitrosylation had occurred was evaluated in order to determine if DNSP-11 was able to bind to nitrosylated GAPDH and prevent it from binding to Siah-1 which would result in a reduction of GAPDH-Siah-1 complex formation and therefore, nuclear transport.

To gain insight into DNSP-11's effects on GAPDH, we evaluated the interaction between DNSP-11; GAPDH, after it was nitrosylated, was evaluated to determine if DNSP-11 binds nitrosylated GAPDH to inhibit the formation of the GAPDH-Siah-1 complex. GAPDH was nitrosylated with GSNO prior to incubation with biotinylated DNSP-11. After nitrosylation of GAPDH, it was incubated with biotinylated DNSP-11 and a pull down assay was performed. This pull down assay showed that binding between biotinylated DNSP-11 to GAPDH after nitrosylation with GSNO was reduced. The reduction in binding that was observed implies that DNSP-11 does not interact with nitrosylated GAPDH.

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Therefore, it does not likely bind to nitrosylated GAPDH to inhibit it from interacting with Siah-1. It also suggests that DNSP-11 is unlikely to bind to nitrosylated GAPDH when it is part of the GAPDH-Siah-1 complex and, therefore inhibiting the nuclear localization of the complex.

The role in DNSP-11 in the nitrosylation of GAPDH was then evaluated. Since nitrosylation of GAPDH is required for binding to Siah-1, if DNSP-11 is able to inhibit the nitrosylation of GAPDH, it could prevent it from binding to Siah-1 and, therefore, reduce apoptosis. Since nitrosylation of GAPDH also results in it losing its metabolic activity, activity assays of GAPDH were used to evaluate the level of GAPDH nitrosylation after treatment with GSNO. It was observed that incubation with DNSP-11 allowed for GAPDH to remain active even in the presence of the nitrosylating agent, GSNO. The ability of DNSP-11 to inhibit nitrosylation of GAPDH could allow for it to prevent the activation of the GAPDH apoptotic pathway. This could contribute to the beneficial cellular effects of DNSP-11 that have been observed.

It was observed that in HEK 293 cells, 100 µM staurosporine was able to elevate the levels of GAPDH in the nuclear fractions of these cells after 24 hours of treatment. In HEK 293 cells treated with either 1 or 10 nM DNSP-11 for 24 hours along with 100 µM staurosporine, there was no significant reduction in nuclear GAPDH levels observed. This indicates that the interaction with the GAPDH apoptotic pathway may not be solely involved in DNSP-11's ability to reduce apoptotic levels. Since such a large number (eleven of sixteen) of the identified DNSP-11 binding partners play a role in metabolism, DNSP-11 may

provide protection by influencing these pathways. There is evidence that

DNSP-11 plays a protective role in mitochondrial mechanisms of cell death.

Additional data from MN9D cells indicates that changes in nuclear GAPDH may

occur at an earlier time points than originally hypothesized. It has been observed

that DNSP-11 is able to reduce nuclear GAPDH levels after treatment with

staurosporine for only 5 minutes. Based on this information, it is probable that

GAPDH mechanism might be an early protective mechanism with other

mitochondrial mechanisms playing a role later in the protection.

V. Conclusion

It has been observed that DNSP-11 is capable of providing anti-apoptotic

effects in cellular toxin models of apoptosis if it is able to reduce apoptotic levels

similarly to GDNF. However, it appears to work by a mechanism that is unique

from mature GDNF. We have shown that DNSP-11 is able to interact *in vitro* to a

variety of proteins, the majority of which have known metabolic functions.

Because of its known role in regulating apoptosis and metabolic pathways, we

investigated the role of DNSP-11 in mediating the GAPDH apoptotic pathway.

While it has been noted that high concentrations of DNSP-11 are able to inhibit

nitrosylation of GAPDH, we did not observe a significant reduction in nuclear

GAPDH in HEK 293 cells. It seems that DNSP-11's interaction with GAPDH may

play a role in the reduction of apoptosis, but is likely not the only mechanism by

which DNSP-11 is able to afford protection from apoptosis. The interaction with

numerous other metabolic proteins indicates that DNSP-11 may play a broader

role in these metabolic pathways.
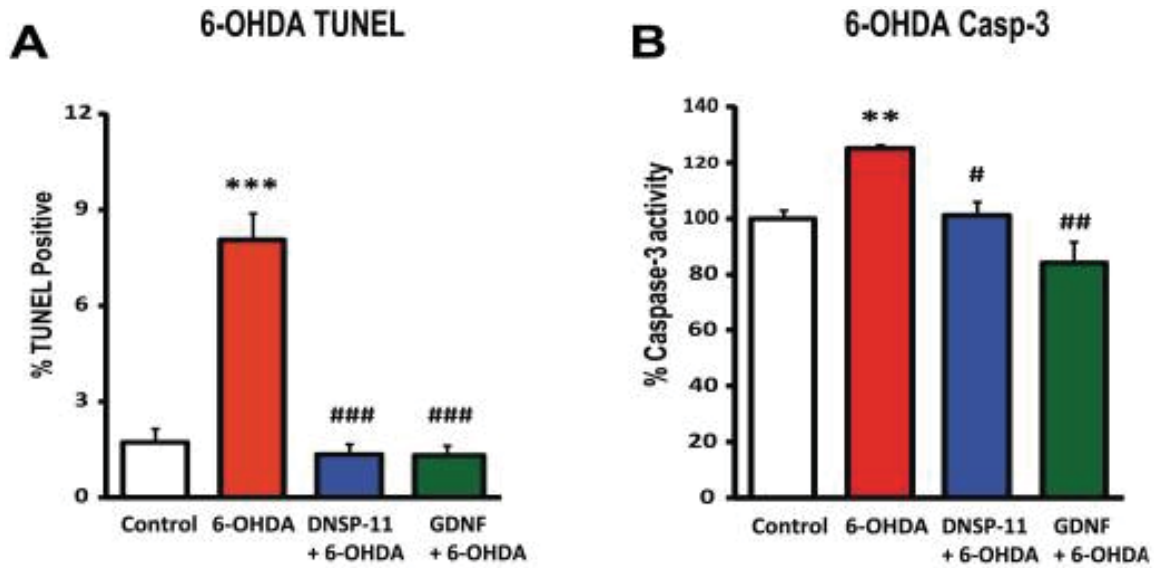
Figure 4.1: Protective effects of DNSP-11 and GDNF against 6-OHDA. Both DNSP-11 and GDNF protect against 6-OHDA toxicity as demonstrated by reductions in TUNEL staining at 24 h (A) and caspase-3 (B) activity at 3 h after 6-OHDA exposure. MN9D dopaminergic cells were incubated for 1 hour with either citrate buffer (control), 1 ng/mL of DNSP-11 or GDNF prior to 100 µM 6-OHDA exposure for 15 min. Data are + SD, one-way ANOVA with Tukey's post hoc analysis, *p<0.05, **p<0.01, ***p<0.001 vs. control; #p<0.05, ##p<0.01, ###p<0.001 vs. 6-OHDA (Modified from Bradley *et al.*, 2010).
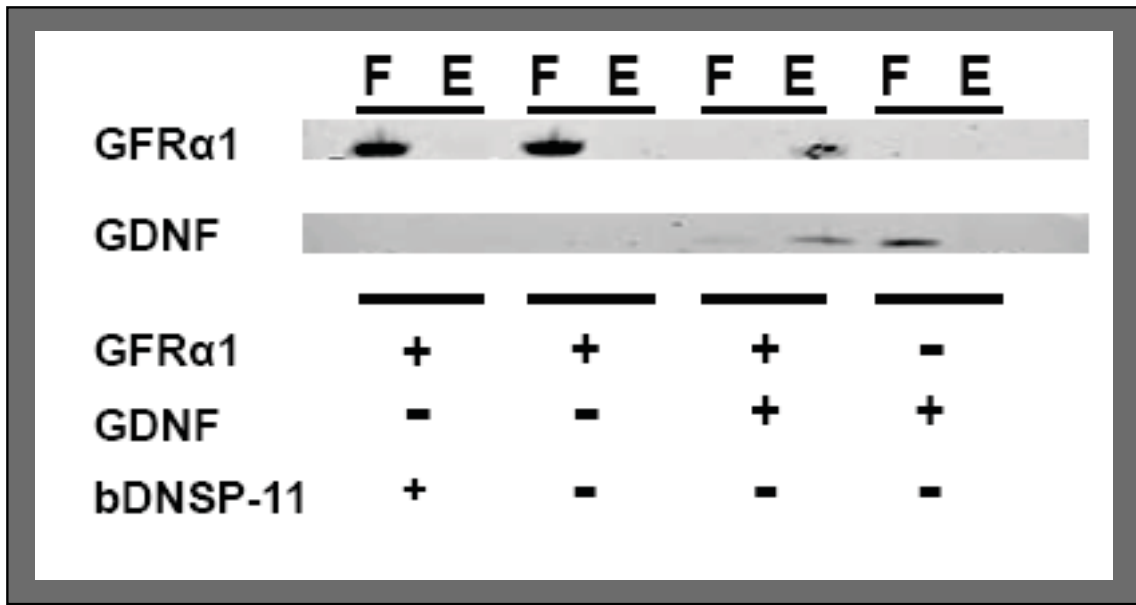
Figure 4.2: DNSP-11 does not bind to GFRα1. When GFRα1 was incubated with bDNSP-11 no interaction was observed as indicated by the GFRα1 being found in the flow through (F) lane and absent in the elution (E) lane. However, incubation of GDNF with GFRα1 resulted in an observed interaction between GDNF and GFRα1 as shown by the presence of both GDNF and GFRα1 in the elution lane.
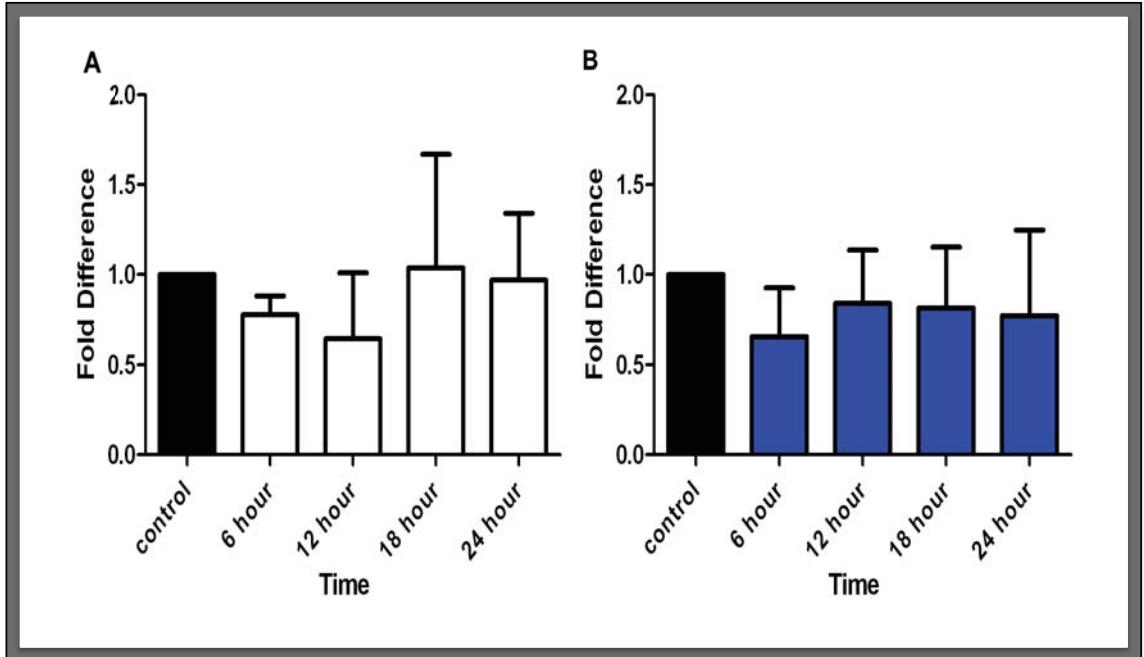
Figure 4.3: Effects of DNSP-11 on GDNF mRNA levels. After treatment of HEK293 cells with 1 nM DNSP-11 for 6, 12, 18, and 24 hours, (A) shows that there was no significant increase in GDNF mRNA levels as measured by real time PCR. Similar results were observed after treatment with 10 nM DNSP-11 for 6, 12, 18, and 24 hours (B).
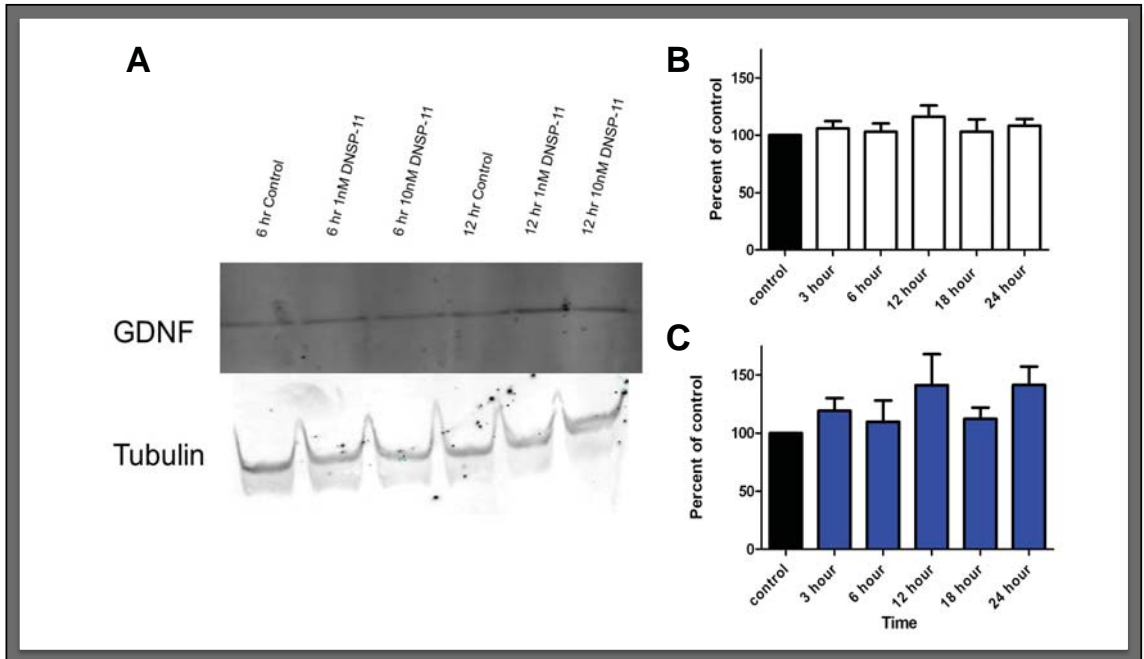
Figure 4.4: Effects of DNSP-11 on GDNF protein levels. (A) shows a Western blot analysis of the GDNF in MN9D after treatment with DNSP-11. (B) shows that there was no significant changes in the levels of GDNF after treatment with 1 nM DNSP-11 for 3,6,12,18, and 24 hours. (C) shows similar results of no increase in GDNF after treatment with 10 nM DNSP-11 for 3, 6, 12, 18, and 24 hours.
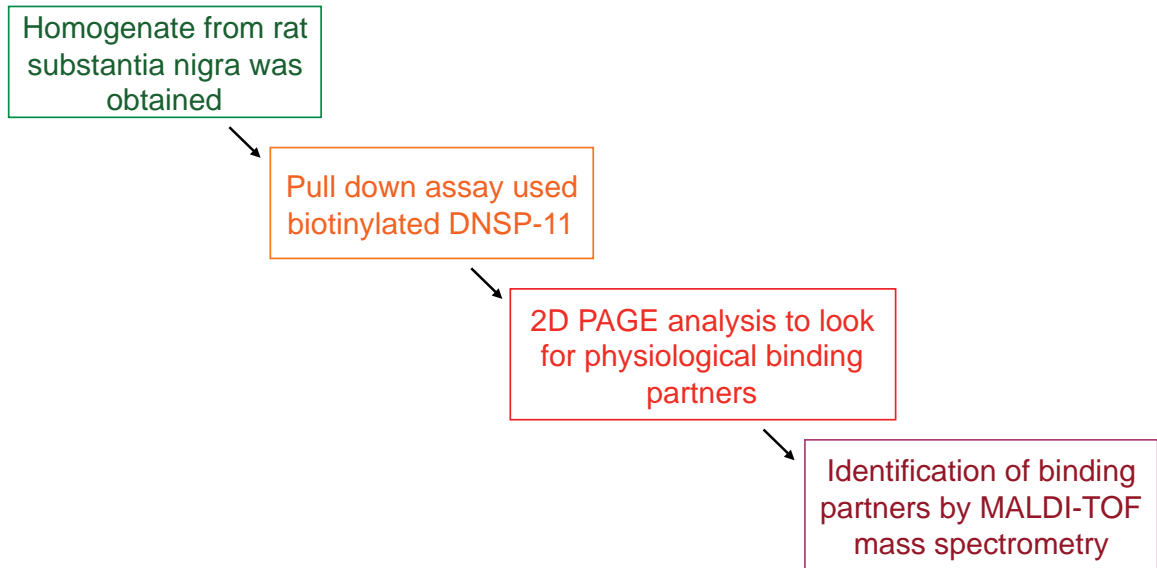
Figure 4.5: Proteomic pull down with biotinylated DNSP-11. A proteomic pull down with biotinylated DNSP-11 was preformed as described here. Homogenate from rat substantia nigra was incubated with biotinylated DNSP-11 and a pull down was performed. The potential binding partners were isolated by 2D PAGE analysis and identified by MALDI-TOF mass spectrometry.
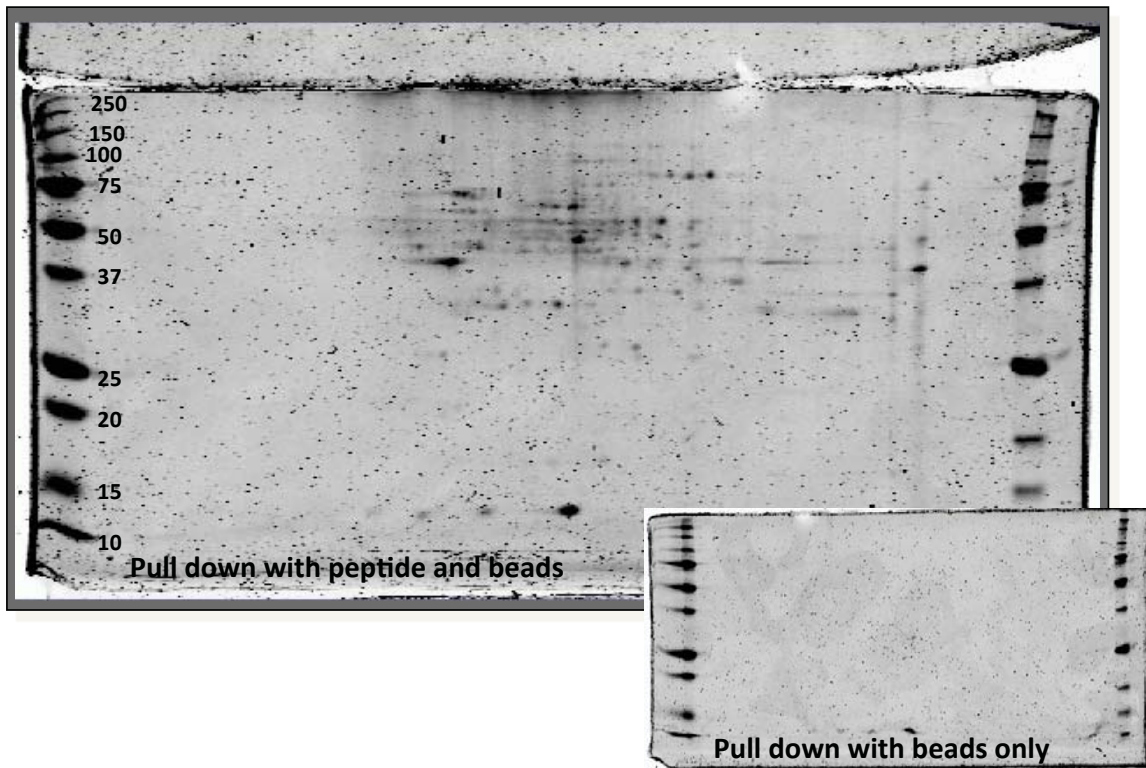
Figure 4.6: Potential binding partners for DNSP-11. The cytosolic fraction isolated from rat substantia nigra was used to carry out a pull down assay. The potential binding partners were separated by 2D PAGE analysis with the gel shown here. The proteins isolated were then submitted for identification by MALDI-TOF mass spectroscopy.

Table 4.1: Potential binding partners for DNSP-11. The potential binding partners of DNSP-11 identified in the cytosolic fraction are shown here along with their primary function. The majority of the proteins identified play a role in metabolism, indicating that DNSP-11 may play a role in mediating metabolic pathways (Modified from Bradley *et al.*, 2010).

Protein	Primary Function
Aconitate Hydralase	Metabolism
Alpha Enolase	Metabolism
Aspartate Aminotransferase	Metabolism
Creatine Kinase	Metabolism
Fructose Bisphosphate Aldolase	Metabolism
Glutamate Dehydrogenase	Metabolism
Glutamine Synthetase	Metabolism
Glyceraldehyde 3-Phosphate Dehydrogenase	Metabolism/Apoptosis
L-Lactate Dehydrogenase B Chain	Metabolism
Malate Dehydrogenase	Metabolism
Pyruvate Kinase M1/M2	Metabolism
Heat Shock Cognate 71kDa Protein	Chaperone
Actin	Cytoskeleton
Tubulin	Cytoskeleton
Dihydropyrimidinase-Related Protein 2	Neuronal Development
Elongation Factor-1 Alpha 2	Translation
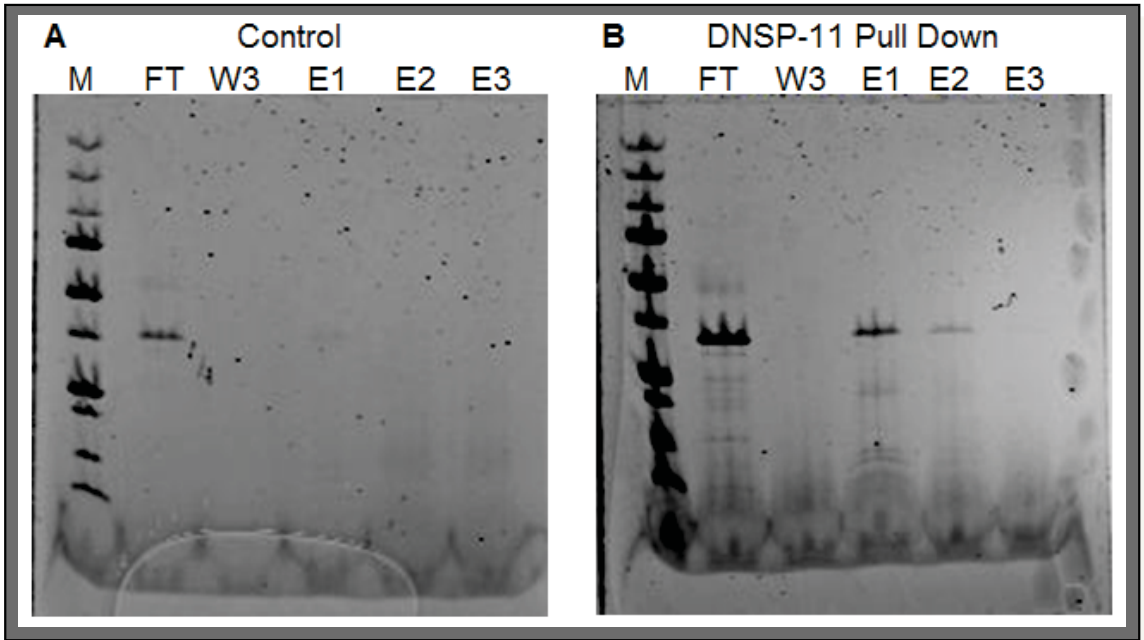
Figure 4.7: DNSP-11's interaction with GAPDH. An in vitro pull down was performed with purified GAPDH and biotinylated DNSP-11 (B). When the pull down was done in the absence of DNSP-11 (A), no GAPDH was pulled down. M: Precision Plus Protein Standard, FT: Flow through, W3: Wash three, E1: Elution one, E2: Elution two, E3: Elution three.
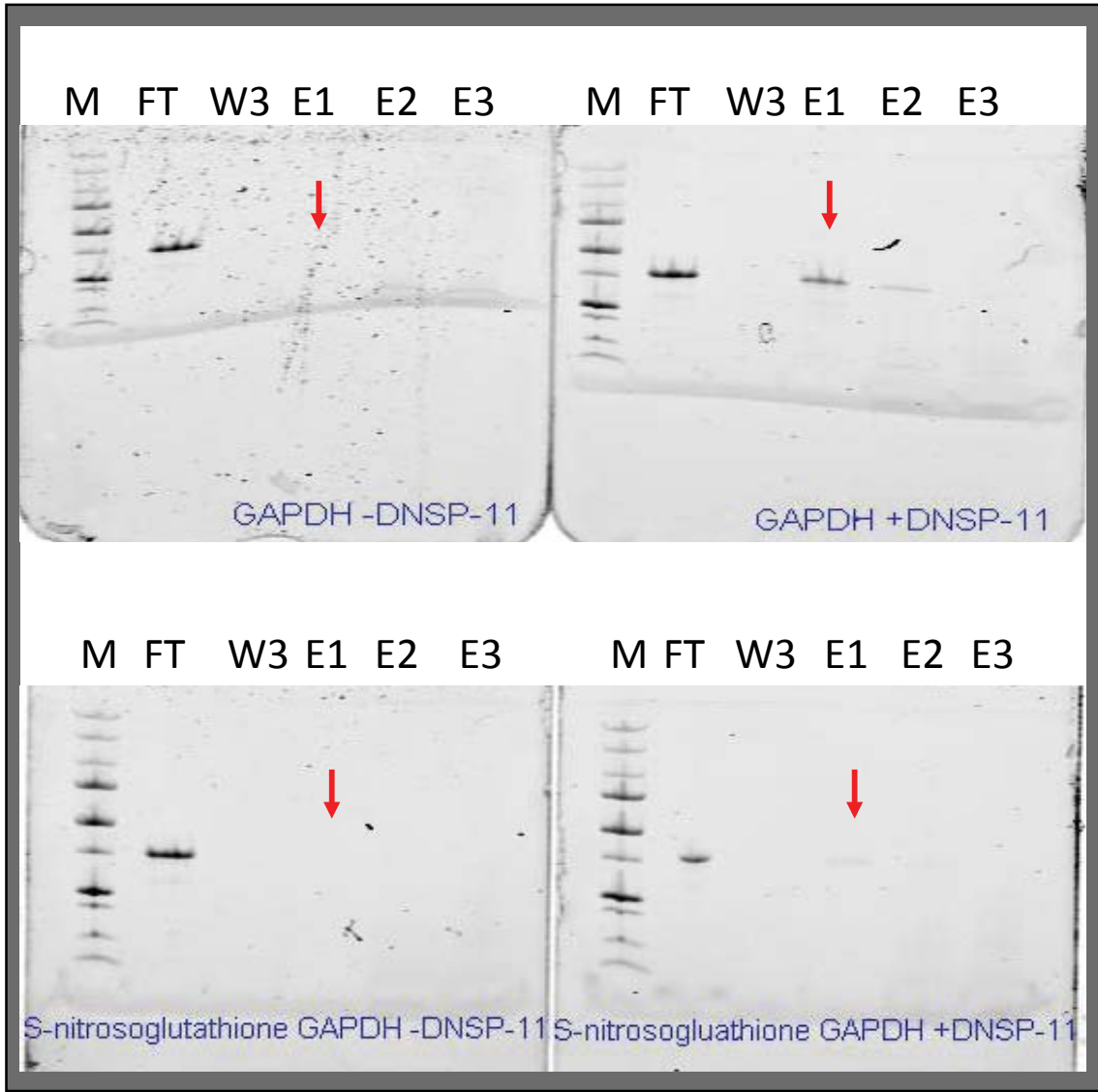
Figure 4.8: Effect of GAPDH nitrosylation on DNSP-11 binding. GAPDH nitrosylation by GSNO was able to reduce the binding of GAPDH to DNPS-11 as show be a reduction in GAPDH present in E3 after treatment with GSNO in the presence of DNSP-11 compared to GAPDH that was not pretreated with DNSP-11. M: Precision Plus Protein Standard, FT: Flow through, W3: Wash three, E1: Elution one, E2: Elution two, E3: Elution three.
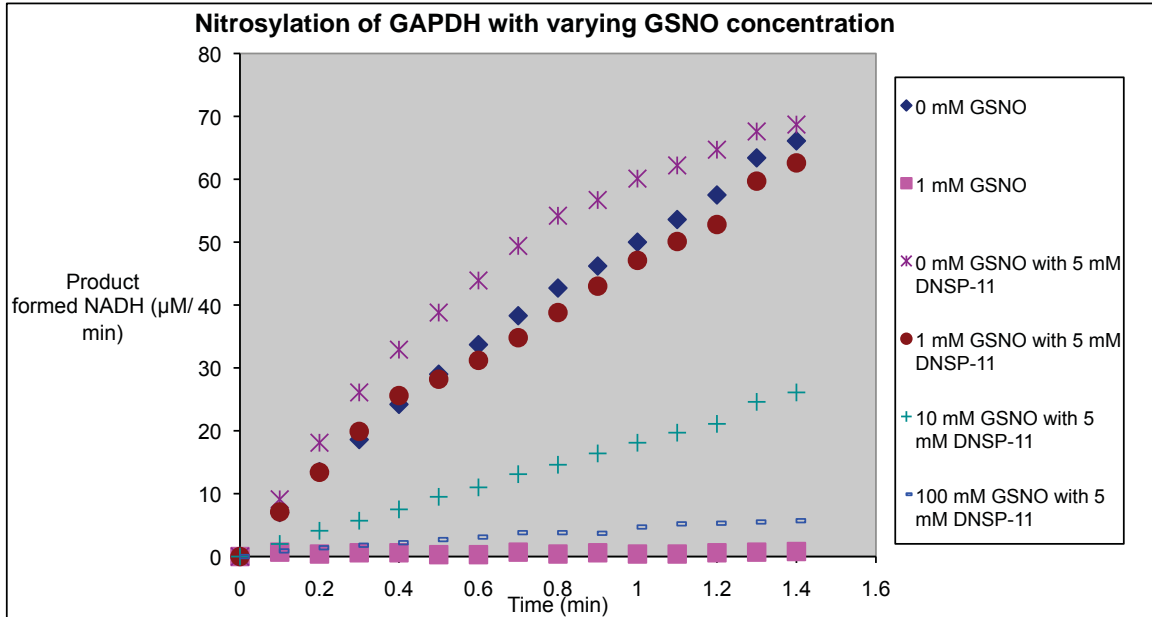
Figure 4.9: Nitrosylation of GAPDH with varying GSNO concentrations. GAPDH incubated alone (blue diamonds) showed enzymatic activity. When GAPDH was pre-incubated with 1 mM of GSNO (pink squares), no GAPDH activity was observed. When GAPDH was incubated with 5 mM DNSP-11 only (pink asterisks) for one hour prior to measuring GAPDH activity, activity was still observed at similar levels as GAPDH that was not incubated with DNSP-11. DNSP-11 was able to increase the amount of GAPDH activity that was observed in the presence of 1 mM GSNO (red circles) to similar levels as what was observed in the absence of GSNO. DNSP-11 was able to partially restore GAPDH activity in the presence of 10 mM GSNO (green crosses), but at the levels of 100 mM GSNO (blue dashes) little GAPDH activity was observed when 5 mM DNSP-11 was present.
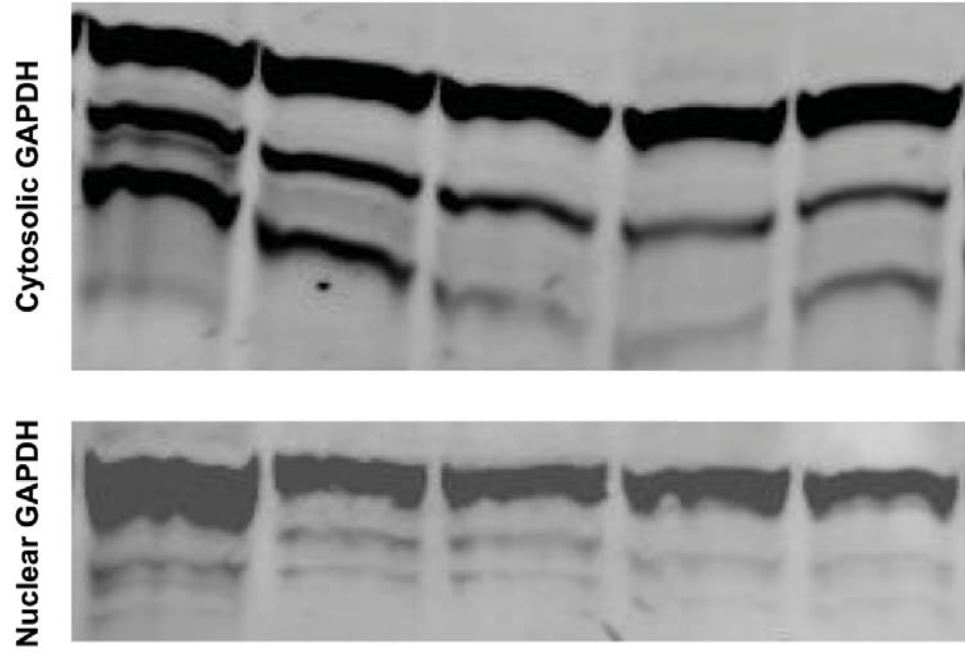
Figure 4.10: Effects of DNSP-11 on nuclear GAPDH levels in MN9D cells. Nuclear GAPDH was reduced in MN9D cells after treatment with 1 or 10 ng/mL DNSP-11 and 20 μM 6-OHDA compared to cells treated only with 20 μM 6-OHDA .
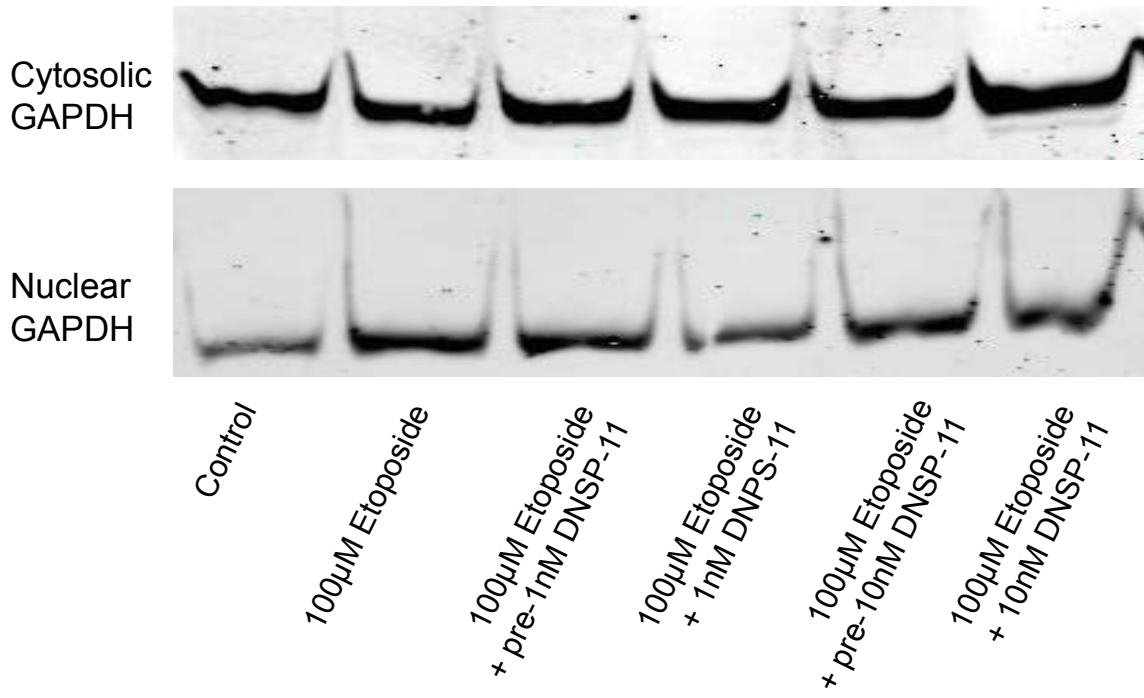
Figure 4.11: Effects of DNSP-11 on nuclear GAPDH levels in HEK293 cells. Western blots showed that treatment of HEK293 cells 100 µM etoposide resulted in an increase in nuclear GAPDH after 24 hours of treatment. However, no significant decrease in nuclear GAPDH was observed when these cells were treated with either 1 or 10 nM of DNSP-11 for 24 hours.
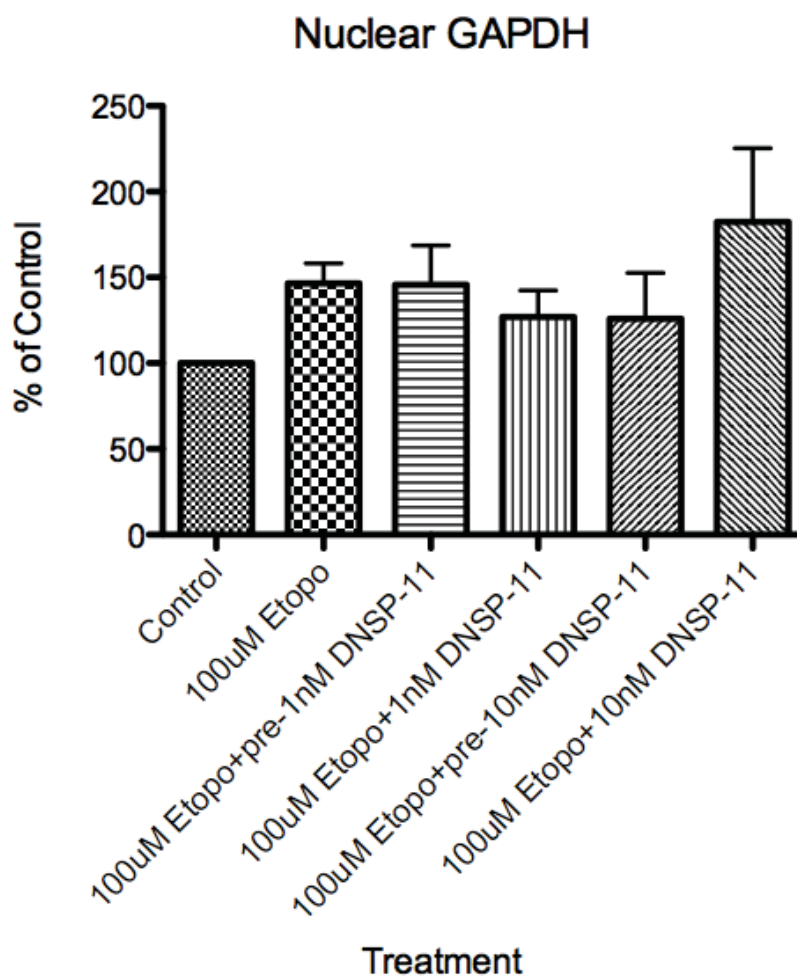
Figure 4.12: Effects of DNSP-11 on nuclear GAPDH levels in HEK293 cells. Western blots showed that treatment of HEK293 cells 100 µM etoposide resulted in an increase in nuclear GAPDH after 24 hours of treatment. However, no significant decrease in nuclear GAPDH was observed when these cells were treated with either 1 or 10 nM of DNSP-11 for 24 hours.
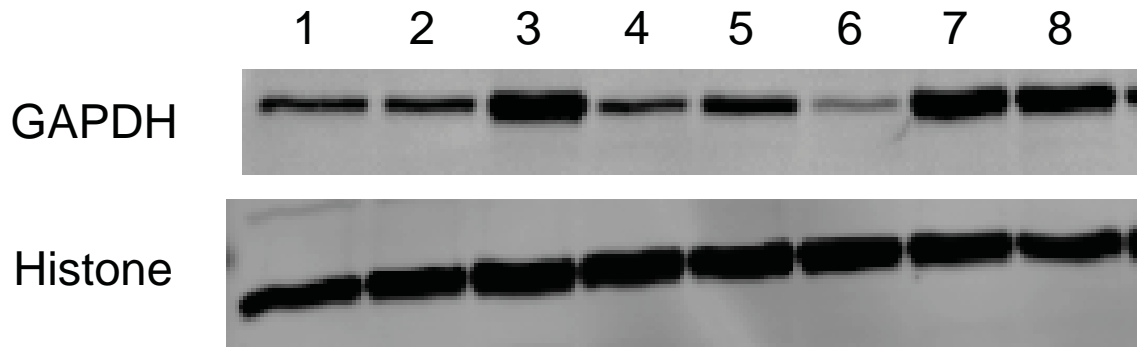
Figure 4.13: Effects of DNSP-11 on nuclear GAPDH levels after 5 minute treatments in MN9D cells. Treatment of MN9D cells with either 8 mM 3-NP, 1 μM staurosporine, or 20 μM 6-OHDA resulted in an increase in nuclear GAPDH. Treatment with 100 nM DNSP-11 after staurosporine treatment resulted in a reduction in nuclear GAPDH. Lane 1: Control, Lane 2: 8 mM 3-NP, Lane 3: 1 μM Staurosporine, Lane 4: 8 mM 3-NP and 100 nM DNSP-11, Lane 5: 1 μM Staurosporine and 100 nM DNSP-11, Lane 6: 100 nM DNSP-11, Lane 7: 20 μM 6-OHDA, Lane 8: 20 M 6-OHDA and 100 nM DNSP-11 (Data obtained by Dr. Turchan-Cholewo).

Nuclear GAPDH in MN9D
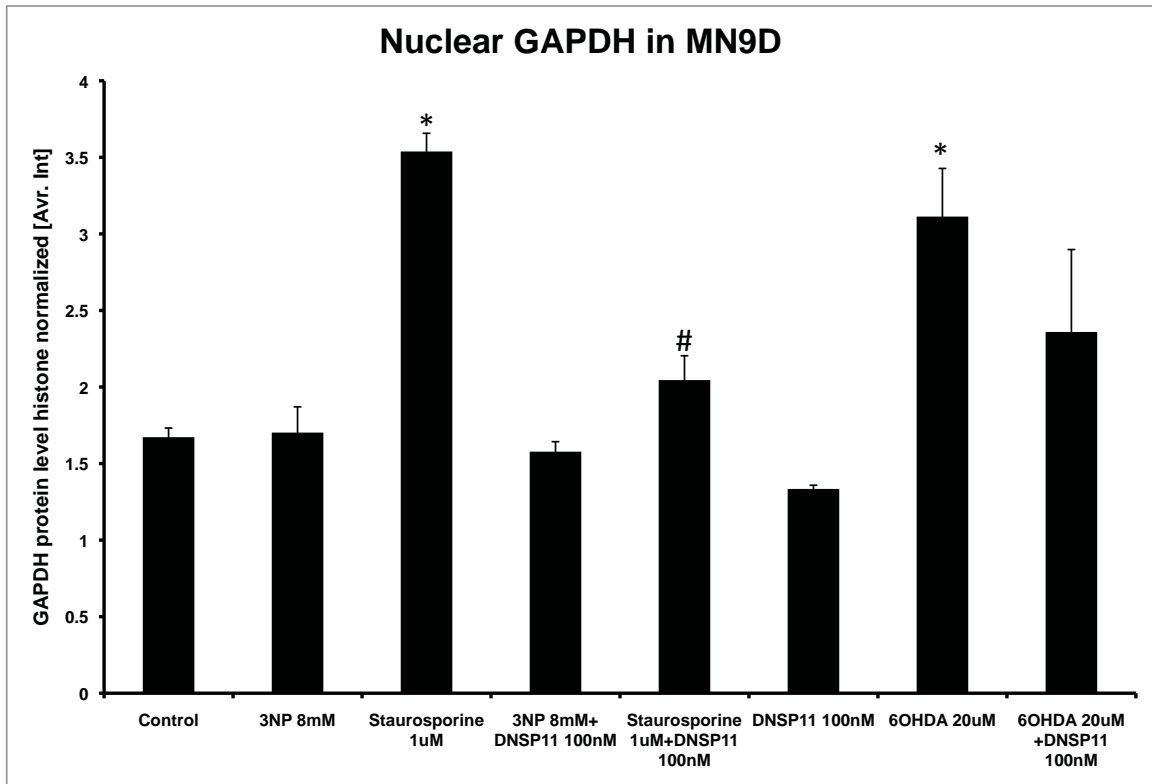
Figure 4.14: Changes in nuclear GAPDH levels in MN9D cells. No changes were observed in cytosolic GAPDH levels after treatment with 8 mM 3-NP, 1 μM staurosporine, or 20 μM 6-OHDA. However, increases in nuclear GAPDH levels were observed after treatment with 1 μM staurosporine or 20 μM 6-OHDA. The levels of nuclear GADH were reduced after treatment with 100 nM DNSP-11. Data are + SD, one-way ANOVA with Tukey's post hoc analysis, *$p<0.05$ vs. control; #$p<0.05$ vs. Staurosporine (Data obtained by Dr. Turchan-Cholewo).

Chapter Five: Conclusions and future directions

I. Introduction

Several obstacles accompany the treatment of neurodegenerative disease. These obstacles are often associated with the delivery of therapeutic agents to the central nervous system. The treatment of Parkinson's disease (PD) has not been an exception. Previous studies evaluating potential treatments for PD, including clinical trials with glial cell line-derived neurotrophic factor (GDNF), have faced issues associated with the delivery of a large protein to the brain (Gill *et al.,* 2003; Slevin *et al.,* 2005; Lang *et al.,* 2006). These complications have resulted in a need to develop molecules that have the same protective effects as GDNF, but that have the potential for less invasive methods for delivery. The development and evaluation of the DNSPs has shown that they possess characteristics that make them viable lead-candidates for potential therapeutics including their size, solubility, stability, and protective effects.

II. Introduction of alternative mechanisms

Data presented here has shown that DNSP-11 is able to protect against apoptosis. The ability of DNSP-11 to reduce apoptosis levels in cellular models, along with the small size and long-term stability of the molecule, make DNSP-11 of interest as a potential therapeutic for PD. As a smaller molecule, DNSP-11 offers an advantage over larger molecules for synthesis and delivery and was shown to be stable for out to one month under a variety of storage conditions,

including -80°C and 37°C. These data also provide information about the lack of aggregation of the peptide in solution. The development of aggregations can prevent the peptides from advancing through development as a potential therapeutic agent because it increases the likelihood that it triggers an immune response. Together these data make the peptide a viable candidate for future development as a potential therapeutic molecule.

Since it has been observed that DNSP-11 is able to provide anti-apoptotic effects, the investigation into the mechanism by which DNSP-11 is able to provide these effects became important. It was determined that DNSP-11 did not function through the same mechanism as mature GDNF - since it was shown to not bind to GFRα1. These data are further supported by the differential protective effects against staurosporine that have been observed (Bradley *et al.*, 2010). DNSP-11 was also shown to not function through a feedback mechanism involving an upregulation of either GDNF mRNA or GDNF protein levels. Sixteen cytosolic proteins were identified as potential binding partners. Of these identified proteins, one, GAPDH, was known to play a role in apoptosis. After further evaluation of the interactions between DNSP-11 and GAPDH, it was observed that DNSP-11 could inhibit nitrosylation of GAPDH; however, it was not able to significantly reduce nuclear localization of GAPDH. This implies that while the interaction with GAPDH may play a contributing role in DNSP-11's anti-apoptotic activity, it is not likely the only mechanism by which DNSP-11 helps to reduce apoptosis.

If DNSP-11 is not providing its anti-apoptotic effects only through interactions with GAPDH, then it is likely that its interactions with other identified binding partners play at least a contributing role, if not the primary role, in DNSP-11's observed anti-apoptotic effects. One possible scenario is that DNSP-11 is able to interact with multiple metabolic binding partners that have been identified through a proteomic screen for a combined anti-apoptotic effect. Additionally, there is evidence that DNSP-11 has mitochondrial effects that are not observed after treatment with GAPDH, indicating that this mechanism is unique for DNSP-11. These mitochondrial effects may allow for an overall reduction in oxidative damage and lead to a reduction in levels of apoptosis. Further evaluation of these potential mechanisms is required to determine what their roles are in DNSP-11's anti-apoptotic activities.

III. Metabolic binding partners

In order to determine the potential binding partners of DNSP-11, a proteomic pull down assay was carried out. This assay identified sixteen proteins as potential binding partners of DNSP-11. The majority of these proteins are known to play a role in cellular metabolism. Because of the number of potential DNSP-11 binding partners that have been identified as playing a role in cellular metabolism, it is likely that DNSP-11, in mediating cellular metabolism, not only interacts with GAPDH, but may play a broader role as well.

While interaction between GAPDH and DNSP-11 were observed and confirmed, it seems likely that this interaction is not the sole source of the

observed anti-apoptotic effect that has been associated with treatment with DNSP-11. This broad mechanism may allow for DNSP-11 to interact with multiple binding partners with each of these interactions contributing in part to the overall observed effect. Therefore, while interactions with GAPDH may not be the primary source of protection, it may play a role in this combined mechanism.

a. Glutamate dehydrogenase

One example of an additional protein that has also been identified as a potential binding partner of DNSP-11 is glutamate dehydrogenase. Glutamate dehydrogenase is located within the mitochondria of cells. This enzyme functions in the interconversion of glutamate to α-ketoglutarate. This reaction also involves the conversion of NAD^+ to NADH. Due to the change in NADH/ NAD^+ levels, this reaction can be monitored through the use of a spectrophotometer.

Studies were carried out looking at DNSP-11's effects on glutamate dehydrogenase activity. These *in vitro* studies involved treating glutamate dehydrogenase with DNSP-11 and then evaluating its kinetic activity. It was observed that the presence of DNSP-11 reduced the activity of glutamate dehydrogenase. This study provides us with evidence that some of the additional potential binding partners that have been identified through the proteomic binding study play a role in mediating the observed anti-apoptotic effects of DNSP-11.

Additional evaluation of the other identified potential binding partners with metabolic functions is required to get a complete picture of the full extent of the anti-apoptotic effects of DNSP-11. These studies can help to further reveal the complex nature of DNSP-11's protective activity. By providing further information about DNSP-11's mechanism, these studies will provide us with the necessary foundation for further studies to evaluate its full potential as a therapeutic agent.

IV. Mitochondrial interactions

It has been observed that DNSP-11 is able to protect from 6-OHDA induced apoptosis, similar to what has been observed in studies with mature GDNF. However, DNSP-11 offers protection from additional toxins that mature GDNF has been shown not to provide. Studies have been carried out evaluating the DNSPs' (DNSP-5, DNSP-11, and DNSP-17) ability to provide protection from several mitochondrial toxins. It was observed that DNSP-11 offered the most significant protection from these toxins, including staurosporine and 3-nitropropionic acid (3-NP). This observation led to the further evaluation of DNSP-11's protection from mitochondrial involvement.

It was observed that 10 ng/mL DNSP-11 was able to protect B65 cells, a dopaminergic cell line, from 1mM staurosporine during a 20 hour treatment by approximately 125% as measured by LIVE/DEAD assay. DNSP-11 was able to significantly reduce the observed staurosporine-induced cytotoxicity. However, treatment with 10 ng/mL GDNF did not provide significant protection from staurosporine-induced cytotoxicity. Similarly, DNSP-11 was able to provide

protection from gramicidin-induced cytotoxicity. DNSP-11 was able to reduce

gramicidin-induced cytotoxicity after treatment with 1mM gramicidin for 20 hours

by approximately 60% compared to the control. In contrast, GDNF was not able

to protect the B65 cells from gramicidin. This study indicates that there are

distinct differences between the mechanisms by which GDNF and DNSP-11

provide their observable beneficial effects, despite being derived from the same

parent molecule.

The release of cytochrome c from the mitochondria is associated with

mitochondrial apoptosis. The release of cytochrome c can be used to measure

the relative levels of the associated mitochondrial apoptosis. Studies looking at

the staurosporine-induced release of cytochrome c after treatment with either

DNSP-11 or GDNF have been carried out. It was observed that treatment with

DNSP-11 was able to reduce the amount of cytochrome c that was released from

the mitochondria. However, no reduction in cytochrome c release was observed

after treatment with mature GDNF. This further indicates that DNSP-11 functions

via a mechanism distinct from mature GDNF. This unique mechanism appears to

involve a role in mediating mitochondrial apoptosis.

These studies indicate that DNSP-11 can offer broad mitochondrial

protection in cellular models. Based on data about the potential binding partners

of DNSP-11, it is likely that DNSP-11's interactions with multiple metabolic

binding partners is responsible for these protective effects. In addition to these

studies, there is data indicating that DNSP-11 is able to affect the mitochondrial

respiration rate, which has also not been previously observed with mature GDNF.

V. Combination of mechanisms

Since significant reductions in the levels of nuclear GAPDH were not observed after treatment with DNSP-11 in HEK-293 cells, it is likely that DNSP-11 does not offer its observed anti-apoptotic effects through this single mechanism. Evidence that DNSP-11 is able to reduce the amount of GAPDH nitrosylation that is observed indicates that DNSP-11 could be altering the GAPDH's interaction with Siah-1 even if this does not result in significant reductions in nuclear GAPDH. Based on this data along with other data looking at DNSP-11's effects on the mitochondria, it is likely that DNSP-11 interacts with multiple partners, not just GAPDH, to provide its anti-apoptotic effects.

Since many of the binding partners that were observed during the proteomic binding assay have known metabolic functions, it may be hypothesized that DNSP-11 has a broader metabolic effect. Through interactions with these proteins, DNSP-11 may influence multiple pathways to provide an overall protective effect. Evidence that DNSP-11 is able to affect mitochondrial pathways by mechanisms that are unique from that of GDNF supports the idea that DNSP-11 is functioning by a mechanism unique from that of GDNF.

VI. Future directions

a. GAPDH

While the interaction with GAPDH does not appear to be the only mechanism by which DNSP-11 is able to offer its protective effects, this interaction does appear to alter the activity of GAPDH. Data presented here

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indicates that DNSP-11 is able to bind to non-nitrosylated GAPDH and alter its activity within the apoptotic pathway. The binding of DNSP-11 to GAPDH reduces the nitrosylation of GAPDH, which is necessary for GAPDH binding to Siah1 to activate apoptosis. It was observed that this interaction was not able to significantly reduce nuclear GAPDH, a marker of this apoptotic pathway, in HEK 293 cells after 24 hour treatment; however, reductions in nuclear GAPDH were observed in MN9D cells after 5 minutes of treatment with staurosporine and DNSP-11. These data indicate that the 24 hour timeframe was to long for the GAPDH mechanism to be effective. Based on this, future studies could be carried out at shorter time periods to determine if GAPDH plays an early role in the observed anti-apoptotic effects of DNSP-11.

i. C150 mutation of GAPDH experiments

When GAPDH is involved in initiating apoptosis, GAPDH is nitrosylated at cysteine 150 (C150) (Hara *et al.*, 2005). This nitrosylation allows for the binding between GAPDH and Siah1 and therefore, the nuclear localization of GAPDH. The nuclear localization of GAPDH is a marker of the apoptotic pathway. Studies to evaluate DNSP-11's interaction with C150 could be carried out to further evaluate its role in mediating this apoptotic pathway. C150 of GAPDH can be mutated and binding studies between the mutated GAPDH and DNSP-11 can performed. These studies will provide information about the importance of C150 in the binding of DNSP-11 to GAPDH. It is hypothesized that this site on GAPDH is involved in the interaction between DNSP-11 and GAPDH since it has

been observed that nitrosylation of GAPDH, which has been shown to occur at C150 (Hara *et al.*, 2005), has been shown to reduce the binding of DNSP-11 to GAPDH.

These studies will provide further insight into the interactions between DNSP-11 and GAPDH. This will allow for a better understanding of how DNSP-11 is able to mediate GAPDH's role in apoptosis and potentially provide insight into why this interaction is not able to completely inhibit nuclear localization of GAPDH.

b. Glutamate dehydrogenase

The identification of DNSP-11's potential binding partners resulted in data indicating that DNSP-11 interacts with a variety of metabolic proteins, including glutamate dehydrogenase (GDH). Additional studies have shown that the interaction between DNSP-11 and GDH is able to reduce the activity of GDH. Further evaluation of GDH's interaction with DNSP-11 can provide insight into how the interaction between DNSP-11 and GDH is able to alter the activity of GDH. By further understanding the role of DNSP-11 in modifying the activity of GDH, insight into how DNSP-11 is able to affect apoptotic pathways can be provided.

c. Evaluation of other binding partners

To date, only two of the potential binding partners identified during the proteomic pull down assay have been further investigated for a potential role in

apoptosis. By evaluating the interaction of DNSP-11 with some of these additional proteins, insight into potential involvement of multiple mechanisms in the observed protective effects of DNSP-11 can be explored.

Additional studies can be carried out to further evaluate the roles of each of these potential binding partners. First, binding studies can be done to determine if DNSP-11 is actually able to interact with each of the potential binding partners. These studies would provide data that would help to determine which interactions should be further evaluated. Once those binding partners have been identified, studies to look at how the interaction with DNSP-11 affects their activity can be performed. If the data indicates that DNSP-11 is able to affect the activity of these binding partners, further studies can provide information about the mechanism of action.

Finally, studies to look at DNSP-11's overall role in metabolism will provide further insight into how the interactions with multiple binding partners can affect the metabolic pathways *in vitro*. The interaction with numerous metabolic proteins indicates that DNSP-11 may play an important role in modifying multiple pathways involved in metabolism. Understanding the modification of metabolic pathways by DNSP-11 may play an important role in providing a more comprehensive understanding of the role of DNSP-11 in apoptosis.

REFERENCES

Airaksinen M.S., Titievsky A., Saarma M. (1999). GDNF family neurotrophic factor signaling: four masters, on servant? Mol Cell Neurosci. 13: 313-325.

Akundi R.S., Zhi L., Sullivan P.G. Bueler H. (2012). Shared and cell type-specific mitochondrial defects and metabolic adaptations in primary cells from PINK1-deficient mice. Neurodegener Dis. Epub.

Alderson R.F., Alterman A.L., Barde Y.A., Lindsay R.M. (1990). Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron. 5:297-306.

Alfano I., Vora P., Mummery R.S., Mulloy B., Rider C.C., (2007). The major determinant of the heparin binding of glial cell-line-derived neurotrophic factor is near the N-terminus and is dispensable for receptor binding. Biochem J. 404: 131-140.

Alibin R.L., Greenamyre J.T. (1992). Alternative excitotoxic hypotheses. Neurology. 42:733-738.

Alim M.A., Ma Q.L., Takeda K., Aizawa T., Matsubara M., Nakamura M., Asada A., Saito T., Kaji H., Yoshii M., Hisanaga S., Ueda K. (2004). Demonstration of a role for alpha-synuclein as a functional microtubule-associated protein. J Alzheimers Dis. 6: 435-442, discussion 443-449.

Altar C.A., Boylan C.B., Jackson C., Hershenson S., Miller J., Wiegand S.J., Lindsay R.M., Hyman C. (1992). Brain-derived neurotrophic factor augments rotational behavior and nigrostriatal dopamine turnover in vivo. Proc Natl Acad Sci USA. 89:11347-11351.

Amberg J.J., Schreier T.M., Gaikowski M.P. (2012). Molecular responses differ between sensitive silver carp and tolerant bighead carp and bigmouth buffalo exposed to rotenone. Fish Physiol Biochem. 38:1379-1391.

Apostolou A., Shen Y., Liang Y., Fang S. (2008). Armet, a UPR-upregulated protein, inhibits cell proliferation and ER stress-induced cell death. Exp Cell Res. 314:2454-2467.

Aquilonius S.M., Hartvig P. (1986). A Swedish county with unexpectedly high utilization of antiparkinsonian drugs. Acta Neurol Scand. 74:379-382.

Arenas E., Trupp M., Akerud P., Ibanez C.F. (1995). GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron. 15:1465-1473.

Arendt T., Bigl V., Arendt A., Tennstedt A. (1983). Loss of neurons in the nucleus basalis of Meynert in Alzheimer's disease, paralysis agitans and Korsakoff's Disease. Acta Neuropathol. 61: 101-108.

Ballard P.A., Tetrud J.W., Langston J.W. (1985). Permanent human parkinsonism due to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): seven cases. Neurology. 35:949-956.

Baloh R.H., Tansey M.G., Lampe P.A., Fahrner T.J., Enomot H., Simburger K.S., Leitner M.L., Araki T., Johnson E.M., Milbrandt J. (1998). Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron. 21: 1291-1301.

Bankiewicz K.S., Eberling J.L., Kohutnicka M., Jagust W., Pivirotto P., Bringas J., Cunningham J., Budinger T.F., Harvey-White J. (2000). Convection-enhanced delivery of AAV vector in parkinsonian monkeys; in vivo detection of gene expression and restoration of dopaminergic function using pro-drug approach. Exp Neurol. 164:2-14.

Bandopadhyay R., Kingsbury A.E., Cookson M.R., Reid A.R. Evans I.M., Hope A.D., Pittman A.M., Lashley T., Canet-Aviles R., Miller D.W., McLendon C., Strand C., Leonard A.J., Abou-Sleimana P.M. Healy D.G., Ariga H., Wood N.W., de Silva R., Revesz T., Hardy J.A., Lees A.J. (2004). The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. Brain. 127: 420-430.

Bartus R.T. (2012). Translating the therapeutic potential of neurotrophic factors to clinical 'proof of concept': a personal saga achieving a career-long quest. Neurobiol Dis. 48: 153-178.

Beal M.F. (1994). Neurochemistry and toxin models in Huntington's disease. Curr Opin Neurol. 7: 542-547.

Bekris L.M., Mata I.F., Zabetian C.P. (2010). The genetics of Parkinson disease. J Geriatr Psychiatry Neurol. 23: 228-242.

Bell L.N. (1997). Peptide stability in solids and solutions. Biotechnol Prog. 13: 342-346.

Benraiss A., Chmielnicki E., Lerner K., Roh D., Goldman S.A. (2001). Adenoviral brian-derived neurotrophic factor induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. J Neurosci. 21:6718-6731.

Bensadoun J.C., Deglon N., Tseng J.L., Ridet J.L., Zurn A.D., Aebischer P. (2000). Lentiviral vectors as a gene delivery system in the mouse midbrain:

cellular and behavioral improvements in a 6-OHDA model of Parkinson's disease using GDNF. Exp Neurol. 164:15-24.

Betarbet R., Sherer T.B., MacKenzie G., Garcia-Osuna M., Panov A.V., Greenamyre J.T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. Nat Neurosci. 3:1301-1306.
Biskup S., Moore D.J., Celsi F., Higashi S., West A.B., Andrabi S.A., Kurkinen K., Yu S.W., Savitt J.M., Waldvogel H.J. Faull R.L. Emson P.C., Torp R., Ottersen O.P., Dawson T.M., Dawson V.L. (2006). Localization of LRRK2 to membranous and vesicular structures in mammalian brain. Ann Neurol. 60: 557-569.

Bobo R.H., Laske D.W., Akbasak A., Morrison P.F., Dedrick R.L., Oldfield E.H. (1994). Convection-enhanced delivery of macromolecules in the brain. Proc Natl Acad Sci USA. 91:2076-2080.

Bonifati V., Fabrizio E., Vanacore N., De Mari M., Meco G. (1995). Familial Parkinson's disease: a clinical genetic analysis. Can J Neurol Sci. 22: 158.

Bonifati V., Rizzu P., va Baren M.J., Schapp O., Breedveld G.J., Krieger E., Dekker M.C., Squitieri F., Ibanez P., Joosse M., van Dongen J.W., Vanacore N., van Swieten J.C. Brice A., Meco G., van Duijn C.M., Oostra B.A., Heutink P. (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science. 299: 256-259.

Bonifati V., Rohe C.F., Breedveld G.J., Fabrizio E., De Mari M., Tassorelli C., Tavella A., Marconi R., Nicholl D.J., Chien H.F., Fincati E., Abbruzzese G., Marini P., De Gaetano A., Horstink M.W., Maat-Kievit J.A., Sampaio C., Antonini A., Stocchi F., Montagna P., Toni V., Guidi M. Dalla Libera A., Tinazzi M., De Pandis F., Fabbrini G., Goldwurm S., de Klein A., Barbosa E., Lopiano L., Martignoni E., Lamberti P., Vanacore N., Meco G., Oostra B.A.; Italian Parkinson Genetic Network. (2005). Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. Neurology 65: 87-95.

Bordeaux M.C., Forcet C., Granger L., Corset V., Bidaud C., Billaud M., Bredesen D.E., Edery P., Mehlen P. (2000). EMBO J. 19:4056-4063.

Borrello M.G., Alberti L., Arighi E., Bongarzone I., Battistini C., Bardelli A., Pasini B., Piutti C., Rizzetti M.G., Mondellini P., Radice M.T., Pierotti M.A. (1996). The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase C gamma. Mol Cell Biol. 16: 2151-2163.

Braak H., Del Tredici K., Bratzke H., Hamm-Clement J., Sandmann-Keil D., Rub U. (2002). Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). J Neurol. 249 Suppl 3: III/1-5.

Braak H., Del Tredici K., Rub U., De Vos R.A., Jansen Steur E.N., Braak E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging. 24: 197-211.

Bradley L.H., Fuqua J., Richardson A., Turchan-Cholwo J., Ai Y., Kelps K.A., Glass J.D., He X., Zhang Z., Grodin R., Littrell O.M., Huettl P., Pomerleau F., Gash D.M., Gerhardt G.A. (2010). Dopamine neuron stimulating actions of a GDNF propeptide. PLos One. 5:e9752.

Bradley L.H., Gash D.M., Gerhardt G.A., Glass J. (2009). Amidated dopamine neuron stimulating peptides for CNS dopaminergic upreguation. US Patent Application. 12/508,916.

Bueler H., (2009). Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease. Exp Neurol. 218: 235-246.

Cass W.A., Manning M.W. (1999). GDNF protection against 6-OHDA-induced reduction in potassium-evoked overflow of striatal dopamine. J Neurosci. 19: 1416-1423.

Cass W.A., Walker D.J., Manning M.W. (1999). Augmented methamphetamine-induced overflow of striatal dopamine 1 day after GDNF administration. Brain Res. 827: 104-112.

Castello P.R., Dreschsel D.A., Patel M. (2007). Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. J Biol Chem. 282: 14186-14193.

Chan-Palay V., Asan E. (1989). Alterations in catecholamine neurons of the locus coeruleus in senile dementia of the Alzheimer type and in Parkinson's disease with and without dementia and depression. J Comp Neurol. 287: 373-392.

Chao M.V., Bothwell M. (2002). Neurotrophins: to cleave or not to cleave. Neuron. 33:9-12.

Chiba K., Trevor A., Castagnoli N. Jr. (1984). Metabolism of the neurotoxic tertiary amine, MPTP, by brain monoamine oxidase. Biochem Biophys Res Commun. 120:574-578.

Choi-Lundberg D.L., Lin Q., Chang Y.N., Chiang Y.L., Hay C.M., Mohajeri H., Davidson B.L., Bohn M.C. (1997). Dopaminergic neurons protected from degeneration by GDNF gene therapy. Science. 275:838-841.

Choi-Lundberg D.L., Lin Q., Schallert T., Crippens D., Davidson B.L., Chang Y.N., Chiang Y.L., Qian L., Bardwaj L., Bohn M.C. (1998). Behavioral and cellular

protection of rat dopaminergic neurons by an adenoviral vector encoding glial cell line-derived neurotophic factor. Exp Neurol. 154:261-275.

Conway K.A., Harper J.D., Lansbury P.T. (1998). Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nat Med. 4: 1318-1320.

Cookson M.R., Lockhart P.J., McLendon C., O'Farrell C., Schlossmacher M., Farrer M.J. (2003). RING finger 1 mutations in Parkin produce altered localization of the protein. Hum Mol Genet. 12: 2957-2965.

Costello S., Cockburn M., Bronstein J., Zhang X., Ritz B. (2009). Parkinson's disease and residential exposure to maneb and paraquat from agricultural applications in the central valley of California. Am J Epidemiol. 169: 919-926.

Creedon D.J., Tansey M.G., Baloh R.H., Osborne P.A., Lampe P.A., Fahrner T.J., Heuckeroth R.O., Milbrandt J., Johnson E.M. Jr. (1997). Neurturin shares receptors and signal transduction pathways with glial cell line-derived neurotrophic factor in sympathetic neurons. Proc Natl Acad Sci USA. 94:7018-7023.

Darios F., Corti O., Lucking C.B., Hampe C., Muriel M.P., Abbas N., Gu W.J., Hirsch E.C., Rooney T., Ruberg M., Brice A. (2003). Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. Hum Mol Genet. 12: 517-526.

Date I., Shingo T., Ohmoto T., Emerich D.F. (1997). Long-term enhanced chromaffin cell survival and behavioral recovery in hemiparkinsonian rats with co-grafted poylmer-encapsulated human NGF-secreting cells. 147: 10-17.

Dawson T.M., Dawson V.L. (2003). Molecular pathways of neurodegeneration in Parkinson's disease. Science 302: 819-822.

Decressac M., Ulusoy A., Mattsson B., Georgievska B., Romero-Ramos M., Kirik D., Bjorklund A. (2011). GDNF fails to exert neuroprotection in a rat a-synuclein model of Parkinson's disease. Brain. 134:2302-2311.

Dekker M.C., van Swieten J.C., Houwing-Duistermaat J.J., Snijders P.J., Boeren D., Hofman A., Breteler M.M., Heutink P., Oostra B.A., van Duijn C.M. (2003). A clinical-genetic study of Parkinson's disease in a genetically isolated community. J Neurol. 250: 1056-1062.

Dekker M.C., Galjaard R.J., Snijders P.J., Heutink P., Oostra B.A., van Duijn C.M. (2004). Brachydactyly and short stature in a kindred with early-onset parkinsonism. Am J Med Genet A. 130A: 102-104.

Deglon N., Tseng J.L., Bensadoun J.C., Zurn A.D., Arsenijevic Y., Pereira de Almeida L., Zufferey R., Trono D., Aebischer P. (2000). Self-inactivating lentiviral vectors with enhanced transgene expression as potential gene transfer system in Parkinson's disease. Hum Gene Ther. 11:179-190.

Depino A.M., Earl C., Kaczmarczyk E., Ferrari C., Besedovsky H., del Rey A., Pitossi F.J., Oertel W.H. (2003). Microglial activation with atypical proinflammatory cytokine expression in a rat model of Parkinson's disease. Eur J Neurosci. 18:2731-2742.

Desai V.G., Feuers R.J., Hart R.W., Ali S.F. (1996). MPP(+)-induced neurotoxicity in mouse is age-dependent: evidened by the selective inhibition of complexes of electron transport. Brain Res. 715: 1-8.

Dhillon A.S., Tarbutton G.L., Levin J.L., Plotkin G.M., Lowry L.K., Nalbone J.T., Shepherd S. (2008). Pesticide/environmental exposures and Parkinson's disease in East Texas. J Agromedicine. 13:37-48.

Dinis-Oliveria R.J., Remiao F., Carmo H., Duarte J.A., Navarro A.S., Bastos M.L., Carvalho F. (2006). Paraquat exposure as an etiological factor of Parkinson's disease. Neurotoxicology. 27:1110-1122.

Factor S.A. (1999). Dopamine agonists. Med Clin North Am. 83:415-443.

Feng L., Wang C.Y., Jiang H., Oho C., Dugich-Djordjevic M., Mei L., Lu B. (1999). Differential signaling of glial cell line-derived neurothrophic factor and brain-derived neurotrophic factor in cultured ventral mesencephalic neurons. Neuroscience. 93:265-273.

Fiandaca M.S., Forsayeth J.R., Dickinson P.J., Bankiewicz K.S. (2008). Image-guided convection-enhanced delivery platform in the treatment of neurological diseases. Neurotherapeutics. 5: 123-127.

Forno L.S., (1996). Neuropathology of Parkinson's disease. J Neuropathol Exp Neurol. 55: 259-372.

Fox C.M., Gash D.M., Smoot M.K., Cass W.A. (2001). Neuroprotective effects of GDNF agains 6-OHDA in young and aged rats. Brain Res. 896: 56-63.

Gash D.M., Zhang Z., Cass W.A., Ovadia A., Simmerman L., Martin D., Russell D., Collins F., Hoffer B.J., Gerhardt G.A. (1995). Morphological and functional effects of intranigrally administered GDNF in normal rhesus monkeys. J Comp Neurol. 363: 345-358.

Gash D.M., Zhang Z., Ovadia A., Cass W.A. Yi A., Simmerman L., Russell D., Martin D., Lapchack P.A., Collins F., Hoffer B.J., Gerhardt G.A. (1996). Functional recovery in parkinsonian monkeys treated with GDNF. Nature. 380: 252-255.

Gash D.M., Zhang Z., Ai Y., Grondin R., Coffey R., Gerhardt G.A. (2005). Trophic factor distribution predicts functional recovery in parkinsonian monkeys. Ann Neurol. 58:224-233.

Gash D.M., Rutland K., Hudson N.L., Sullivan P.G., Bing G., Cass W.A., Pandya J.D., Liu M., Choi D.Y., Hunter R.L., Gerhardt G.A., Smith C.D., Slevin J.T., Prince T.S. (2008). Trichloroethylene: Parkinsonism and complex 1 mitochondrial neurotoxicity. Ann Neurol 63: 184-192.

Gasmi M., Herzog C.D., Brandon E.P., Cunningham J.J., Ramirez G.A., Ketchum E.T., Bartus R.T. (2007). Striatal delivery of neurturin by CERE-120, and AAV2 vectore for the treatment of dopaminergic neuron degeneration in Parkinson's disease. Mol Ther. 15: 62-68.

Gasmi M., Brandon E.P. Herzog C.D., Wilson A., Bishop K.M., Hofer E.K., Cunningham J.J., Printz M.A., Kordower J.H., Bartus R.T. (2007). AAV2-mediated delivery of human neurturin to the rat nigrostriatal system: long-term efficacy and tolerability of CERE-120 for Parkinson's disease. Neurobiol Dis. 27: 67-76.

Gaspar P., Gray F. (1984). Dementia in idiopathic Parkinson's disease. A neuropathological study of 32 cases. Acta Neuropathol. 64: 43-52.

George J.M. (2002). The synucleins. Genome Biol. 3: 3: reviews3002.1-reviews3002.6.

Greenamyre J.T. Sherer T.B., Betarbet R., Panov A.V. (2001). Complex I and Parkinson's disease. IUBMB Life. 52: 135-141.

Gill S.S., Patel N.K., Hotton G.R., O'Sullivan K., McCarter R., Bunnage M., Brooks D.J., Svendsen C.N., Heywood P. (2003). Direct brain infusion of glial cell line-derived neurotophic factor in Parkinson disease. Nat Med 9:589-595.

Gimenez F., Krauze M.T., Valles F., Hadaczek P., Bringas J., Sharma N., Forsayeth J., Bankiewicz K.S. (2011). Image-guided convection-enhanced delivery of GDNA protein into monkey putamen. Neuroimage. 54:189-195.

Goldman S.M., Quinlan P.J., Ross G.W., Marras C., Meng C., Bhudhikanok G.S., Comyns K., Korell M., Chade A.R., Kasten M., Priestley B., Chou K.L., Fernandez H.H., Cambi F., Langston J.W., Tanner C.M. (2012). Solvent exposures and Parkinson disease risk in twins. Ann Neurol. 71: 776-784.

Gorell J.M., Johnson C.C., Rybicki B.A., Peterson E.L., Richardson R.J. (1998). The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living. Neurology. 50:1346-1350.

Granieri E., Carreras M., Casetta I., Govoni V., Tola M.R., Paolino E., Monetti V.C., De Bastiani P. (1991). Parkinson's disease in Ferrara, Italy, 1967 through 1987. Arch Neurol. 48:854-857.

Greenamyre J.T., Sherer T.B., Betarbet R., Panov A.V. (2001). Complex I and Parkinson's disease. IUBMB Life. 52:135-141.

Greene J.G., Greenamyre J.T. (1996). Manipulation of membrane potential modulates malonate-induced strital excitotoxicity in vivo. J Neurochem. 66: 637-643.

Greenfield J.G., Bosanquet F.D. (1953). The brain-stem lesions in Parkinsonism. J Neurol Neurosurg Psychiatry. 16: 213-226.

Grondin R., Zhang Z., Yi A., Cass W.A., Maswood N., Andersen A.H., Elsberry D.D., Klein M.C., Gerhardt G.A., Gash D.M. (2002). Chronic, controlled GDNF infusion promotes structural and functional recovery in advanced parkinsonian monkeys. Brain. 125: 2191-2201.

Gu S., Huang H., Bi J., Yao Y., Wen T. (2009). Combined treatment of neurotrophin-3 gene and neural stem cells is ameliorative to behavior recovery of Parkinson's disease rat model. Brain Res. 1257: 1-9.

Gu W.J., Corti O., Araujo F., Hampe C., Jacquier S., Lucking C.B., Abbas N., Duyckaerts C., Rooney T., Pradier L., Ruberg M., Brice A. (2003). The C289G and C418R missense mutations cause rapid sequestration of human Parkin into insoluble aggregates. Neurobiol Dis. 14: 357-364.

Hamilton J.F., Morrison P.F., Chen M.Y., Harvey-White J., Pernaute R.S., Phillips H., Oldfield E., Bankiewicz K.S. (2001). Heparin coinfusion during convection-enhanced delivery (CED) increases the distribution of the glial-derived neurotrophic factor (GDNF) ligand family in rat striatum and enhances the pharmacological activity of neurturin. Exp Neurol. 168: 155-161.

Hara M.R., Agrawal N., Kim S.F., Cascia M.B., Fujimuro M., Ozeki Y., Takahashi M., Cheah J.H., Tankou S.K., Hester L.D., Ferris C.D., Hayward S.D., Snyder S.H., Sawa A. (2005). S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. Nat Cell Biol. 7:665-667.

Hara M.R., Thomas B., Casci M.B., Bae B.I., Hester L.D., Dawson V.L., Dawson T.M., Sawa A., Snyder S.H. (2006). Neuroprotection by pharmacologic blockade of the GAPDH death cascade. Proc Natl Acad Sci USA. 103: 3887-3889.

Hardy J., Cai H., Cookson M.R., Gwinn-Hardy K., Singleton A. (2006). Genetics of Parkinson's disease and parkinsonism. Ann Neurol. 60: 389-398.

Hatano Y., Li Y., Sato K., Asakawa S., Yamamura Y., Tomiyama H., Yoshino H., Asahina M., Kobayashi S., Hassin-Baer S., Lu C.S., Ng A.R., Rosales R.L., Shimizu N., Toda T., Mizuno Y., Hattori N. (2004). Novel PINK1 mutations in early-onset parkinsonism. Ann Neurol. 56: 424-427.

Hatano T., Kubo S., Imai S., Maeda M., Ishikawa K., Mizuno Y., Hattori N. (2007). Leucine-rich repeat kinase 2 associates with lipid rafts. Hum Mol Genet. 16: 678-690.

Henderson C.E., Phillips H.S., Pollock R.A., Davies A.M., Lemeulle C., Armanini M., Simmons L., Moffet B., Vandlen R.A., Simmons L., et al. (1994). GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science. 266:1062-1064.

Herbert M.A., Gerhardt G.A. (1997). Behavioral and neurochemical effects of intranigral administration of glial cell line-derived neurotrophic factor on aged Fischer 344 rats. J Pharmacol Exp Ther. 282: 760-768.

Herzog C.D., Dass B., Holden J.E., Stansell J., Gasmi M., Tuszynski M.H., Bartus R.T>, Kordower J.H. (2007). Striatal delivery of CERE-120, an AAV2 vector encoding human neurturin, enhances activity of the dopaminergic nigrostriatal system in aged monkeys. Mov Disord. 22: 1124-1132.

Hoffer B.J., Hoffman A., Bowenkamp K., Hudson J., Martin D., Lin L.F., Gerhardt G.A. (1994). Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. Neurosci Lett. 182:107-111.

Horowski R., Horowski L., Vogel S., Poewe W., Kielhorn F.W. (1995). An essay on Wilhelm von Humboldt and the shaking palsy: first comprehensive description of Parkinson's disease by a patient. Neurology. 45: 565-568.

Hou J.G., Lin L.F., Mytilineou C. (1996). Glial cell line-derived neurotrophic factor exerts neurotrophic effects on dopaminergic neurons in vitro and promotes their survival and regrowth after damage by 1-methyl-4-phenylpyridinium. J Neurochem. 66:74-82.

Howells D.W., Porritt M.J., Wong J.Y., Batchelor P.E., Kalnins R., Hughes A.J., Donnan G.A. (2000). Reduce BDNF mRNA expression in the Parkinson's disease substantia nigra. Exp Neurol. 166:127-135.

Hubble J.P., Cao T., Hassanein R.E., Neuberger J.S., Koller W.C. (1993). Risk factors for Parkinson's disease. Neurobiology 43: 1693-1697.

Hung H.C., Lee E.H. (1996). The mesolimbic dopaminergic pathway is more resistant that the nigrostriatal dopaminergic pathway to MPTP and MPP+ toxicity: role of BDNF gene expression. Brain Res Mol Brain Res. 41:14-26.

Hurtig H., Joyce J., Sladek J.R., Trojanowski J.Q. (1989). Postmortem anaylsis of adrenal-medulla-to caudate autograft in a patient with Parkinson's disease. Ann Neurol. 25: 607-614.

Hyman C., Hofer M., Barde Y.A., Juhasz M., Yancopoulos G.D., Squinto S.P., Lindsay R.M. (1991). BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature. 350: 230-232.

Hyman C., Juhasz M., Jackson C., Wright P., Ip N.Y., Lindsay R.M. (1994). Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the bentral mesencephalon. J Neurosci 14: 335-347.

Ibanez C.F. (1998). Emerging themes in structural biology of neurotrophic factors. Trends Neurosci. 21:438-444.

Immonen T., Alakuijala A., Hytonen M., Sainio K., Poteryaev D., Saarma M., Pasternack M., Sariola H. (2008). A proGDNF-related peptide BEP increases synaptic excitation in rat hippocampus. Exp Neurol. 210:793-796.

Jankovic J., Stacy M. (2007). Medical management of levodopa-associated motor complications in patients with Parkinson's disease. CNS Drugs. 21:677-692.

Jing S., Wen D., Yu Y., Holst P.L., Luo Y., Fang M., Tamir R., Antonio L., Hu Z., Cupples R., Louis J.C., Hu S., Altrock B.W., Fox G.M. (1996). GDNF-induced activation of the ret protein tyrsine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell. 85:1113-1124.

Kajiwara K., Byrnes A.P., Charlton H.M., Wood M.J., Wood K.J. (1997). Immune responses to adenoviral vectors during gene transfer in the brain. Hum Gene Ther. 8:253-265.

Kelps K.A., Turchan-Cholewo J., Hascup E.R., Taylor T.L., Gash D.M., Gerhardt G.A., Bradley L.H. (2011). Evaluation of the physical and in vitro protective activity of three synthetic peptides derived from the pro-and mature GDNF sequence. Neuropeptides. 45:213-218.

Kearns C.M., Gash D.M. (1995). GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo. Brain Res. 672:104-111.

Kearns C.M., Cass W.A., Smoot K., Kryscio R., Gash D.M. (1997). GDNF protection against 6-OHDA: time dependence and requirement for protein synthesis. J Neurosci. 17: 111-118.

Kent S.B. (1988) Chemical synthesis of peptides and proteins. Annu Rev Biochem. 57:709-715.

Khan N.L., Jain S., Lynch J.M., Pavese N., Abou-Sleiman P., Holton J.L., Healy D.G., Gilks W.P., Sweeney M.G., Ganguly M., Gibbons V., Gandhi S., Vaughn J., Eunson L.H., Katzenschlager R., Gayton J., Lennox G., Revesz T., Nicholl D., Bhatia K.P., Quinn N., Brooks D., Lees A.J., Davis M.B., Piccini P., Singleton A.B., Wood N.W. (2005). Mutations in the gene LRRK2 encoding dardarin (PARK8) cause familial Parkinson's disease: clinical, pathological, olfactory and functional imaging and genetic data. Brain. 128: 2786-2796.

Kirik D., Rosenblab C., Bjorklund A., Mandel R.J. (2000). Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J Neurosci. 20:4686-4700.

Klein R.D., Sherman D., Ho W.H., Stone D., Bennett G.L., Moffat B., Vandlen R., Simmons L., Gu Q., Hongo J.A., Devaux B., Poulsen K., Armanini M., Nozaki C., Asai N., Goddard A., Phillips H., Henderson C.E., Takahashi M., Rosenthal A. (1997). Nature. 387: 717-721.

Klein R.L., Muir D., King M.A., Peel A.L., Zolotukhin S., Moller J.C., Kruttgen A., Heymach J.V. Jr., Muzyczka N., Meyer E.M. (1999). Long-term actions of vector-derived nerve growth factor or brain-derived neurotrophic factor on choline acetyltransferase and Trk receptor levels in the adult rat basal forebrain. Neuroscience. 90:815-821.

Knusel B., Winslow J.W., Rosenthal A., Burton L.E., Seid D.P., Nikolics K., Hefti F. (1991). Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci USA. 88: 961-965.

Koller W., Veter-Overfield B., Gray C., Alexander C., Chin T. (1990). Environmental risk factors in Parkinson's disease. Neurology. 40: 1218-1221.

Kordower J.H., Bloch J., Ma S.Y., Chu Y., Palfi S., Roitberg B.Z., Emborg M., Hantraye P., Deglon N., Aebischer P. (1999). Lentiviral gene transfer to the nonhuman primate brain. Exp Neurol. 160:1-16.

Kordower J.H., Emborg M.E., Bloch J., Ma S.Y., Chu Y., Leventhal L., McBride J., Chen E.Y., Palfi S., Roitberg B.Z., Brown W.D., Holden J.E., Pyzalski R., Taylor M.D., Carvey P., Ling Z., Trono D., Hantraye P., Deglon N., Aebischer P., (2000).

Neurodegeneration prevented by lentiviral vector delivery delivery of GDNF in primate models of Parkinson's disease. Science. 290: 767-773.

Kordower J.H., Herzog C.D., Dass B., Bakay R.A. Stansell J., Gasmi M., Bartus R.T. (2006). Delivery of neurturin by AAV2 (CERE-120)-mediated gene transfer provides structural and functional neuroprotection and neurorestoration in MPTP-treated monkeys. Ann Neurol. 60: 706-715.

Kordower J.H., Bjorklund A. (2013). Trophic factor gene therapy for Parkinson's disease. Mov Disord. 28: 96-109.

Kotzbauer P.T., Lampe P.A., Heuckeroth R.O., Golden J.P., Creedon D.J., Johnson E.M. Jr. Milbrandt J. (1996). Neurturin, a relative of glial-cel-line-derived neurotrophic factor. Nature. 384:467-470.

Krauze M.T., Mcknight T.R., Yamashita Y., Bringas J., Noble C.O., Saito R., Geletneky K., Forsayeth J., Berger M.S., Jackson P., Park J.W., Bankiewicz K.S. (2005). Real-time visualization and characterization of liposomal delivery into the monkey brain by magnetic resonance imaging. Brain Res Brain Res Protoc. 16:20-26.

Kreutzberg G.W. (1996). Microglia:a sensor for pathological events in the CNS. Trends Neurosci. 19:312-318.

Krieglstein K., Suter-Crazzolara C., Unsicker K. (1995). Development of mesencephalic dopaminergic neurons and the transforming growth factor-beta superfamily. J Neural Transm Suppl. 46: 209-216.

Kubo S.I., Kitami T., Noda S., Shimura H., Uchiyama Y., Asakawa S., Minoshima S., Shimizu N., Mizuno Y., Hattori N. (2001). Parkin is associated with cellular vesicles. J Neurochem. 78: 42-54.

Lamballe F., Klein R., Barbacid M. (1991). trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell. 66:967-979.

Lang A.E., Gill S., Patel N.K., Lozano A., Nutt J.G., Penn R., Brooks D.J., Hotton G., Moro E., Heywood P., Brodsky M.A., Burchiel K., Kelly P., Dalvi A., Scott B., Stacy M., Turner D., Wooten V.G., Elias W.J., Laws E.R., Dhawan V., Stoessi A.J., Matcham J.,
Lorigados L., Alvarez P., Pavon N., Serrano T., Blanco L., Macias R., Coffey R.J., Traub M. (2006). Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson disease. Ann Neurol. 59:459-466.

Langston J.W., Langston E.B., Irwin I. (1984). MPTP-induced parkinsonism in human and non-human primates-clinical and experimental aspects. Acta Neurol Scand Suppl. 100: 49-54.

Lapchak P.A., Araujo D.M., Hilt D.C., Jiao S., Collin F., Miyoshi Y., Yi A., Zhang Z., Gash D.M. (1998). Topographical distribution of [^{125}I]-glial cell line-derived neurotrophic factor in unlesioned and MPTP-lesioned rhesus monkey brain following a bolus intraventricular injection. Brain Res. 789: 9-22.

Lazzarini A.M., Myers R.H., Zimmerman T.R., Mark M.H., Golbe L.I., Sage J.I., Johnson W.G., Duvoisin R.C. (1994). A clinical genetic study of Parkinson's disease: evidence for dominant transmission. Neurology. 44: 499-506.

Levi-Montalcini R., Hamburger V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J Exp Zool. 116:321-361.

Levivier M., Przedborski S., Bencsics C., Kang U.J. (1995). Intrastriatal implantation of fibroblasts genetically engineered to produce brain-derived neurotrophic factor prevents degeneration of dopaminergic neurons in a rat model of Parkinson's disease. J Neurosci. 15: 781-7820.

Levy O.A., Malagelada C., Greene L.A. (2009). Cell death pathways in Parkinson's disease: proximal triggers, distal effectors, and final steps. Apoptosis. 14: 478-500.

Lin L.F., Doherty D.H., Lile J.D., Bektesh S., Collins F. (1993). GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science. 160: 1130-1132.

Lin L.F., Zhang T.J., Collins F., Armes L.G. (1994). Purification and initial characterization of rat B49 glial cell line-derived neurotrophic factor. J Neurochem. 63: 758-768.

Lindahl M., Poteryaev D., Yu L., Arumae U., Timmusk T., Bongarzone I., Aiello A., Pierotti M.A., Airksinen M.S., Saarma M. (2000). Human glial cell line-derived neurotrophic factor recpetor alpha 4 is the recpetor for persephin and is predominantly expressed in normal and malignant thyroid medullary cells. J Biol Chem. 276:9344-9351.

Lindholm P., Voutilainen M.H., Lauren J., Peranen J., Leppanen V.M., Andressoo J.O., Lindahl M., Janhunen S., Kilkkinen N., Timmusk T., Tuominen R.K., Saarma M. (2007). Novel neurotrophic factor CDNF protects and rescues midbrain dopamine neurons in vivo. Nature. 448:73-77.

Lindholm P., Peranen J., Andressoo J.O., Kalkkinen N., Kokaia Z., Lindvall O., Timmusk T., Saarma M. (2008). MANF is widely epressed in mammalian tissues and differently regulated after ischemic and epileptic insults in rodent brain. Mol Cell Neurosci. 39:356-371.

Lindner M.D., Winn S.R., Baetge E.E., Hammang J.P., Gentile F.T., Doherty E., McDermott P.E., Frydel B., Ullman M.D., Schallert T., Emerich D.F. (1995). Implantation of encapsulated catecholamine and GDNF-producing cells in rats with unilateral dopamine depletions and parkinsonian symptoms. Exp Neurol. 132: 62-76.

Lindvall O., Wahlberg L.U. (2008). Encapsulated cell biodelivery of GDNF: A novel clinical strategy for neuroprotection and neuroregeneration in Parkinson's disease? Exp Neurol. 209: 82-88.

Litteljohn D., Mangano E., Clarke M., Bobyn J., Moloney K., Hayley S. (2010). Inflammatory mechanisms of neurodegeneration in toxin-based models of Parkinson's disease. Parkinsons Dis. 2001: 713517.

Lonser R.R., Gogate N., Morrison P.F., Wood J.D., Oldfield E.H. (1998). Direct convective delivery of macromolecules to spinal cord.J Neurosurg. 89:616-622.

Lopez A., Munoz A., Guerra M.J., Labandeira-Garcia J.L. (2001). Mechanisms of the effects of exogenous levodopa on the dopamine-denervated striatum. Neuroscience. 103: 639-651.

Lorigados L., Soderstrom S., Ebendal T. (1992). Two-site enzyme immunoassay for beta NGF applied to human patient sera. J Neurosci Res. 32: 329-339.

Ludolph A.C., He F., Spencer P.S., Hammerstad J., Sabri M. (1991). 3-Nitropropionic acid-exogenous animal neurotoxin and possible human striatal toxin. Can J Neurol Sci. 18: 492-498.

Lucking C.B., Durr A., Bonifati V., Vaughan J., De Michele G., Gasse T., Harhangi B.S., Meco G., Denefle P., Wood N.W., Agid Y., Brice A.; French Parkinson's Disease Genetics Study Group; European Consortium on Genetic Susceptibility in Parkinson's Disease. (2000). Association between early-onset Parkinson's disease and mutations in the parkin gene. N Engl J Med. 342: 1560-1567.

Mandel R.J., Spratt S.K., Snyder R.O., Leff S.E. (1997). Midbrain injection of recombinant adeno-associated virus encoding rat glial cell line-derived neurotrophic factor protects nigral neurons in a progressive 6-hydroxydopamine-induced degeneration model of Parkinson's disease in rats. Proc Natl Acad Sci USA. 94:14083-14088.

Mandel R.J., Snyder R.O., Leff S.E. (1999) Recombinant adeno-associated viral vector-mediated glial cell line-derived neurotrophic factor gene transfer protects nigral dopamine neurons after onset of progressive degeneration in a rat model of Parkinson's disease. Exp Neurol. 160:47-64.

Marks W.J., Ostrem J.L., Verhagen L., Starr P.A., Larson P.S., Bakay R.A., Taylor R., Cahn-Weiner D.A., Stoessl A.J., Olanow C.W., Bartus R.T. (2008). Safety and tolerability of intraputaminal delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: an open-label, phase I trial. Lancet Neurol. 7: 400-408.

Mata I.F., Wedemeyer W.J., Farrer M.J., Taylor J.P., Gallo K.A. (2006). LRRK2 in Parkinson's disease: protein domains and functional insights. Trends Neurosci. 29: 286-293.

Mayo Clinic. (2012). Parkinson's disease. http://www.mayoclinic.com/health/parkinsons-disease/DS00295/DSECTION=treatments-and-drugs.

Menegon A., Board P.G., Blackburn A.C., Mellick G.D., Le Couteur D.G. (1998). Parkinson's disease, pesticides, and glutathione transferase polymorphisms. Lancet. 352: 1344-1346.

Merrifield R. B. (1963). Solid phase peptide synthesis. J AM Chem Soc. 85:2149-2154.

Milbrandt J., de Sauvage F.J., Fahrner T.J., Baloh R.H, Leitner M.L., Tansey M.G., Lampe P.A., Heuckeroth R.O., Kotzbauer P.T., Simburger K.S., Golden J.P., Davies J.A., Vejsada R., Kato A.C., Hynes M., Sherman D., Nishimura M., Wang L.C., Vandlen R., Moffat B., Klein R.D., Poulsen K., Gray C., Garces A., Johnson E.M. Jr., et al. (1998). Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron. 20:245-253.

Mizobuchi N., Hoseki J., Kubota H., Toyokuni S., Nozaki J., Naitoh M., Koizumi A., Nagata K. (2007). ARMET is a soluble ER protein induced by the unfolded protein response via ERSE-II element. Cell Struct Funct. 32: 41-50.

Mogi M., Togari A., Kondo T., Mizuno Y., Komure O., Kuno S., Ichinose H., Nagatsu T. (1999). Brain-derived growth factor and nerve growth factor concentrations are decreased in the substantia nigra in Parkinson's disease. Neurosci Lett. 270: 45-48.

Monahan P.E., Samulski R.J. (2000). AAV vectors:is clinical success on the horizon? Gene Ther. 7:24-30.

Moore D.J., Dawson V.L., Dawson T.M. (2003). A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. J Neurochem. 87: 1558-1567.

Mouatt-Prigent A., Muriel M.P., Gu W.J., El Hachimi K.H., Lucking C.B., Brice A., Hirsch E.C. (2004). Ultrastructural localization of parkin in the rat brainstem, thalamus and basal ganglia. J Neural Transm. 111: 1209-1218.

Mori H., Kondo T., Yokochi M., Matsumine H., Nakagawa-Hattori Y., Miyake T., Suda K., Mizuno Y. (1998). Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology. 51: 890-892.

Morrison P.F., Laske D.W., Bobo H., Oldfield E.H., Dedrick R.L. (1994). High-flow microinfusion:tissue penetration and pharmacodynamics. Am J Physiol. 266:292-305.

Morrison P.F., Lonser R.R., Oldfield E.H. (2007). Convective delivery of glial cell line-derived neurotrophic factor in the human putamen. J Neurosurg. 107: 74-83.

Mortiboys H.J., Scaefer J., Reichmann H., Jackson S. (2007). Mitochondrial dysfunction in Parkinson's disease-revisited. Neurol Neurochir Pol. 41:150-159.

Najim al-Din A.S., Wriekat A., Mubaidin A., Dasouki M., Hiari M. (1994). Pallido-pyramidal degeneration, supranuclear upgaze paresis and dementia: Kufor-Rakeb syndrome. Acta Neurol Scand. 89: 347-352.

Nakano I., Hirano A. (1984). Parkinson's disease: neuron loss in the nucleus basalis without concomitant Alzheimer's disease. J Neuropathol Exp Neurol. 15: 415-418.

Nasr P., Carbery T., Geddes J. (2009). N-Methyl-d-aspartate receptor antagonists have variable affect in 3-nitropropionic acid toxicity. Neurochem Res. 34: 490-498.

Nguyen A.D., Herzog H., Sainsbury A. (2011). Neuropeptide Y and YY: important regulators of energy metabolism. Curr Opin Endocrinol Diabetes Obes. 18:56-60.

Nicklas W.J., Vyas I., Heikkila R.E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sci. 36: 2503-2508.

Nykjaer A., Lee R., Teng K.K., Jansen P., Madsen P., Nielsen M.S., Jacobsen C., Kliemannel M., Schwarz E., Willnow T.E., Hempstead B.L., Petersen C.M. (2004). Sortilin is essential for proNGF-induced neuronal cell death. Nature. 427:843-848.

Olney J.W. (1990). Excitotoxicity: an overview. Can Dis Wkly Rep. 16 Suppl 1E: 47-57.

Olson L., Ayer-LeLiever C., Ebendal T., Eriksdotter-Nilsson M., Ernfors P., Henschen A., Hoffer B., Giacobini M., Mouton P., Palmer M, et al. (1990). Grafts, growth factors and grafts that make growth factors. Prog Brain Res. 82:55-66.

Padgett C.M., Whorton A.R. (1995). S-nitrosoglutathione reversibly inhibits GAPDH by S-nitrosylation. Am J Physiol. 269:739-749.

Paisan-Ruiz C., Jain S., Evans E.W., Gilks W.P., Simon J., van der Brug M., Lopez de Munain A., Aparicio S., Gil A.M., Khan N., Johnson J., Martinex J.R., Nicholl D., Carrera I.M., Pena A.S., de Silva R., Lees A., Marti-Masso J.F., Pere-Tur J., Wood N.W., Singleton A.B. (2004). Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron. 44: 595-600.

Palfi S., Ferrante R.J., Brouillet E., Beal M.F., Dolan R., Guyot M.C., Peschanski M., Hantraye P. (1996). Chronic 3-Nitropropionic acid treatment in baboons replicates the cognitive and motor deficits of Huntington's disease. J Neurosci. 16: 3019-3025.

Pandey N., Schmidt R.E., Galvin J.E. (2006). The alpha-synuclein mutation E46K promotes aggregation in cultured cells. Exp Neurol. 197: 515-520.

Panov A., Dikalov S., Shalbuyeva N., Taylor G., Sherer T., Greenamyre J.T. (2005). Rotenone model of Parkinson disease: multiple brain mitochondria dysfunctions after short term systemic rotenone intoxication. J Biol Chem. 280: 42026-42035.

Parkash V., Leppanen V.M., Virtanen H., Jurvansuu J.M., Bespalov M.M., Sidorova Y.A., Runeberg-Roos P., Saarma M., Goldman A. (2008). The structure of glial cell line-derived neurotrophic factor-coreceptor complex: insights into RET signaling and heparin binding. J Biol Chem. 283: 35164-35172.

Parkinson J. (2002). An essay on the shaking palsy. 1817. J Neuropsychiatry Clin Neurosci. 14: 223-236, discussion 222.

Parkinson's Disease Foundation. (2013). Statistics on Parkinson's. http://www.pdf.org/en/parkinson_statistics.

Patel N.K., Gill S.S. (2007). GDNF delivery for Parkinson's disease. Acta Neurochir Suppl. 97: 135-154.

Peel A.L., Klein R.L. (2000). Adeno-associated virus vectors:activity and applications in the CNS. J Neurosci Methods. 98:95-104.

Peterson D.I., Price M.L., Small C.S. (1989). Autopsy findings in a patient who had an adrenal-to-brain transplant for Parkinson's disease. Neurology. 39: 235-238.

Petrova P., Raibekas A., Pevsner J., Vigo N., Anafi M., Moore M.K., Peaire A.E., Shridhar V., Smith D.I., Kelly J., Durocher Y., Commissiong J.W. (2003). MANF: a new mesencphalic, astrocyte-derived neurotrophic factor with selectivity for dopaminergic neurons. (2003). J Mol Neurosci. 20:173-188.

Piltonen M., Bespalov M.M., Ervasti D., Matilainen T., Sidorova Y.A., Rauvala H., Saarma M., Mannisto P.T. (2009). Heparin-binding determinants of GDNF reduce its tissue distribution but are beneficial for the protection of nigral dopaminergic neurons. Exp Neurol. 219: 499-506.

Powell M.F. (1994). Peptide stability in aqueous parenteral formulations. In Cleland J.L.; Langer R, editors. Formulation and delivery of proteins and peptides. American Chemical Society. 100-117.

Przedborski S., Jackson-Lewis V. (1998). Mechanisms of MPTP toxicity. Mov Disord. 13 Suppl 1:35-38.

Przedborski S., Jackson-Lewis V., Djaldetti R., Liberatore G., Vila M., Vukosavic S., Almer G. (2000). The parkinsonian toxin MPTP: action and mechanism. Restor Neurol Neurosci. 16: 135-142.

Racette B.A., Criswell S.R., Lundin J.I., Hobson A., Seixas N., Kotzbauer P.T., Evanoff B.A., Perlmutter J.S., Zhang J., Sheppard L., Checkoway H. (2012). Increased risk of parkinsonism associated with welding exposure. Neurotoxicology. 33:1356-1361.

Ramaswamy S., Soderstrom K.E., Kordower J.H. (2009). Trophic factors therapy in Parkinson's disease. In: Verhaagen J., Hol E.M., Huitenga I., Wijnholds J., Bergen A.B., Boer G.J., Swaab D.F., editors. Progress in Brain Research. Elsevier. 201-216.

Ramirez A., Heimbach A., Grundemann J., Stiller B., Hampshire D., Cid L.P., Goebel I., Mubaidin A.F., Wriekat A.L., Roeper J., Al-Din A., Hillmer A.M., Karsak M., Liss B., Woods C.G., Behrens M.I., Kubisch C. (2006). Herditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nat Genet. 38: 1184-1191.

Richardson J.R., Quan Y., Sherer T.B., Greenamyre J.T., Miller G.W. (2005). Paraquat neurotoxicity is distinct from that of MPTP and rotenone. Toxicol Sci. 88: 193-201.

Rickard S.M., Mummery R.S., Mulloy B., Rider C.C. (2003). The binding of human glial cell line-derived neurotrophic factor to heparin and heparin sulfate: importance of 1-O-sulfate groups and effect on its interaction with its receptor, GFRa1. 13: 419-426.

Rybicki B.A., Johnson C.C., Uman J., Gorell J.M. (1993). Parkinson's disease mortality and the industrial use of heavy metals in Michigan. Mov Disord. 8: 87-92.

Saarma M. (2000). GDNF-a stranger in the TGF-beta superfamily? Eur J Biochem. 267: 6968-6971.

Salvatore M.F., Zhang J.L., Large D.M., Wilson P.E., Gash C.R., Thomas T.C., Haycock J.W., Bing G., Stanford J.A., Gash D.M., Gerhardt G.A. (2004). Striatal GDNF administration increases tyrosine hydroxylase phosphorylation in the rat striatum and substantia nigra. J Neurochem. 90: 245-254.

Salvatore M.F., Ai Y., Fischer B., Zhang A.M., Grondin R.C., Zhang Z., Gerhardt G.A., Gash D.M. (2006). Point source concentration of GDNF may explain failure of phase II clinical trial. Exp Neurol. 202: 497-505.

Sanicola M., Hession C., Worley D., Carmillo P., Ehrenfels C., Walus L., Robinson S., Jaworski G., Wei H., Tizard R., Whitty A., Pepinsky R.B., Cate R.L. (1997). Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins. Proc Natl Acad Sci USA. 10: 6238-6243.

Sariola H., Saarma M. (2003). Novel functions and signalling pathways for GDNF. J Cell Sci. 116: 3855-3862.

Schoenberg B.S., Osuntokun B.O., Adeuja A.O., Bademosi O., Nottidge V., Anderson D.W., Haerer A.F. (1988). Comparison of the prevalence of Parkinson's disease in back populations in the rural United States and in rural Nigeria: door-to-door community studies. Neurology. 38:645-646.

Sherer T.B., Betarbet R., Greenamyre J.T. (2002). Environment, mitochondria, and Parkinson's disease. Neuroscientist. 8:192-197.

Sherer T.B., Fiske B.K., Svendsen C.N., Lang A.E., Langston J.W. (2006). Crossroads in GDNF therapy for Parkinson's disease. Mov Disord. 21: 136-141.

Shimura H., Hattori N., Kubo S.I., Mizunao Y., Asakawa S., Minoshima S., Shimizu N., Iwai K., Chiba T., Tanaka K., Suzuki T. (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet. 25: 302-305.

Silan V., Falini A., Strada O., Pizzuti A., Pezzoli G., Motti E.D., Vegeto A., Scarlato G. (1990). Effect of nerve growth in adrenal autographs in parkinsonism. Ann Aeurol. 27:341-342.

Silvestri L., Caputo V., Bellacchio E., Atorino L., Dallapiccola B., Valente E.M., Casari G. (2005). Mitochodrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. Hum Mol Genet. 14: 3477-3492.

Slevin J.T., Gerhardt G.A., Smith C.D., Gash D.M., Kryscio R., Young B. (2005). Improvement of bilateral motor functions in patients with Parkinson disease through the unilateral intraputaminal infusion of glial cell line-derived neurotrophic factor. J Neurosurg. 102:216-222.

Spillantini M.G., Schmidt M.L., Lee V.M., Trojanowski J.Q., Jakes R., Goedert M. (1997). Alpha-synuclein in Lewy bodies. Nature. 388: 839-840.

Svenson L.W., Platt G.H., Woodhead S.E. (1993). Geographic variations in the prevalence rates of Parkinson's disease in Aiberta. Can J Neurol Sci. 20:307-311.

Takahashi H., Ohama E., Suzuki S., Horikawa Y., Ishikawa A., Morita T., Tsuji S., Ikuta F. (1994). Familial juvenile parkinsonism: clinical and pathologic study in a family. Neurology. 44: 437-441.

Tan E.K., Yew K., Chua E., Puvan K., Shen H., Lee E., Puong K.Y., Zhao Y., Pavanni R., Wong M.C., Jamora D., de Sliva D. Moe K.T., Woon F.P., Yuen Y., Tan L. (2006). PINK1 mutations in sporadic early-onset Parkinson's disease. Mov Disord. 21: 789-793.

Tanner C.M. (1992). Occupational and environmental causes of parkinsonism. Occup Med. 7: 503-513.

Taylor H., Barua N., Bienemann A., Wyatt M., Castrique E., Foster R., Luz M., Fibiger C., Mohr E., Gill S. (2013). Clearance and toxicity of recombinant methionyl human glial cell line-derived neurotrophic factor (r-metHu GDNF) following acute convection-enhanced delivery into the striatum. PLoS One. 8:e56186.

Teng H.K., Teng K.K., Lee R., Wright S., Tevar S., Almeida R.D., Kermani P., Torkin R., Chen Z.Y., Lee F.S., Kraemer R.T., Nykjaer A., Hempstead B.L. (2005). ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin. J Neurosci. 23:5455-5463.

Tessarollo L., Tsoulfas P., Martin-Zanca D., Gilbert D.J., Jenkins N.A., Copeland N.G., Parada L.F. (1993). trkC, a receptor for neurotrophin-3, is widely expressed in the developing nervous sytem and in non-neuronal tissues. Development. 118:463-475.

Thomas B., Beal M.F. (2007). Parkinson's disease. Hum Mol Genet. 16 Spec No. 2: R183-194.

Thomas B. (2009). Parkinson's disease: from molecular pathways in disease to therapeutic approaches. Antioxid Redox Signal. 11: 2077-2082.

Thorne R.G., Frey W.H. (2001). Delivery of neurotrophic factors to the central nervous system: Pharmacokinetic considerations. Clin Pharmacokinet. 40: 907-946.

Tomac A., Lindqvist E, Lin LF, Ogren SO, Young D, Hoffer BJ, Olson L. (1995). Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. Nature.40:276-284.

Treanor J.J., Goodman L., de Sauvage F., Stone D.M. Poulsen K.T., Beck C.D., Gray C., Armanini M.P., Pollock R.A., Hefti F., Phillips H.S., Goodard A., Moore M.W., Buj-Bello A., Davies A.M., Asai N., Takahashi M., Vandlen R., Henderson C.E., Rosenthal A. (1996). Characterization of a multicomponent receptor for GDNF. Nature. 382:80-83.

Trupp M., Arenas E., Fainziber M., Nilsson A.S., Sieber B.A., Grigoriou M., Kilkenny C., Salazar-Grueso E., Pachnis V., Arumae U. (1996). Functional receptor for GDNF encoded by the c-ret protooncogene. Nature. 381:785-789.

Turchan-Cholewo J. *et al.* unpublished.

Uversky V.N. (2003). A protein-chameleon: conformational plasticity of alpha-synuclein, a disordered protein involved in neurodegenerative disorders. J Biomol Struct Dyn. 21: 211-234.

Valente E.M., Bentivoglio A.R., Dixon P.H., Ferraris A., Ialongo T., Frontali M., Albanese A., Wood N.W. (2001). Localization of a novel locus for autosomal recessive earlly-onset parkinsonism, PARK6, on human chromosome 1p35-p36. Am J Hum Genet. 68: 895-900.

Valente E.M., Abou-Sleiman P.M., Caputa V., Muqit M.M., Harvey K., Gispert S., Ali Z., Del Turco D., Bentivoglio A.R., Healy D.G., Albanese A., Nussbaum R., Gonzalez-Maldonado R., Deller T., Salvi S., Cortelli P., Gilks W.P., Latchman D.S., Harvey R.J., Dallapiccola B., Auburger G., Wood N.W. (2004). Herediatry early-onset Parkinson's disease caused by mutations in PINK1. Science. 304:1158-1160.

Van Weering D.H., Bos J.L. (1998). Signal transduction by the receptor tyrosine kinase Ret. Recent Results Cancer Res. 154:271-281.

von Coelln R., Dawson V.L., Dawson T.M. (2004). Parkin-associated Parkinson's disease. Cell Tissue Res. 318: 175-184.

Voutilainen M.H., Back S., Porsti E., Toppinen L., Lindgren L., Lindholm P., Peranen J., Saarma M., Tuominen R.K. (2009). Mesencephalic astrocyte-derived neurotrophic factor is neurorestorative in rat model of Parkinson's disease. J Neurosci. 29: 9651-9659.

Wang C., Tan J.M., Ho M.W., Zaiden N., Wong S.H., Chew C.L., Eng P.W., Lim T.M., Dawson T.M., Lim K.L. (2005). Alterations in the solubility and intracellular localization of parkin by several familial Parkinson's disease-linked point mutations. J Neurochem. 93: 422-431.

Wang L., Muramatsu S., Lu Y., Ikeguchi K., Fujimoto K., Okada T., Mizukami H., Hanazono Y., Kume A., Urano F., Ichinose H., Nagatsu T., Nakano I., Ozawa K. (2002). Delayed delivery of AAV-GDNF prevents nigral neurodegeneration and promotes functional recovery in a rat model of Parkinson's disease. Gen Ther. 9: 381-389.

West A.B., Moore D.J., Biskup S., Bugayenko A., Smith W.W., Ross C.A., Dawson V.L., Dawson T.M. (2005). Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. Proc Natl Acad Sci USA. 102: 16842-16847.

Wichmann T., Dostrovsky J.O. (2011). Pathological basal ganglia activity in movement disorders. Neuroscience 198: 232-244.

Williams D.R., Hadeed A., al-Din A.S., Wreikat A.L. Lees A.J. (2005). Kufor Rakeb disease: autosomal recessive, levodopa-responsive parkinsonism with pyramidal degeneration, supranuclear gaze palsy, and dementia. Mov Disord 20:1264-1271.

Wood M.J., Charlton H.M., Wood K.J., Kajiwara K., Byrnes A.P. (1996). Immune responses to adenovirus vectors in the nervous system. Trends Neurosci. 19:497-501.

Worby C.A., Vega Q.C., Zhao Y., Chao H.H., Seasholtz A.F. Dixon J.E. (1996). Glial cell line-derived neurotrophic factor signals through the RET receptor and activates mitogen-activated protein kinase. J Biol Chem. 271: 23619-23622.

Yin D., Valles F.E., Fiandaca M.S., Bringas J., Gimenez F., Berger M.S., Forsayeth J., Bankiewicz K.S. (2011). Optimal region of the putamen for image-guided convection-enhanced delivery of therapeutics in human and non-human primates. Neuroimage. 54 Suppl 1:S196-203.

Yoshimoto Y., Lin Q., Collier T.J., Frim D.M., Breakefield X.O., Bohn M.C. (Astrocytes retrovirally transduced with BDNF elicit behavioral improvement in a rat model of Parkinson's disease. Brain Res. 691: 25-36.

Zhang Z., Miyoshi Y., Lapchak P.A., Collins F., Hilt D., Lebel C., Kryscio R., Gash D.M. (1997). Dose response to intraventricular glial cell line-derived neurotrophic factor administration in parkinsonian monkeys. J Pharmacol Exp Ther. 282: 1396-1401.

Zhang L., Shimoji M., Thomas B., Moore D.J., Yu S.W., Marupudi N.I., Torp R., Torgner I.A., Ottersen O.P., Dawson T.M., Dawson V.L. (2005). Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis. Hum Mol Genet. 14: 2063-2073.

Zigova T., Pencea V., Wiegand S.J., Luskin M.B. (1998). Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. Mol Cell Neurosci. 11: 234-245.

Zimprich A., Biskup S., Leitner P., Lichtner P., Farrer M., Lincoln S., Kachergus J., Hulihan M., Uitti R.J., Calne D.B., Stoessl A.J., Pfeiffer R.F., Patenge N., Carbajal I.C., Vieregge P., Asmus F., Muller-Myhsok B., Dickson D.W., Meitinger T., Strom T.M., Wxzolek Z.K., Gasser T. (2004). Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron. 44: 601-607.

VITA
KRISTEN A. KELPS

EMPLOYMENT
Assistant Professor of Biology, Alderson Broaddus University
August 2011-present

EDUCATION
Ph.D., Anatomy and Neurobiology, University of Kentucky, Lexington, KY, December 2013
Thesis: Molecular and Cellular Characterization of the Dopamine Neuron Stimulating Peptides
Mentor: Dr. Luke Bradley
GPA: 4.0

B.S, Biology, High Point University, High Point, NC, May 2005
Summa Cum Laude, GPA: 3.98

B.A., Mathematics, High Point University, High Point, NC, May 2005
Summa Cum Laude, GPA: 3.98

RELATED WORK EXPERIENCE
REU: Fungal Genomics and Computational Biology
University of Georgia, Athens, GA
May 2004-July 2004
Supervisors: Dr. Jonathon Arnold, Professor
                Dr. Michael Terns, Associate Professor

Certified Learning Assistant, High Point University, High Point, NC
        January 2002-May 2005
Supervisor: Mr. Craig Curty, Assistant Director of Academic Services

TEACHING EXPERIENCE
ANA 209-Principles of Human Anatomy, Graduate Teaching Assistant, University of Kentucky,
Spring 2007
Course Director: Dr. Brian MacPherson
This was an online course for pre-professional students. I was responsible for managing the online discussion board, holding review sessions, and administering exams.

ANA110-Anatomy & Physiology for Nursing II, Graduate Teaching Assistant, University of
Kentucky, Spring 2010
Course Director: Dr. Pamela Stein
This course is part of a two-semester anatomy and physiology for approximately 200 prenursing students. In this course, I was responsible for giving lectures, answering

student questions/e-mails, and assisting with exam preparation, administration, and grading.
Lectures given: Digestive System-Large Intestine, March 1, 2010
    Urinary System-Kidneys, March 3, 2010
    Male Reproductive System, March 26, 2010
    Male Reproductive System, March 29, 2010
    Male Reproductive System, March 31, 2010

ANA 109-Anatomy & Physiology for Nursing I, Graduate Lecturer, University of Kentucky, Fall 2010
Course Director: Dr. April Richardson
Lectures given: Cellular Organelles, August 27, 2010
    Nervous System-CSF, Brain Stem, Cerebellum, Spinal Cord, Blood
     Supply, October 22, 2010

HONORS
American Society for Biochemistry and Molecular Biology Hill Day 2011, March 2011

Lyman T. Johnson Academic Year Fellowship, University of Kentucky, 2010-2011

Therapeutic Strategies for Neurodegeneration Training Grant, University of Kentucky, 2008-2010

Lyman T. Johnson Academic Year Fellowship, University of Kentucky, 2007-2008

Graduate School Academic Year Fellowship, University of Kentucky, 2005-2006

Junior Marshal, High Point University, 2004

Alpha Chi Honors Society, High Point University, 2003-2005

Cardinal Award in Chemistry, High Point University, 2002-2003

   University Fellowship Scholarship, High Point University, 2001-2005

Alumni Memorial Scholarship, High Point University, 2002-2005

Louis Adams Alumni Scholarship, High Point University, 2001-2002

MANUSCRIPTS
Kelps KA, Turchan-Cholewo J, Hascup ER, Taylor TL*, Gash DM, Gerhardt GA, and Bradley LH. Evaluation of the Physical and *In Vitro* Protective Activity of Three Synthetic Dopamine Neuron Stimulating Peptides. Neuropeptides (2011), doi:10.1016/j.npep.2011.03.003

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, and Gerhardt GA. 2010 Dopamine Neuron Stimulating Actions of a GDNF Propeptide. PLoSONE5(3): e9752. doi:10.1371/journal.pone.0009752
This paper was named a 'Paper of the Week' by the Michael J. Fox Foundation

PD Online Research.

* Mentored Undergraduate Student

CONFERENCE PRESENTATIONS

Kelps KA, Turchan-Cholewo J, Taylor TL, Gash DM, Gerhardt GA, and Bradley LH. 2011. Characterization of the Intrinsic Molecular and Protective Properties of the Synthetic Dopamine Neuron Stimulating Peptides. CCRS Spring Conference 2011. April 21. Lexington, KY.

Kelps KA, Turchan-Cholewo J, Taylor TL, Gash DM, Gerhardt GA, and Bradley LH. 2011. Evaluation of the Molecular and Cellular Protective Properties of the Synthetic Dopamine Neuron Stimulating Peptides. Bluegrass Society for Neuroscience Spring Conference. March 31. Lexington, KY.
This poster received an award for Outstanding Poster.

Turchan-Cholewo J, Kelps KA, Gash DM, Gerhardt GA, and Bradley LH. 2010. The mitochondrial neuroprotective effects of Dopamine Neuron Stimulating Peptide 11. 40th Annual Meeting of the Society for Neuroscience. November 14-17. San Diego, CA.

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, and Gerhardt GA. 2010. Antiparkinsonian actions of a GDNF propeptide. 40th Annual Meeting of the Society for Neuroscience. November 14-17. San Diego, CA.

Bradley LH, Kelps KA, Turchan-Cholewo J. 2010. The mitochondrial protective effects of Dopamine Neuron Stimulating Peptide-11. Experimental Biology. April 24-28. Anaheim, CA.

Kelps KA, Turchan-Cholewo J, Bradley LH. 2010. Dopamine Neuron Stimulating Peptide-11's antiapoptotic functions. Bluegrass Society for Neuroscience Spring Conference. March 17. Lexington KY.

Kelps KA, Turchan-Cholewo J, Bradley LH. 2009. Antiapoptotic effects of a GDNF Propeptide. 39th Annual Meeting of the Society for Neuroscience. October 17-21. Chicago, IL.
This poster was recognized as a 'Hot Topics' meeting poster by the Michael J. Fox Foundation.

Kelps KA, Turchan-Cholewo J, Bradley LH. 2009. Antiapoptotic actions of DNSP-11. Bluegrass Society for Neuroscience Spring Conference. March 18. Lexington KY.

Kelps KA, Turchan-Cholewo J, Bradley LH. 2009. Antiapoptotic effects of DNSP-11. CCTS Spring Conference 2009. April 23. Lexington, KY.

Hauser, KF, Kelps KA, Bruce-Keller AJ, Knapp PE, and El-Hage N. 2006. A central role for astroglia in opioid-HIV-1 mediated neurodegeneration. Internatl. Narcotics Res. Conf. (INRC) July 12, St. Paul/Minneapolis, MN.

PUBLISHED ABSTRACTS
Bradley LH, Kelps KA, Turchan-Cholewo J. 2010. The mitochondrial protective effects of Dopamine Neuron Stimulating Peptide-11. FASEB J. 24:lb468.

MENTORED STUDENTS
      Alyssa Fountain, Undergraduate, Agricultural Biotechnology, Fall 2010-Present
      Tiffany Taylor, Undergraduate, Biology, Summer 2010-Present
      Erin Miller, Graduate Rotation Student, Anatomy & Neurobiology, Fall 2008
      Katie Mattinson, Graduate Rotation Student, Anatomy & Neurobiology, Fall 2007
      Sudipa Chowdhury, Undergraduate, Chemistry, 2007-2009