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STUDIES OF THE EFFECTS OF DOPAMINE NEURON STIMULATING PEPTIDES IN RODENT MODELS OF NORMAL AND DYSFUNCTIONAL DOPAMINERGIC SYSTEMS

Joshua Lee Fuqua
University of Kentucky, jfuqua@wfubmc.edu

ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

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

By
Joshua Lee Fuqua

Lexington, Kentucky

Director: Dr. Greg A. Gerhardt, Professor of Anatomy and Neurobiology

Lexington, Kentucky

2010

Abstract of Dissertation

STUDIES OF THE EFFECTS OF DOPAMINE NEURON STIMULATING
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DOPAMINERGIC SYSTEMS

A theoretical post-translational processing model of the proprotein form of glial cell line-derived neurotrophic factor (GDNF) likely produces three biologically active peptides. The three prospective peptides formed are 5, 11, and 17 amino acid peptides, entitled dopamine neuron stimulating peptide -5 (DNSP-5), -11 (DNSP-11), and -17 (DNSP-17), respectively. The DNSPs were hypothesized to increase dopaminergic neuron function because of their relationship to GDNF: a molecule with established neurotrophic actions on dopaminergic neurons. The DNSPs have the potential to provide a therapeutic molecule similar to GDNF, but with increased ease of delivery and improved bioavailability.

Neurochemical effects of DNSPs were examined in the nigrostriatal pathway of normal Fischer 344 rats, and DNSP-11 was found to be the most effective in increasing dopamine neurochemical function. Striatal microdialysis, four weeks after a single intranigral administration of DNSP-11, showed significant increases in the baseline concentrations of dopamine, DOPAC, and HVA. In addition, both, potassium and d-amphetamine-evoked dopamine overflow were significantly increased.

DNSP-11 was delivered intranigrally to aged Fischer 344 rats to examine DNSP-11's ability to improve dopaminergic function in aged dopamine neurons. DNSP-11 affected striatal dopaminergic function 28 days after treatment by decreasing baseline concentrations of dopamine and evoked dopamine release.

Investigation of DNSP-11 continued, using two models of neurotoxin-induced dopamine neuron loss that model cell loss associated with Parkinson's disease. The neuroprotective properties of DNSP-11 were evaluated by delivering DNSP-11 prior to the neurotoxic insult. DNSP-11 treatment was unable to protect dopaminergic neurons, but significantly increased dopamine metabolism. In a model of severe dopamine neuron loss, DNSP-11 treatment significantly improved apomorphine-induced rotation behavior, indicative of alterations in the function of nigrostriatal dopaminergic neurons. Subsequent

examination of dopamine content within the SN revealed significant increases in dopamine and DOPAC levels by DNSP-11.

Taken together, DNSP-11 treatments modified dopamine neurochemistry in all investigated rodent models. The observed long-term alterations of dopamine neurochemistry by DNSP-11 and subsequent behavioral changes support a potential use for DNSP-11 as a therapeutic for dopaminergic cell loss. Increased dopaminergic function by DNSP-11 is evidence for the novel concept that peptides contained within the prodomain of trophic factors can have neurotrophic actions.

Joshua Lee Fuqua

2/3/2010

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By

Joshua Lee Fuqua

Greg A. Gerhardt
Director of Dissertation

Jane Joseph
Director of Graduate Studies

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DISSERTATION

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Dedication

This dissertation is dedicated to my grandad, Leamon Fuqua, and my aunt, Doris Gilbert, both of whom lost battles with neurodegenerative disease. Their experience has inspired me to work to find a way to help others like them and the families that love them.

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Chapter One: Introduction

Overview

Parkinson's disease (PD) is a neurodegenerative disease characterized by the progressive loss and dysfunction of dopaminergic neurons. Increasingly debilitating motor disturbances are associated with the disruption of dopaminergic neurons (Wichmann and DeLong, 2006). Currently, clinical therapies for PD focus on increasing the concentration of the lost neurotransmitter, dopamine. Over time, neurotransmitter replacement therapies become less effective at managing the motor disruptions associated with PD because they fail to stop or reverse the neuronal loss (Goudreau, 2006). A neurotrophic factor, glial cell-line derived neurotrophic factor (GDNF), has previously shown the ability to restore and enhance the function of dopaminergic neurons (Hoffer et al., 1994; Gash et al., 1996; Hebert et al., 1996; Slevin et al., 2005). GDNF combats the underlying neuronal dysfunction associated with PD, but difficulties with delivery and potential toxic effects halted ongoing clinical trials.

An effort was made to identify a molecule with similar functional effects as GDNF with greater ease of administration and bioavalability. Therefore, a post-translational processing model of the proprotein form of GDNF was developed that may produce three bioactive peptides (Glass, 2004). The three peptides, known as dopamine neuron stimulating peptides (DNSPs), should have improved bioavailability and be more readily synthesized and modified than their parent molecule, GDNF. However, investigation of their functional impact on dopaminergic neurons still needed to be examined *in vivo*. Rodent models were used to test DNSPs' functional effects on dopaminergic neurons, focusing on the nigrostriatal pathway. Within this dissertation: I established the DNSPs ability to modify dopaminergic function, identified the DNSP most effective at altering dopaminergic function, and provided evidence for the DNSPs' ability to improve dopamine neuron function in dysfunctional dopaminergic systems. The goal of this dissertation is to aid in the development of a therapeutic molecule that can stop or reverse the dopaminergic neuron loss and dysfunction associated with

PD. Therefore, results achieved with the DNSPs were compared to previous results with GDNF because the neurotrophic actions of GDNF on dopaminergic neurons are considered the "gold standard" (Peterson and Nutt, 2008).

Dopamine: A Brief History

Dopamine (DA) was first synthesized in 1910, but 40 years passed before it was discovered in mammalian tissue and almost another 10 years before it was accepted as a neurotransmitter. DA, or 3-hydroxytyramine, is a catecholamine derived from the amino acid tyrosine and was discovered in the sheep heart and adrenal medulla (Goodall, 1951). At the time DA was discovered, in mammalian tissue, it was theorized to function primarily as an intermediate in the production of the neurotransmitters, epinephrine, and norepinephrine (NE) (Blaschko, 1942). But by the late 1950's, Carlsson and others began to demonstrate that the dopamine precursor L-3,4-dihyroxyphenylalanine (L-dopa) alleviated catalepsy related to reserpine, a vesicular monoamine transporter (VMAT) blocker (Carlsson et al., 1957). The activity of L-dopa renewed interest in DA, and shortly afterward, DA was discovered in the mammalian brain (Montagu, 1957). Two years later in 1959 the distribution of DA was characterized in the CNS of humans and determined to be concentrated in extrapyramidal neurons (Sano et al., 1959). Hornykiewicz expanded upon studies of DA in the human CNS and provided the first evidence of DA depletion in the striatum and substantia nigra (SN) in Parkinson's disease (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1963). Since these discoveries, PD has been characterized by the loss of DA in the nigrostriatal pathway. By 1969, the combinatorial PD therapeutic L-dopa and carbidopa, a peripheral dopa decarboxylase inhibitor, began use as the primary treatment for PD. Neurotransmitter replacement therapy became widely accepted and L-dopa / carbidopa is the most commonly used today, now known as Sinemet ®.

Dopamine Systems

Knowledge of the function and regulation of DA in the CNS has been continually advanced since its discovery in 1951 and will be discussed

throughout this chapter. There are four primary dopaminergic systems in the CNS: mesocortical, mesolimbic, tuberoinfundibular, and nigrostriatal (Cooper et al., 2003). The tuberoinfundibular system is part of the neuroendocrine regulation of the release of prolactin from the anterior pituitary gland. Hormone regulation is a distinct function of the tuberoinfundibular system relative to other dopaminergic systems and necessitates only short to intermediate length dopaminergic projections (Björklund et al., 2005). The mesocortical dopaminergic system is the primary dopaminergic input to the prefrontal cortex from the midbrain nuclei, specifically the ventral tegmental area (VTA) and SN. Evidence supports that the mesocortical system is involved in higher cognitive functions such as attention direction, stimulus prioritization, and "working memory." Dysfunctions within the mesocortical dopaminergic projections are potentially related to the cognitive deficits observed in schizophrenia and attention deficit hyperactivity disorder (ADHD) (Iversen, 2009). Mesolimbic and mesocortical systems have similar originating nuclei in the VTA and SN but the mesolimbic system projects to more posterior structures such as the nucleus accumbens and amygdala. The mesolimbic system has been implicated in motivation and reward with emphasis on a role in drug abuse and other addictive behaviors (Iversen, 2009). The nigrostriatal system at its most basic level is considered to be the dopaminergic projections from the SN that travel through the medial forebrain bundle (mfb) to the striatum, but projections from the VTA to the striatum travel in the same fiber bundle and project to similar areas with overlapping activity (Borrelli and Di Chiara, 2002).

Dopaminergic neuron function is involved in multiple aspects of behavior and cognition, and the projections from the three long dopaminergic systems, mesolimbic, mesocortical, and nigrostriatal, overlap providing some functional redundancy between the systems. The nigrostriatal system is involved in the motor deficits associated with PD and therefore is the system of primary interest in this dissertation.

Striatum

Striatum is a term that includes the caudate nucleus and putamen, which in primates are distinct structures separated by the internal capsule. In the rat, the caudate nucleus and putamen are indistinguishable because the sparsely organized internal capsule provides no structural division. The resulting structure is a combination of the caudate nucleus and putamen striated with white matter fiber tracts and therefore, labeled the striatum (Mueller et al., 1989). In the rat, the striatum is subdivided into the dorsal and ventral areas based on the innervating fibers and the divergence of function. The DA afferents to the dorsal striatum classically are considered to be from the SN with primary involvement in motor movement. The ventral striatum includes the nucleus accumbens and olfactory tubercule. The primary dopaminergic input to the ventral striatum arises from the VTA and is involved in reward behavior (van Domburg and ten Donkelaar, 1991).

The striatum primarily consists of medium spiny neurons (~95 %) with GABAergic efferents divided between the SN reticulata (SNr) and internal segment of the globus pallidus (GPi) or external segment of the globus pallidus (GPe) (Figure 1.1). Within the striatum interneuronal connections are made by large aspiny acetylcholinergic neurons and medium aspiny GABAergic neurons (Nicola et al., 2000). Both interneuronal populations interact with the medium spiny neurons, but they also receive input from striatal afferents (Smith and Kieval, 2000). The striatum is innervated and modulated by dopaminergic neurons of the SN and glutamatergic neurons from the cortex and thalamus (Tepper et al., 2007). The glutamatergic and dopaminergic striatal afferents form synapses with interneuron types as well as medium spiny neurons. The dopaminergic afferents, in particular, innervate the acetylcholinergic interneurons and the glutamatergic afferents innervate the GABAergic interneurons with some overlap (Di Chiara et al., 1994). The DA input from the SN directly alters interneuron and efferent neuron function of the striatum providing strong modulatory control over the GABAergic efferents of the striatum.

The neuronal populations within the striatum are compartmentalized by specific afferent and efferent neurons into two areas divided immunohistochemically by the absence or presence of acetylcholinesterase (Graybiel and Ragsdale, 1978). The two distinct areas within the striatum have been termed the patch or the matrix. The patch, initially termed the striosome, lacks acetylcholinesterase and therefore maintains a high concentration of acetylcholine and is primarily innervated by limbic associated areas such as the hippocampus. The matrix, initially termed the extrastriosomal matrix, has a high concentration of acetylcholinesterase maintaining a choline rich environment and is the primary innervation area for cortical and thalamic glutamatergic projections (Gerfen, 1992). The DA projections from the SN primarily innervate the patch areas, which are most concentrated in the dorsolateral striatum and decline in a graded manner moving ventromedially within the striatum (Björklund et al., 2005). With the study of the compartmentalization of the striatum and mapping of cortical projections, there has been a shift in the division of the striatum (Gerfen, 1992). The striatum is now divided into three primary divisions: the limbic, associative, and motor. The limbic area is the ventral portion of the striatum and the motor area consists of the dorsolateral striatum. The new division of the striatum, the associative area, is in the central striatum between the limbic and motor areas (Borrelli and Di Chiara, 2002). The significance of this division of the striatum is that the midbrain projections follow a similar layout with the medial VTA projecting to the ventral striatum with few patch compartments and the lateral SN projecting to the dorsolateral striatum with a large number of patch compartments. The limbic area in the central striatum is a mixture of VTA and SN projections and contains a mixture of matrix and patch compartments (Joel and Weiner, 2000). The large degree of heterogeneity exhibited by midbrain DA innervations provides sampling from both the VTA and SN to varying degrees within all areas of the striatum. The heterogeneity of the striatum and multiple afferent and efferent fibers adds complexity to a study, but the SN projections to the striatum and subsequent motor area are well defined and can be targeted for study.

The primary DA innervation to the motor areas in the striatum arises in the SNc forming the nigrostriatal pathway. The dopaminergic projections target the patch areas in the dorsolateral striatum and modulate motor movement by innervating striatal interneurons and afferent fibers to the globus pallidus and the SNr. Therefore, the SN as a whole provides striatal efferents and receives afferent projections from the striatum, making it critical in the modulation of motor movement.

Substantia Nigra

As discussed above, the SN is divided into two distinct areas, the SNc and SNr. The SNc is a densely packed band of cell bodies containing approximately 44% of the cell bodies in the SN (Poirier et al., 1983). The SNr is ventral to the SNc and is sparsely populated with neuronal cell bodies. The SNr does maintain a dense innervation of GABAergic projections from the striatum and glutamatergic innervation from the subthalamic nucleus (STN) (Tepper et al., 2007). The SNr also receives dopaminergic input from a small population of SNc cell bodies that send fingerlike dendritic arborizations into the neighboring reticulata (Smith and Kieval, 2000). The SNr's GABAergic axonal projections primarily innervate the thalamus but also interact with the SNc altering dopaminergic release and activation. The SNc is the primary area containing dopaminergic neurons within the SN, and in addition to connections to the SNr, the SNc has axonal projections to the striatum, cortex, and amygdala (Loughlin and Fallon, 1983, 1984). Most of the dopaminergic output of the SN is directed to the striatum but the dopaminergic neurons are modulated through GABAergic input from the SNr and feedback from the striatum. The loss of dopaminergic neurons in the SN disrupts the function of multiple systems and in the basal ganglia circuitry, GABAergic fibers are released from the regulatory effects of DA, negatively modulating related motor movement.

The Basal Ganglia: Direct and Indirect Pathways

The nigrostriatal pathway is involved in the modulation and initiation of motor movement but does not project directly to the cortical motor centers. The

influence of the nigrostriatal pathway on motor movement is related to the downstream effects of striatal projections on the thalamus, which directly influences cortical motor output (Figure 1.1). The nigrostriatal dopaminergic projections can modulate the thalamocortical input through two pathways, the direct pathway and indirect pathway (Blandini et al., 2000). Both pathways originate in the nigrostriatal dopaminergic projections and differentiate through the DA receptor subtypes on their innervating post-synaptic striatal neuron. The direct pathway signals primarily through the D_1 family of DA receptors, a stimulatory receptor discussed later within this chapter. The indirect pathway signals primarily through the D_2 family of DA receptors, an inhibitory receptor discussed later within this chapter. The activated post-synaptic neurons of the direct pathway in the striatum innervate the GPi, which directly regulates the thalamocortical projections through GABAergic inhibitory effects (Nicola et al., 2000; Björklund et al., 2005). The inhibited post-synaptic neurons of the indirect pathway innervate the subthalamic nucleus (STN) and this inhibits the excitatory output from the STN on the GPi (Tepper et al., 2007).

A hallmark of PD is the loss of the dopaminergic input into the striatum from the SN and with the loss, a lack of activation occurs in the direct pathway and subsequent release of the GPi from inhibition. The loss of DA input in the indirect pathway disinhibits the STN increasing the activation of the GPi. The GPi released from direct pathway inhibition and stimulated by the indirect pathway causes increased inhibition of thalamocortical projections and thereby reduces the glutamatergic input to the cortices from the thalamus (Blandini et al., 2000). The reduction in cortical glutamate signaling manifests behaviorally through hypokinesia (DeLong and Wichmann, 2007).

The simplified model system of the cortico-basal ganglia-cortical circuit has recently been under scrutiny for its lack of encompassing all basal ganglia inputs and interconnections and when modeling the dysfunctional systems of PD it does not encompass all affected areas (Braak et al., 2004). For review of a more complete model of the basal ganglia interactions including spinal cord and neocortical motor centers please refer to Braak and Del Tredici, 2008. The author
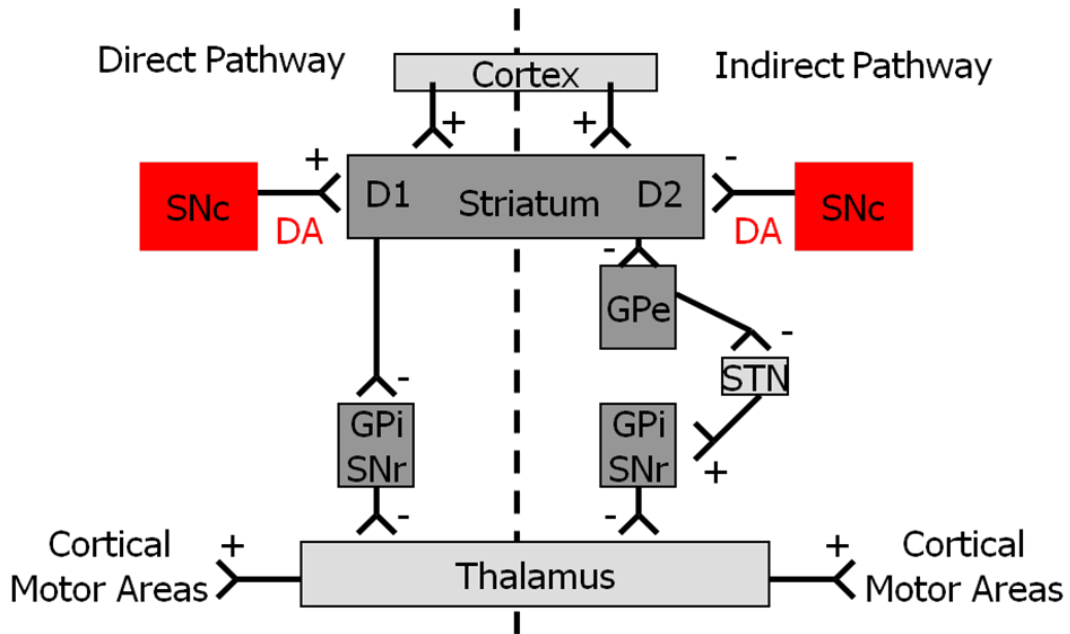
Figure 1.1 The Direct and Indirect Pathways of the Basal Ganglia

A simplified model of the basal ganglia demonstrating the divergence of the direct and indirect pathways within the striatum adapted from Blandini, 2000. The direct and indirect pathways diverge within the striatum through SN DA signaling on two distinct groups of receptors, D_1 and D_2. The D_1 receptors provide a stimulatory signal on the post-synaptic neuron and the D_2 receptor provides an inhibitory signal on the post-synaptic neuron. The expression of the receptors localizes them to the neurons projecting through the subsequent pathways. This model of basal ganglia function does not exhibit the complex interconnections of the basal ganglia and other motor centers, but demonstrates the involvement of the nigrostriatal pathway in motor output.

+ post-synaptic stimulatory effects and **–** post-synaptic inhibitory effects

concedes the model system of the direct and indirect pathways described above is not complete, but the connection between the SN and motor output is apparent using this model and demonstrates the necessity of studying the nigrostriatal pathway related to movement dysfunction.

Dopamine Synthesis, Release, Uptake, and Metabolism

DA, the primary neurotransmitter of the nigrostriatal pathway, is synthesized from tyrosine to DA through a two-step synthesis (Figure 1.2). Tyrosine is a naturally occurring non-essential amino acid that can be synthesized from phenylalanine. Tyrosine is converted to L-dopa through an enzymatic reaction with tyrosine hydroxylase (TH) and the co-factors tetrahydrobiopterin and Fe^{2+}. The production of L-dopa by TH is the rate-limiting step in DA production (Carlsson et al., 1972; Haavik and Toska, 1998). L-dopa is converted to DA by L-aromatic amino acid decarboxylase (L-AADC), which is regulated by protein production. TH activity is regulated by the phosphorylation of 4 serine residues enhancing its affinity for the cofactor tetrahydrobiopterin (Kumer and Vrana, 1996). Synthesized DA is packaged into vesicles by the vesicular monoamine transporter 2 where it remains until a release event. Modulation of TH function and the synthesis of DA alter available DA for release and the functional signaling of dopaminergic neurons.

DA release is initiated by an action potential that depolarizes the membrane potential and allows calcium influx through voltage-gated calcium channels. Calcium interacts with vesicles and initiates vesicle docking and release of DA (Figure 1.3). DA release into the synaptic cleft binds to post synaptic receptors or presynaptic autoreceptors. There are five isoforms of the post-synaptic DA receptor divided between two families. DA receptors of both families are G protein coupled receptors that modulate protein kinase A (PKA) through the stimulation or inhibition of adenylate cyclase. The D_1 family of receptors activates adenylate cyclase and the D_2 family of receptors down regulates adenylate cyclase (Wooten, 1997). The modulation of PKA by adenylate cyclase directly modifies the activity of DARPP-32 (Dopamine and cAMP regulated phosphoprotein of 32 kDa).
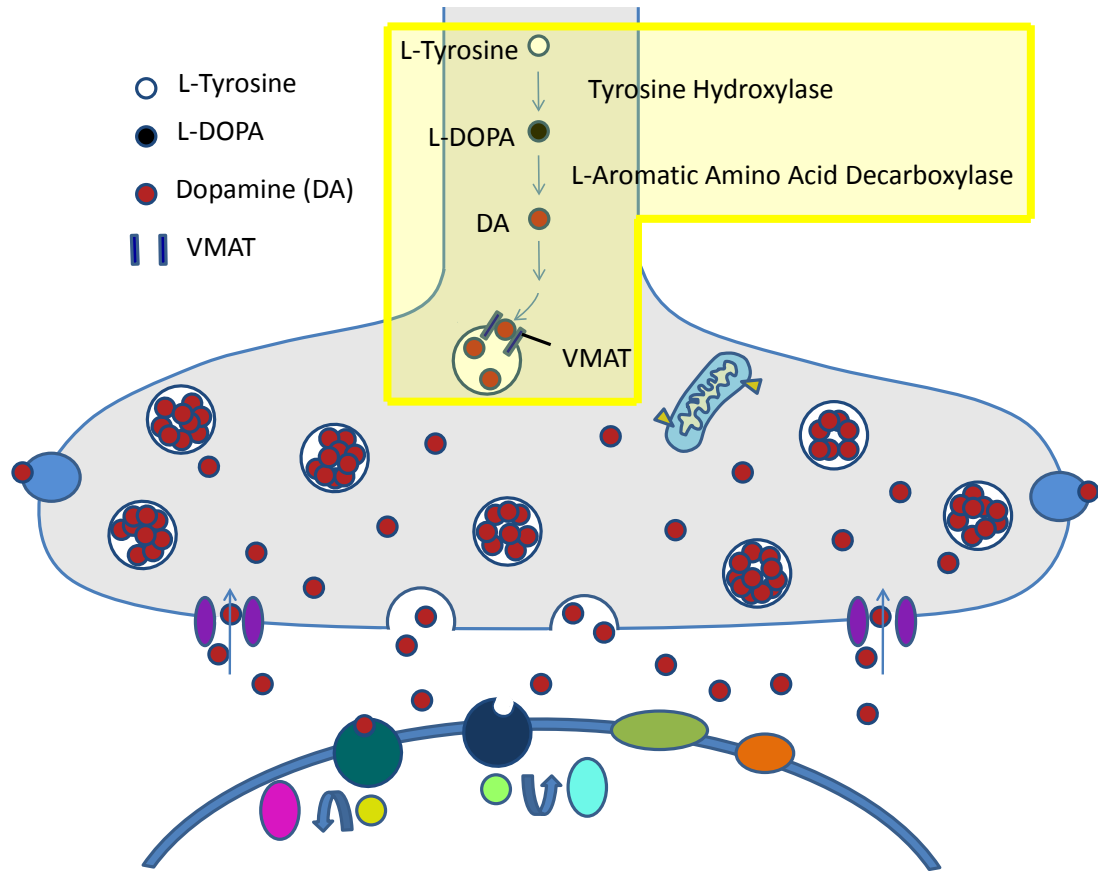
Figure 1.2 Dopamine Synthesis

L-Tyrosine is converted to L-dopa by TH and undergoes conversion to DA by L-AADC. The dopamine molecule is then packaged into vesicles through the vesicular monoamine transporter. The packaged dopamine is ready to be released into the synapse.
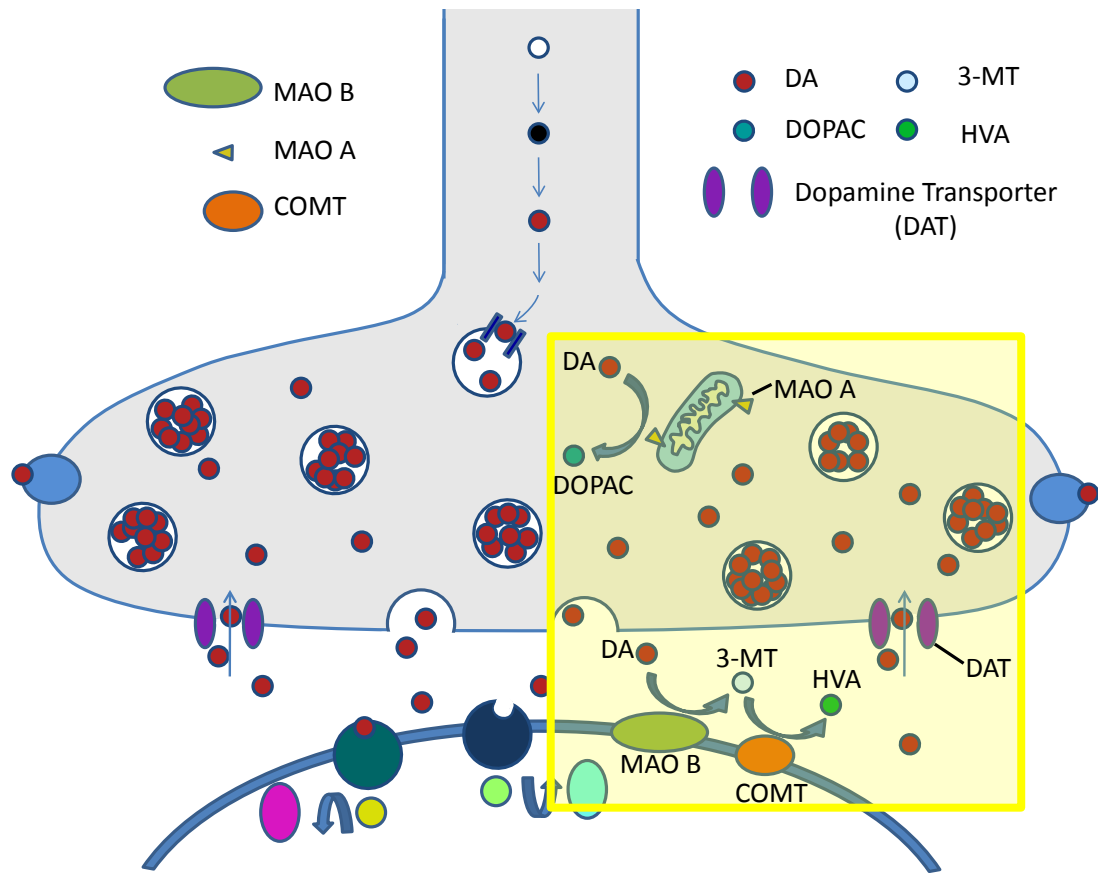
Figure 1.3 Dopamine Receptor Interactions

DA signals post-synaptically through D1 and D2 family receptors. Both receptor types are G protein coupled and modulate adenylate cyclase. D1 like receptors activate adenylate cyclase and D2 like receptors inhibit adenylate cyclase. DA signals presynaptically through D2 / D3 like autoreceptors modulating DA synthesis, release, and firing rate.

Activated DARPP-32 inhibits the activity of Protein Phosphatase-1 (PP-1) (Hemmings et al., 1984; Hemmings et al., 1989). Therefore, D_1 like receptors stimulate adenylate cyclase increasing DARPP-32 activity and inhibit PP-1. Whereas, D_2-like receptors inhibit adenylate cyclase thereby decreasing DARPP-32 activity and increasing the activity of PP-1 (Nishi et al., 1999). DA receptor activation modulates ion influxes and protein activity (Ouimet et al., 1988; Surmeier et al., 1995). The modification of protein activity can modify receptor or channel expression and function leading to alterations in synaptic plasticity. The two different receptor families lead to the ability of DA signaling to provide inhibitory or stimulatory effects based on which post-synaptic receptor is activated (Lindskog et al., 1999)

Presynaptic receptors on dopaminergic neurons are considered autoreceptors and fall into the D2 / D3 family of receptors (Feuerstein, 2008). DA autoreceptors primarily regulate three areas of DA signaling: synthesis, release, and firing rate. The regulation of synthesis and release is performed by autoreceptors located on the axon terminal and firing rate is regulated by autoreceptors on the soma or dendrites (Lindgren et al., 2001). DA autoreceptors provide a mechanism for DA to self regulate when the function of the system becomes altered.

In the nigrostriatal pathway, the DA signal is primarily terminated by the re-uptake through the dopamine transporter (DAT) in an ion dependent manner (Figure 1.4) (Hoffman et al., 1998). DAT is located at the periphery of the presynaptic nerve terminal in the nigrostriatal pathway and functions to remove DA from the extracellular space before it escapes the synaptic cleft (Kuhar et al., 1990; Graham and Langer, 1992). A portion of the DA that is transported into the neuron terminal by DAT is repackaged into vesicles for release, but cytosolic enzymes can also metabolize cytosolically available DA.

The DA signal is also terminated through the metabolism of DA. Enzymes for DA metabolism are located extrasynaptically and intrasynaptically. Therefore, DA in the cytosol or extracellular space has the potential to be metabolized. DA
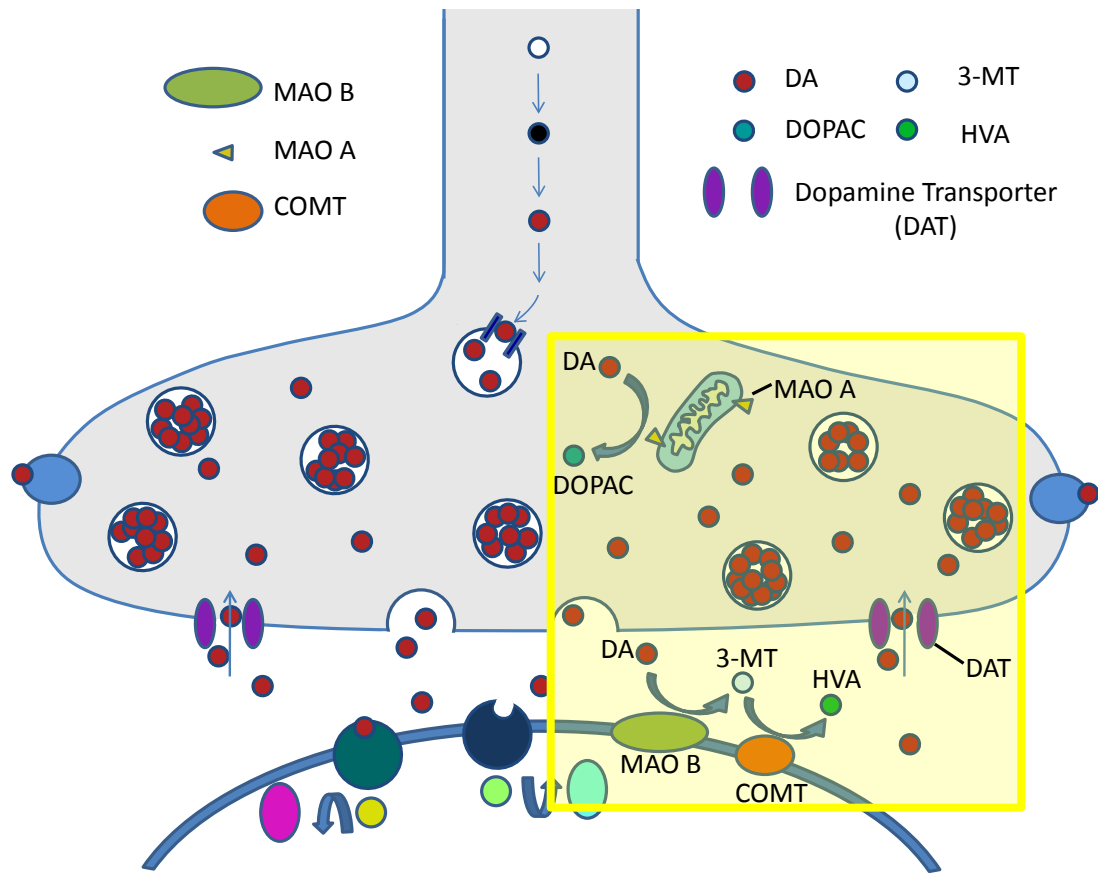
Figure 1.4 Dopamine Reuptake and Metabolism

Dopamine is removed from the synapse through reuptake by the DAT or metabolism by extraneuronal enzymes. DA can be metabolized intraneuronally by MAO A and ALDH or extraneuronally by COMT and/or MAO B. Metabolism of DA by MAO / ALDH produces DOPAC and metabolism by COMT produces 3-MT, which can be converted to HVA by MAO.

metabolites include 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), and 3-methoxytyramine (3-MT). In the rat the primary metabolite of DA is DOPAC, but in primates HVA is the primary metabolite (Kopin, 1994). There are three enzymes primarily responsible for the metabolism of DA monoamine oxidase (MAO), aldehyde dehydrogenase (ALDH), and catechol-*o*-methyltansferase (COMT).

MAO is divided into two categories, A and B, based on localization and substrate preference. In the striatum MAO-A is primarily localized to the outer mitochondrial membrane in dopaminergic neurons and MAO-B is localized extraneuronally (Kopin, 1985; Shih et al., 1999). Both forms of MAO catalyze the conversion of DA to the corresponding aldehyde upon which ALDH acts. ALDH is localized to the cytoplasm and inner mitochondrial leaflet and functions to oxidize aldehydes to their corresponding acids (Tank et al., 1981). In this manner, DA is converted to DOPAC. COMT is primarily a membrane bound enzyme in the CNS and is responsible for the metabolism of DA to 3-MT. Generally, 3-MT is only present in small quantities because it readily interacts with MAO and ALDH to form HVA (Iversen, 2009). The modification of the metabolic processes/enzymes modifies DA availability for release and signaling. The degree of DA signaling activity is dependent on synthesis, packaging, release, reuptake, and metabolism, and alterations of these processes modify the function of the entire nigrostriatal pathway and potentially the entire basal ganglia.

Parkinson's Disease

In 1817 PD was first described by the man from whom its name is derived, James Parkinson (Reprint: Parkinson, 2002). However, the disease can be traced back to approximately 1500 – 2500 B.C. PD is divided into two subcategories: idiopathic and familial, based on the pathogenesis of the disease. The etiology of idiopathic PD is generally theorized to involve genetic and environmental components that begin the progressive neuronal loss demonstrated by the disease (Thomas, 2009). Familial PD is caused by genetic mutations and related protein dysfunctions. Familial PD presents with similar symptoms as idiopathic PD, but has an earlier age of onset (Bajaj et al., 1998).

The focus of this dissertation is on therapeutic interventions in idiopathic PD, which may also have implications on familial PD.

The primary risk factor for development of idiopathic PD is age, with 4% of the population over age 65 experiencing parkinsonism. There are four cardinal motor features related to PD; resting tremor, bradykinesia, rigidity, and postural instability (Wichmann and DeLong, 2003). The motor symptoms can become debilitating as the disease progresses, but cognitive symptoms such as depression and dementia can manifest as well (Kummer and Teixeira, 2009; Lohle et al., 2009). Greater than 47% of patients diagnosed with PD exhibit depression symptoms often predating their motor symptoms. The cognitive decline associated with PD is related to the age of the individual; 65% of patients over age 85 exhibit signs of dementia (Davis, 2002). The disruption of basal ganglia function by the dysfunction of the nigrostriatal pathway is assumed the primary cause of the motor manifestations, but it is not the primary cause of the psychiatric and cognitive alterations associated with PD.

The hallmark of PD is a loss of the dopaminergic neurons in the nigrostriatal pathway with greater than a 60% reduction of dopaminergic neurons in the SN (Gibb and Lees, 1991). Dopaminergic neuronal loss in conjunction with dysfunction of the remaining neurons contributes to an 80% or greater reduction in DA content. A pathological hallmark of PD is the presence of Lewy bodies, protein aggregates, which are generally deposited in the cell bodies of dopaminergic neurons. These protein aggregates are thought to contribute to the neuronal loss and dysfunction because they coincide with areas of the most cell death (Bethlem and Den Hartog Jager, 1960; Gibb and Lees, 1989). The loss of dopaminergic neurons extends beyond the SN but is not as severe in any other region of the brain. Other areas in the brain also demonstrate damage, especially other midbrain catecholaminergic neurons, such as the locus coeruleus and dorsal raphe (Braak et al., 2003; Braak et al., 2004; Braak and Del Tredici, 2008). PD inexorably progresses generally over 10 - 20 years with continued neuron loss in the nigrostriatal pathway. As the disease progresses, multiple neurotransmitter systems begin to experience neuronal loss extending to cortical

systems (Hawkes et al., 2009). Intervention strategies for PD range from surgical interventions like deep brain stimulation to the continued use of L-dopa, a chemical replacement therapy. Drug therapies for PD will be discussed in more detail later in this chapter but primarily focus on restoring dopaminergic function in the nigrostriatal pathway. Restoration of the DA deficit in the nigrostriatal pathway is known to alleviate some of the motor symptoms associated with PD. Therefore, our studies will focus on the modulation of dopaminergic function, which could potentially alleviate some of the motor symptoms related to PD.

Rodent Models of Dopaminergic Dysfunction

Rodent models of dopaminergic dysfunction have been developed to aid in the understanding of the progression and development of PD as well as to test potential therapeutics. Numerous methods are currently employed to produce animals that model components of PD. One of the oldest and most widely used models uses 6-hydroxydopamine (6-OHDA) to produce dopaminergic neuronal loss (Ungerstedt, 1973; Ungerstedt et al., 1974; Ungerstedt, 1976). Catecholaminergic neurons are targeted by 6-OHDA by neuronal uptake through the catecholamine transporters such as the norepinephrine transporter or the DAT. 6-Hydroxydopamine induced cell death is mediated by the autooxidation of 6-OHDA producing quinones, peroxide, superoxide and hydroxyl radicals (Sachs and Jonsson, 1975; Schwarting and Huston, 1996). Molecules produced from 6-OHDA are highly reactive and can damage proteins, disrupt the mitochondrial membrane, and damage DNA. Neurotoxin damage induces degeneration of the dopaminergic neurons that peaks at approximately 2 weeks after the injection and stabilizes by 4 weeks (Leak and Zigmond, 2008). Cell death induced by 6-OHDA can be used to model the neuronal loss demonstrated in PD. To mimic the cell loss at different stages of PD, 6-OHDA-induced cell death can be modulated through the site and volume of the delivered neurotoxin. 6-Hydroxydopamine induced neuronal loss does not produce Lewy bodies or progress in a similar manner to PD, but can provide a model with similar dopaminergic neuron loss and correlative motor impairments. The 6-OHDA

model provides controllable, specific dopaminergic cell loss that is well characterized.

Discovery of genetic linkages to PD and the development of technology to produce genetically modified mice converged to allow the production of multiple mouse models of various components of PD (Polymeropoulos et al., 1996; Matsumine et al., 1997). Genetic models of PD are generally related to dysfunction of mitochondria, protein degradation, and reactive oxygen species regulation. Genetic models of PD do not generally produce the neuronal loss in the nigrostriatal dopaminergic system, associated with PD (Goldberg et al., 2003; Von Coelln et al., 2004; Kitada et al., 2007). These mouse models provide the opportunity to investigate genetic components that could be contributing to the development of PD.

Many of the genetic models do not show dramatic dopaminergic dysfunction and are not responsive to DA replacement therapeutics. Neurotoxin induced denervation models demonstrate responsiveness to L-dopa and provide a loss of dopaminergic neurons in the nigrostriatal pathway equivalent to that of PD (Robertson and Robertson, 1988, 1989; Kirik et al., 1998; Lindgren et al., 2007). Therefore, in the studies within this dissertation I used 6-OHDA induced models of DA denervation.

Parkinson's Disease Drug Therapies

DA therapeutic drugs generally focus on the replacement of the lost neurotransmitter. There are multiple ways to enhance or replace DA in the CNS such as blocking metabolism of DA, increasing DA synthesis, or using DA receptor agonists to provide post-synaptic stimulation (Iversen, 2009). DA replacement therapy by administration of some form of L-dopa / carbidopa is the most common treatment of PD and has been used extensively with positive results since its inception in the late 1960s (Diaz and Waters, 2009). L-dopa therapy has been implicated in the development of dyskinesias after long-term therapy with the short half-life of the drug contributing to the motor fluctuations. Also, L-dopa therapy commonly begins to lose effectiveness after 5 years as the neuronal loss progresses (Goudreau, 2006; Muller and Russ, 2006). Other

therapies focusing on MAO or COMT inhibition rely on an intact dopaminergic synapse to produce DA and as primary therapies become ineffective as the neuronal loss increases (Cedarbaum et al., 1990). These therapies can increase the effectiveness of L-dopa therapy by modulating its breakdown and increasing the half-life, but their reliance on L-dopa eventually renders them ineffective. DA receptor agonists can also be used as monotherapies in early symptomatic stages of PD but generally are used in conjunction with L-dopa to maintain a steady activation of DA receptors (Nutt, 1998). In later stages, most of the drug therapies for PD are dependent on co-delivery with L-dopa for lasting beneficial effects. Treatment of PD is still maintained through a 40-year old drug replacement therapy that does little to modify the progression of the disease. In this dissertation, the focus will be on treating the underlying problem of progressive neuronal loss to restore function to the nigrostriatal pathway.

GDNF Development as a Potential Therapeutic

GDNF was identified in 1993 from a glial cell line and shown to have neurotrophic properties on midbrain dopaminergic neurons (Lin et al., 1993). GDNF is a member of the transforming growth factor-β superfamily, characterized by seven cysteine residues and mature protein dimerization. GDNF is the originator of the subfamily of GDNF family ligands including Neurturin, Artemin, and Persephin (Peterziel et al., 2007). GDNF family ligands signal through a shared receptor family the GDNF family receptor α (GFRα) 1 – 4, which are coupled to Ret tyrosine kinase. GDNF primarily signals through GFRα-1 activating Ret tyrosine kinase and signaling downstream through the Erk/MAPK pathway (Sariola and Saarma, 2003).

GDNF demonstrated potential as a therapeutic for PD by enhancing and / or partially restoring dopaminergic neuron function in rodent models of dopaminergic dysfunction (Hoffer et al., 1994; Wang et al., 1996; Hebert and Gerhardt, 1997; Rosenblad et al., 2000; Kirik et al., 2001). In the normal Fischer 344 rat, a single intranigral delivery of GDNF increased basal concentrations of DOPAC and HVA in the striatum. Simultaneously, potassium and d-amphetamine evoked releases were also increased within the striatum (Hebert et

al., 1996). Intranigral GDNF delivery in aged rats increased basal concentrations of DA, DOPAC, and HVA, while increasing potassium and d-amphetamine evoked DA release (Hebert and Gerhardt, 1997). Hoffer *et al.* showed GDNF treatment reduces apomorphine-induced rotation behavior and increases DA and DOPAC content in the SN of rat with greater than a 90% loss of dopamine. GDNF partially restored dopaminergic function in dysfunctional model systems, the aged rat, and dopamine depleted model systems, the lesioned rat. GDNF improves neurochemical function, which leads to improved behavioral performance in rodents. The positive effects of GDNF treatment on rodent dopaminergic systems provided the necessary supporting evidence to begin investigation in non-human primates.

Intraventricular treatment of parkinsonian non-human primates with GDNF greatly improved behavioral and neurochemical function with increased neuronal sprouting within the SN (Gash et al., 1996; Zhang et al., 1997; Grondin et al., 2002). GDNF treatment: increased dopamine content within the SN, VTA, and globus pallidus, improved the parkinsonian features of bradykinesia, postural instability, and rigidity, and increased TH+ positive neuron size and density in the SN. Initially, GDNF entered into phase I clinical trials using intraventricular delivery, but was unable to alter PD related behavioral deficits. Intraventricular delivery of GDNF still demonstrated biological activity through its multiple side effects such as nausea, Lhermite's sign, inappropriate sexual behavior, hallucinations, and depression (Kordower et al., 1999; Nutt et al., 2003). Because of the positive preliminary work with GDNF, it was concluded that the intraventricular delivery system was the primary problem and other methods and areas of delivery needed to be explored.

Intranigral delivery of GDNF in normal non-human primates increased activity levels and increased potassium-evoked dopamine release. Investigation of tissue neurochemical levels in the same animals demonstrated increases in DA and HVA in the SN and VTA (Gash et al., 1995). Intraputamenal delivery was determined to be an effective means of delivery with distribution of GDNF a key component of successful treatment (Grondin et al., 2002; Ai et al., 2003; Gash et

al., 2005). Intraputamenal delivery of GDNF in parkinsonian non-human primates improved motor function, increased striatal tissue levels of DA, DOPAC, and HVA, and increased TH+ neuron density within the striatum (Grondin et al., 2002). In the aged non-human primate intraputamenal GNDF treatment, increased TH+ fiber density in both the SN and striatum further validated the putamen as an effective delivery site (Ai et al., 2003).

Increased knowledge on delivery and distribution spurred the reintroduction of GDNF to phase I clinical testing. In two different phase I trials, GDNF showed positive behavioral results with only minor difficulties primarily related to the catheter delivery system (Gill et al., 2003; Slevin et al., 2005). GDNF progressed into phase II clinical trials, which were abandoned after a lack of positive clinical results, and potential neurotoxic effects on the cerebellum within the non-human primate (Lang et al., 2006; Hovland et al., 2007). Technical differences in drug-delivery between the phase II trial and both phase I trials were possible reasons for the discrepancy in results. Differences in delivery and distribution of GDNF within the putamen could have potentially reduced the efficacy of GDNF to combat PD. Therefore, a small molecule with neurotrophic actions similar to GDNF, which can be administered orally or nasally, with no toxic effects, could be of great value in the treatment of PD.

Bioactive Peptide Formation and Neurotrophic Factors

Nerotrophins are generally large complex molecules with molecular properties limiting their bioavailability. The potential of neurotrophins to combat neurodegenerative disease has led to intense investigation of their function, but more recently, the proregions of growth factors such as BDNF and NGF have demonstrated functional significance. Small bioactive molecules are of interest in the development of a potential therapeutic because it should have increased bioavailability and therefore, increased functional impact. Post-translational processing of proBDNF to mature BDNF allows the classically defined signaling action TrkB activation, which regulates cell survival, differentiation, and signaling function. However, when proBDNF is secreted without post-translational modification, apoptosis can be induced through the p75^{NTR}/ sortilin complex

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(Teng et al., 2005a). The endoproteolytic cleavage of proNGF also regulates receptor affinities for p75^{NTR} or TrkA. Therefore, the functional effects of BDNF or NGF are regulated by the post-translational processing of the precursor protein, which shows that a single proprotein may have differential effects enacted through processing (Lee et al., 2001; Nykjaer et al., 2004). The proteolytic cleavage of the proNGF molecule at dibasic cleavages sites releases two bioactive peptides from the prodomain of NGF that bind tyrosine kinase receptors similarly to their precursor protein and provide neuroprotection against excitotoxic lesions (Dicou et al., 1997; Dicou, 2006). Neuropeptide proenkephalin A has biological activity in opiod neurotransmission, but when post-translationally processed at dibasic cleavage sites, studies show the production of multiple bioactive peptides from the precursor protein with variable effects on cell signaling and neurotransmission. The post-translational processing of precursor proteins to produce multiple bioactive peptides, that may have therapeutic value, is evidence of the production of diverse signaling molecules originating from a single proprotein (Haskins et al., 2004; Bernay et al., 2009).

A theoretical model of the post-translational processing of GDNF predicts the production of three peptides of which one has been proven to have bioactivity, Brain Excitatory Peptide (BEP) (Glass, 2004; Immonen et al., 2008) (Figure 1.4). Immonen et al. demonstrated rodent BEP could increase excitatory post-synaptic potentials of the rat hippocampal slice. We have synthesized the human homolog of the three peptides and we have designated these as Dopamine Neuron Stimulating Peptides (DNSPs).

GDNF, the DNSPs' precursor protein, has exhibited profound effects in the rodent dopaminergic system; therefore, we will begin our initial characterization of the DNSPs examining dopaminergic neurotransmission in the nigrostriatal pathway. Investigation of GDNF has yielded many positive results in protecting and increasing function of neurons in rodent and cell culture models of dopaminergic loss (Lin et al., 1993; Hoffer et al., 1994; Kearns and Gash, 1995; Tomac et al., 1995b). The successful translation of such a trophic factor to a therapy would provide a significant advancement in the ability to treat

MKLWDVVAVCLVVLLHTASAFPLPAGKRPPE
APAEDRSLGRRRAPFALSSDSNMPEDYPDQ
FDDVMDFIQATIKRLKRSPDKQMAVLPRRE
RNRQAAAANPENSRGKGRRGQRGKNRGCV
LTAIHLNVTDLGLGYETKEELIFRYCSGSCD
AAETTYDKILKNLSRNRRLVSDKVGQACCR
PIAFDDDLSFLDDNLVYHILRKHSAKRCGCI

Signal Peptidase →

FPLPAGKRPPE
APAEDRSLGRRRAPFALSSDSNMPEDYPDQ
FDDVMDFIQATIKRLKRSPDKQMAVLPRRE
RNRQAAAANPENSRGKGRRGQRGKNRGCV
LTAIHLNVTDLGLGYETKEELIFRYCSGSCD
AAETTYDKILKNLSRNRRLVSDKVGQACCR
PIAFDDDLSFLDDNLVYHILRKHSAKRCGCI

↓ "Paired Basics-Specific" endopeptidase

FPLPAGKR**/**PPE
APAEDRSLGRR**/**RAPFALSSDSNMPEDYPDQ
FDDVMDFIQATIK**RLKR**SPDKQMAVLPRRE
RNRQAAAANPENSRGKGRRGQRGKNRGCV
LTAIHLNVTDLGLGYETKEELIFRYCSGSCD
AAETTYDKILKNLSRNRRLVSDKVGQACCR
PIAFDDDLSFLDDNLVYHILRKHSAKRCGCI

← Carboxypeptidase H

FPLPAG
PPEAPAEDRSLG

↓ Peptidylglycine Amidating Monooxygenase

FPLPA-amide } **DNSP-5**
PPEAPAEDRSL-amide } **DNSP-11**

Mature GDNF {

↓ **Proteolytic Processing enzymes of Constitutive Pathway**

SPDKQMAVLPRRE
RNRQAAAANPENSRGKGRRGQRGKNRGCV
LTAIHLNVTDLGLGYETKEELIFRYCSGSCD
AAETTYDKILKNLSRNRRLVSDKVGQACCR
PIAFDDDLSFLDDNLVYHILRKHSAKRCGCI

Mature GDNF After Signaling??

↓ "Paired Basics-Specific" endopeptidase

SPDKQMAVLPRR/E
RNRQAAAANPENSRGKGRR/GQRGKNRGCV
LTAIHLNVTDLGLGYETKEELIFRYCSGSCD
AAETTYDKILKNLSRNRRLVSDKVGQACCR
PIAFDDDLSFLDDNLVYHILRKHSAKRCGCI

← Carboxypeptidase H

ERNRQAAAANPENSRGKG

↑ Peptidylglycine Amidating Monooxygenase

ERNRQAAAANPENSRGK-amide } **DNSP-17**

Figure 1.5 Theoretical Post Translational Processing of GDNF

The proprotein form of GDNF is theorized to be processed to DNSP-11 and DNSP-5 through post-translational modification through multiple enzyme interactions. GDNF is potentially modified post-signaling by enzymatic reactions to DNSP-17. All of the DNSPs are amidated, potentially altering *in vivo* stability.

neurodegenerative disease by preventing neuronal loss and/or restoring function of damaged neurons (Peterson and Nutt, 2008). Negative results provided by clinical investigation of GDNF are accounted for by the difficulties with delivery, distribution, or specificity (Lang et al., 2006; Salvatore et al., 2006; Hovland et al., 2007; Morrison et al., 2007). DNSPs have the potential to rectify many of the difficulties associated with GDNF by their ease of manufacturing and potential administration through oral or nasal routes. In addition, modifications can be made to the DNSPs to increase distribution and specificity. However, the effects of the DNSPs on DA neurons must first be understood to determine if and what modifications may be necessary.

Thesis Outline

Hypothesis: Dopamine Neuron Stimulating Peptides will modulate DA neurochemistry in the nigrostriatal pathway analogous to GDNF.

GDNF has been established as the most effective neurotrophic factor at increasing the functional capacity of dopaminergic neurons and is the "gold standard" to which other neurotrophins are compared. However, GDNF has not been without problems, especially concerning bioavailability and delivery methods. The DNSPs may have similar effects on dopaminergic neurons as GDNF and would potentially provide increased bioavailability and ease of delivery. Therefore, the effects of the DNSPs on DA neurochemical function needed to be assessed and compared to previous results with GDNF. The primary endpoints of interest were neurochemical measures using whole tissue dopamine content or microdialysis collected dopamine concentrations. Behavioral and immunohistochemical techniques were applied to explore the effects of DNSP altered DA function and were considered supporting data for the neurochemical measures. All data were examined relative to previous results with GDNF providing a benchmark for the effectiveness of the DNSPs.

In this thesis, I will discuss the investigation of the DNSPs and their effects on dopamine neurochemistry in the nigrostriatal pathway. The goal of these studies was to determine if any of the DNSPs can modulate dopaminergic neurotransmission analogous to GDNF and characterize their effects. To

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investigate the DNSPs effects on the damaged dopaminergic system, multiple models with varying degrees of dopaminergic dysfunction were treated with the DNSPs and their neurochemical function examined. In Chapter 3, the dosage of the combined delivery of all three DNSPs was investigated, as was the activity of the individual DNSPs on dopaminergic function. Analysis of 3- 6 month Fischer 344 rats 28 days after receiving intranigral DNSP delivery showed increased baseline concentrations of DA, DOPAC, and HVA as well as increased K^+ and d-amphetamine evoked DA release as measured by microdialysis. All concentrations of the DNSP mixture altered DA neurochemistry, but the 30 µg dose delivered to the SN was found to be most effective. Effects of treatment with the individual DNSPs were then investigated to minimize confounding factors within the data. DNSP-11 produced significant modifications of DA in the striatum 28 days after treatment. DNSP-11 increased baseline concentrations of DA, DOPAC, and HVA and increased evoked DA release by K^+ and d-amphetamine. Acute effects of DA were also investigated in the normal Fischer 344 rat because the rat homolog was demonstrated to stimulate increases in post-synaptic potentials   (Immonen et al., 2008). Multiple concentrations of DNSP-11 were delivered in the striatum via reverse microdialysis with no change in DA concentration until the 1 mM concentration of DNSP-11 stimulation increased the basal concentrations of DA with no change in metabolite concentrations. DNSP-11 was effective in modulating DA neurochemistry in the normal rats, which demonstrated the functional activity of DNSP-11.

In Chapter 4, the aged (24-month) Fischer 344 rat was used as two models of dopaminergic dysfunction. Aged rats received a bilateral infusion of DNSP-11 and DA neurochemistry was examined by striatum microdialysis and whole tissue neurochemical analysis. DNSP-11 decreased the baseline concentration of DA, DOPAC, and K^+ evoked release of DA. Modulation of DA by DNSP-11 maintained the premise of functional activity by the prodomain molecule but was not evidentiary of a therapeutic capacity.

Chapter 5 continues the investigation of DNSP-11 in a model of dopaminergic dysfunction using 6-OHDA neurotoxin to create neuroprotection

and neurorestoration study paradigms. In the neuroprotection model, DNSP-11 did not provide neuroprotective effects but did modulate neurochemistry by enhancing DA metabolism. In the neurorestoration model, DNSP-11 enhanced or restored function to dopaminergic neurons 28 days after a single injection to the SN. DNSP-11 reduced apomorphine-induced rotation behavior and increased DA and DOPAC nigral tissue content. A reduction in rotation behavior was the first documented occurrence of a behavioral modification by DNSP-11 treatment. Similar to previous results with GDNF, DNSP-11 was seen to increase function of normal and dysfunctional DA neurons over 4 weeks after a single intranigral administration.

Chapter Two: Methods

Chemicals

All chemicals were purchased from Fisher Scientific (Fisher Chemical Fairlawn, NJ) except for liquid chromatography grade chemicals purchased from Sigma-Aldrich (St. Louis, MO). The DNSPs were synthesized and purified by W.M. Keck Foundation, Yale University (New Haven, CT).

Animals and Procedures

Male Fischer 344 (F344) rats were used in all experiments to reduce hormone interference with neurochemical function, and cellular interactions of the DNSPs. F344 rats are an inbred rat strain, which reduces the variability of study measurements due to minimization of genetic variation. Prior work shows GDNF increased dopaminergic function in the F344 rat and provides precedence for trophic activity and is a benchmark for DNSP activity (Hoffer et al., 1994; Bowenkamp et al., 1995; Hebert et al., 1996; Hebert and Gerhardt, 1997). F344 rats are commonly used as a model of aging and have significant reductions in motor functions that are related to dopaminergic dysfunction (discussed in detail in Chapter 1). Therefore, aged F344 rast (older than 22 months) were used as a model of dopaminergic dysfunction in the studies described within this dissertation (Shimokawa et al., 1993). All rats were obtained from Harlan Laboratories Inc. (Indianapolis, IN), but the aged (24 months old) rats originate from the National Institute on Aging Colony. Animals were housed in the Department of Laboratory Animal Research Facility on a 12 hr light/dark cycle with food and water provided *ad libitum*. All behavioral and neurochemical measures were performed in the 12 hr light portion of the cycle. All animal procedures were reviewed and approved by our Institutional Animal Care and Use Committee (IACUC) and conform to AAALACI guidelines.

Surgical Procedures

Survival surgical procedures were performed under continuous delivery of 1.5% - 2.5% isoflurane in a sterilized laminar flow hood. Sterilization and pain

management techniques were followed according to protocols approved by IACUC. After all survival surgeries animals were allowed to recover in their home cage and provided with pain relief for 72 hours or until euthanasia using 5 mg/kg carprofen subcutaneously delivered every 24 hours.

Non-survival surgical procedures were performed under urethane (1.25 mg/kg).

Infusion Delivery of DNSPs

The skin above the skull was reflected and a single burr hole drilled to allow access to two points. The following stereotaxic coordinates from bregma were used to deliver the infusion solutions: Point #1 Tooth Bar (TB): -2.3mm, anterior posterior (AP): -5.6 mm, medial lateral (ML): -1.7 mm, dorsal ventral (DV): -7.3 mm and Point #2 TB: -2.3 mm, AP: -5.6 mm, ML: -2.5 mm, DV: -6.8 mm (Hebert et al., 1996; Paxinos and Watson, 2005) (Figure 2.1A). DNSPs were dissolved in pH 5 Citrate Buffer (150 mM NaCl and 10 mM Sodium Citrate). The DNSP solution or citrate buffer vehicle was delivered unilaterally to the nigral cell bodies through the burr hole to the two aforementioned stereotaxic coordinates using a 22-gauge tip style 3 Hamilton syringe (Hamilton Company USA, Reno, NV) connected to a 25 µL gas tight syringe. The individual performing the infusion was blinded to the content of the delivered solution. The doses for the DNSPs are detailed in each chapter. An infusion rate of 0.25 µL/min over 10 minutes was controlled by a KD Scientific model 100 infusion pump (KD Scientific Inc., Holliston, MA) to deliver 2.5 µL of the blinded treatment solution to the two points dorsal to the SN. A total of 5 µL of solution was delivered to the SN. A five minute wait period between needle insertion and solution delivery was carried out to allow normalization of the tissue. A 10 minute wait period between solution delivery and needle removal was carried out to prevent concentrating the solution in the needle track. Delivery to the second infusion point used the same burr hole and followed similar methodology as the first point. Bone wax was used to cover the burr whole and the animals surgical site was closed using dissolvable sutures.
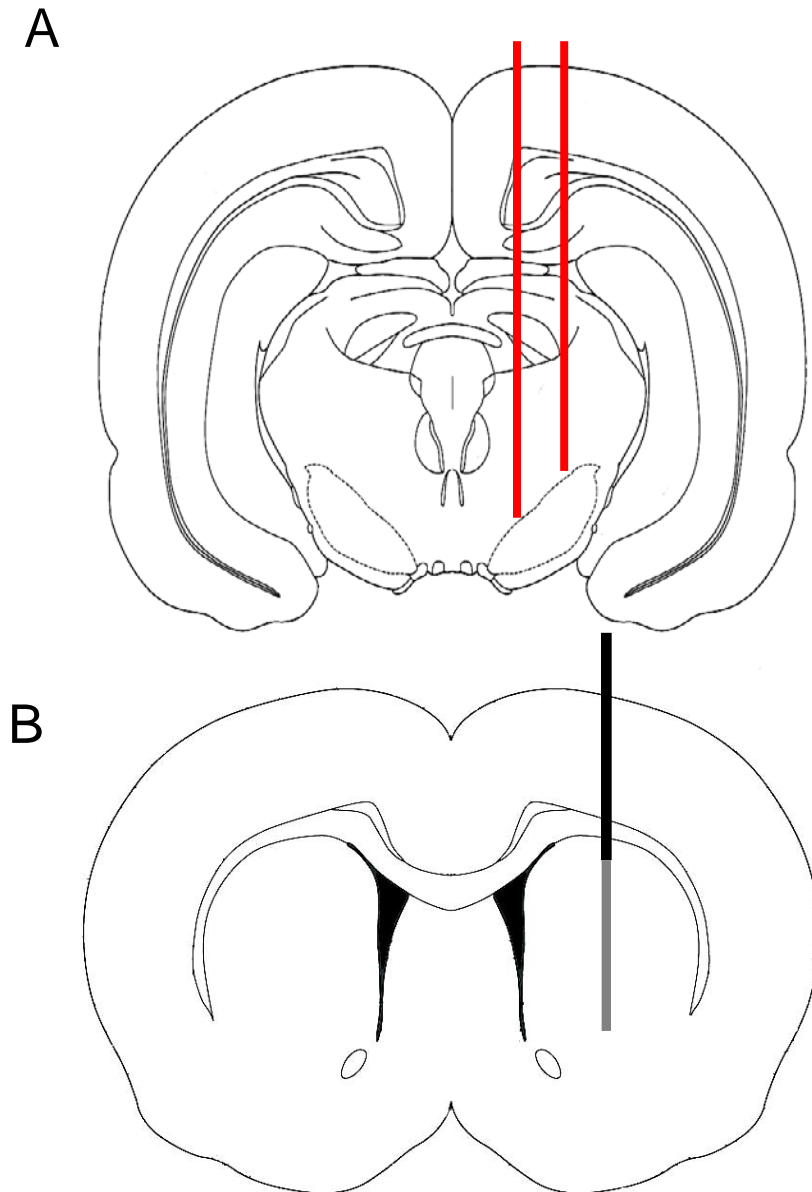
Figure 2.1 Neuroanatomical Location of SN Infusions and Microdialysis Probes

A) (RED) Representation of the path of needle insertion used to deliver the DNSPs dorsal to the dopaminergic cell bodies located within the SN. B) The microdialysis probe membrane (GREY) spans the dorsal / ventral length of the striatum sampling the extracellular space at the dopaminergic terminals. Modified from (Paxinos and Watson, 2005).

Delivery of 6- Hydroxydopamine to the Medial Forebrain Bundle and Infusion of DNSP-11 or Vehicle

An extensive DA lesion was produced by lesioning the nigrostriatal pathway with 6-OHDA. Adult F344 (3-6 month) rats were administered desipramine (30 mg/kg i.p.) in physiological saline at the onset of the procedure to maintain dopaminergic neuron specific toxicity. Rats were placed in a stereotaxic frame, the skin above the skull reflected, and a single burr hole was drilled into the skull. The burr hole was oriented to allow access to two injection sites along the MFB with the following stereotaxic coordinates: Point #1 TB: -2.4 mm, AP: -4.4 mm, ML: -1.2 mm, DV: -7.1 mm, Point #2 TB: +3.4 mm, AP:-4.0 mm, ML: -0.8 mm, DV: -8.0 mm (Lundblad et al., 2002; Paxinos and Watson, 2005). 6-OHDA was injected into the MFB using a 3.6 µg/µL 6-OHDA•HCl solution with 0.9% NaCl and 0.02% ascorbic acid. The 6-OHDA solution was injected using a 10 µL 22-gauge tip style 3 Hamilton syringe. The first point targeted neurons projecting from the SN (A9 pathway) with an injection of 2.5 µL of the 6-OHDA solution and the second point targeted neurons projecting from the ventral tegmental area (A10 pathway) with an injection of 2.0 µL of the 6-OHDA solution. The burr hole was covered with bone wax and the surgical site was closed using dissolvable sutures. The lesion was allowed to progress for four weeks and the animal's health was closely monitored. Four weeks after the lesions, the severity of the lesion was tested using apomorphine-induced (0.05 mg/kg s.c.) rotation behavior, which will be discussed in detail later in this chapter.

Five weeks after the unilateral 6-OHDA delivery, animals were grouped based on their prescreen rotation values while maintaining equal apomorphine induced rotation behavior between groups. Animals received either a 100 µg/5 µL DNSP-11 solution in citrate buffer or the vehicle solution, citrate buffer, with the solution content blinded to the person performing the infusion. An infusion protocol was followed identical to the previous section, Infusion Delivery of DNSPs, with delivery to the SN ipsilateral to the delivery of 6-OHDA. An infusion rate of 0.25 µL/min over 10 minutes was used to deliver 2.5 µL of the blinded

treatment solution to each of the two points for a total of 5 µL delivered to the SN. All rats' apomorphine-induced rotation behavior was assessed weekly for four weeks. One week after the last rotation session animals were sacrificed and their SN and striatum dissected out for analysis of neurochemical content. Both rotation behavior and neurochemical content analysis will be discussed more thoroughly in subsequent sections within this chapter.

Striatal Delivery of 6- Hydroxydopamine and DNSP-11

Normal Young (3-6 month) Fischer 344 rats were anesthetized under isoflurane and treated in a blinded manner with 100µg/3µL of DNSP-11 or 3 µL of citrate buffer vehicle. Infusion treatment was delivered over 3 minutes using a 10 µL 22-gauge tip style 3 Hamilton syringe placed into the brain at the following stereotaxic coordinate within the striatum; TB: 0.0 mm, AP: +1.0 mm, ML: -3.0 mm, DV: -5.0 mm (Kirik et al., 2000; Paxinos and Watson, 2005). After drug infusion treatment, a five-minute wait period was used to allow diffusion of the delivered solution and the animal was allowed to recover from anesthesia.

Six hours after infusion delivery of vehicle or DNSP-11, animals were anesthetized again and injected with 20µg/3µL of 6-OHDA•HCl in physiological saline containing 0.02% ascorbic acid. The 6-OHDA was infused over three minutes to the same striatal coordinates as the treatment using a10 µL 22-gauge tip style 3 Hamilton syringe. A five-minute wait period was carried out after infusion to allow diffusion of 6-OHDA and then the incision was closed using dissolvable sutures.

Microdialysis

Microdialysis can be used to sample the content of the extracellular space and simultaneously deliver solutions of interest (Figure 2.2). Simultaneous delivery and collection allows the quantification of multiple neurochemicals under multiple conditions (Hocht et al., 2007). Microdialysis uses a porous membrane on the tip of a hollow steel shaft allowing for diffusion of molecules from the extracellular space into the fluid flowing through the probe (Ungerstedt and Hallstrom, 1987).

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Courtesy of CMA Microdialysis
http://www.microdialysis.se/products
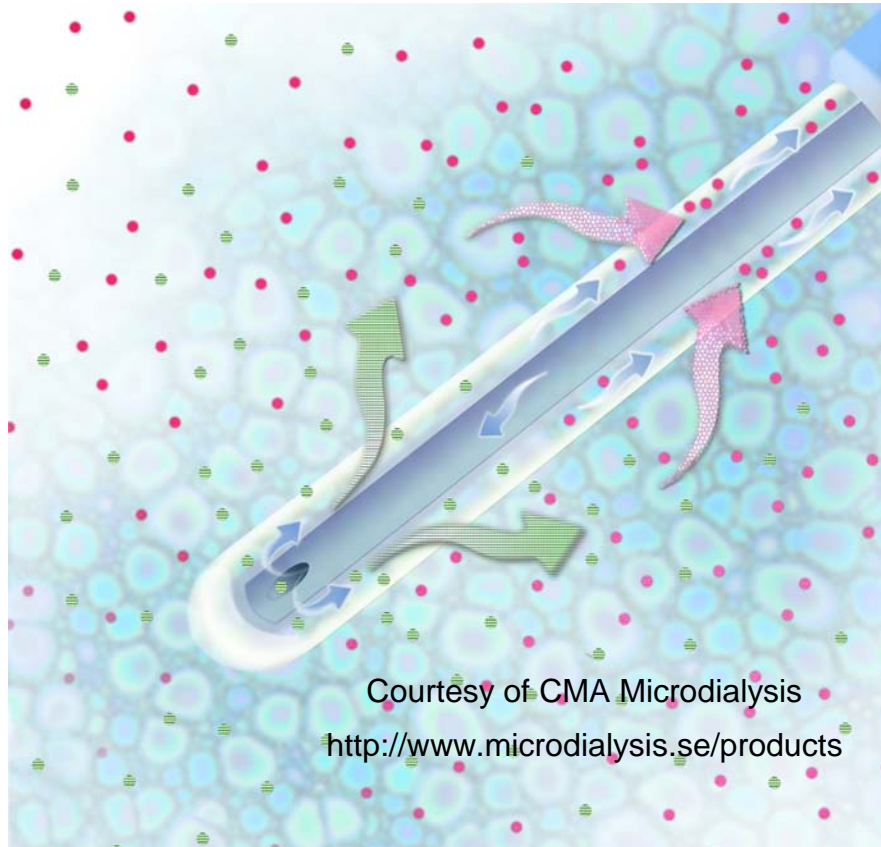
Figure 2.2 Microdialysis Membrane Function

Microdialysis provides delivery of the perfusion solution (GREEN) and simultaneous sampling of the extracellular neurochemicals (PINK) relative to their respective concentration gradients.
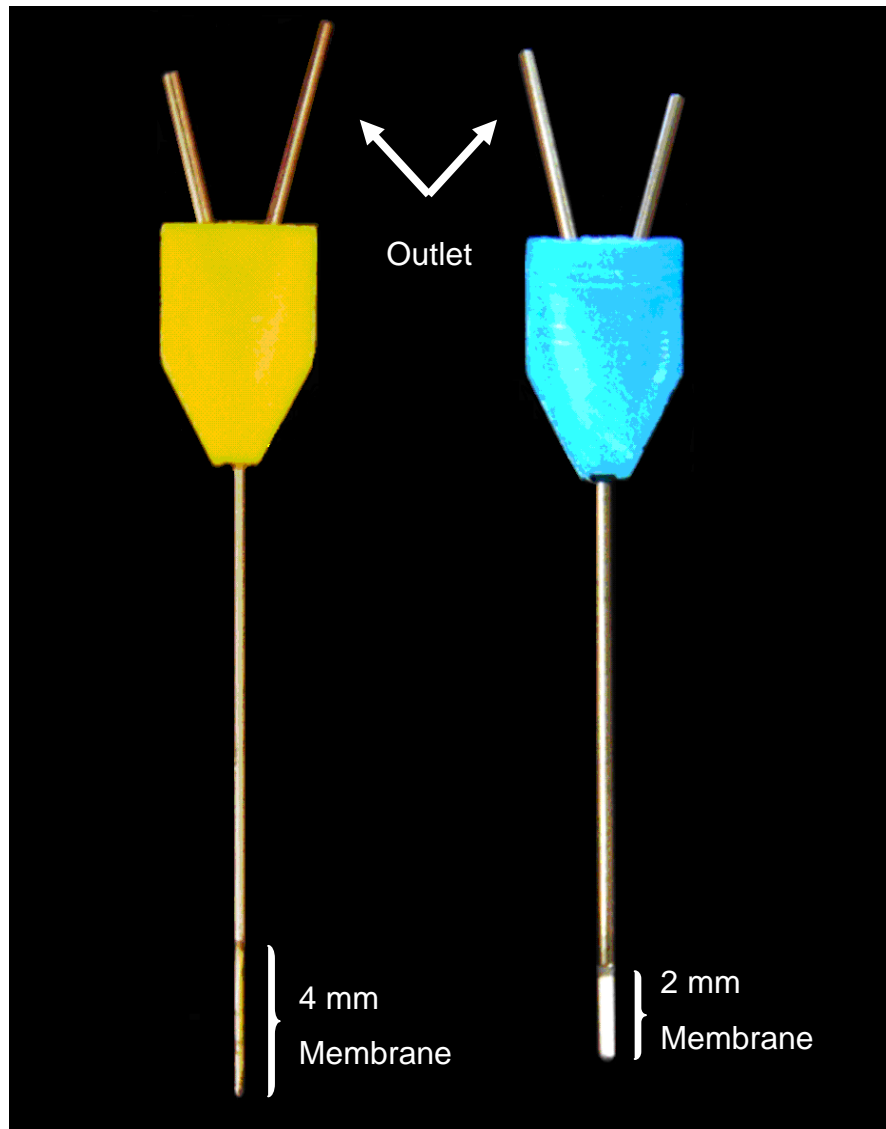
Figure 2.3 Microdialysis Probes Images and Descriptions

Depiction of CMA 11 and CMA 12 microdialysis probes. The CMA 11 probe has a 4mm Cuprophane membrane with a 6,000 Dalton molecular weight cut-off and 0.38 mm shaft diameter. The CMA 12 probe has a 2mm Polyarylethersulfone membrane with a 20,000 Dalton molecular weight cut-off and a 0.64mm shaft diameter.

In the studies discussed in this document two types of microdialysis probes were used, CMA 11 and CMA 12 (Figure 2.3), with their primary variation being membrane length and molecular cut-off. The membrane length was changed based upon the brain structure of interest and the molecular weight cut-off was varied based upon the size of the molecules of interest. Rate of recovery and delivery of molecules of interest is relative to the concentration gradient of the chemicals delivered and those being sampled. Recovery and sampling rates can be manipulated by modifying the sample collection time and fluid flow rate. A balance between optimal recovery, sample volume, and collection time needs to be achieved to maintain sensitivity and resolution to neurochemical changes (Li et al., 2006).

Microdialysis Probe Preparation and Probe Recovery Determination

A KD Scientific model 230 pump in conjunction with 1mL Hamilton syringes (point style 3) were connected to a CMA 11 microdialysis probe via FEP tubing. Microdialysis probes were flushed with 70% ethanol for a minimum of 4-5 minutes to remove the glycerol packing solution from the probe membrane. Probes were then flushed with deionized water for 4-5 minutes to remove the ethanol and prevent salt crystal formation. Probes were soaked in deionized water until preparations were made to determine the percent recovery rate for the neurochemicals of interest. The percent of recovery for each probe was determined by perfusing the probes with artificial cerebral spinal fluid (aCSF) (124 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4) as the probe was submerged in a solution containing standardized concentrations of the following neurochemicals: 2×10^{-7} M NE, 1×10^{-7} M DOPAC, 2×10^{-7} M DA, 1×10^{-7} M HVA, 2×10^{-7} M 5-hydroxytryptamine (5-HT), and 4×10^{-7} M 5-hydroxyindoleacetic acid (5-HIAA). Neurochemical concentrations were measured by high performance liquid chromatography coupled with electrochemical detection (HPLC-EC). The percentage of neurochemicals in the collected solution relative to the standard solution yields the neurochemical percent recovery.

Microdialysis in DNSP pretreated rats

Twenty-eight days after treatment with DNSPs, animals were anesthetized with urethane (1.25 mg/kg i.p.) and placed in a stereotaxic frame. A craniotomy was performed to place the CMA 11 microdialysis probe within the striatum using the following stereotaxic coordinates: from bregma AP: +1.5 mm ML: -2.3 mm DV: -8.0 mm (Hebert et al., 1996; Hebert and Gerhardt, 1997; Paxinos and Watson, 2005)(Figure 2.1B). Perfusion solutions included aCSF, 100 mM K^+ aCSF (26 mM NaCl, 100 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4), or 250 µM d-amphetamine aCSF all within the pH range of 7.2 -7.4. All solutions were perfused at a rate of 1 µL/min with a sampling time of 20 minutes yielding 20 µL fractions. The first 20-minute fraction after placement of the probe within the striatum was discarded because of variability due to damaging synapses upon lowering the probe into the brain. **(Sample Time 0 - 100 min.)** Five total fractions were collected, and the perfusate solution was switched to the 100 mM K^+ aCSF using a CMA liquid switch (CMA 110). **(Sample Time 100 – 120 min.)** The 100 mM K^+ aCSF solution was perfused for 20 minutes. **(Sample Time 120 – 200 min.)** Upon completion of fraction 6, the solution was switched back to aCSF for fractions 7-10. **(Sample Time 200 – 220 min.)** After fraction 10 was collected, the perfusate was switched to d-amp aCSF for 20 minutes. **(Sample Time 220 – 320 min.)** Fraction 11 was collected and the perfusate was switched back to aCSF for the remainder of the experiment, fractions 12 – 16. Dialysate samples were immediately frozen (-70 °C) after collection and thawed just prior to HPLC-EC analysis.

Baseline neurochemical concentrations were calculated for all data sets from the average of samples 2-4. The high K^+ solution and d-amp containing solutions were used to evoke DA release and were compared across treatment groups. The 100 mM K^+ solution stimulates neurotransmitter release from pre-synaptic terminals and can provide evidence of changes in vesicle number and neurotransmitter content. The d-amp containing solution interacts with the DA transporter reversing DA transport and causing neurotransmitter release in a calcium independent manner. D-amphetamine-induced neurotransmitter release

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provides evidence of changes in cytosolic DA content and / or the DA transporter. Overall, the microdialysis design we use provides insights into the tonic release of DA (baseline), calcium and sodium-dependent release of DA (100 mM K$^+$), and calcium-independent release of DA (250 μM d-amphetamine).

Reverse Microdialysis of DNSP-11

F344 rats (3-6 month old) were used for acute DNSP-11 studies discussed in Chapter 3, but were given no pretreatment with DNSP-11. Animals were anesthetized with urethane (1.25 mg/kg i.p.) and placed in a stereotaxic frame. A craniotomy was performed allowing the microdialysis probe to be placed into the striatum using the following stereotaxic coordinates: From bregma AP: +0.5 mm ML: -3.0 mm DV: -5.5 mm. A KD Scientific model 230 pump in conjunction with 1 mL Hamilton syringes (point style 3) was connected to CMA 12 microdialysis probes (Figure 2.3) via FEP tubing. Microdialysis probes had a 2.0 mm membrane length and 20,000 Dalton cut-off providing delivery of the perfusate and sampling of the extracellular fluid. Perfusion solutions include aCSF and varying concentrations of DNSP-11 (3 μM – 1 mM) dissolved in aCSF. All solutions were sterile filtered and adjusted to a pH range of 7.2 -7.4. A sampling rate of 1 μL/min was used with a sampling time of 20 min yielding 20 μL of volume for each fraction. Artificial cerebral spinal fluid was perfused for a total of 30 minutes and the dialysate discarded. After 30 minutes of aCSF perfusion, dialysate sample collection began every 20 minutes. **(Sample Time 0 – 80 min.)** Fractions 1-4 were collected with aCSF as the perfusate. Immediately following the collection of sample 4, the perfusate solution was switched to DNSP-11 in aCSF using a CMA liquid switch (CMA 110). **(Sample Time 80-160 min.)** Fractions 5-8 were collected using aCSF containing DNSP-11 as the perfusion solution. **(Sample Time 160-220 min.)** After the collection of fraction 8, the perfusate solution was switched back to aCSF for the remainder of the experiment, fractions 8 – 11. The neurochemical baseline with aCSF perfusion solution was determined by the average of fractions 1-4 (0 – 80 min) and the change from baseline caused by DNSP-11 was determined from the average of fractions 5-8 (80 – 160 min). Probe recoveries ranged from 25 – 35% with an

average of 31.2 ± 0.5%. The raw data were analyzed by a two-way ANOVA and fraction 8 was used to produce a dose response curve, analyzed by a one-way ANOVA.

Measurements of Spontaneous Locomotion

Spontaneous motor activity and movement velocity was assessed by automated activity chambers (Omnitech Electronics, Digiscan Animal Activity Monitor, Columbus, OH) in 3-6 month old F344 rats treated with 30 µg/5 µL of DNSP-11. The automated activity chamber is a 40 x 40 acrylic box with infrared beams mounted horizontally every 2.5 cm at both 2 cm and 10 cm from the floor (Figure 2.4). For a single testing session, the animal spent 6, ten minute periods in the automated activity chamber with the number of beam breaks being recorded by a computer (Hebert and Gerhardt, 1997). Motor activity was represented by the total distance the animal travels in the specified sampling period and average movement speed, which was the distance traveled divided by time. All animals were habituated to the activity chambers through four weekly testing sessions before treatment. Animals were subsequently divided into two groups with comparable spontaneous activity and movement velocity values. Animals received DNSP-11 or vehicle as per the previously discussed infusion protocol with personnel performing the infusion blinded to the treatment. Both groups underwent weekly testing sessions for four weeks after treatment comparing total distance and movement speed. Data were analyzed by a two-way ANOVA with Bonferroni's post-hoc tests comparing DNSP-11 and citrate vehicle treatment over the five testing sessions.

Figure 2.4 Assessment of Spontaneous Locomotor Activity

We used the activity monitors sold by Omnitech to assess spontaneous locomotion. Horizontal projecting lasers allow quantification of the movement speed and distance traveled based on the interruption of the projected beams.

Assessment of Drug-Induced Rotation Behavior

Drug-induced rotation behavior produced by d-amphetamine and apomorphine injections has been used as a method for measuring nigrostriatal dopaminergic function in unilateral lesioned rats. Ungerstedt and Arbuthnott introduced rotation behavior as a method to study nigrostriatal DA activity and hypothesized the behavior was related to imbalances in DA receptor activation between the two hemispheres (Ungerstedt and Arbuthnott, 1970). The asymmetrical activation of DA receptors has been demonstrated to be responsible for rotation behavior with changes in DA receptor function after lesion (Sonsalla et al., 1988). Apomorphine and amphetamine rotations have been shown to correlate with loss of dopaminergic function, but low-dose (0.05 mg/kg) apomorphine is highly sensitive to dopaminergic loss above a 90% loss threshold and post-synaptic dopamine receptor supersensitivity. Amphetamine has a linear relationship with functional DA loss making it less sensitive, but has value for predicting mild dopaminergic functional loss (Hudson et al., 1993). In the studies discussed within this dissertation, both apomorphine and amphetamine rotation behavior was utilized based primarily upon the lesion model being used and the sensitivity of rotation behavior provided by each drug. Apomorphine-induced rotations were used in conjunction with the extensive nigrostriatal lesion and amphetamine rotations were used in conjunction with the striatal lesion.

Apomorphine-Induced Rotational Behavior

MFB lesion severity was assessed before DNSP treatment using subcutaneous injections of 0.05 mg/kg apomorphine to induce rotational behavior measured by SDI Rotation software. A thoracic harness connected the rats to a counting head that interfaced with the computer and quantified the number of ipsi- and contralateral rotations (Figure 2.5). Rotation behavior was quantified for two hours in five-minute intervals after apomorphine was administered. Only animals exhibiting >200 rotations in the first hour contralateral to the lesion, which correlates to a greater than 90% lesion of the nigrostriatal pathway, proceeded to further treatment and testing (Hoffer et al., 1994). Beginning one

week after DNSP treatment apomorphine (0.05 mg/kg) induced rotation behavior was assessed again once per week for four weeks. Because of the pharmacological properties of apomorphine, only the first hour of rotation behavior was analyzed. The subsequent DNSP treatment group data were normalized to the control group and analyzed using a one-way ANOVA with Bonferroni's post-hoc tests.

Amphetamine-Induced Rotational Behavior

Striatal lesioned animals generally have a milder loss of dopaminergic projections and are less sensitive to apomorphine-induced rotational behavior. Therefore, lesion progression was assessed by amphetamine-induced rotational behavior. Striatal lesion animals were monitored weekly from week three – week six after treatment with 6-OHDA and DNSP-11. After the 6-OHDA and DNSP-11 treatment, two weeks was allowed to pass to permit progression of the dopaminergic cell loss (Kirik et al., 2000). At each behavioral recording session, an animal was placed in a thoracic harness, received an intraperitoneal injection of 2.5 mg/kg d-amphetamine, and placed in a rotometer bowl. SDI Rotation software quantified rotational behavior in five-minute bins over a two- hour recording session. Data from the first 20 minutes and last 40 minutes of the recording session were discarded and the remaining one-hour block of stable rotational behavior was analyzed by a two-way ANOVA with Bonferroni's post-hoc tests comparing mean rotational behavior between treatment groups across all testing sessions.

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Figure 2.5 Quantitation of Rotation Behavior

To assess rotation behavior, animals were placed into rotometer bowls and affixed to a thoracic harness. Animals received an injection of apomorphine (0.05 mg/kg) or d-amphetamine (2.5 mg/kg) based upon the study paradigm. The harness was connected to a counting head that interfaced with a computer and quantified the direction and number of rotations exhibited by an animal.

Cylinder Test for Assessment of Asymmetrical Paw Usage

A modified version of the cylinder test developed by Schallert et al. was used to monitor asymmetric changes in the bilateral nigrostriatal dopamine pathways (Schallert et al., 2000). In a darkened room, rats were placed in a 40 cm tall x 16 cm diameter clear plexiglass cylinder and their movements were recorded using a Cannon mini-DVD video recorder for four minutes (Figure 2.6). The darkness was used to enhance their activity level and provide greater contrast in the video recordings of the rat movements. Rearing behavior was analyzed from the video recordings by a blinded observer. Right and left forelimb use was distinguished upon rearing and upon landing from the reared posture. Forelimb use was quantified one week before DNSP-11 and 6-OHDA lesion treatment and at weeks 2 and 4 after treatment (Lundblad et al., 2002). The four-minute long recording session were viewed by a blinded observer and paw usage was quantified by counting the number of times each paw was placed on the cylinder walls upon rearing and upon landing. Data was then normalized to the right paw usage (intact hemisphere) and analyzed by a one-way ANOVA with Bonferroni's post-hoc tests.

Brain Tissue Dissection and Preparation for Neurochemical Analyses for Assessment of the DNSPs' effects on DA Function

Animals were decapitated while under urethane anesthesia or after euthanasia, using CO_2 asphyxiation and their brains removed. The brains were placed in an ice-cold rat brain matrix where they were sliced into 1 mm sections. Visual confirmation of the microdialysis probe placements in the striatum was achieved and tissue punches were taken from the dorsolateral striatum using a 14-gauge blunt tip needle. The SN was dissected out of the most representative sections (Figure 2.7). Tissue was placed in pre-weighed microfuge tubes, weighed, and recorded. Samples were then frozen on dry ice snow. They were stored at -70° C (Hudson et al., 1995).

Figure 2.6 Modified Schallert's Cylinder Test for Studies of Motor Asymmetry

A) Modified Schallert's cylinder test using a Cannon mini-DVD recorder to monitor the animals paw use.

B) The cylinder test assessed asymmetric use of forelimbs after striatal lesion.
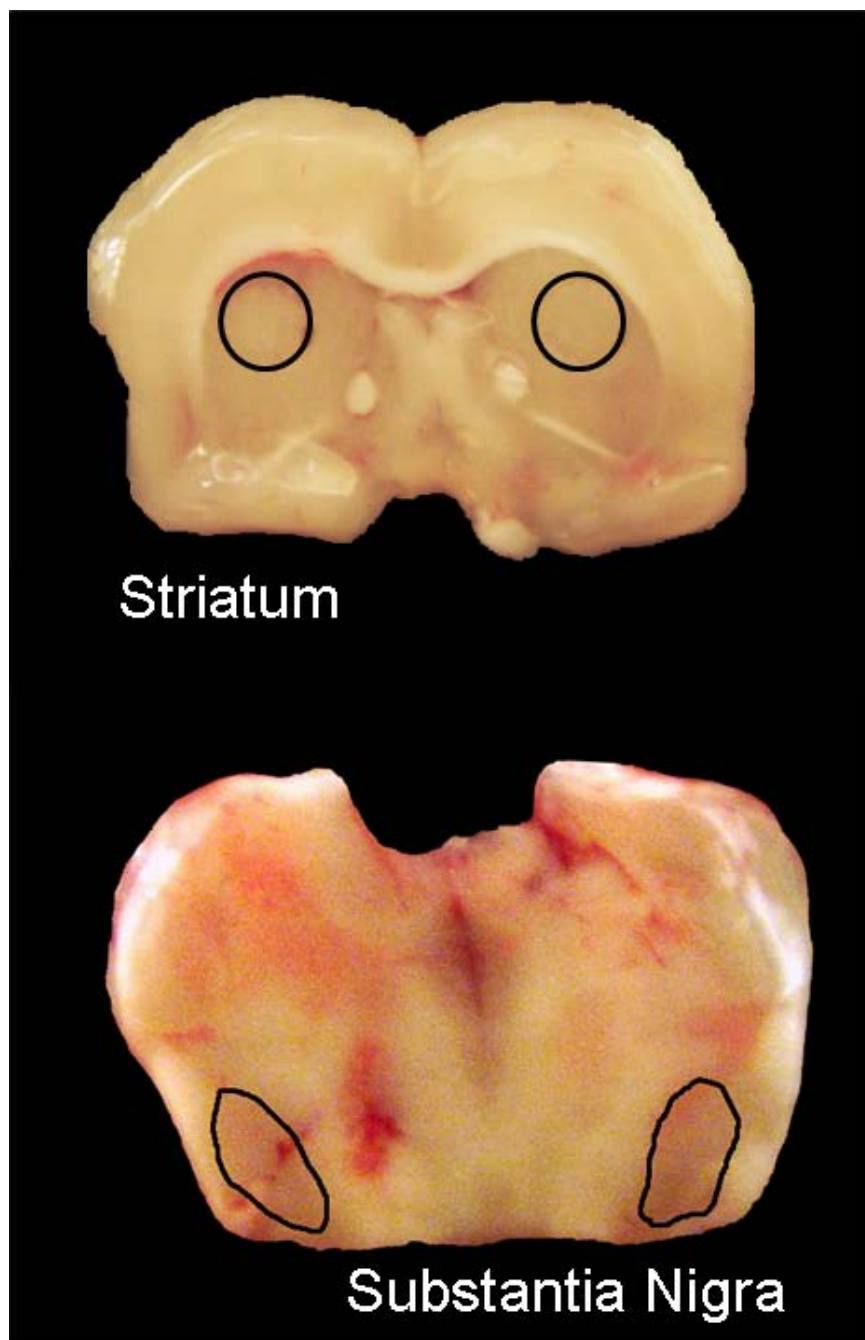
Figure 2.7 Representative Images of Brain Tissue Slices Showing Regions for Neurochemical Analysis

The above 1 mm tissue slices are representative photographs of the areas dissected for HPLC-EC neurochemical analyses and its metabolites. Note the striatal tissue punches were taken from the dorsolateral striatum (motor striatum) and the nigral tissue was taken from the anterior SN (near infusion points).

Tissue Processing for Immunohistochemical studies of TH in DNSP Treated Rats

Tyrosine hydroxylase is an enzyme found in catecholaminergic neurons that converts tyrosine to L-DOPA, and is utilized in dopamine, norepinephrine, and epinephrine production. Labeling and staining for TH allows catecholamine-producing neurons to be distinguished within a larger population of neurons. Depending on the brain region, specificity for DA neurons can be assumed when a small component of the neuron population is noradrenergic or adrenergic relative to the DA neuron population. There is an increased affinity for TH found in dopaminergic neurons over TH in noradrenergic neurons (Cuello et al., 1983). The sensitivity for TH staining visualization increases in models of dopaminergic cell loss because there is a decrease in the variability. TH staining allows changes in the number or morphology of DA neurons to be visualized also providing evidence of neuronal sprouting and/or restoration.

Tissue slicing and staining procedures were performed by Yi Ai, MD in the lab of Don M. Gash, Ph.D. (Department of Anatomy and Neurobiology, University of Kentucky Medical Center). Animals were deeply anesthetized with isoflurane and transcardially perfused with heparinized phosphate buffer (0.1 M, PB) followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4). Brains were collected and post-fixed overnight at 4 $^\circ$C in 0.1 M PB with 4% paraformaldehyde (pH 7.4). Brains were cryoprotected in increasing gradients of phosphate-buffered sucrose (15% & 30%) at 4 $^\circ$C until they sunk. Coronal sections (30-μm-thick) through the rostrocaudal extent of the brain were cut on a frozen sliding microtome. Slices were collected in a cryoprotectant solution and stored at –20 $^\circ$C until immunocytochemical processing.

A series of one in every 12th section was immunostained for TH. Endogenous peroxidase was blocked with 0.2% hydrogen peroxide for 10 minutes. Free-floating sections were incubated with primary anti-TH antibody (1:2000, Chemicon) overnight at 4 $^\circ$C and then exposed to biotinylated horse anti-mouse secondary antibody (1:1000, Vector) for 1 hour. Sections were then incubated in the avidin-biotin-peroxidase complex using the Elite ABC Vectastain

Kit (Vector). Visualization of TH immunoreactivity was achieved by using 3,3'diaminobenzidine as the chromogen with nickel enhancement. In addition, two series of adjacent sections were processed for hematoxylin and eosin (H&E) and cresyl violet (Nissl) staining for pathological evaluation.

HPLC-EC Analysis of Dialysate Fractions

Dialysate fractions were analyzed by HPLC-EC using a pH 4.1 citrate-acetate buffer mobile phase with 4% methanol and 0.34 mM 1-octane-sulfonic acid. At a flow rate of 1.7 mL/ min, analytes of interest were separated using a C18 column (4.6 mm x 75 mm, 3 μm particle size, Shiseido CapCell Pak UG120, Shiseido Co., LTD., Tokyo, Japan) (Figure 2.8). molecules were detected by flowing through a 100 mV conditioning cell before reaching the dual channel coulometric detector (ESA model 5014B dual analytical cell) with E_1 = +300 mV and $E_{2=}$ -250 mV. Levels of DA, DOPAC, and HVA were quantified based on peak area and retention times of known standard concentrations of the analytes. This protocol was slightly modified from original work by Hall *et al.* 1989.

HPLC-EC Detection and Analysis of Brain Tissue Neurochemical Content

The subsequent description of tissue preparation and respective HPLC-EC was performed by Stewart Surgener. Whole tissue levels of DA, DOPAC, HVA, 5-HIAA, and 5-HT were determined bilaterally in the striatum and SN for both DNSP-11 and citrate buffer vehicle groups. Dissected tissue was prepared for analysis by addition of an internal standard, dihydroxybenzylamine (DHBA), to provide an index of recovery. Tissue was then sonicated in cold citrate-acetate buffer mobile phase (pH 4.1) and centrifuged at 16,000 X g for 10 min. 50 µL of the subsequent supernatant was injected into the HPLC-EC system. Separation of the analytes was achieved by a C18 column (4.6 mm x 75 mm, 3 μm particle size, Shiseido CapCell Pak UG120, Shiseido Co., LTD., Tokyo, Japan). The mobile phase flow rate was 2.0 mL/min. Detection was carried out using a dual channel coulometric detector (ESA model 5011A dual analytical cell) with potentials of E_1 = +350 mV and $E_{2=}$ -250 mV.

Figure 2.8 HPLC-EC System

High performance liquid chromatography was used to separate and analyze dialysis fractions. Above is an example of the HPLC-EC system, the pump (far left) provides flow of the mobile phase carrying injected samples throughout the system. The autosampler (center) provides automation of sample delivery and precise control over injected sample volumes. The column organizer (far right) houses the C18 column, conditioning cell, and detector cell allowing control of the temperature and environment in which a sample is separated and detected.

The peak area and the retention times of the standards were used to quantify the levels of the analytes of interest in the tissue. A more detailed review of this protocol can be found in the original work by Hall *et al.*, 1989.

Brain tissue neurochemical levels were reported as total nanograms in sample per gram of the brain tissue wet weight (ng/g wet weight). Tissue neurochemical content was analyzed by a one-way ANOVA or Student's t-test depending on the number of groups. The neurochemical content was utilized to calculate a ratio of DA metabolism, called the turnover ratio. Turnover ratios were calculated from the total DA metabolite content (DOPAC + HVA) divided by the DA content. Therefore, the ratio changes as metabolism changes and can be used as an indicator of modified metabolic function. Data for each neurotransmitter of interest was analyzed by either a one-way ANOVA with Bonferroni's post-hoc tests (more than two treatment groups) or an unpaired two-tailed t-test (two treatment groups).

Mitochondrial Isolation and Respiration Assessment

Mitochondrial isolation and respiration studies were performed by Jignesh Pandya, Ph.D. in the lab of Patrick Sullivan, Ph.D. (Department of Anatomy and Neurobiology, University of Kentucky Medical Center). The mitochondrial isolation procedure was performed at 4^0C. Rats were anesthetized by CO_2 exposure, their brains were quickly removed, and soaked in a petri dish containing mitochondrial isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 20 mM HEPES, 1 mM EGTA; pH adjusted to 7.2 with KOH) . The striatum was dissected out of each brain and homogenized with mitochondrial isolation buffer containing 1 mM EGTA. The homogenized tissue was then transferred to microcentrifuge tubes and centrifuged 2 X at 1,300g for 3 minutes. The resulting supernatant was collected into another set of microcentrifuge tubes (while the pellet was saved as the nuclear pellet) and centrifuged at 13,000g for 10 min. The supernatant was saved as the cytosolic fraction where as the resulting pellet contained enriched crude mitochondria with a small synaptosomal portion. The pellet was further resuspended in 0.5 ml of isolation buffer containing 1mM EGTA and placed in a nitrogen cell disruption

chamber for 10 min at a pressure of 1200 psi (Brown et al., 2004). Application of nitrogen cell disruption to the pellet released trapped mitochondria from synaptosomes due to high pressure. The suspension was then placed on top of a ficoll density gradient (7.5% ficoll was layered over 10.0% ficoll solution) and centrifuged at 100,000g for 30 min using a Beckman ultracentrifugation. The purified mitochondrial pellet after ficoll centrifugation was resuspended in isolation buffer without EGTA in an Eppendorf tube where the top ficoll layers were discarded. The purified mitochondrial suspension contained synaptoneurosomes, which was centrifuged at 10,000g for 10 minutes to give washing after ficoll isolation. The resulting final pellet was resuspended with isolation buffer without EGTA to a concentration of approximately 10 mg/ml and placed on ice for further assessment.

A Clark type oxygen electrode inside a thermostatically sealed chamber was used to measure oxygen consumption of mitochondria in response to several mitochondrial substrates. The slope of the oxygen consumption was used to calculate mitochondria respiration rates in response to each substrate. To measure rate of oxygen consumption, mitochondrial protein of approximately 200 - 300 μg/ml was placed in respiration buffer (125 mM KCl, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 20 mM HEPES and 0.1% BSA, pH 7.2) within the sealed chamber(Sullivan et al., 2004c; Sullivan et al., 2004b; Sullivan et al., 2004a). The mitochondrial solution was allowed to equilibrate for 1 minute and state 2 respiration was induced by the addition of complex I substrates; 5 mM pyruvate and 2.5 mM malate. Two additions of 120 nmol adenosine 5'- diphosphate (ADP) were used to determine state III respiration followed by the determination of state IV respiration using 1 μM oligomycin. Mitochondrial state V respiration, which is the maximum electron transport, was induced by uncoupling the electron transport chain with 1 μM FCCP. Complex I was inhibited by 0.8 μM rotenone allowing the assessment of complex II driven electron transport with the addition of 10 mM succinate (Pandya et al., 2009). Mitochondrial function was quantified by measuring all the states as mentioned earlier along with the respiration control ratio RCR (State III oxygen consumption / State IV oxygen consumption). All

rates, including State III, State IV, and RCR, were calculated and compared using a two-way ANOVA or two-tailed unpaired t-test.

Data Analyses

All data sets were analyzed using Graph Pad Prism 4. To test for outliers, a Grubb's test was used and outliers were removed from data sets prior to further analysis. Bonferroni's post-hoc tests, if needed, were used to analyze data sets after either a one-way or a two-way analysis of variance (ANOVA). In all data sets with two groups an unpaired two-tailed Student's t-test was used to assess statistical significance, which was defined as a $p < 0.05$ for all analyses.

Chapter Three: Dopamine Neuron Stimulating Peptides Dose and Effects in Normal Rats

Hypothesis: DNSPs increase DA synthesis and metabolism in the nigrostriatal pathway of young adult F344 rats.

Introduction

DNSPs are novel molecules theorized to be formed from the proprotein form of GDNF. The amino acid sequence illustrates a large degree of sequence homology across multiple species (rat, mouse, human) making it likely that the DNSPs have biological activity (Chapter 1). Immonen et al. demonstrated DNSP-11 has a high degree of sequence homology with neuropeptide Y and provided evidence of a biological effect on the excitatory post-synaptic potentials in hippocampal brain slices (Immonen et al., 2008). Multiple trophic factors such as BDNF and NGF also have biological activity of their prodomains (Lee et al., 2001).

The parent molecule of the three DNSPs, GDNF, is a trophic factor that has previously demonstrated a profound effect on the rat dopaminergic system, specifically the nigrostriatal pathway. GDNF delivered to the SN of 3-6 month old F344 rats modulates striatum neurochemistry. 28 days after delivery of GDNF microdialysis neurochemical measures applied within the striatum showed increased basal concentrations of DOPAC and HVA, potassium-evoked DA release, and d-amphetamine-evoked DA release. Neurochemical changes associated with GDNF treatment translate to increases in motor movement (Hebert et al., 1996).

Because GDNF is the progenitor molecule of the DNSPs, we hypothesized that the DNSPs would increase DA levels and / or increase DA release and / or metabolism within the nigrostriatal pathway. If the DNSPs could provide modulatory effects similar to GDNF and minimize the negative aspects associated with GDNF discussed in Chapter 1, they could provide the basis for DA therapeutic molecules, but until the studies described in this chapter were performed, the DNSPs had not been tested *in vivo.* Before we could begin thoroughly testing our hypothesis, a dose response study was needed. GDNF's

dosing range varies between 10 µg and 100 µg per injection depending on the route of administration and animal model being tested (Hoffer et al., 1994; Cass et al., 2000; Fox et al., 2001; Kirik et al., 2001). Therefore, DNSPs were administered in a similar dosing range to determine if an effect on DA neuron function was produced by the DNSPs.

The site of DNSP delivery was also a concern because, in the past, GDNF has been delivered to multiple brain areas; striatum, SN, and ventricles. Significant side effects and a lack of penetrance have been demonstrated with intraventricular delivery of GDNF (Lapchak et al., 1998; Kordower et al., 1999). GDNF is normally expressed in low levels in the striatum and when exogenously applied to the striatum is retrogradely transported to the nigral cell bodies (Stromberg et al., 1993; Tomac et al., 1995a). GDNF was previously demonstrated to increase DA and DA metabolism in young adult F344 rats when delivered to the SN consequently we used a similar delivery paradigm (Hebert et al., 1996). In previous studies with GDNF, the entirety of the nigrostriatal pathway was considered and therefore delivery occurred at the cell bodies and the primary endpoint focused on the striatal projections. In most DA release paradigms of the nigrostriatal pathway, the terminal fields in the striatum are considered the primary site of DA release. Therefore, we used striatal microdialysis measures of DA and DA metabolism 28 days after the infusion as the primary endpoint measure to ensure detection of neurochemical changes similar to those seen initially with GDNF.

A dose response was performed using a mixture of the DNSPs, but we began infusing rats with a single DNSP to minimize the number of variables in our experimental design and enhance our ability to interpret the experimental results. Effects from the DNSP mixture would prove difficult to elucidate because of the complexity of multiple drug interactions. Our understanding of the endogenous release of the DNSPs was limited at the time and aspects of synergistic interplay between the DNSPs were unknown. We hypothesized that at least one of the DNSPs would be effective at increasing the basal concentrations of DA, DOPAC, and HVA as well as increasing evoked DA

release within the nigrostriatal pathway. The most effective dose, 30 μg, was determined from the previous study and used in delivering each peptide to the SN.

After striatal microdialysis showed significant neurochemical modulation by DNSP-11, a behavioral testing paradigm was designed to determine if the neurochemical changes would provide behavioral modification. Modification of motor movement would be the primary goal of a potential PD therapy because the observed neurochemical changes would lack clinical value without corresponding behavioral changes. Spontaneous motor movement was used to assess baseline motor movements focusing on movement speed and total distance traveled. The assessment of spontaneous motor movement in young adult F344 rats lacks the sensitivity drug induced behavior can provide but offers a comparison of movement between treatment groups.

The 28 day timing between DNSP delivery and sampling was determined because previous GDNF studies illustrate the 28 day time course to be most effective (Hudson et al., 1995; Kearns et al., 1997). Applied GDNF has an intraparenchymal life span of approximately seven days (Tomac et al., 1995a; Lapchak et al., 1996). GDNF has been demonstrated to have acute effects on excitability by modulating K^+ channels increasing excitability (Yang et al., 2001). DNSP-11 is not detected within the extracellular space two hours after exogenous application (Bradley et al., 2009 (Submitted)). The large discrepancy between the *in vivo* half-lives of GDNF and DNSP-11 and the acute stimulatory effect on excitability led to the hypothesis that DNSP-11 may exhibit acute effects on DA neurochemistry. To further corroborate DNSP-11's potential to have acute effects Immonen *et al.* demonstrated the rat homolog of DNSP-11 acutely increases excitatory post-synaptic potential in hippocampal slices (Immonen et al., 2008). To investigate potential acute effects by DNSP-11, reverse microdialysis was used to allow delivery of DNSP-11 while simultaneously sampling the neurochemical content of the extracellular space.

DNSPs result from the processing of the proGDNF molecule. Mature GDNF and the DNSPs originate from the same molecule therefore similarities in

target or function are likely. GDNF demonstrated great possibilities as a potential therapeutic for diseases effecting dopaminergic neurons, but ultimately fell short. The goal of these studies was to begin determining the potential of the DNSPs to be dopaminergic therapeutics. Using the assumption that the DNSPs and GDNF would share similarities in target or function these studies began the characterization of the effects of the DNSPs *in vivo*.

Methods

Studies of the DNSP Mixture

Equal mass quantities (1:1:1) of DNSP-5, DNSP-11, and DNSP-17 were dissolved together in pH 5 citrate buffer vehicle. Three concentrations of the DNSP mixture were used for delivery 10 µg/ 5 µL, 30 µg/ 5 µL, and 100 µg/ 5 µL. The 10 µg/ 5 µL solution contained 10 µg of DNSP-5, 10 µg of DNSP-11, and 10 µg of DNSP-17 (1:1:1 by mass). The other two solutions were made in a similar manner. Erin Rutherford, Ph.D. in the lab of Greg A. Gerhardt, Ph.D. infused 3-6 month old F344 rats with one of three concentrations of the DNSP Mixture or citrate buffer vehicle in a manner similar to the description provided in Chapter 2: Infusion Delivery of DNSPs. 28 days after infusion delivery Barry M. Joyce, Ph.D. in the lab of Greg A. Gerhardt, Ph.D. performed microdialysis as described in Chapter 2: Microdialysis (Table 3.1A). Animals were euthanized and their brains collected for either tissue neurochemistry or immunohistochemistry following methods described in Chapter 2. At the time the study was performed, I was assisting with the procedures and gaining technical experience. For all of the data generated in this study I performed all the data analyses.

Studies of individual DNSPs

Solutions of 30 µg/ 5 µL of DNSP-5, DNSP-11, DNSP-17, or citrate buffer vehicle were delivered unilaterally in a blinded manner to the SN of 3-6 month F344 rats. To maintain consistency in DNSP delivery between studies Erin Rutherford, Ph.D. in the lab of Greg A. Gerhardt, Ph.D. infused 3-6 month old F344 rats using a similar protocol as discussed in Chapter 2. 28 days after infusion treatment, microdialysis was performed in the ipsilateral striatum following the same protocol as described in Chapter 2 (Table 3.1B). After the completion of microdialysis sampling, animals were euthanized and their brains collected for TH immunohistochemistry. Staining focused on the DNSP-11 and vehicle treatment groups.

Table 3.1 Timeline of DNSP studies in Normal F344 Rats

A

Day 0	Infusion with 10 µg, 30 µg, 100µg DNSP Mixture or Citrate Buffer Vehicle (n=8)
Day 28	Str Microdialysis followed byTH Immunohistichemistry + Tissue Neurochemistry

B

Day 0	Infusion with DNSP-5, DNSP-11, DNSP-17 or Citrate Buffer Vehicle (n=6)
Day 28	Str Microdialysis + TH Immunohistichemistry

C

Day 0	Infusion with DNSP-11 or Citrate Buffer Vehicle (n=8)
Day 7	Assessment of Spontaneous Movement Activity
Day 14	Assessment of Spontaneous Movement Activity
Day 21	Assessment of Spontaneous Movement Activity

A) A 10 µg, 30 µg, or 100 µg mixture of the DNSPs or citrate buffer vehicle was infused at day 0 and 28 days later microdialysis was performed in the striatum. Half the animals were used for tissue neurochemistry and half for tyrosine hydroxylase immunohistochemistry.

B) 30 µg of DNSP-5, DNSP-11, DNSP-17, or citrate buffer vehicle was infused into the SN of a normal F344 rat at day 0 and 28 days later underwent striatum microdialysis followed by tyrosine hydroxylase immunohistochemistry in animals from the vehicle treatment group and from the DNSP-11 treatment group.

C) Animals were habituated to movement boxes. 30 µg of DNSP-11 was infused unilaterally into the SN and one week later spontaneous movement activity was assessed weekly for 3 weeks after the infusion.

After the completion of the above study, 3-6 month old F344 rats received 30 μg/ 5 μL of DNSP-11 and underwent motor movement evaluation weekly for 3 weeks (Table 3.1 C).

Data Analyses

All data sets were analyzed in accordance with the methods discussed in Chapter 2: Methods. Variability within the recovery rates of microdialysis probes ranged from 10 – 32 % and averaged 22.6 ± 1.3 % required data to be corrected using the recovery rates collected at the beginning of every experiment. Microdialysis data were analyzed by a two-way ANOVA with Bonferroni's post-hoc tests. Baseline levels were calculated from samples 2-4 and averaged for each group then analyzed with a one-way ANOVA. Striatal and SN tissue DA content was analyzed by a one-way ANOVA and immunohistochemistry underwent qualitative visual examination. Statistical significance was defined as $p < 0.05$.

Acute effects of DNSP-11

Please refer to Chapter 2 for all methods used to collect and analyze data related to the acute effects of DNSP-11.

Results

DNSP Mixture Dose Response

To establish an effective dosing range for the mixture of the DNSPs (1:1:1 by weight, DNSP-5, DNSP-11, DNSP-17) striatal microdialysis studies 28 days after infusion delivery revealed significant increases in evoked DA release (Figure 3.1). A differential response to the 100 mM K^+ was seen in the groups treated with the DNSP mixture solution (Table 3.2). D-amphetamine application produced changes related to DNSP mixture treatment (Table 3.2). Increased K^+ and d-amphetamine-evoked release of DA were observed at all concentrations of the DNSP mixture relative to the vehicle treatment group ($F_{(3,15)}$=3.878, p=0.0094). No significant differences were observed between DNSP mixture treatment concentrations in relation to evoked DA release.

The baseline DA concentration was significantly elevated only in the 10 µg DNSP mixture treatment group when compared to vehicle (Table 3.2). The 100 µg DNSP mixture treatment was significantly lower than both the 10 and 30 µg DNSP mixture treatments, but was not significantly lower than vehicle treatment. Baseline concentrations of DOPAC were consistently elevated in all groups treated with the DNSP mixture relative to vehicle treatment (Table 3.2). Baseline HVA concentrations were significantly augmented by the 10 and 30 µg DNSP mixture treatment groups (Table 3.2) (Figure 3.2).

Brain tissue neurochemical content was used to gain insight into metabolic activity by determining turnover ratios for DA in both the striatum and SN. Brain tissue neurochemical content in the striatum and SN of either hemisphere was not significantly altered after treatment with any concentration of the DNSP mixture (Table 3.3). Turnover ratios relate DA concentrations to the concentration of its metabolites DOPAC and HVA providing insights into metabolic function. No significant changes in turnover for the SN ($F_{(3, 15)}$ = 1.17, p = 0.23) or striatum ($F_{(3, 15)}$ = 1.09, p = 0.38) were observed relative to the vehicle treatment group (Figure 3.3).
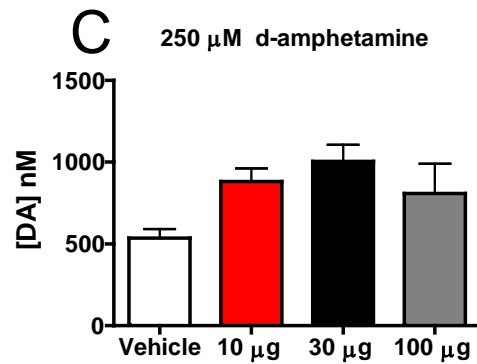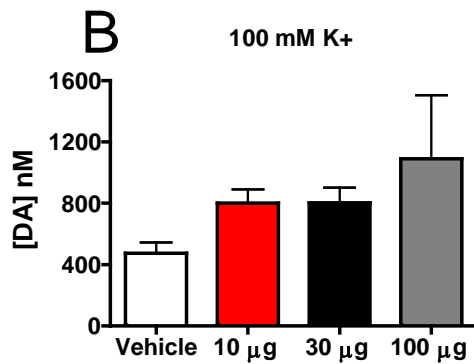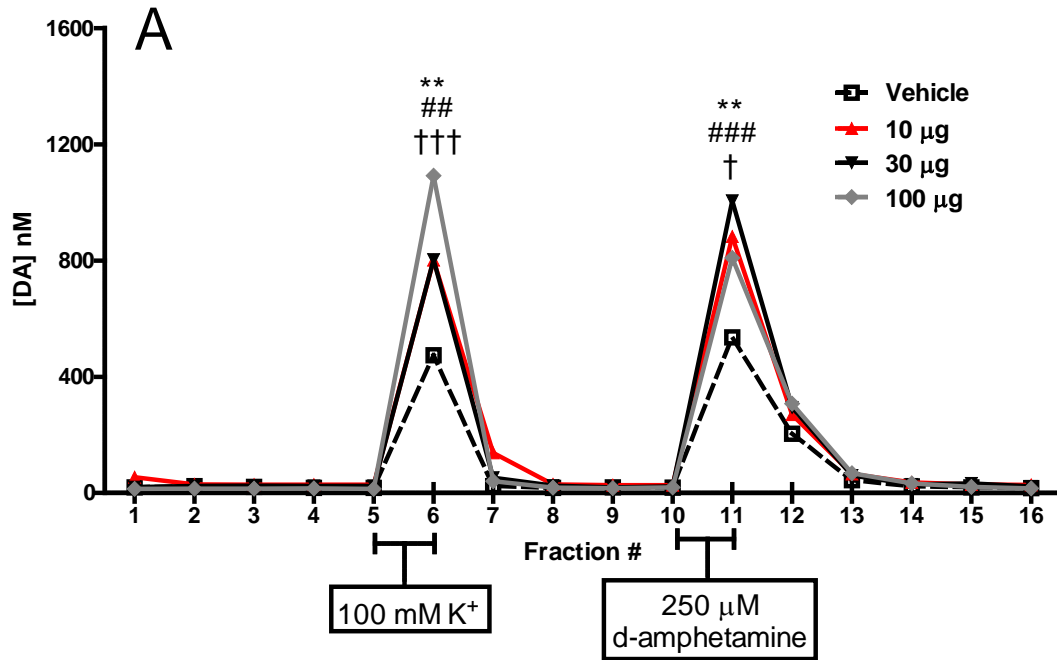
Figure 3.1 Microdialysis Studies of Resting and Sitmulus-Evoked DA Release after DNSP Mixture Treatment

(A) DA concentration throughout striatum microdialysis 28 days after infusion treatment delivery showed significant increases in evoked release of DA by K^+ (B) and d-amp (C) at all amounts of the DNSP mixture (10 μg, 30 μg, and 100 μg) relative to vehicle when analyzed by a two-way ANOVA with Bonferroni's post-hoc test ($F_{(3,15)}=3.878$, $p=0.0094$). ** $p < 0.01$ (Vehicle vs. 10 μg DNSP Mixture), ## $p < 0.01$, ### $p < 0.0001$ (Vehicle vs. 30 μg DNSP Mixture), † $p < 0.05$, ††† $p < 0.0001$ (Vehicle vs. 100 μg DNSP Mixture). Data Points: Mean ± SEM, n = 8.

Table 3.2 Extracellular Levels of DA and DA Metabolites 28 days after DNSP Mixture Infusion into the SN

Perfusion Solution, Fraction #	Analyte	Analyte Concentration (nM) 28 days after Infusion with DNSP Mixture:				F and p-values for ANOVA
		Citrate Buffer	10 µg	30 µg	100 µg	
aCSF, 2 – 4	DA	20.71 ± 2.25	28.79* ± 1.79	24.53 ± 2.13	15.11 ± 1.34	$F_{(3,82)}$=8.57, $p < 0.0001$
	DOPAC	3790.0 ± 241.0	6072.0*** ± 388.9	6448.0### ± 322.3	6258.0††† ± 519.9	$F_{(3,82)}$=10.1, $p < 0.0001$
	HVA	1422 ± 144.1	2104.0* ± 197.5	2031.0# ± 133.5	1927.0 ± 124.5	$F_{(3,82)}$=4.20, $p < 0.0081$
100 mM K+, 6	DA	474.0 ± 70.3	801.7** ± 89.1	802.9## ± 99.56	1092.0††† ± 414.6	$F_{(3,15)}$=3.88, p=0.0094
250 µM d-amp, 11	DA	535.1 ± 55.83	882.3** ± 79.66	1005.0### ± 100.6	809.2† ± 181.9	$F_{(3,15)}$=3.88, p=0.0094

The table contains all DA related neurochemical parameters sampled by striatal microdialysis. * $p < 0.05$, *** $p < 0.0001$ (Vehicle vs. 10 µg DNSP mixture), # $p < 0.05$, ### $p < 0.0001$ (Vehicle vs. 30 µg DNSP mixture), ††† $p < 0.0001$ (Vehicle vs. 100 µg DNSP mixture). Data: Mean ± SEM, n = 8.
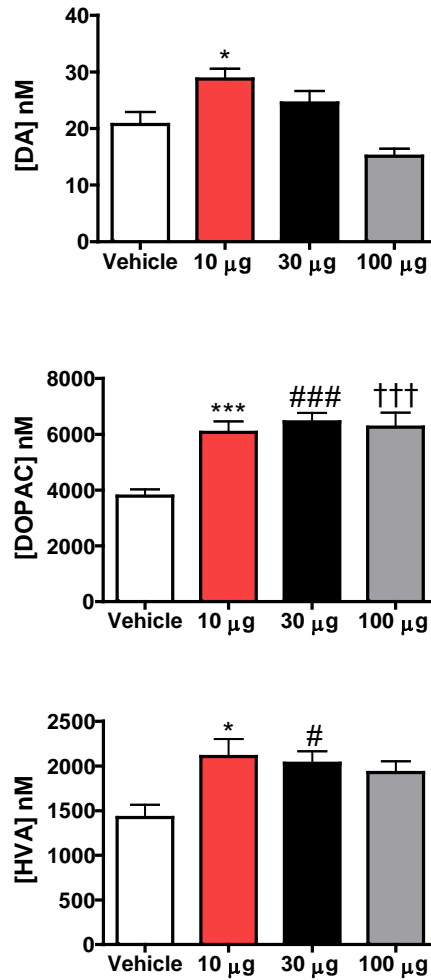
Figure 3.2 Baseline Neurochemical Concentrations after DNSP Mixture Treatment

Baseline concentrations of DA, DOPAC, and HVA 28 days after infusion of the DNSP mixture (10 µg, 30 µg, or 100µg) or citrate buffer vehicle was delivered unilaterally to the SN. Each neurochemical data set was analyzed by a one-way ANOVA with Bonferroni's post-hoc tests. * $p < 0.05$, *** $p < 0.0001$ (vehicle vs. 10 µg DNSP mixture), # $p < 0.05$, ### $p < 0.0001$ (vehicle vs. 30 µg DNSP mixture), ††† $p < 0.0001$ (vehicle vs. 100 µg DNSP mixture). Data Points: Mean ± SEM, n = 8.

Table 3.3 Brain Tissue Neurochemical Content after DNSP Mixture Delivery

Brain Area	Analyte	Treatment				F and p values for one-way ANOVA
		Vehicle (ng/g)	10 µg DNSPs (ng/g)	30 µg DNSPS (ng/g)	100 µg DNSPs (ng/g)	
Lft Str	DA	4111 ± 303.0	4461 ± 568.6	3414 ± 665.5	6105 ± 773.7	$F_{(3, 15)} = 3.41$, p = 0.06
	DOPAC	10782 ± 752.3	12647 ± 2586	15140 ± 2917	12988 ± 2116	$F_{(3, 15)} = 0.66$, p = 0.54
	HVA	444.0 ± 42.85	404.4 ± 43.05	392.4 ± 66.15	477.6 ± 36.34	$F_{(3, 15)} = 0.60$, p = 0.65
Lft SN	DA	62.09 ± 28.98	239.0 ± 104.0	96.78 ± 16.05	200.4 ± 41.93	$F_{(3, 15)} = 1.82$, p = 0.19
	DOPAC	189.1 ± 98.32	566.6 ± 154.9	572.6 ± 139.7	608.8 ± 156.1	$F_{(3, 15)} = 1.68$, p = 0.21
	HVA	27.97 ± 11.39	59.85 ± 13.38	18.38 ± 5.917	39.02 ± 10.00	$F_{(3, 15)} = 2.99$, p = 0.06
Rt Str	DA	4014 ± 263.1	3832 ± 1096	3175 ± 653.5	3843 ± 751.3	$F_{(3, 15)} = 0.22$, p = 0.88
	DOPAC	10114 ± 228.4	10794 ± 1920	12979 ± 2315	12385 ± 2453	$F_{(3, 15)} = 0.75$, p= 0.41
	HVA	448.5 ± 40.06	387.6 ± 56.03	399.6 ± 62.84	380.2 ± 42.10	$F_{(3, 15)} = 0.82$, p = 0.35
Rt SN	DA	35.09 ± 16.42	90.47 ± 22.79	72.95 ± 16.24	83.19 ± 33.26	$F_{(3, 15)} = 0.95$, p = 0.44
	DOPAC	189.1 ± 98.32	566.6 ± 154.9	572.6 ± 139.7	608.8 ± 156.1	$F_{(3,15)}=0.42$, p=1.01
	HVA	11.95 ± 2.027	46.87 ± 11.14	25.37 ± 8.045	34.88 ± 7.939	$F_{(3, 15)} = 2.90$, p = 0.07

The table contains the tissue levels of DA, DOPAC, and HVA in their respective tissues 28 days after right SN delivery of the DNSP mixture or vehicle. The F and p values for the one-way ANOVAs are included on the far right portion of the table. Data: Mean ± SEM, n = 6.

Qualitative analysis of the TH positive staining revealed no changes in fiber density or morphology in the striatum. Both the 10 µg and 30 µg doses of the DNSP mixture appear to increase fiber and cell body density in the SN when compared to vehicle treated animals (Figure 3.4). In summary, the 10 µg DNSP mixture increased all microdialysis related parameters relative to the vehicle treatment group and potentially increased TH positive neuron density in the SN but was not seen to alter tissue related neurochemistry. The 30 µg dose of the DNSP mixture significantly increased K^+- and d-amp- evoked DA release as well as increased baseline concentrations of DA metabolites, DOPAC and HVA, when compared to the vehicle. However, the 30 µg dose was not seen to significantly affect baseline DA concentrations or DA tissue content, but possibly increased TH fiber density. The 100 µg dose was the least effective in eliciting a neurochemical response, but still was able to increase K^+- and d-amp- evoked DA release and baseline HVA concentrations. The 100 µg DNSP mixture was not seen to significantly alter baseline DA or HVA concentrations relative to the vehicle treatment groups and provided no discernible changes in tissue neurochemistry or TH immunohistochemistry.

Effects of Individual DNSPs

After establishing an effective dose range for the DNSP mixture on DA related neurochemistry, responses to individual DNSPs (Individual DNSPs include separate 30 µg solution of DNSP-5, DNSP-11, and DNSP-17) needed to be assessed. Baseline neurochemical concentrations and evoked DA release were monitored by striatal microdialysis 28 days after infusion with individual DNSPs. Baseline DA concentrations (Table 3.4) were significantly increased by DNSP-11 treatment. No other treatment groups displayed any significant changes on baseline DA concentrations. Baseline concentrations of DOPAC (Table 3.4) were significantly higher in the DNSP-11 treatment group relative to vehicle and DNSP-5 treatment groups. DNSP-11 and DNSP-17 both significantly increased baseline HVA (Table 3.4) concentrations when compared to DNSP-5 or vehicle.
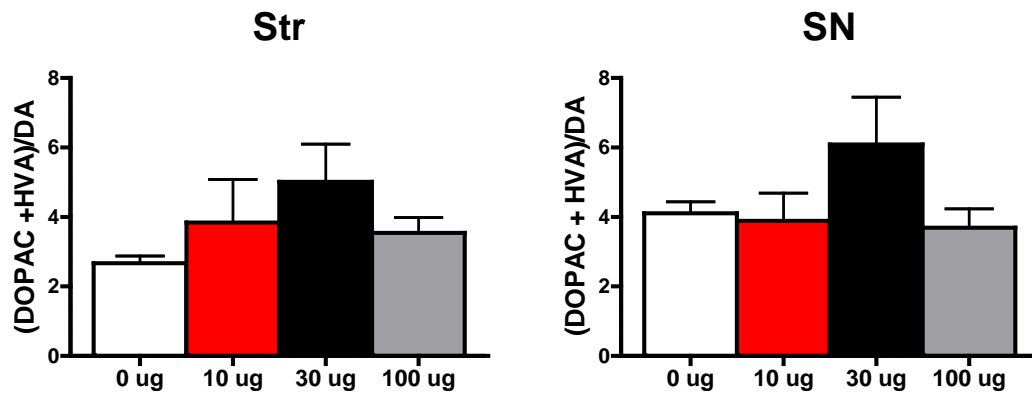
Figure 3.3 DA Turnover Ratios after DNSP Mixture Treatment

DA, DOPAC, and HVA content of striatal and SN tissue was used to provide a ratio focusing on DA metabolism. The above graphs demonstrate no significant changes in the DA turnover ratios of the SN ($F_{(3, 15)}$ = 1.17, p = 0.23) or striatum ($F_{(3, 15)}$ = 1.09, p = 0.38) when analyzed by a one-way ANOVA. Data: Mean ± SEM, n = 6.
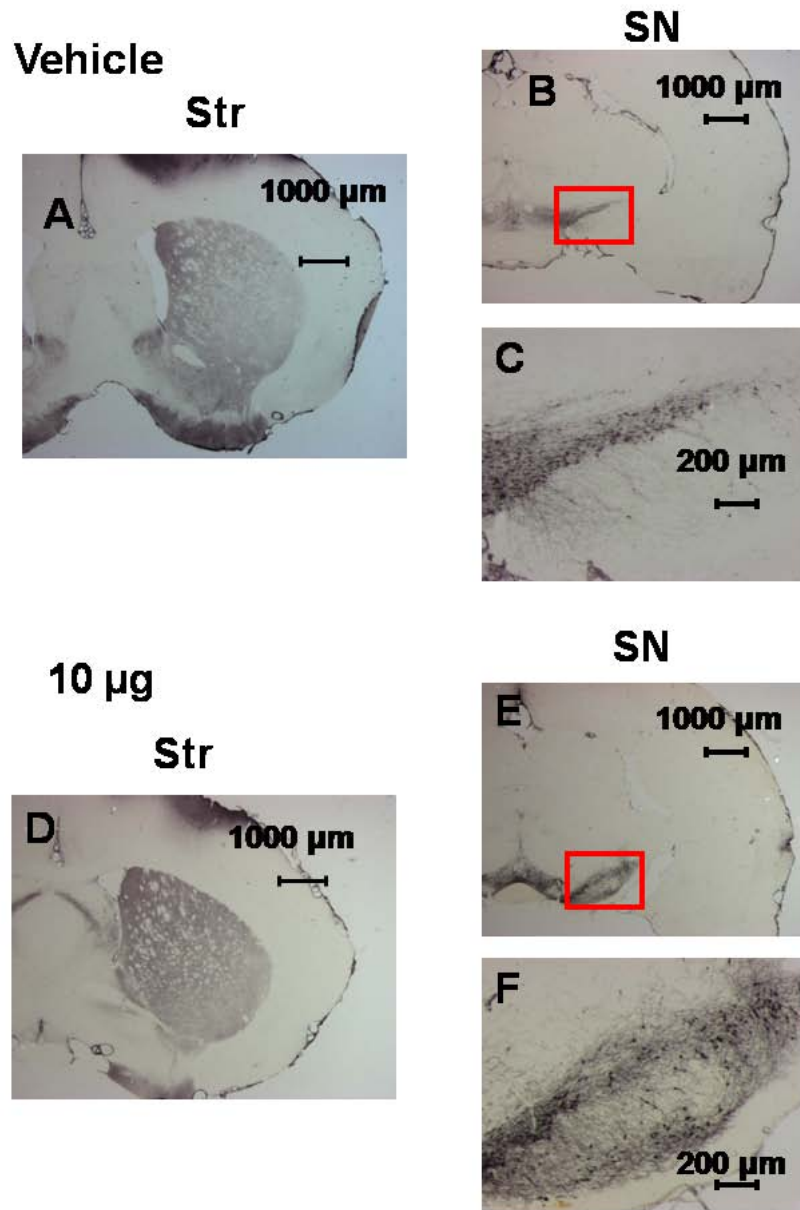
Figure 3.4 TH Immunohistochemistry of the Nigrostriatal Pathway after DNSP Mixture Treatment

Representative slices stained for TH immunohistochemistry. Continued and explained on the next page.
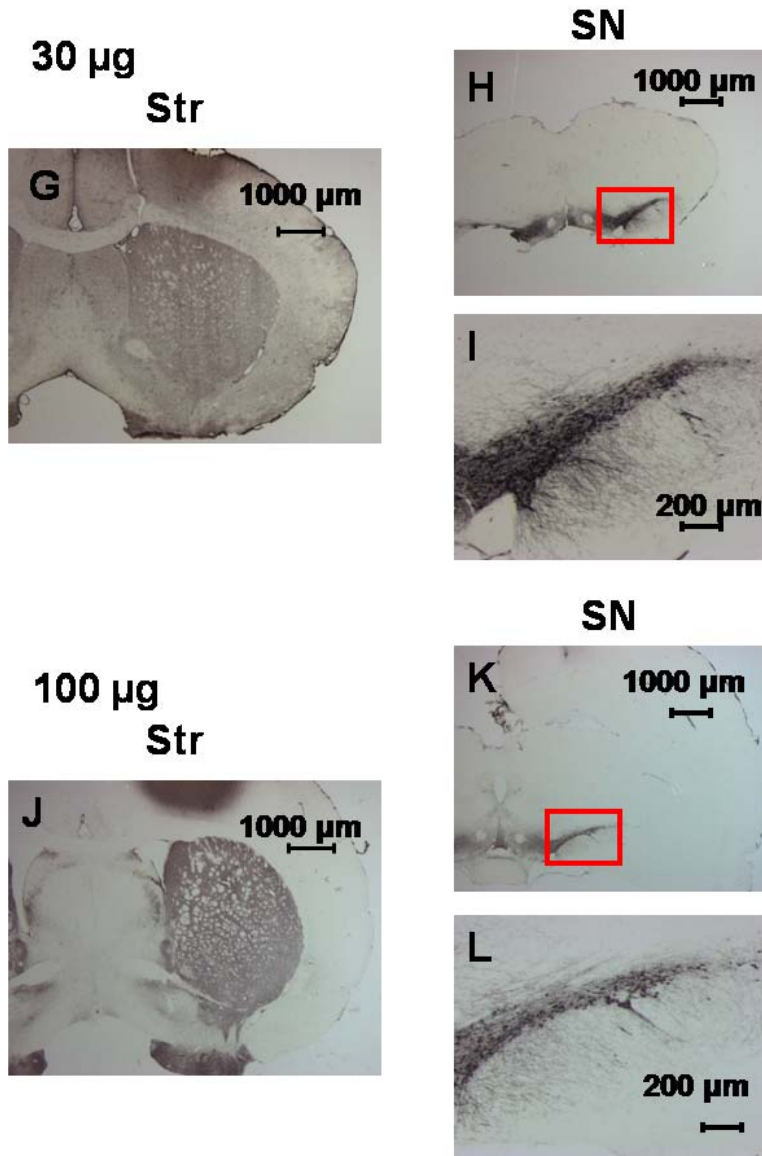
Figure 3.4 Continued

All DNSP mixtures were characterized as well as citrate buffer vehicle. Photographs A, B, D, E, G, H, J, and K were taken at 10 X magnification and C, F, I, and L were taken at 50 X magnification. Relative to vehicle (C), the 10 μg DNSP mixture (F) increased the number of SN cell bodies and the 30 μg DNSP mixture (I) increased fiber density within the SN. No changes were seen in the staining of the 100 μg treatment group. In addition, the striatum of all four groups was observed and determined too similar to determine any differences (n=2).

Table 3.4 Extracellular Levels of DA and DA Metabolites 28 days after DNSP Infusion into the SN

Perfusion Solution, Fraction #	Analyte of Interest	Analyte Concentration (nM) 28 days after Infusion with:				F and p-values for ANOVA
		Citrate Buffer	DNSP-5	DNSP-11	DNSP-17	
aCSF, 2 – 4	DA	26.00 ± 2.69	43.10 ± 4.17	45.84* ± 7.70	41.26 ± 3.10	$F_{(3,59)}$=2.87 p=0.0483
	DOPAC	3555 ± 337.5	4325 ± 429.5	6544** ± 836.2	5472 ± 274.6	$F_{(3,59)}$=6.30 p=0.0009
	HVA	2419 ± 250.7	2532 ± 355.1	4516** ± 501.8	3931## ± 249.6	$F_{(3,59)}$=8.15 p=0.0001
100 mM K^+, 6	DA	541.7 ± 125.1	419.4 ± 47.75	690.4* ± 97.06	685.2 ± 60.34	$F_{(3,15)}$=9.48 p< 0.0001
250 µM d-amp, 11	DA	603.5 ± 129.1	508.0 ± 45.97	772.1* ± 103.8	783.7## ± 64.21	$F_{(3,15)}$=9.48 p < 0.0001

The table contains all DA related neurochemical parameters sampled by striatal microdialysis. * $p < 0.05$,** $p < 0.01$ Vehicle vs. DNSP-11, # p , 0.05 ## p , 0.01 Vehicle vs. DNSP-17. Data: Mean ± SEM, n = 6.

DNSP-11 was able to increase the baseline concentrations of DA, DOPAC, and HVA, while DNSP-17 increased baseline HVA concentrations. DNSP-5 produced no effect on baseline concentrations of DA, DOPAC, or HVA neurochemistry in this study (Figure 3.5).

Twenty-eight days after DNSP treatment, K^+ and d-amp-stimulated DA release was investigated. DNSP-11 treatment increased DA release in response to K^+ stimulation, while DNSP-5 and DNSP-17 treatment produced no significant changes in DA release evoked by K^+ (Table 3.4). DNSP-11 and DNSP-17 both increased d-amp-evoked DA release when compared to the vehicle treatment group, but DNSP-5 lacked the ability to produce any significant changes (Table 3.4).

A qualitative analysis of immunohistochemical staining was performed to reveal potential differences between DNSP-11 and vehicle treatment. Though a relatively small n of two hinders any definitive conclusions there is the possibility for more intense staining in the striatum and an increased number of dopaminergic fibers in the SN of the DNSP-11 treatment group (Figure 3.7).

In summary DNSP-5 was not seen to alter any of the measured parameters and DNSP-17 only altered baseline HVA and d-amp-evoked DA release. DNSP-11 produced the most robust effects on DA-related neurochemical parameters: increasing evoked DA release with K^+ and d-amp, as well as increasing baseline concentrations of DA, DOPAC, and HVA. DNSP-11 demonstrated a potential impact on TH-positive neurons and because of the robust neurochemical effects; behavioral consequences to DNSP-11 treatment were examined.

To measure possible behavioral changes associated with DNSP-11 treatment, movement speed and distance traveled were determined weekly for 3 weeks after unilateral SN administration of DNSP-11 or vehicle. Analysis of total distance traveled ($F_{(1,70)}$ = 2.734, p = 0.1027) showed no significant alterations between the DNSP-11 treatment groups (Table 3.5 and Figure 3.8). Movement speed ($F_{(1,70)}$ = 2.652, p = 0.1079) was not significantly affected by DNSP-11
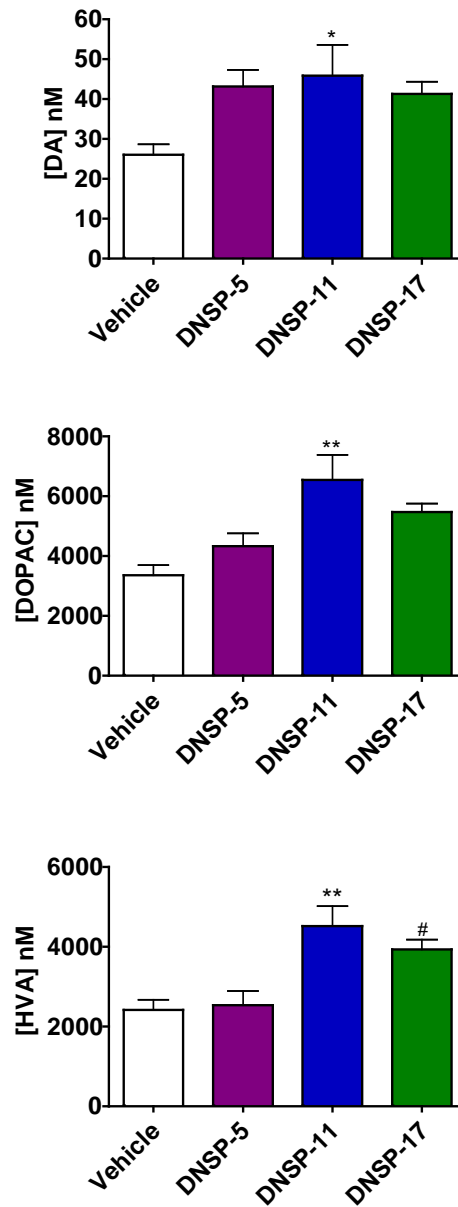
Figure 3.5 Microdialysis Measures of Baseline DA and Metabolite Concentrations 28 Days after DNSP Infusion Treatment

Baseline microdialysis concentrations of DA ($F_{(3,59)}$=2.873 , p=0.0483), DOPAC ($F_{(3,59)}$=6.301 , p=0.0009), and HVA ($F_{(3,59)}$=8.153 , p=0.0001) after treatment with vehicle, DNSP-5, DNSP-11, or DNSP-17 analyzed by a one-way ANOVA with Bonferroni's post-hoc tests. * p < 0.05,** p < 0.01 Vehicle vs. DNSP-11, # p , 0.05 Vehicle vs. DNSP-17. Data: Mean ± SEM, n = 6.
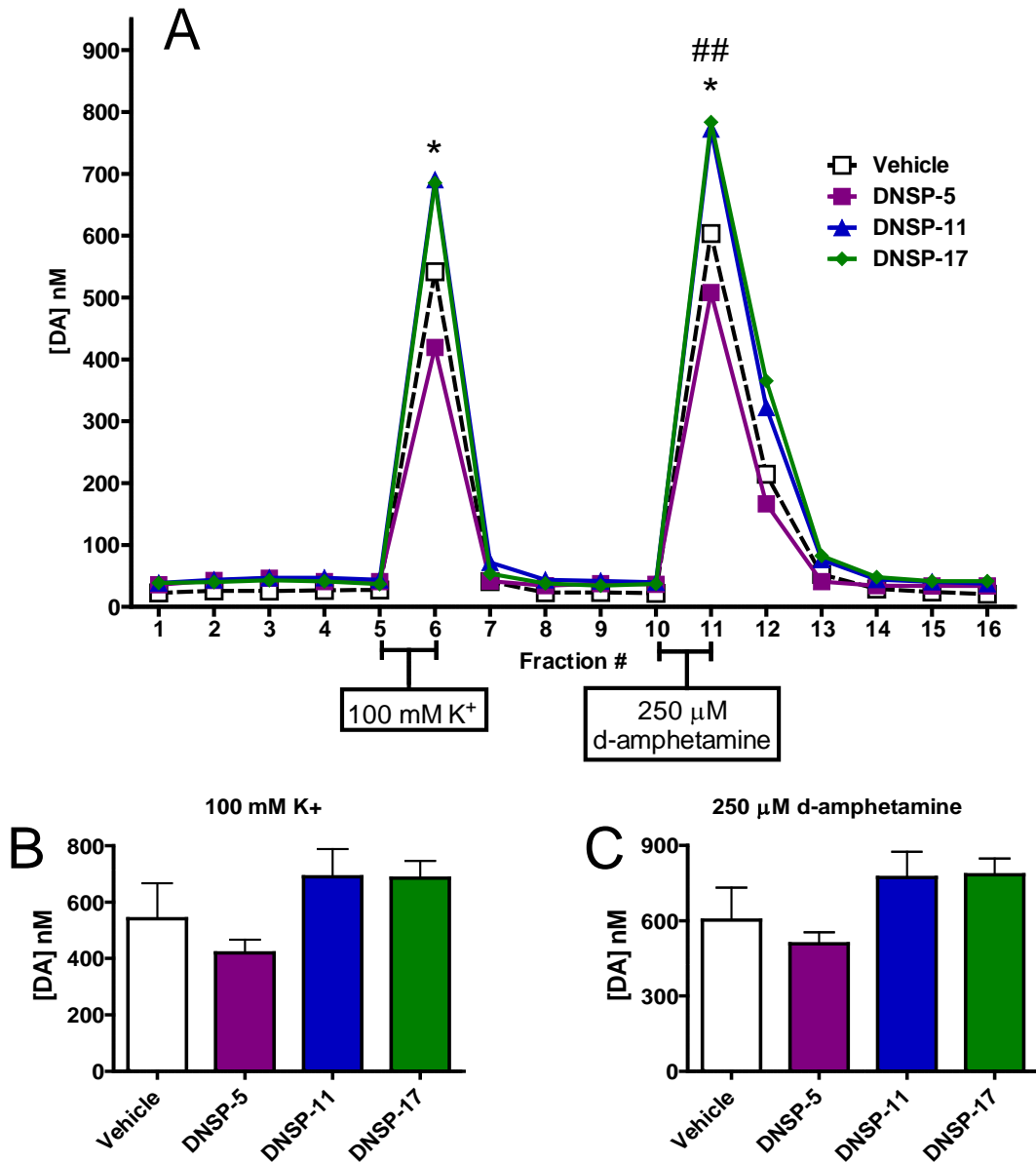
Figure 3.6 Microdialysis Studies of Resting and Sitmulus-Evoked DA Release after Individual DNSP Treatment

Extracellular levels of DA, 28 days after unilateral SN treatment with 30 μg of DNSP-5, DNSP-11, DNSP-17, or vehicle. Significant increases were observed from K^+-evoked DA release after DNSP-11 treatment and from d-amp-evoked release after DNSP-11 and DNSP-17 treatment $F_{(3,59)}=2.873$, p=0.0483). Analyzed using a two-way ANOVA with Bonferroni's post-hoc tests * p < 0.05 vehicle vs. DNSP-11, ## p < 0.01 vehicle vs. DNSP-17. Mean ± SEM, n = 6.
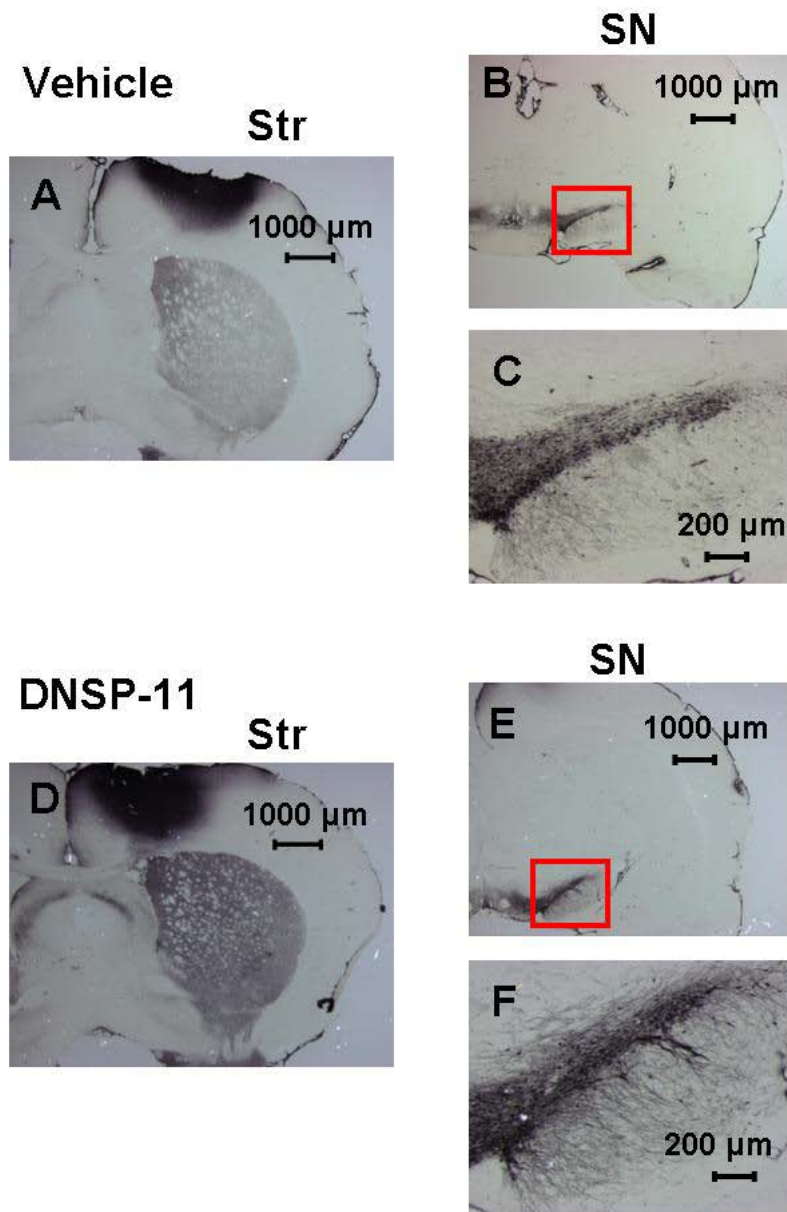
Figure 3.7 TH Immunohistochemistry after DNSP-11 or Vehicle Treatment

Representative TH immunohistochemistry of the striatum and SN 28 days after DNSP-11 or vehicle treatment. Pictures A, B, D, and E are all at 10 X magnification. Pictures C and F are at 50 X magnification. The DNSP-11 treated striatum (D) showed more intense staining and the DNSP-11 treated SN (F) exhibits thicker dendritic arborization (n=2).

Table 3.5 Total Distance Traveled after DNSP-11 Treatment

	Distance Traveled (cm)	
	Vehicle Treatment Group	DNSP-11 Treatment Group
Week 1	2335.13 ± 331.33	3234.38 ± 409.96
Week 2	2291.63 ± 227.13	2905.75 ± 388.99
Week 3	2201.13 ± 445.11	1931.62 ± 373.82
Week 4	1704.13 ± 405.51	2090.37 ± 432.60
Week 5	1754.50 ± 164.35	2031.50 ± 364.85
F and p values for Two-Way ANOVA	$F_{(1,70)} = 2.734$, $p = 0.1027$	

The total distance traveled of habituated normal F344 rats was determined by using movement boxes and no significant difference was observed after DNSP-11 treatment relative to the vehicle treatment group.

treatment relative to vehicle treatment (Table 3.6 and Figure 3.8). Unilateral administration of DNSP-11 produced significant neurochemical increases, but did not appear to alter immunohistochemical staining or behavior in normal F344 rats.

Acute effects of DNSP-11

DNSP-11 was delivered acutely within the striatum through reverse microdialysis after a baseline neurochemical concentration was collected with aCSF (Figure 3.9). Two-way ANOVA analysis of microdialysis recordings of DA ($F_{(6, 395)}$ = 26.26, $p < 0.0001$), DOPAC ($F_{(6, 395)}$ = 10.76, $p < 0.0001$), and HVA ($F_{(6, 395)}$ = 5.45, $p < 0.0001$) showed significant differences, but Bonferroni's post-hoc tests determined the only significant neurochemical change occurred in the DA concentration. DA was significantly increased with the application of 1 mM DNSP-11 (Sample 5: 46.09 ± 15.98 nM, Sample 6: 44.01 ± 13.62 nM, Sample 7: 38.63 ± 9.72 nM, Sample 8: 35.87 ± 8.24 nM) in aCSF from samples 5 – 8 when compared to the same sample numbers while applying aCSF (Sample 5: 13.84 ± 2.056 nM, Sample 6: 13.09 ± 1.82 nM, Sample 7: 11.83 ± 1.90 nM, Sample 8: 11.97 ± 2.18 nM). Extracellular DA was increased by 1 mM DNSP-11, but produced no significant effects on DOPAC or HVA. Reverse microdialysis delivery of DNSP-11 in concentrations ranging from 3 – 300 µM produced no acute alterations in DA or DA related metabolites.

Table 3.6 Movement Speed after DNSP-11 Treatment

	Movement Speed (cm/sec)	
	Vehicle Treatment Group	DNSP-11 Treatment Group
Week 1	7.760 ± 0.216	7.678 ± 0.111
Week 2	7.772 ± 0.297	7.664 ± 0.240
Week 3	7.782 ± 0.161	7.543 ± 0.302
Week 4	7.830 ± 0.185	7.481 ± 0.185
Week 5	8.020 ± 0.424	7.519 ± 0.216
F and p values for Two-Way ANOVA	$F_{(1,70)} = 2.652$, p = 0.1079	

Movement speed was calculated from the movement time and distance traveled data collected by movement boxes. DNSP-11 treatment produced no observable effect on movement speed in the normal F344 rats. Data: Mean ± SEM, n = 8.
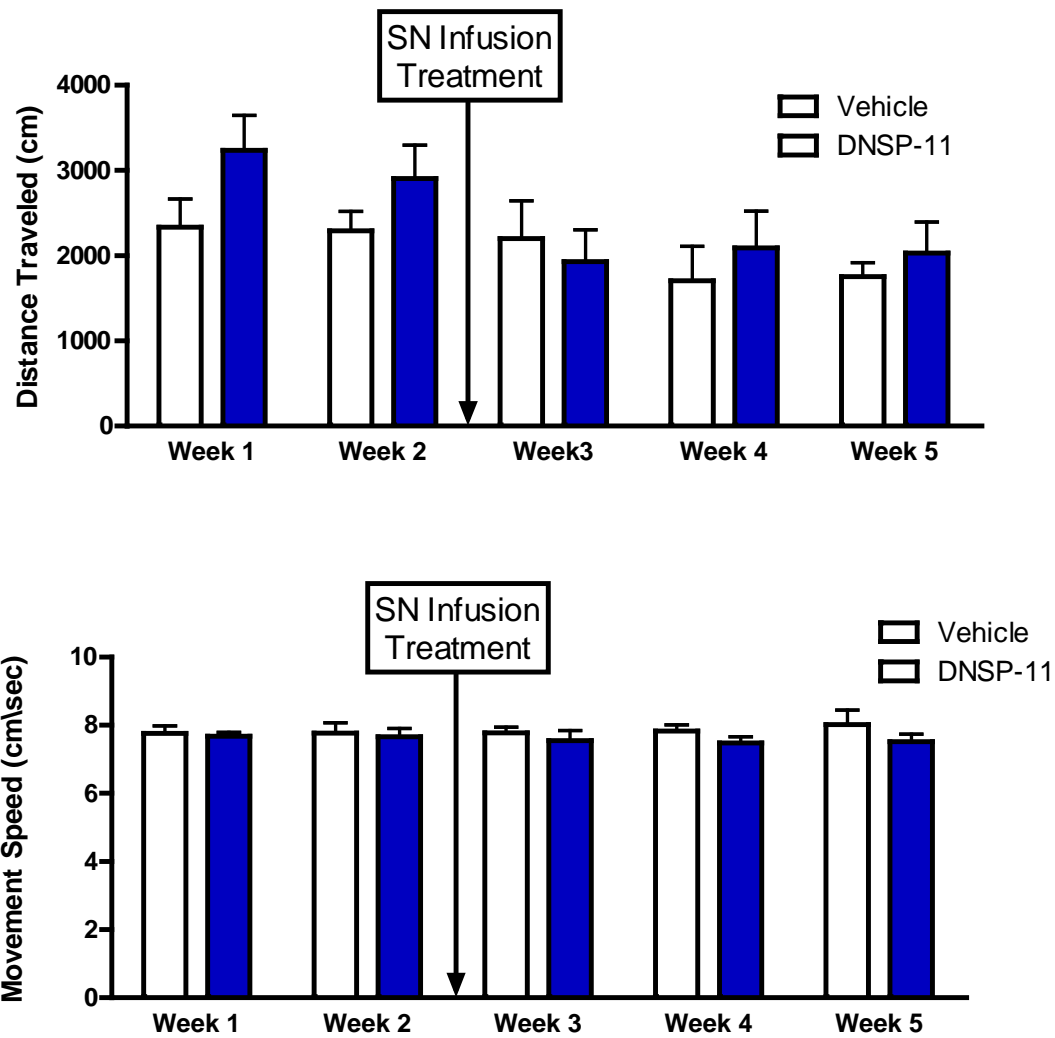
Figure 3.8 Spontaneous Movement after DNSP-11 Treatment

Spontaneous motor movement assessed weekly after unilateral DNSP-11 treatment illustrated no significant behavioral changes in movement speed ($F_{(1,70)}$ = 2.652, p = 0.1079) or distance traveled ($F_{(1,70)}$ = 2.734, p = 0.1027) when analyzed by a two-way ANOVA. Data: Mean ± SEM, n = 8.
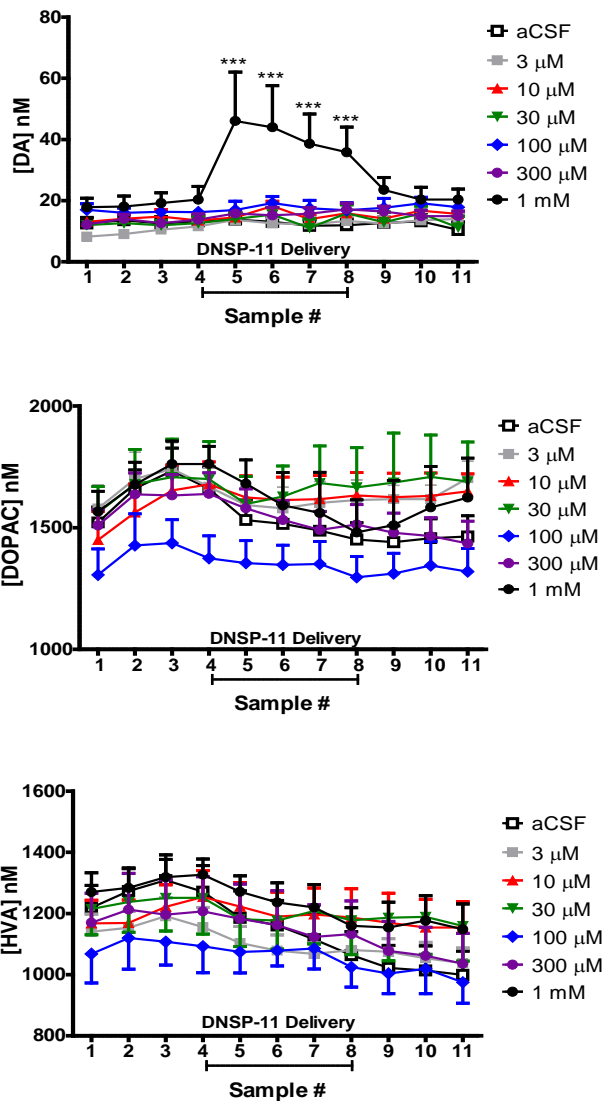
Figure 3.9 Microdialysis Measures of DA, DOPAC, and HVA during Acute DNSP-11 Delivery

DNSP-11 was delivered to the striatum by reverse microdialysis with the following concentrations: 3 µM, 10 µM, 30 µM, 100 µM, 300 µM, 1 mM. DA, DOPAC, and HVA values were analyzed with a two-way ANOVA with Bonferroni's post-hoc tests. Acute treatment with 1 mM DNSP-11 produced significant increases in DA. (DA: $F_{(6, 395)} = 26.26$, $p < 0.0001$, DOPAC: $F_{(6, 395)} = 10.76$, $p < 0.0001$, HVA $F_{(6, 395)} = 5.45$, $p < 0.0001$) *** $p < 0.0001$ with Bonferroni's post-hoc test 1mM DNSP-11 vs. aCSF. Data: Mean ± SEM, n = 8.

Discussion

DNSPs Enhance Dopaminergic Function

Enhancement of DA neurochemical function was observed in the studies using the DNSP mixture. All doses of the DNSP mixture were seen to increase the function of the normal dopaminergic system. Increased DA release in response to a high K^+ solution was observed from all three concentrations of the DNSP mixture. The enhanced evoked DA response relative to the vehicle treatment group showed the DNSP mixture's ability to modulate DA release, possibly through the manipulation of vesicle packaging, vesicle number, or vesicle binding. The most probable mechanism for increased evoked DA release would be to increase DA synthesis, which would thereby increase the availability of DA for packaging. GDNF increases the vesicle content of DA through enhanced synthesis and the DNSP mixture could use a similar mechanism to enhance DA release (Pothos et al., 1998).

Increased availability of DA in the cytosol for packaging could potentially explain changes in d-amp-evoked DA release. A d-amp solution was delivered via reverse microdialysis that causes a reversal of the dopamine transporter and release of cytosolic DA to the extracellular space where it is then collected. The enhanced DA release response to d-amp elicited by the DNSP mixture treatment could be attributed to an increase in cytosolically available DA. Elevated cytosolic DA in conjunction with increased K^+ evoked DA release established increases in DA synthesis as the most probable mechanism.

All concentrations of the DNSP mixture increased baseline concentrations of DA metabolites. Modulation of the metabolites is most likely related to a change in DA synthesis. Enhanced DA synthesis has the potential to cause the increase in DA metabolites demonstrated within this study. Increasing cytosolic DA would augment the availability of DA to metabolic enzymes thereby escalating the concentration of metabolites produced. Higher DA baseline concentrations caused by 10 μg DNSP mixture would increase DA available for metabloism. Baseline DA concentrations are primarily a measure of tonic DA release, which is generally modified by reducing reuptake by the DA transporter

or modulating spontaneous DA release events. Changes in the DAT are unlikely because ongoing studies in our laboratory have revealed no change in reuptake mechanisms after DNSP treatment (Unpublished data, Meagan Littrell). To increase basal output without manipulating reuptake, the dopaminergic neuron would have to increase the frequency or concentration of spontaneous DA release (Goto et al., 2007). Acute DNSP delivery studies demonstrate an increase in the excitatory post-synaptic potential , as well as an increase in the frequency of action potentials (Immonen et al., 2008). Acute post-synaptic changes could be related to presynaptic modifications of the concentration and frequency of tonic DA release that could potentiate into long-term modulation of tonic DA. Elevated extracellular DA levels could be related to long-term alterations in the concentration and frequency of tonic DA firing.

Striatal and SN tissue content of DA, DOPAC, or HVA were unchanged after DNSP mixture treatment. The lack of significant tissue neurochemical data is surprising because of the apparent changes in DA synthesis measured by microdialysis. Much of the data has a high degree of variability likely related to multiple factors such as damage from the microdialysis probe upon removal, variability in the dissections, and too low of an *n*. Variability in tissue neurochemical measures resulted in variability of metabolic turnover ratios. This variability in the neurochemical measures eliminated any significance that could have been demonstrated by both the tissue content and the turnover ratios.

Immunohistochemical staining showed no visible distinctions between the striatum of any treatment group. A lack of distinguishable differences in TH staining could be related to a lack of effect on the number of TH staining neurons or the effect was not robust enough to overcome the variability within an intact striatum. However, the SN of both the 10 µg DNSP mixture and 30 µg DNSP mixtures demonstrate potential enhancement in cell bodies and fiber outgrowth respectively. GDNF enhanced TH positive fiber outgrowth in multiple models of dopaminergic dysfunction and the DNSP mixture has the potential to similarly increase neuronal sprouting (Ebendal et al., 1995; Ai et al., 2003). Normal young adult F344 rats are an impractical model to visualize TH staining related effects

because the animal maintains an intact nigrostriatal pathway leading to a large number of dopaminergic fibers and less sensitivity. The lack of sensitivity of TH staining in the normal F344 rat can be solved in future studies by using models with dopaminergic cell loss to minimize the variability of the pathway before DNSP treatment.

GDNF and the DNSPs have similar effects on K^+ and d-amp evoked DA release, which in GDNF's case was related to changes in DA synthesis due to modification of TH phosphorylation (Salvatore et al., 2004). Changes in evoked DA release and baseline metabolite concentrations are comparable to previous work with GDNF but do not explain the increases in baseline DA exhibited at the 10 µg DNSP dose. Increases in DA synthesis with GDNF did not increase baseline DA in the normal young adult rat (Hebert et al., 1996). The DNSP mixture mimics functional aspects of GDNF but has additional functional alterations such as the increased baseline DA concentrations. Therefore, DNSP related functional changes are potentially mediated through multiple mechanisms one of which could be the same as GDNF. The DNSP mixture's effects could also be mediated through an entirely different mechanism than GDNF, but with overlap between their signaling cascades.

Mechanistic aspects of the DNSPs would have been difficult to elucidate using the DNSP mixture because of potential interactions between the three peptides. DNSPs could modify different aspects of dopaminergic function or they could act in a synergistic manner to increase activity and functional changes. Therefore, the next phase in our studies of the DNSPs was to determine the effects from the individual peptides on dopaminergic function. The 10 µg and 30 µg dose both demonstrated effects on evoked DA release and baseline metabolite concentrations, but the 10 µg DNSP mixture dose was the only dose that exhibited the ability to modify baseline concentrations of DA. As a result of the DNSPs synergistic effects in cell culture, we decided to use a 30 µg dose of the individual DNSPs in further studies (Unpublished Data, Bradley Lab).

In summary, the DNSP mixture increased DA synthesis and in turn augmented DA metabolism. Potential changes in TH positive fiber density by two

different DNSP mixture doses demonstrated possible modifications of dopaminergic neuron numbers and morphology. The tissue neurochemistry showed no significant effects likely due to a small n combined with a high degree of variability. Therefore, these studies provided a potential dose range for future studies and more importantly demonstrated similar effects between DNSPs and GDNF on nigrostriatal dopaminergic neurons.

DNSP-11 Enhances Neuronal Function

Neurochemical changes 28 days after treatment with 30 μg of a single DNSP was assessed in normal F344 rats. DNSP- 5 alone did not alter DA related neurochemistry as measured by microdialysis, but DNSP-17 treatment enhanced d-amp-evoked DA release and it increased baseline HVA. DNSP-17 potentially alters synthesis causing changes in cytosolic and extracellular DA. Although unlikely, another potential mechanism could be that DNSP-17 interacts with the DAT. Modifications of DAT function could increase extracellular DA for metabolism to HVA and simultaneously increase sensitivity to d-amp evoked DA release (Sulzer et al., 1993).

DNSP-11 was the only DNSP to exhibit significant changes on all DA related neurochemical parameters. Effects on DA neurochemistry are similar between the 30 μg DNSP-11 dose and the 10 μg DNSP mixture dose. Neurochemical effects by DNSP-11 can be most easily explained by a modulation in DA synthesis (significantly increased K^+ and d-amp evoked DA release), which would lead to corresponding alterations in DA metabolism (increased baseline DOPAC and HVA). DNSP-11, alone, produced the distinguishing feature of augmented baseline DA by modulating tonic DA release. The 30 μg dose of DNSP-11 provided the same modifications of the nigrostriatal dopaminergic pathways as the DNSP mixture.

DNSP-11 potentially enhances TH fiber density in the SN and striatum. The large number of TH positive fibers in the young adult F344 rats can cause dfficulties interpreting the TH immunohistochemical staining. Changes in the number of TH positive fibers were not readily discernible because of variability within each group. GDNF previously exhibited robust enhancement of fiber

density in the SN even after neurotoxic insult (Stromberg et al., 1993; Tomac et al., 1995b; Grondin et al., 2002). Therefore, DNSP-11 may mimic more than just GDNF's neurochemical effects. DNSP-11 may enhance fiber density and outgrowth similar to previous results with GDNF. Although the possibility remains that DNSP-11 does not affect TH positive neuron number or outgrowth, qualitative investigation showed a potential increase in fiber density in both the SN and striatum. DNSP-11 potentially altered TH staining and significantly enhanced DA synthesis and metabolism in a manner similar to GDNF. Evidence of increased TH fiber number adds to the possibility that DNSP-11's neurochemical effects could be related to neuron number as well as increases in synthesis and metabolism. Enhancement of the DA baseline concentration was not previously reported with GDNF, which was able to increase TH fiber density. DSNP-11 increased baseline concentrations of DA distinguishing its effect from those of GDNF. Therefore, DNSP-11 enhances dopaminergic function with possible increases in DA fiber density. DNSP-11 mediated increases in DA neurochemistry directed future work to focus primarily on DNSP-11 and its ability to modulate DA neurochemistry.

To determine if the neurochemical changes provided by DNSP-11 could modify motor movement we examined locomotor function after DNSP-11 treatment. DNSP-11 did not alter measures of total distance traveled or movement speed at weeks 1-3 after unilateral DNSP-11 treatment. GDNF was previously seen to modify spontaneous motor movement parameters at 1 and 3 weeks after infusion (Hebert et al., 1996). The lack of significant effect of DNSP-11 treatment on behavior may stem from the lack of sensitivity of the spontaneous movement test due to unilateral treatment of the animals and bilateral behavioral endpoints. Neurochemical effects of unilaterally delivered DNSP-11 may not be strong enough to alter bilateral motor movement.

In summary, the 30 µg doses of DNSP-5 and DNSP-17 were not as effective in altering DA neurochemistry as DNSP-11, but individual dose responses would need to be carried out before ruling them ineffective in modulating neurochemistry. Synergistic effects of the DNSPs are possible.

Therefore, future studies aimed at enhancing the potency or maximum effect need to be determined before DNSP-5 and DNSP-17 can be declared nonfunctional. However, future studies of DNSP-5 and DNSP-17 are beyond the scope of this dissertation. The 30 µg dose of DNSP-11 was effective in eliciting a DA neurochemical response and produced potential evidence of increases in TH positive neuron number. Similar to GDNF, DNSP-11 modified DA synthesis and metabolism 28 days after a single infusion. DNSP-11 did increase baseline levels of DA providing evidence of alternative mechanisms of action than GDNF.

Acute Delivery of 1 mM DNSP-11 Induces DA Release

DNSP-11 delivered by reverse microdialysis to the striatum significantly increased DA at a 1mM concentration of DNSP-11. The 1 mM DNSP-11 perfusion solution concentration is equivalent to approximately 24 µg of DNSP-11 delivered every 20 minutes. In using microdialysis to deliver the solution, the recovery rate of DNSP-11 needed to be accounted for to determine the concentration of DNSP-11 reaching the extracellular space. Due to detection limitations of DNSP-11 we were unable to accurately determine the probe recoveries, but summaries of probe recoveries for similar sized peptides range from 8 – 12% (Kehr, 1991). Assuming a 10% recovery rate, a concentration of approximately 100 µM DNSP-11 evoked DA release.

The amount of DA release was similar to those previously described using dopamine transporter blockers, cocaine or GBR 12909 (Nomikos et al., 1990). DNSP-11 may block the reuptake of DA by binding the dopamine transporter allowing DA to build up in the extracellular space. A transporter blockade would have minimal effect on DA metabolites, which remain relatively stable when acutely treated with DNSP-11. The lack of a DA neurochemical effect at lower concentration is expected from a dopamine transporter blocker because of the large number of dopamine transporters in the dorsal striatum that must be overcome to cause DA to build up in the extracellular space (Cass et al., 1992). In summary, only the 1mM concentration of DNSP-11 increases extracellular DA potentially through a blockade of the dopamine transporter or an increase in the K^+ channel sensitivity.

Chapter Summary

In the studies discussed in this chapter, the DNSP mixture was determined to be effective in increasing DA synthesis, metabolism, and tonic release. The 10 μg and 30 μg concentrations of the DNSP mixture exhibited the potential to alter the number of TH neurons, but subsequent work continued to focus on neuronal function and therefore neurochemistry. A 30 μg DNSP dose was determined to be the most effective dose for use in future studies. The 30 μg dose of DNSP-11 was most effective in eliciting DA neurochemical increases when compared to DNSP-5 or DNSP-17. DNSP-11 elicited a substantial and lasting increase of DA synthesis, metabolism, and tonic release. Neurochemical changes exhibited by DNSP-11 are similar to those previously seen in normal F344 rats with GDNF (Table 3.7). The exception to the similarities to GDNF is DNSP-11's ability to modulate tonic DA release. A difference in the DA neurochemical effects implies a difference in mechanism between GDNF and DNSP-11. In conjunction with the neurochemical modulation, potential increases in DA fiber density were seen after DNSP-11 treatment, but unilateral DNSP-11 delivery was unable to produce significant changes in behavior. Visualization of changes in TH neuron number in normal animals was difficult because of the lack of sensitivity and therefore firm conclusions cannot be made from relatively few animals. Behavioral measures showed DNSP-11 treatment was unable to modify spontaneous locomotor movement. Acute DNSP-11 treatment at a high concentration showed the potential for promoting the release of DA possibly through the blockade of the dopamine transporter. DNSP-11 increased DA synthesis, metabolism, tonic release, and acutely increased extracellular DA all within the normal F344 rat. In addition to the effect on neurochemistry, DNSP-11 potentially enhances DA neuron fiber density. Neurochemical modulation of the magnitude demonstrated by DNSP-11 is rare and in conjunction with potential alterations of DA neuron outgrowth could establish a basis for future therapeutics. Therefore, subsequent studies discussed in this dissertation will focus on the exploration of the limitations of DNSP-11's effects by using varying models of dopaminergic dysfunction.

Table 3.7 Comparison of the Effects of DNSPs and GDNF on the Nigrostriatal Pathway of Normal F344 rats

	DNSP-5	DNSP-11	DNSP-17	GDNF (Hebert et al. 1996)
Baseline DA	▬	⬆	▬	▬
Baseline DOPAC and HVA	▬	⬆⬆	▬ ⬆	⬆
Evoked DA overflow	▬	⬆	▬ ⬆	⬆⬆
Movement speed at 3 weeks	NA	▬	NA	⬆
Distance traveled at 3 weeks	NA	▬	NA	⬇

Comparison of the effects of DNSP-5, DNSP-11, DNSP-17, and previous studies of GDNF. Similarities between the effects of DNSP-11 and GDNF can be seen, with GDNF having a larger magnitude effect on evoked DA release and DNSP-11 affecting baseline DA levels.

Chapter Four: DNSP-11 induced Functional Recovery in the Aged Rat

Hypothesis: DNSP-11 will enhance or restore dopaminergic neuron function in the aged rat

Introduction

The previous chapter illustrated DNSP-11's ability to enhance DA synthesis and metabolism in the normal F344 rat. To begin to understand DNSP-11's potential as a therapeutic molecule, its action in a model of dopaminergic dysfunction must be characterized. Aged F344 rats provide a relevant model of dopaminergic dysfunction. The aged rat has a small percentage of cellular loss (<30%) but high degree of dopaminergic dysfunction with deficits in DA release, uptake, and synthesis (Fearnley and Lees, 1991; Hebert and Gerhardt, 1997, 1999). Alterations in DA release and uptake coincide with reductions in motor movement (Yurek et al., 1998). The aged rat is a model of dopaminergic dysfunction in which a high degree of neurons are alive but dysfunctional. Therefore, DNSP-11 could interact with a large degree of dysfunctional neurons with a goal of restoring their dopaminergic neurochemical function. Aged rats exhibit a continual progression of dopaminergic dysfunction providing a model to test DNSP-11's potential restorative properties. As an intervention strategy, DNSP-11 needed to improve the function of the nigrostriatal pathway. Normal aging exhibits many of the continually progressing deficits and dysfunctions associated with PD (Fearnley and Lees, 1991).

Analogous to PD and other models of dopaminergic dysfunction, aging induces behavior and motor movement deficits. By 18 months of age and continuing throughout their remaining life span, F344 rats have reduced motor activity to 70 − 80% of young adult (3-6 month) motor activity (Lapchak et al., 1997; Hebert and Gerhardt, 1998). The decline in motor movement is unlikely associated with changes in DA content, which is a point of contention in the field reduced by approximately 0 - 30 % in rodents at 22 months of age (Morgan et al., 1987; Stanford and Gerhardt, 2001; Rollo, 2009). The possible reduction in content could be related to changes in synthesis, explained by the 55% reduction in TH activity due to increased oxidative inactivation (De La Cruz et al., 1996). A

decline in TH activity could reduce DA synthesis and thereby reduce content however; the changes in DA content alone are not large enough to induce the reported motor deficits. In PD, a reduction in the DA content is thought to be in the 60 – 80% range before motor impairment can be visualized (Braak et al., 2004; Rollo, 2009). Further investigation of the age related dopaminergic dysfunction found a decreased capacity for release and uptake of DA. The reduced dopamine transporter function is related to the decline in plasma membrane expression of DAT (Hebert and Gerhardt, 1999; Salvatore et al., 2003). Changes in DA content, synthesis, and uptake regulation seen in aged rats provides a uniquely challenging system with multiple components of normal dopaminergic function yet still exhibiting dysregulation.

Many of the age-related deficits of the nigrostriatal dopaminergic system have been related to mitochondrial dysfunction due to mitochondrial DNA damage over time (Reeve et al., 2008). The increased bioenergetic burden of dopaminergic neurons within the nigrostriatal pathway increases susceptibility to age-related mitochondrial DNA damage that leads to dysfunction and increased production of reactive oxygen species (Banerjee et al., 2009). Reactive oxygen species further damage the mitochondrial membranes and induce apoptosis of dopaminergic neurons (Arthur et al., 2009). Dopaminergic neuron loss in the SN has been approximated at 36% in humans over the age of 85 and a portion has been attributed to apoptosis due to increases in reactive oxygen species (Rudow et al., 2008). GDNF reduces the concentration of reactive oxygen species, which could reduce apoptosis of dopaminergic neurons (Smith and Cass, 2007). The GDNF-induced reduction in reactive oxygen species coincides with increased neuroprotection from reactive oxygen species generating neurotoxins, 6-OHDA, in aged animals (Fox et al., 2001). GDNF's neuroprotective properties could be related to the inhibition of mitochondrial induced apoptosis or an overall modulation of mitochondrial function (Mograbi et al., 2001). Therefore, efficiency of mitochondrial function in the aged animal was compared using mitochondrial respiration rates of vehicle and DNSP-11 treatment groups.

GDNF bilaterally increases basal extracellular DA, motor movement, and unilaterally enhances K^+ and d-amp evoked DA release in the aged nonhuman primate (Grondin et al., 2003b). In the aged rat, GDNF treatment increases spontaneous movement parameters, total distance traveled and movement speed, 3 weeks after treatment. Striatal baseline concentrations of DA, DOPAC, HVA, and evoked DA release (K^+ or d-amp) were elevated in the aged rat 28 days after GDNF treatment. GDNF treatment also increased SN tissue content of DA, DOPAC, and HVA in the aged rat (Hebert and Gerhardt, 1997). Many of the neurochemical changes are likely related to changes in dopaminergic synthesis caused by the increased content of phosphorylated TH after GDNF treatment (Salvatore et al., 2004). The enhancement of TH function by GDNF alleviates some of the strain put on the system by the age induced oxidative damage to active TH. Neurochemical parameters are modified by GDNF in the aged animal and tonic release is increased by enhancing the firing rate of movement related neurons (Stanford et al., 2007). GDNF enhances neurochemical and electrophysiological function of nigrostriatal dopaminergic neurons in the aged rat.

Dopaminergic dysfunction in the aged animal provides a test bed to determine the ability of DNSP-11 to intervene and restore a dysfunctional dopaminergic system. The aged rat models dopaminergic dysfunction, and DNSP-11 can act upon the large number of surviving neurons. Positive results in the aged rat can provide further validation of DNSP-11 as the basis for dopaminergic therapeutics. I hypothesize DNSP-11 will improve dopaminergic neurochemical function and increase mitochondrial function.

Methods

Bilateral Infusion of DNSP-11 or Vehicle into the SN

Aged (24-month-old) Fischer 344 rats were treated with DNSP-11 or citrate buffer vehicle delivered bilaterally to the SN. The individual performing the infusion was blinded to the treatment the animals received. A total of 30 µg/5µL of DNSP-11 was delivered to the aged rat's SN at a rate of 0.25 µL/min. A similar protocol was followed as discussed in Chapter 2, and the same coordinates were used to treat two SN points within each hemisphere.

Bilateral Microdialysis Studies

Twenty-seven days after infusion treatment, bilateral microdialysis was performed under isoflurane anesthesia. Bilateral microdialysis uses a microdialysis probe placed in the striatum of each hemisphere to determine the extracellular neurotransmitter concentrations in both hemispheres of an aged rat. A CMA 11 probe with a 2 mm probe length was used to deliver and sample fluid to and from the dorsal striatum using the following stereotaxic coordinates; (TB: -2.3 mm, AP: +1.5 mm, ML: +2.3 mm, DV: -6.0 mm). The bilateral microdialysis technique used the same methodology as describe in Chapter 2 for probe recoveries, solution delivery, and sample collection. Animals were allowed to recover for two days after microdialysis to allow clearance of the anesthesia before euthanizing and harvesting tissue for mitochondrial, neurochemical, and TH immunohistochemical studies (Table 4.1).

Euthanasia and Tissue Dissection

Two days after bilateral microdialysis, animals were euthanized with CO_2 asphyxiation and the brain sectioned into quadrants. The striatum was removed from both anterior quadrants of the brain; one of which was used to assess mitochondrial respiration and the other for neurochemical analysis. The SN from one posterior quadrant was removed for analysis of mRNA contingent upon positive neurochemical or mitochondrial results. The remaining brain quadrant was post-fixed for immunohistochemical staining. For more details on the

Table 4.1 Study Design for determining DNSP-11's Restorative Effects in Aged F344 rats

DAY 0	Bilateral Infusion (n = 10)
DAY 27	Bilateral Microdialysis
DAY 29	CO_2 Euthanasia and Dissection SN - mRNA Analysis (Canceled) SN - TH Immunohistochemistry striatum - Tissue Neurochemistry striatum - Mitochondrial Respiration

To determine DNSP-11's effects in aged F344 rats, vehicle or DNSP-11 was infused bilaterally into the SN. Twenty-seven days later, bilateral microdialysis was performed under isoflurane anesthesia to determine neurochemical effects of DNSP-11. Animals were allowed to recover from isoflurane anesthesia for two days and then euthanized with CO_2.

methodology used for the assessment of TH staining, tissue neurochemical content, and mitochondrial respiration please refer to Chapter 2.

Data Analyses

Microdialysis data were not corrected for probe recoveries because all microdialysis probes were prescreened before use and only used if the recoveries were in the range of 12 -18% (Probe recovery Averaged 16 ± 0.5 %, n=12). Microdialysis data collected from each hemisphere were treated independently and analyzed by a two-way ANOVA with Bonferroni's post-hoc tests. Baseline neurochemical concentrations were determined from samples 2 through 4 for each animal and analyzed with an unpaired two-tailed Student's t-test. Tissue neurochemistry and mitochondrial respiration data was analyzed by a two-tailed Student's t-test comparing DNSP-11 and vehicle treatment groups.

Results

Microdialysis Measured Baseline and Evoked DA Concentrations

Bilateral microdialysis was performed 27 days after bilateral DNSP-11 or vehicle infusion. DA release was evoked using 100 mM K^+ and 250 µM d-amp (Table 4.2 and Figure 4.1). DNSP-11 treatment ($F_{(1,16)}$ = 4.02, p = 0.0454) significantly reduced K^+-evoked DA release but did not alter d-amp-evoked DA release. Baseline DA, DOPAC, and HVA concentrations were calculated from microdialysis samples 2-4 (Table 4.2). DNSP-11 treatment significantly decreased baseline concentrations of DA and DOPAC in aged F344 rats (Figure 4.2).

Striatal Tissue Neurochemical Content

One striatum from each animal was used for HPLC-EC neurochemical analysis. DA, DOPAC, and HVA content were analyzed for each sample and the average neurochemical level for each group was analyzed by a two-tailed Student's t-test (Table 4.3). No significant differences between treatment groups were found in the striatal tissue content of DA, DOPAC, or HVA. Neurochemical content was used to calculate the DA turnover ratio (DA/(DOPAC + HVA)). DA turnover ratios relate DA synthesis to DA metabolism, but no significant differences were observed between the vehicle and DNSP-11 treatment groups (Figure 4.3).

Mitochondrial Respiration

Striatal mitochondria were isolated, purified, and their oxygen consumption measured in reaction to multiple substrates. Substrates were used to isolate and test the function of specific portions of the electron transport chain. The RCR exemplifies the efficiency of mitochondrial function by providing a ratio of oxygen consumption during the production of ATP and during the blockade of ATP synthesis. The RCR for the vehicle treatment group was 12.03 ± 0.96 and the DNSP-11 treatment group was 11.33 ± 1.17. When the data was analyzed by a two-tailed Student's t-test, no significant difference in RCR score was detected
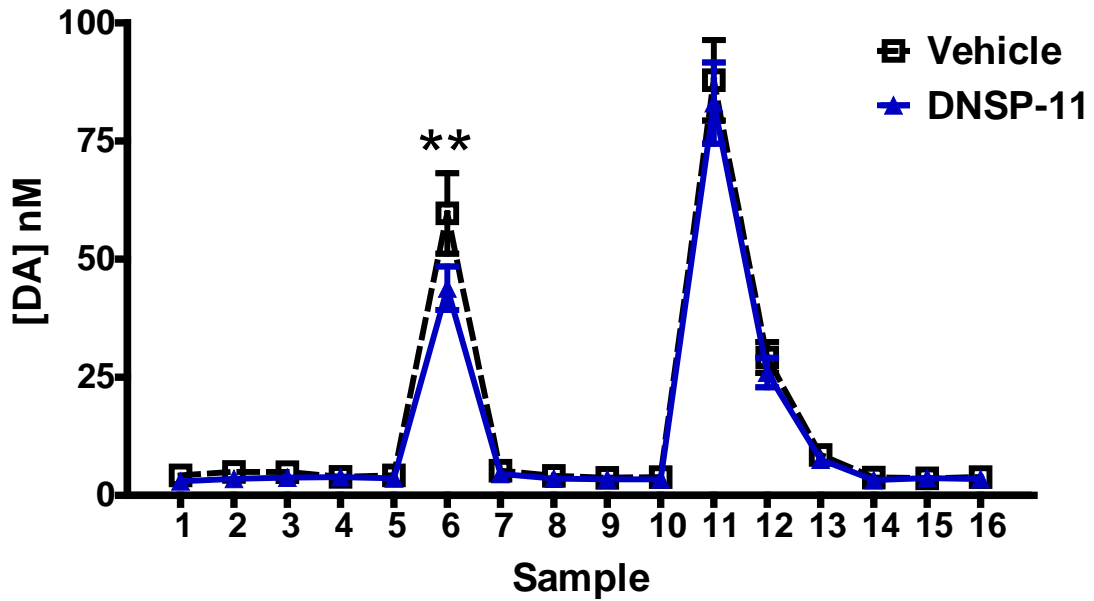
Figure 4.1 Microdialysis Measures of Striatal Extracellular DA in the Aged F344 Rat

DNSP-11 was seen to decrease resting DA levels and decreased K^+-evoked DA release. Striatal DA concentration sampled through microdialysis 27 days after DNSP-11 or vehicle SN infusion. DA concentration data was analyzed by a two-way ANOVA with Bonferroni's post-hoc tests to compare across treatment groups ($F_{(1,16)}$ = 4.02, p = 0.0454). Significant decreases in K^+ evoked DA release were observed after DNSP-11 treatment (**p < 0.01). Data Points: Mean ± SEM, n = 16.

Table 4.2 Extracellular Concentrations of Microdialysis Measured Neurochemicals

Perfusion solution and Sample #	Neurochemical Analyte	Vehicle Treatment Analyte Concentration (nM)	DNSP-11 Treatment Analyte Concentration (nM)	t and p values for Student's t-test or Bonferroni's post-hoc tests
aCSF Samples 2 - 4 (Baseline)	DA	4.62 ± 0.32	3.72 ± 0.24*	$t_{(32)}$= 2.19, p = 0.031
	DOPAC	903.8 ± 42.25	727.8 ± 35.03*	$t_{(32)}$= 3.16, p = 0.0021
	HVA	621.9 ± 33.5	562.6 ± 31.03	$t_{(32)}$= 1.29, p = 0.202
100 mM K^+ Sample 6	DA	59.70 ± 8.522	43.85 ± 4.61**	$t_{(32)}$= 3.91, p < 0.01
250 µM D-amp Sample 11	DA	87.87 ± 8.53	83.03 ± 8.59	$t_{(32)}$= 1.19, p > 0.05

DNSP-11 treatment significantly decreased K^+ evoked DA release and baseline levels of DA and DOPAC. Neurochemical concentrations were collected through bilateral microdialysis 27 days after DNSP-11 or vehicle treatment. Baseline concentrations of DA, DOPAC, and HVA were analyzed by a two-tailed t-test and evoked DA release was analyzed by a two-way ANOVA with Bonferroni's post-hoc tests. (*p < 0.05, **p <0.01) Data: Mean ± SEM, n = 10.
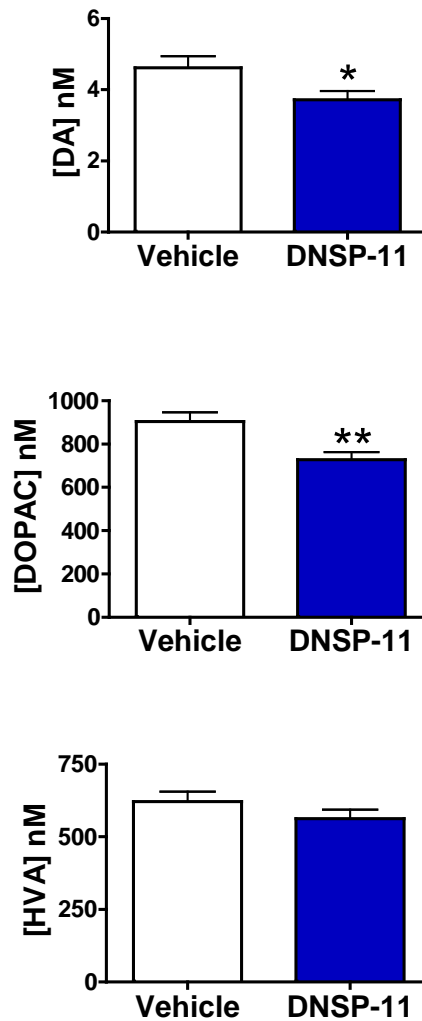
Figure 4.2 Reduction of Baseline Extracellular Neurochemical Concentrations of DA and DOPAC

Baseline microdialysis concentrations were calculated from the neurochemical concentration in microdialysis fractions 2-4. Data were analyzed with a two-tailed Student's t-test to determine differences between treatment groups. The baseline DA ($t_{(32)}$= 2.19, p = 0.031) and DOPAC ($t_{(32)}$= 3.16, p= 0.0021) concentrations were significantly reduced after DNSP-11 treatment relative to the vehicle treatment group (* p < 0.05, ** p < 0.01). The HVA ($t_{(32)}$= 1.29,p = 0.202) concentration demonstrated no significant changes between treatment groups. Data: Mean ± SEM, n = 10.

Table 4.3 Striatal Tissue Neurochemical Content 29 days after SN Infusion Treatment

Tissue Sample	Neurochemical Analyte	Vehicle Group Neurochemical Content (ng/g)	DNSP-11 Group Neurochemical Content (ng/g)	t and p values for Student's t test
Intact Striatum	DA	19399 ± 950.6	18086 ± 602.1	$t_{(20)}$= 1.11, p = 0.28
	DOPAC	4995 ± 844.5	4623 ± 702.4	$t_{(20)}$= 1.11, p = 0.28
	HVA	1553 ± 90.1	1492 ± 70.1	$t_{(20)}$= 0.518, p = 0.61

The striatal neurochemical content 29 days after SN delivery of DNSP-11 or vehicle. The striatum tissue was dissected and DA, DOPAC, and HVA neurochemical levels were determined from known standards. Data were analyzed using a two-tailed t-test with no significant differences between treatment groups. Data: Mean ± SEM, n = 10.
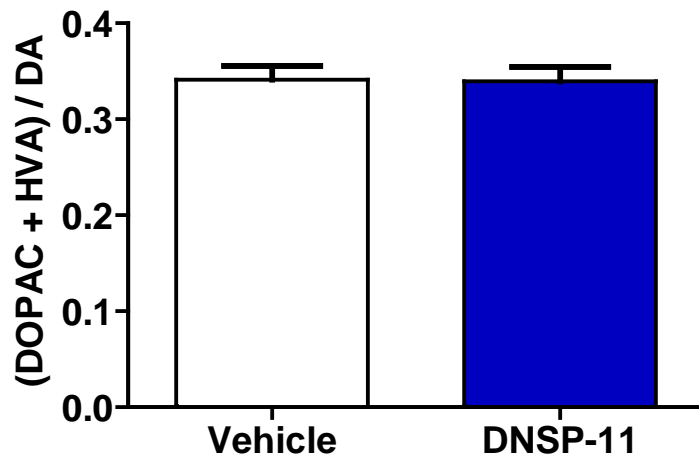
Figure 4.3 Striatal DA Turnover Ratios in the Aged F344 Rat following DNSP-11 Treatment

The ratio of DA to its metabolites is used to represent any changes in the metabolism of DA. The ratio graphically represented above was not statistically different between treatment groups when analyzed by a two-tailed Student's t-test ($t_{(20)}$ = 0.0801 p = 0.937). Data: Mean ± SEM, n = 10.

between groups (Figure 4.4). Oxygen consumption rates from all mitochondrial respiration states were compared between treatment groups by a two-way ANOVA with Bonferroni's post-hoc tests (Data Not Shown). No significant difference between DNSP-11 and vehicle treatment groups was identified through statistical analysis.

SN TH Immunohistochemistry

One of the SN from each animal was harvested for TH immunohistochemistry to examine any qualitative changes in TH fiber density or morphology. Visual comparison of the TH stained SN from each treatment group revealed no pronounced differences in the density or morphology of the TH positive neurons (Figure 4.5).
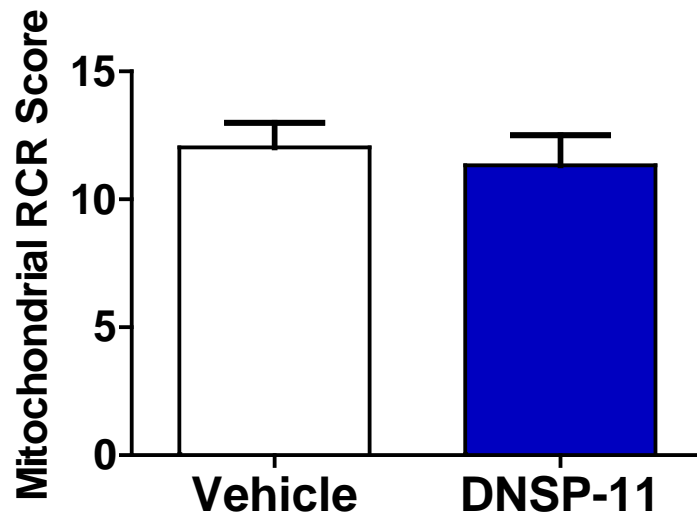
Figure 4.4 Striatal Mitochondrial Respiratory Control Ratio

No differences in the mitochondrial control ratios were observed between DNSP-11 and vehicle treatment. The mitochondrial respiratory control ratio represents the efficiency and function of the mitochondria in the striatum 29 days after DNSP-11 or vehicle treatment. Comparison of the two treatment groups was made using a two-tailed Student's t-test ($t_{(20)}$ = 0.4695, p = 0.64). Analysis of the data revealed no significant difference between the treatment groups. Data: Mean ± SEM, n = 10.
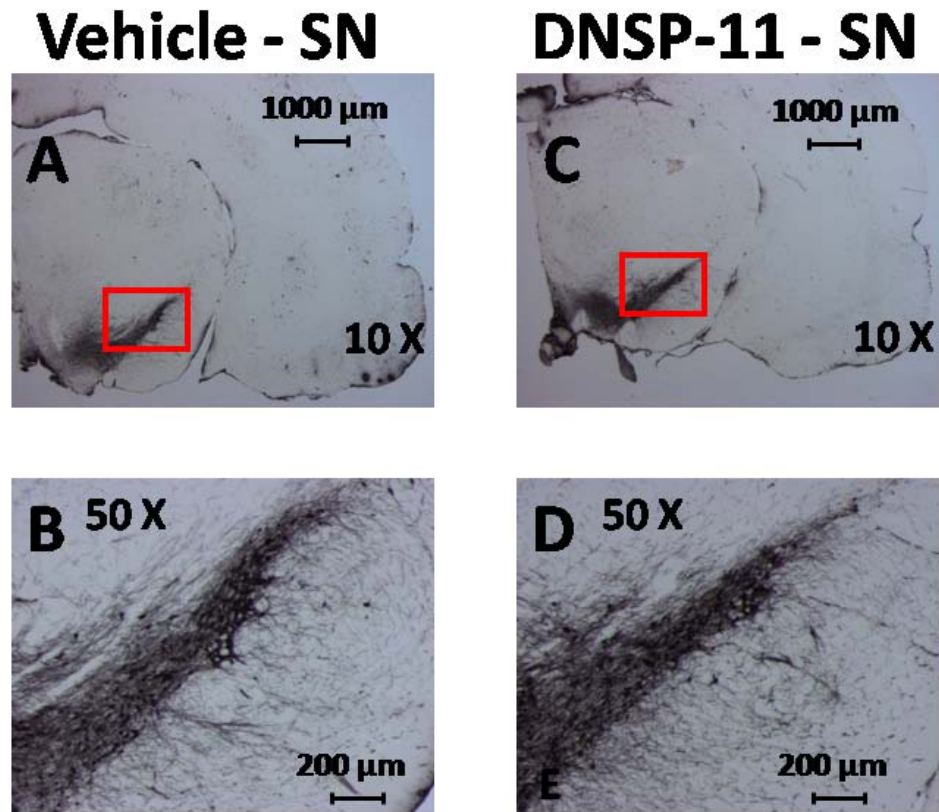
Figure 4.5 TH Staining 29 days after DNSP -11 Infusion to the SN

TH immunohistochemistry in the SN of the aged rat 29 days after DNSP-11 (C and D) or vehicle treatment (A and B) revealed no observable differences in TH positive cell body or fiber density. Photographs A and C were taken under 10 x magnification while B and D were under 50 x magnification (n = 10).

Discussion

DNSP-11 treatment in the aged rat produced small (20-30%) significant decreases in microdialysis-measured striatal neurochemistry. Baseline concentrations of DA and DOPAC were significantly decreased by DNSP-11 along with a significant reduction in K^+-evoked DA release. Neurochemical changes measured in the striatal extracellular space showed potential modulation of synthesis, metabolism, or release. The previous chapter demonstrated DNSP-11's potential to modulate DA synthesis and therefore the most probable explanation would be a decrease in synthesis related to the dose of DNSP-11. GDNF affects TH activity, through phosphorylation and at higher doses, reduces TH content and DA synthesis (Rosenblad et al., 2000; Salvatore et al., 2004). GDNF showed a bilateral effect on aged animal neurochemistry (Grondin et al., 2002). Therefore, bilateral dosing of DNSP-11 could modify the dose response range and produce a decrease in DA synthesis and a subsequent decline in DOPAC, the primary metabolite in the rat. A decline in DA synthesis by DNSP-11 would diminish baseline DA, baseline DOPAC, and K^+ evoked DA release, but DNSP-11 would also be expected to decrease d-amp evoked release. There was no observable difference in TH staining of the SN between treatments, though not performed quantitatively, a large reduction in TH content caused by excessive DNSP-11 would be anticipated to be visible in the TH staining density. Modifications in DA synthesis were not observable in TH staining or d-amp-evoked DA release and striatal tissue neurochemical data showed no change in DA, DOPAC, or HVA. Therefore, alternative mechanisms for modifying baseline and evoked DA release needed to be examined.

Brain tissue neurochemical data in conjunction with microdialysis data provided evidence that synthesis and metabolism of DA are unchanged after DNSP-11 treatment. Therefore, the release of DA must be modified to achieve the changes in DA and DOPAC observed by microdialysis after DNSP-11 treatment. To reduce basal DA and DOPAC the frequency or size of release must be affected. In the previous chapter, DNSP-11 increased baseline DA possibly through increased DA synthesis (Pothos et al., 1998; Yang et al., 2001).

Less DA and DOPAC were observed in the extracellular space in the aged animals as well as decreased K+ evoked DA release, which could be related to decreased DA packaging. However, decreased DA packaging with no significant changes in synthesis should increase cytosolically available DA, which would be observed through d-amp-evoked DA release. Therefore, a reduction in K^+ evoked release would not be expected unless the membrane hyperpolarization reduces the number of terminals recruited to release DA.

Alterations in neurochemical function between treatment groups may be related to the isoflurane anesthetic interactions with functional changes related to DNSP-11 treatment. Isoflurane can increase baseline concentrations of DA and decrease evoked DA release potentially mediated through D_2 autoreceptor interactions (Tsukada et al., 1999). Isoflurane alters the turnover ratio of DA through the internalization of DAT (Byas-Smith et al., 2004). Isoflurane modifications of the DA transporter and autoreceptor could interact with modifications caused by DNSP-11 to mask or reverse overall neurochemical effects. If DNSP-11 increased baseline DA and evoked DA release, the subsequent application of isoflurane could interact with the autoreceptors causing a down regulation of the elevated DA. Therefore, DA concentrations elevated by DNSP-11 treatment could be decreased below vehicle treatment without modifying DA synthesis.

Mitochondrial function was not significantly modified by DNSP-11 treatment, which could be related to a lack of an effect on mitochondrial function. But cell culture data would suggest DNSP-11 can modulate susceptibility to mitochondrial toxins and modulate mitochondria membrane potential (Unpublished data, Luke Bradley). Therefore, it is possible isoflurane anesthesia 2 days before tissue collection may have affected the mitochondrial function. Isoflurane depolarizes the mitochondrial membrane and the two day wash out period between anesthesia delivery and tissue collection may not provide a long enough time for those effects to dissipate (Bains et al., 2009). The depolarization event occurs through the reversal or uncoupling of ATP synthase and may reduce ATP production and create an energy shortage within the neurons

(Ljubkovic et al., 2007). Therefore, isoflurane-mediated modifications in mitochondrial function could mask the differences in mitochondrial function between treatment groups.

DNSP-11 showed negative effects on DA neurochemistry in the aged F344 rat and may not have positive neurochemical effects in the aged animal. DNSP-11 may not be able to reverse the dysfunction demonstrated by the aged dopaminergic system in DAT localization, TH activity, or DA content. GDNF provides restoration of the dysfunctional dopaminergic system in the aged rat by enhancing tissue content and baseline concentrations of DA, DOPAC, and HVA. DNSP-11 decreased the DA and HVA baseline concentrations and had no effect on the neurochemical content of the striatum. GDNF elevates evoked DA release induced by K^+ or d-amp and DNSP-11 decreased K^+ evoked DA release without any effect on d-amp evoked DA release (Hebert and Gerhardt, 1997). DNSP-11 was unable to modulate mitochondrial function and GDNF has been implicated in stabilizing mitochondrial function through the blockade of mitochondrial induced apoptosis and reduction in reactive oxygen species.

Chapter Summary

GDNF and DNSP-11 have contrasting effects in the aged rat (Table 4.4). GDNF affects almost every aspect of DA neurochemical function in the aged animal while DNSP-11 showed minimal effects. DNSP-11 decreased baseline levels of DA, baseline levels of DOPAC, and K^+ evoked DA release. No effects were observed by DNSP-11 on mitochondrial function, TH staining, or tissue neurochemistry. Differences in the effects of DNSP-11 and GDNF could be related to modifications in the study design, specifically isoflurane anesthesia, or differences in the signaling and function of DNSP-11. Isoflurane modifies the dopaminergic system by interacting with the D_2 autoreceptor and DAT, which could mask or reverse changes related to DNSP-11 treatment. It is unknown how isoflurane interacts with trophic factor like-modulation of the dopaminergic system and cannot be determined from these studies.

Table 4.4 Comparison of the Effects of DNSP-11 and GDNF in Aged F344 rats

	DNSP-11	GDNF (Hebert and Gerhardt 1997)
Baseline DA and DOPAC concentrations	↓	↑
K$^+$ evoked DA	↓	↑↑
D-amphetamine evoked DA	—	↑↑
Striatal DA and metabolite content	—	↓

A comparison of the effects of DNSP-11 and previous work with GDNF shows drastically different effects on the aged dopaminergic system in F344 rats. GDNF robustly increases evoked DA release while DNSP-11 reduces K$^+$-evoked release and has no effect on d-amp-evoked DA release. Both DNSP-11 and GDNF affect baseline concentrations of DA and DOPAC, but DNSP-11 decreases baseline DA and DOPAC and GDNF increases baseline DA and DOPAC. The differential effects of DNSP-11 and GDNF show a possible divergence in mechanism of action between DNSP-11 and GDNF.

Therefore, DNSP-11 may restore the dopaminergic system but needs to be thoroughly tested using another anesthetic. DNSP-11 minimally modified the dopaminergic system of the aged F344 rat. The reduction in dopaminergic function is potentially related to an excessive dose of DNSP-11 or the anesthesia's effects on the dopaminergic system. This study raises many questions related to anesthesia function, contrasting effects of GDNF and DNSP-11, and DNSP-11 function but DNSP-11 did manage to modify neurochemistry in the aged rat. DNSP-11 has exhibited neurochemical effects in the normal F344 rat and the aged F344 rat. The neurochemical changes observed in all studies made it necessary to continue investigation of DNSP-11's ability to modulate the dopaminergic system.

Chapter Five: Neuroprotection and Restoration in the 6 – Hydroxydopamine – lesioned rat by DNSP-11

Hypothesis: DNSP-11 will provide neuroprotective and neurorestorative effects on dopaminergic neuron function.

Introduction

In [Chapter 3](), DNSP-11 enhanced DA neurochemistry and potentially increased dopaminergic neuron fiber density in normal rats. In [Chapter 4](), DNSP-11 decreased DA neurochemistry in the aged rat. The neurochemical and potential neuropil changes associated with DNSP-11 treatment, show DNSP-11 could provide the basis for future therapeutics against diseases affecting dopaminergic function. Results with DNSP-11 showed it can enhance function in normal rats and has the potential to restore and/or enhance the age-related dysfunctional dopaminergic system. A true assessment of its potential as a therapeutic for dopaminergic dysfunction occurs in model systems demonstrating a loss of function. At this time, there are two competing theories on the treatment of dopaminergic functional loss with trophic molecules: neurorestoration and neuroprotection.

Neurorestoration is based primarily on the ability of trophic factors to induce neuronal sprouting and restore neuron function by modifying synthesis and metabolism (Lang and Obeso, 2004; Gash et al., 2007). In a neurorestoration paradigm, behavioral deficits are already established and the correlative neurochemical loss has already reached symptomatic levels. In this case, restoration of neuronal function needs to be achieved to enhance dopaminergic function and reduce behavioral symptoms. Restoration of neuronal function is considered a difficult task and has only been demonstrated in the laboratory by a few molecules (Dauer, 2007). A possibly more attainable intervention strategy would be to stop the progression of dopaminergic neuron loss.

Neuroprotection is an intervention strategy based primarily on stopping the progression of dopaminergic cell and functional loss. A key to the effectiveness of a neuroprotection based treatment strategy is early detection (Braak et al.,

2004). Upon detection of a disease reducing dopaminergic function, a therapeutic intervention would begin to stop the continued loss of function but would not restore any of the preceding loss. Therefore, early detection strategies in conjunction with a neuroprotective agent would prevent the symptomatic phase of the disease but otherwise would not fully alleviate established neurochemical and cellular deficits. Both neuroprotection and neurorestoration strategies are flawed, but a combination of both neuroprotection and neurorestoration would be the most beneficial for alleviation of symptoms and subsequent progression (Olanow et al., 2003).

In this chapter, two model systems were used in two different study designs to assess DNSP-11's ability to protect and restore dopaminergic function. Assessment of DNSP-11's neuroprotective properties used a model delivering 6-OHDA to the dopaminergic terminals in the striatum. This method provides consistent loss of dopaminergic function by reducing dopaminergic fiber numbers and dopamine content in the striatum and SN by 60 -80% (Kirik et al., 1998). The reduction in DA content provides correlative stable behavioral deficits that are both drug induced and spontaneous. Behavioral changes can then be used to provide evidence of DA related changes in function. Both the cell loss and neurotransmitter loss demonstrated by this model of dopaminergic dysfunction correlates well with the cell loss and neurotransmitter loss demonstrated in early stage PD (Kirik et al., 1998). A key to assessing neuroprotection with this model of dopaminergic cell loss is pretreatment with DNSP-11. DNSP-11 was delivered to the striatum six hours before 6-OHDA allowing DNSP-11 to establish its effects before being challenged by the neurotoxin. The goal of this study was to determine DNSP-11's neuroprotective properties against neurotoxin induced cell death.

An alternative model of dopaminergic cell loss is one that delivers 6-OHDA to the MFB, which reduces DA function by greater than 90% (Schwarting and Huston, 1996). The severe loss of dopaminergic function reduces the baseline levels of dopaminergic function, fiber content, and behavior increasing sensitivity of endpoint measures to neurochemical and neuropil alterations.

Extreme cellular loss and subsequent loss of dopaminergic function induced by the MFB lesion provides a model system relevant to end stage PD (Kirik et al., 1998). In this model, restoration of dopaminergic function is necessary to demonstrate any significant behavioral recovery. The dopaminergic loss is so severe in this model system that there are very few dopaminergic neurons for a therapy to target. Therefore, to induce any restoration of neurochemical or behavioral deficits, the therapeutic must be able to robustly modify function of a very limited population of neurons. DNSP-11 therapy was delivered to the SN after dopaminergic neuron loss had stabilized for 5 weeks providing a study paradigm to assess restoration. In a similar manner to previous GDNF studies, the dose was increased to 100 μg from the 30 μg used in normal animals. Behavioral and neurochemical measures of restoration were the focus of these studies but TH positive neuron density was also assessed. The goal of the study was to determine the restorative effects of DNSP-11 after neurotoxin induced dopaminergic cell loss.

The neuroprotective effects of GDNF have been studied thoroughly, and GDNF protects against multiple neurotoxins (Kirik et al., 2000; Sawada et al., 2000; Cass et al., 2006). GDNF requires delivery approximately 6 hours before a neurotoxic insult, allowing protein synthesis, and providing neuroprotection (Kearns and Gash, 1995; Kearns et al., 1997). The area of administration does modify the effectiveness of GDNF in protecting against dopaminergic neuron loss. When GDNF is administered into the SN and the 6-OHDA into the striatum there is only protection of the cell bodies and no neuroprotection of the axons. However, when both GDNF and 6-OHDA are delivered to the striatum, and GDNF is given adequate time to modulate protein synthesis, there is protection of both the axons and cell bodies (Kirik et al., 2000). In the design of our studies, adequate time was allowed for protein synthesis between DNSP-11 and 6-OHDA delivery, according to previous work with GDNF (Kearns and Gash, 1995; Kearns et al., 1997). To ensure the most robust response, both 6-OHDA and DNSP-11 were delivered to the striatum increasing the possibility of exhibiting an effect on both the axons and cell bodies.

In multiple neurorestoration testing paradigms, GDNF has produced significant and lasting effects on DA neurochemistry, behavior, and fiber density (Tomac et al., 1995b; Grondin and Gash, 1998). In the most similar testing paradigms to the one discussed in this chapter, GDNF demonstrated a lasting reduction in apomorphine-induced rotation behavior and increased neurochemical content in the SN (Hoffer et al., 1994). Reductions in drug induced rotation behavior began 1 week after infusion and lasted for four weeks. GDNF significantly increased SN DA and DOPAC content four weeks after treatment. GDNF increased the number of cell bodies and neurites in the SN (Bowenkamp et al., 1995). Similar results on DA neurochemistry, behavior, and neurons were hypothesized to occur with DNSP-11 treatment.

DNSP-11 increased the function of an intact dopaminergic system and had similar effects to GDNF in the normal animal. In conjunction with the effects of DNSP-11 on the aged animal and previous work with GDNF, we hypothesized DNSP-11 would have both neuroprotective and neurorestorative effects. These two studies would reveal DNSP-11's true potential as the basis for therapeutic molecules against dopaminergic dysfunction.

Methods

Please refer to [Chapter 2](#) for a detailed review of all methods used within this chapter.

DNSP-11 Restoration

[DNSP-11 or vehicle was infused](#) to F344 rats after they had demonstrated a greater than 90% reduction in dopaminergic function through [apomorphine induced rotation behavior](#). Rotational behavior was assessed for 4 weeks after treatment and striatal and SN tissue was taken 5 weeks after the infusion treatment. Tissue was taken primarily for neurochemical analysis, but two animals exhibiting representative rotational behavior from each group were used for TH staining (Table 5.1A).

DNSP-11 Neuroprotection

F344 rats were tested in a modified cylinder test to assess baseline paw usage for each animal. A week later, animals were treated with DNSP-11 or vehicle within the striatum and 6 hours later were treated with 6-OHDA to the same striatum coordinates. The lesion was allowed to progress for two weeks after which time [asymmetrical paw use](#) and [amphetamine induced rotation behavior](#) was assessed. Behavioral measures were monitored for 4 weeks and the animals were euthanized and brain tissue taken. Tissue was primarily used to assess changes in [neurochemical content](#). Two animals exhibiting representative rotation behavior from each group were taken for [TH staining](#) (Table 5.1 B).

Data Analyses

[Data analyses](#) were performed using an unpaired two-tailed Student's t-test for all tissue neurochemical data, and behavioral data was analyzed by two-way ANOVA with Bonferroni's post-hoc tests. Apomorphine-induced rotational behavior was represented as a percentage of the vehicle for simplification and was analyzed by a one-way ANOVA with Bonferroni's post-hoc tests.

Table 5.1 Study Design for Assessment of DNSP-11-induced Restoration and Neuroprotection

A

Week 0	6-OHDA MFB Lesion
Week 4	Apomorphine Lesion Assessment
Week 5	SN Infusion Treatment with Vehicle or DNSP-11 (n=10)
Week 6	Apomorphine Rotation Behavior
Week 7	Apomorphine Rotation Behavior
Week 8	Apomorphine Rotation Behavior
Week 9	Apomorphine Rotation Behavior
Week 10	Tissue Dissection for Neurochemical Analysis + TH immunohistocehmistry

B

Week -1	Modified Cylinder Test
Week 0	Str Infusion Treatment with Vehicle or DNSP-11 (n=12)
Week 0 + 6 hours	6-OHDA Str Lesion
Week 3	Amphetamine Rotation Behavior + Modified Cylinder Test
Week 4	Amphetamine Rotation Behavior
Week 5	Amphetamine Rotation Behavior + Modified Cylinder Test
Week 6	Amphetamine Rotation Behavior
Week 7	Tissue Dissection for neurochemical analysis + TH immunohistocehmistry

A) The study design used to test neurorestorative effects by DNSP-11. After neurotoxin delivery, the dopaminergic cell loss was allowed to stabilize before treatment with DNSP-11. Recovery from the cell loss was the primary comparator between the two groups.

B) The study design used to test neuroprotective effects produced by DNSP-11. Delivery of DNSP-11 preceded neurotoxin delivery and the progression and extent of deficit produced by the neurotoxin was the primary comparator between the two groups.

Results

Rotational Behavior and Paw Placement in Neuroprotection Study

Amphetamine induced rotation behavior was assessed three weeks after the injection of DNSP-11 or vehicle and 6-OHDA. No significant difference was observed between the vehicle and DNSP-11 treated groups at week 3 or any of the subsequent weeks that followed ($F_{(1,22)}$ = 0.18, p = 0.676) (Figure 5.1).

Schallert's cylinder test provided data differentiating paw use before and after 6-OHDA delivery. The data depicts hemisphere specific changes in motor movement and used within animal controls comparing the movement in the right paw (intact hemisphere) to movement in the left paw (lesioned hemisphere). Data were normalized to the right paw, intact hemisphere, usage and illustrated no significant changes between the DNSP-11 and vehicle treatment groups ($F_{(1,22)}$ = 2.32, p = 0.142) (Figure 5.2).

DA Neurochemistry in Neuroprotection Studies

Seven weeks after DNSP-11 or vehicle treatment and 6-OHDA delivery, animals were euthanized and tissues were dissected from the striatum and SN. Neurochemical analysis by HPLC-EC determined the neurochemical content of the tissue and no significant statistical differences were observed in DA, DOPAC, or HVA content in the SN or striatum (Table 5.2).

The turnover ratio was calculated to provide insight into metabolic aspects of DA (Figure 5.3). The only area to exhibit significant increases in DA metabolism was the area DNSP-11 was delivered, the right striatum ($t_{(13)}$ = 2.46, p = 0.019). DNSP-11 increased the turnover ratio in the right striatum from 2.373 ± 0.19 to 3.272 ± 0.26. All other areas of interest did not show any significant changes in the ratio of metabolites (Rt SN: ($t_{(12)}$ = 0.356, p = 0.729), Lft striatum: ($t_{(12)}$ = 0.752, p = 0.467), Lft SN: ($t_{(12)}$ = 0.986, p = 0.344).

Neuroprotection Study TH Immunohistochemistry

Qualitative visual analysis of TH staining within the SN and striatum revealed no observable differences between the treatment groups (Figure 5.4).
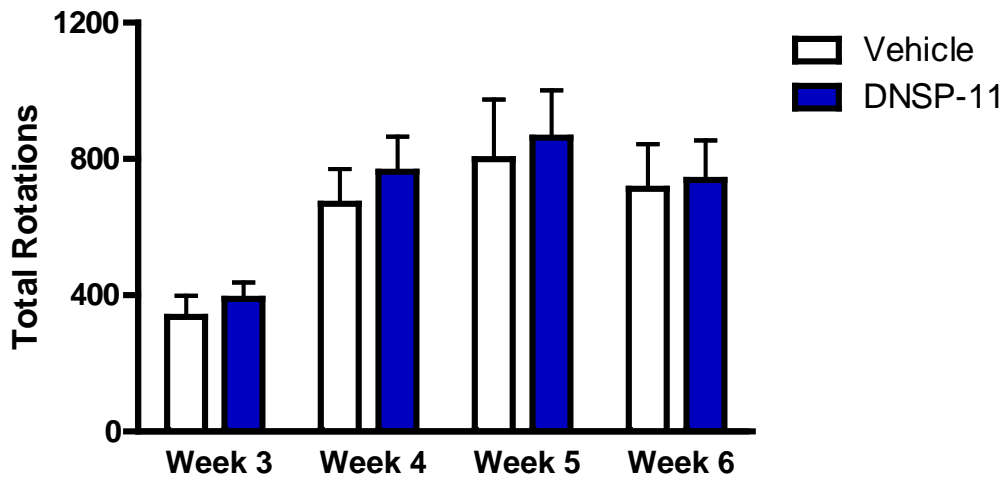
Figure 5.1 Amphetamine-Induced Rotation Behavior beginning 3 weeks after Treatment with DNSP-11 or Vehicle and 6-OHDA

Total rotation behavior collected weekly beginning 3 weeks after neurotoxin / treatment delivery and assessed for a total of 4 weeks. Data were analyzed by a two-way ANOVA for repeated measures with Bonferroni's post-hoc tests comparing differences between each group ($F_{(1,22)}$ = 0.18, p = 0.676). No significant differences were observed between DNSP-11 and vehicle treatment groups (n=12).
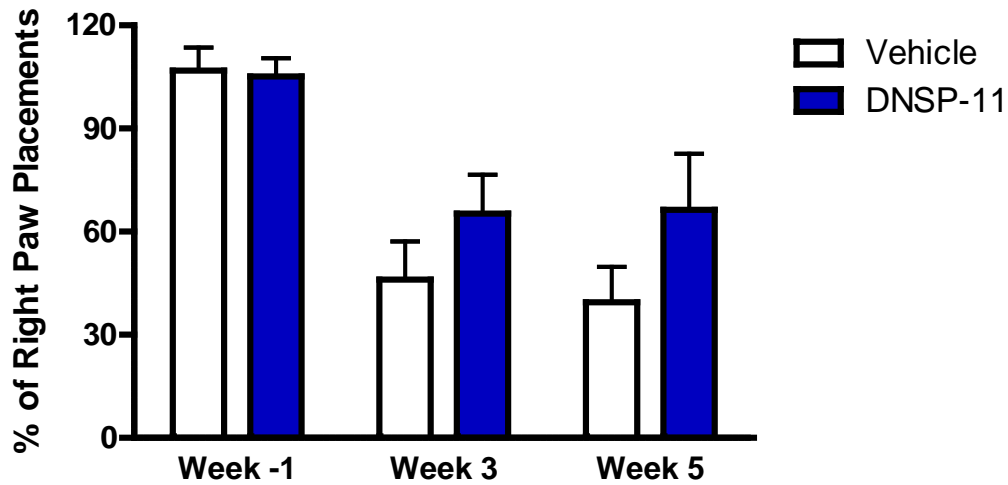
Figure 5.2 Percentage of Right Paw Placement before and after Striatal Treatment of DNSP-11 or Vehicle and 6-OHDA

Paw placement was assessed once before treatment and 6-OHDA delivery and twice afterward. Data are shown as a percentage of the intact hemisphere's paw usage allowing for changes due to habituation and reduction in activity. Analysis of the data were performed by a two-way ANOVA for repeated measures ($F_{(1,22)}$ = 2.32, p = 0.142) with Bonferroni's post-hoc tests comparing DNSP-11 to vehicle treatment groups. No significant differences were seen between treatment groups (n=12).

Table 5.2 Neurochemical Content of the striatum and SN, 7 weeks after striatum Treatment of DNSP-11 or Vehicle and 6-OHDA Delivery

Tissue Sample	Neurochemical Analyte	Vehicle Group Neurochemical Content (ng/g)	DNSP-11 Group Neurochemical Content (ng/g)	t and p values for Student's t test
Lesioned SN	DA	170.4 ± 34.1	195.8 ± 39.1	$t_{(17)} = 0.484$, p = 0.634
	DOPAC	69.38 ± 17.21	62.13 ± 12.17	$t_{(17)} = 0.349$, p = 0.731
	HVA	164.3 ± 31.25	333.5 ± 71.12	$t_{(17)} = 0.052$, p = 2.095
Lesioned striatum	DA	514.4 ± 103.7	486.4 ± 77.64	$t_{(17)} = 0.220$, p = 0.827
	DOPAC	984.1 ± 228.4	1296 ± 247.3	$t_{(17)} = 0.369$, p = 0.910
	HVA	230.2 ± 34.17	247.9 ± 27.43	$t_{(17)} = 0.409$, p = 0.685
Intact SN	DA	469.4 ± 38.22	481.5 ± 85.93	$t_{(17)} = 0.123$, p = 0.903
	DOPAC	160.7 ± 16.73	164.7 ± 22.71	$t_{(17)} = 0.889$, p = 0.142
	HVA	156.1 ± 31.61	191.2 ± 34.21	$t_{(17)} = 0.747$, p = 0.465
Intact striatum	DA	1879 ± 186.7	2560 ± 334.2	$t_{(17)} = 1.673$, p = 0.103
	DOPAC	1656 ± 213.4	2063 ± 235.4	$t_{(17)} = 1.257$, p = 0.216
	HVA	316.1 ± 39.75	349.9 ± 35.08	$t_{(17)} = 0.640$, p = 0.526

DA related neurochemical content in the striatum and SN of both the intact and lesioned hemispheres 7 weeks after DNSP-11 or vehicle treatment and 6-OHDA Delivery. No significant changes in DA related tissue neurochemistry after treatment were detected comparing DNSP-11 and vehicle treatment groups with an unpaired two-way Student's t-tests. The t and p values for each t-test can be found in the table above along with mean ± SEM values for the neurochemical content (n=10).
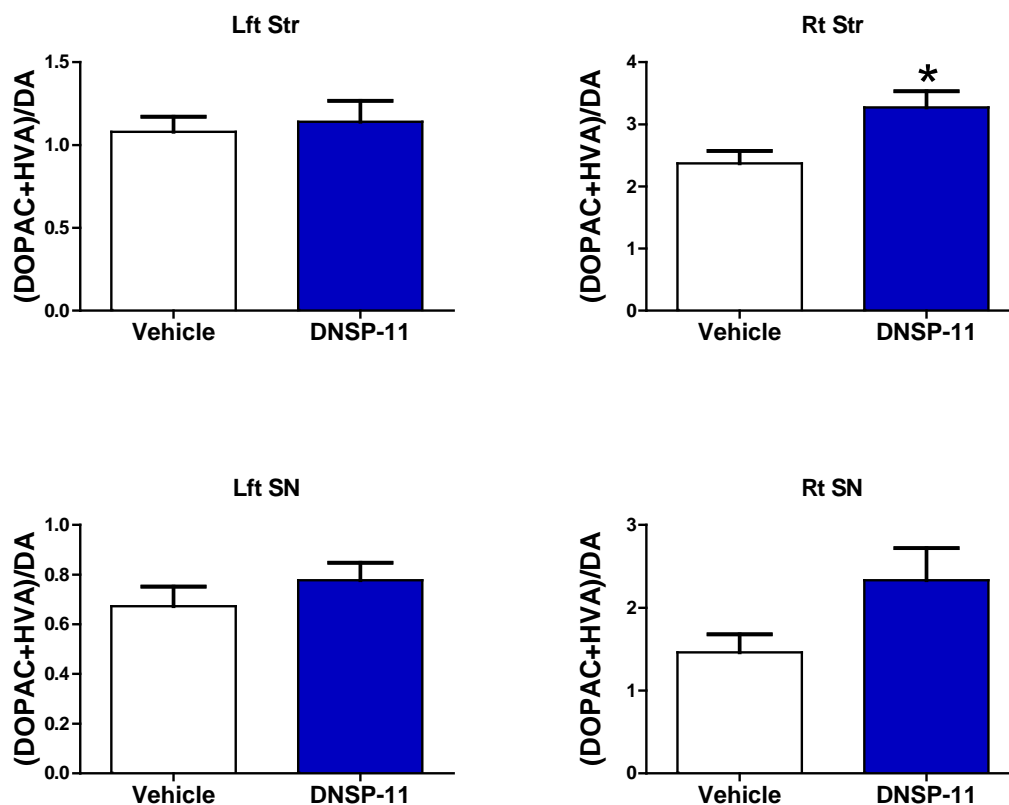
Figure 5.3 Ratio of DA metabolism 7 weeks after DNSP-11 or vehicle treatment and 6-OHDA delivery

Turnover ratios comparing DNSP-11 and vehicle treatment in the striatum and SN of both hemispheres. All data sets were analyzed with a two-tailed Student's t-test determining significant increases in DA metabolism by DNAP-11 treatment within the Rt striatum. Rt SN: ($t_{(17)}$ = 1.45, p = 0.17), Rt striatum: ($t_{(17)}$ = 2.46, p = 0.019), Lft SN: ($t_{(17)}$ = 1.47, p = 0.16), Lft striatum: ($t_{(17)}$ = 0.256, p = 0.80), * p < 0.05. Data: Mean ± SEM, n = 10.
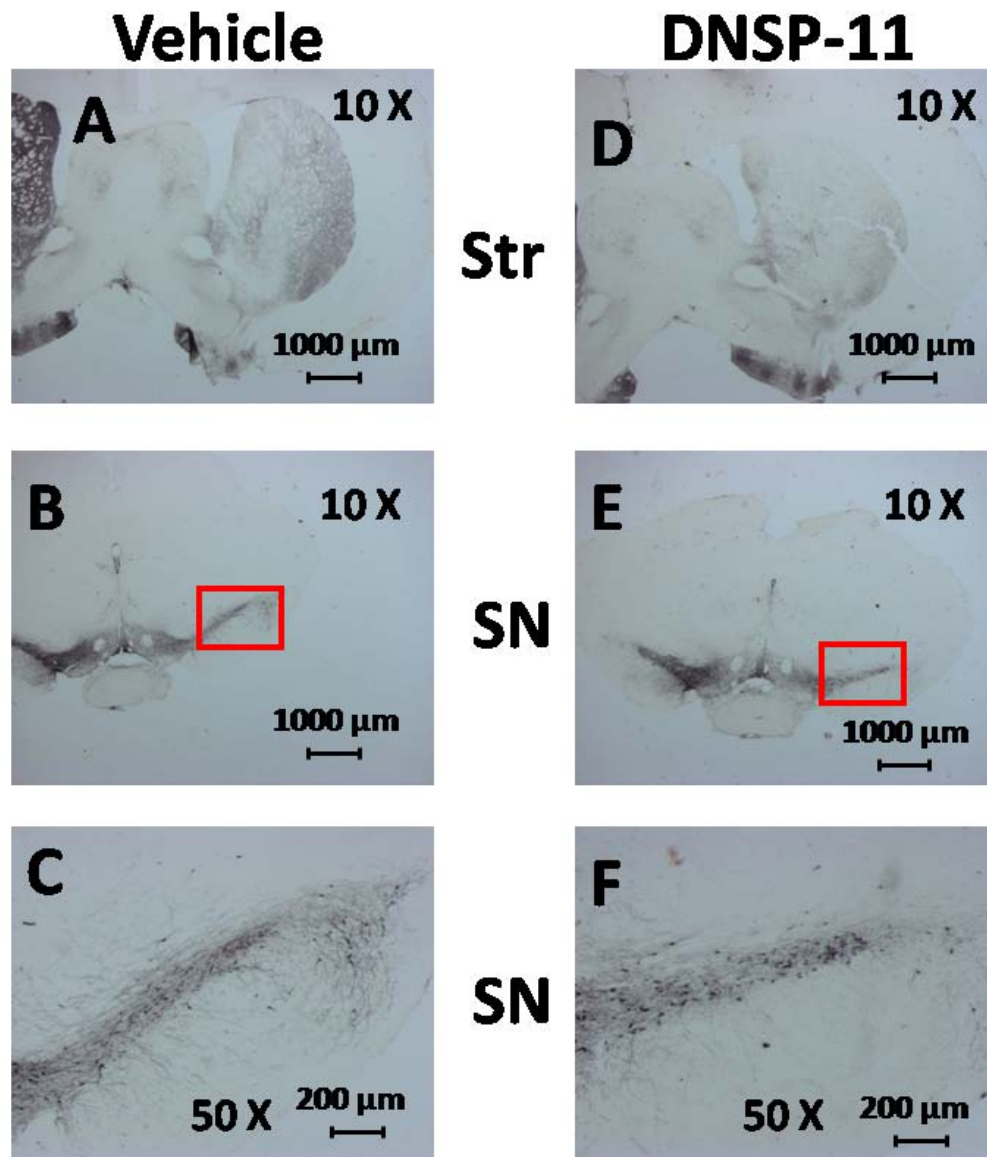
Figure 5.4 TH Immunohistochemistry in the SN and striatum 7 weeks after treatment with DNSP-11 or vehicle and 6-OHDA

TH positive staining after DNSP-11 or vehicle treatment showed a lack of qualitative differences between the vehicle and DNSP-11 treatment groups in either striatum or SN brain regions 7 weeks after treatment delivery. Slices A, B, D, and E were observed under 10 x magnification, while C and F were visualized at 50 x magnification (n=2).

Severe MFB Lesion in the F344 Rat

6-OHDA was delivered to two points along the MFB and provided a severe lesion with a minimum 90% reduction in DA content in both the striatum and SN. When comparing the intact hemisphere to the lesioned hemisphere in vehicle treated animals, there was a reduction in striatum DA from 7601 ± 310.9 to 60.85 ± 11.85 ng/g, a 99.2% reduction, and in the SN DA content decreased from 1637 ± 269.5 ng/g to 34.65 ± 6.44 ng/g a reduction of 97.9% (Figure 5.5). Analysis of the DA reduction showed both the striatum ($t_{(14)}$ = 5.94, p < 0.0001) and SN ($t_{(14)}$ = 24.23, p < 0.0001) had significant loss of DA content.

Apomorphine Induced Rotational Behavior

Apomorphine was utilized to induce a turning behavior that has been correlated with DA content within the SN and striatum (Hudson et al., 1993). Apomorphine-induced rotational behavior was used as a screening tool to separate animals into two groups, vehicle and DNSP-11 treatment, with equal drug induced rotation behavior. Before treatment (Pre), the DNSP-11 group had 95.0 ± 7.8% of the number of vehicle group rotations. One week after infusion treatment, apomorphine-induced rotation behavior in the DNSP-11 treatment group had significantly decreased to 56.9 ± 7.3% of the vehicle group (Figure 5.6). The reduction in rotational behavior was seen at all four time points after DNSP-11 treatment (Week 2: 50.63 ± 10.48% Week 3: 61.43 ± 6.88% Week 4: 61.08 ± 6.38%, $F_{(4,39)}$ = 4.807, p= 0.0005).

6-OHDA Tissue Neurochemistry

The level of apomorphine-induced rotation behavior has been shown to inversely correlate with DA content, but to validate the hypothesized changes in neurochemistry, HPLC-EC methods were used to measure the neurochemical content of tissue samples from the SN and striatum of all animals 5 weeks after treatment. Tissue DA levels in the lesioned SN of the vehicle treatment group was 34.65 ± 6.44 ng/g and the DNSP-11 treatment group was significantly increased to 59.10 ± 7.31 ng/g ($t_{(13)}$= 2.521, p=0.0265) (Table 5.3).
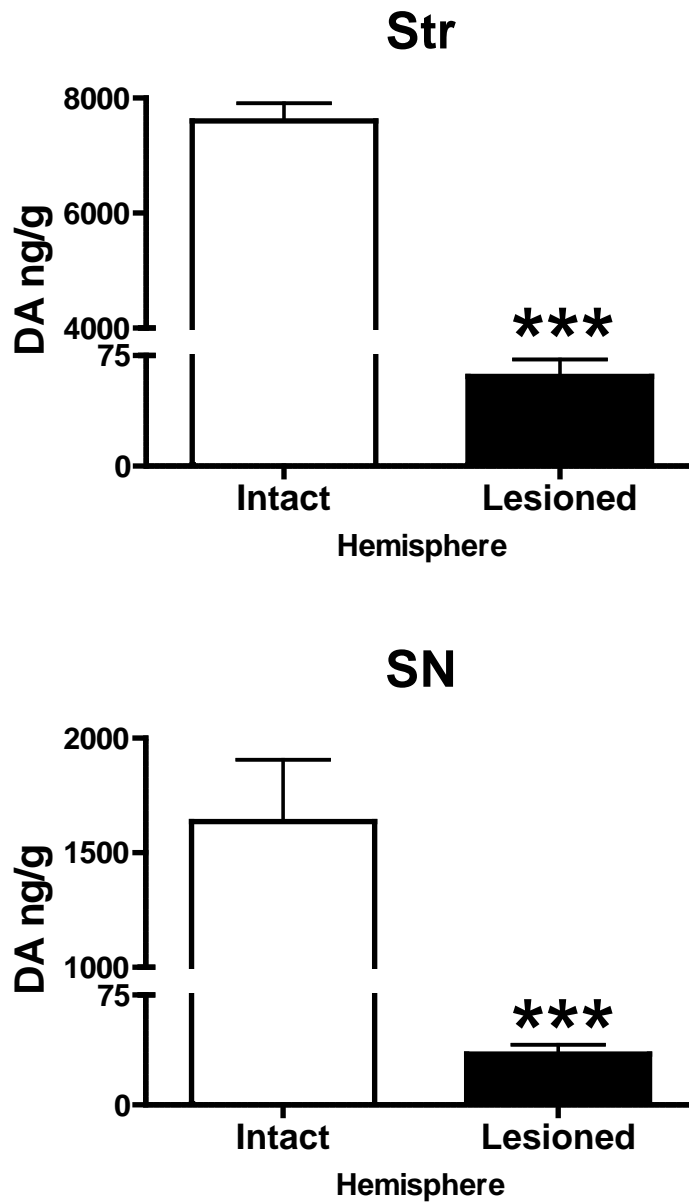
Figure 5.5 6-OHDA-induced striatal and SN reductions in DA content

Comparison of the DA content in the striatum and SN of vehicle treated animals demonstrating the effects of the 6-OHDA MFB lesion. A highly significant reduction of DA content was observed in both the SN ($t_{(14)}$ = 5.94, p < 0.0001) and striatum ($t_{(14)}$ = 24.23, p < 0.0001) when analyzed by a two-tailed Student's t-test. *** p < 0.0001 Intact vs. lesion. Data: Mean ± SEM, n = 8.
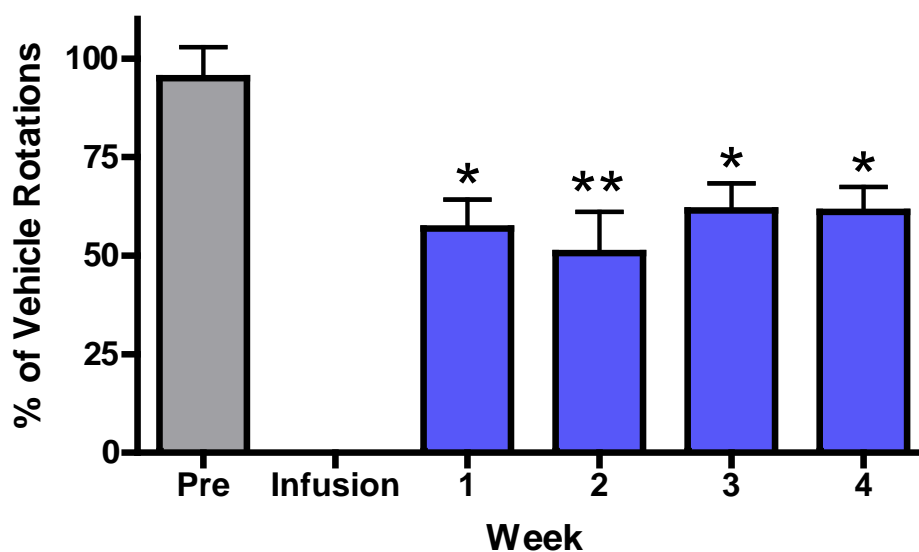
Figure 5.6 Reduction in Apomorphine Induced Rotation Behavior after DNSP-11 Treatment

Percentage of vehicle rotational behavior showing significant reductions in apomorphine-induced rotation behavior by DNSP-11 at all 4 time points after infusion treatment. Data was analyzed by a One-way ANOVA for repeated measures ($F_{(4,39)} = 4.807$, p= 0.0005) with Bonferroni's post-hoc tests to compare all weeks rotation behavior with the prescreen rotation behavior * p < 0.05, * p < 0.01 (n=8).

Table 5.3 Neurochemical content of the SN and striatum 5 weeks after infusion treatment with DNSP-11 or Vehicle

Tissue Sample	Neurochemical Analyte	Vehicle Group Neurochemical Content (ng/g)	DNSP-11 Group Neurochemical Content (ng/g)	t and p values for Student's t test
Lesioned SN	DA	34.65 ± 6.44	59.10 ± 7.31*	$t_{(13)}$= 2.521, p=0.0265
	DOPAC	7.10 ± 1.40	16.48 ± 4.01*	$t_{(13)}$= 2.330, p=0.0364
	HVA	40.66 ± 17.74	42.41 ± 11.52	$t_{(13)}$ =0.083, p = 0.935
Lesioned striatum	DA	60.85 ± 11.58	61.37 ± 10.21	$t_{(14)}$= 0.034, p=0.974
	DOPAC	33.99 ± 8.43	45.01 ± 9.52	$t_{(14)}$= 0.868, p=0.400
	HVA	20.80 ± 6.33	16.75 ± 2.13	$t_{(14)}$= 0.606, p = 0.554
Intact SN	DA	1637 ± 269.5	2137 ± 354.1	$t_{(13)}$ = 1.124, p =0.280
	DOPAC	355.7 ± 90.50	276.8 ± 50.88	$t_{(13)}$ = 0.760, p = 0.460
	HVA	116.2 ± 21.27	136.8 ± 35.01	$t_{(13)}$ = 0.502, p = 0.624
Intact striatum	DA	7601 ± 310.9	9056 ± 707.7	$t_{(14)}$= 0.081, p= 1.883
	DOPAC	1716 ± 145.6	2335 ± 165.9*	$t_{(14)}$= 2.806, p= 0.0140
	HVA	686.2 ± 55.39	738.3 ± 70.05	$t_{(14)}$= 0.584, p= 0.569

Striatum and SN DA, DOPAC, and HVA content from tissue collected 5 weeks after treatment with DNSP-11 or vehicle. All neurochemical content in DNSP-11 treated animals was analyzed relative to the neurochemical content of the vehicle treated animals using a two-tailed Student's t-test *p < 0.05. Data: Mean ± SEM, n = 8.

DOPAC levels in the vehicle treatment group were 7.10 ± 1.40 ng/g while DOPAC levels in the DNSP-11 treatment group were significantly increased to 16.5 ± 4.0 ng/g ($t_{(13)}$= 2.33, p=0.0364) (Table 5.3). No significant changes in HVA were detected in the lesioned SN (Table 5.3). The striatum of the lesioned hemisphere demonstrated no DA related neurochemical changes. The intact striatum, contralateral from the SN in which DNSP-11 was administered, showed significant changes in DOPAC between the vehicle treatment group with a level of 1716 ± 145.6 ng/g and the DNSP-11 treatment group with a significantly increased level of 2335 ± 165.9 ($t_{(14)}$= 2.806, p=0.0140). No significant changes in neurochemistry were detected in the intact SN.

DA metabolism in the SN and striatum was calculated by dividing the total DA metabolite (DOPAC + HVA) content of an area by the DA content. There were no differences between turnover or metabolism ratios between the DNSP-11 and vehicle treated groups (Figure 5.7).

Neurorestoration Study TH Immunohistochemistry

A qualitative analysis of TH staining in each treatment group revealed potential increases in fiber density within the striatum and possible increases in the number of cell bodies and fiber density within the SN of DNSP-11 treated animals (Figure 5.8). Only two animals from each group were used for TH immunohistochemistry but consistent increases in TH positive neurons were observed in the DNSP-11 treatment group. However, the magnitude of the increases in TH immunohistochemistry varied between DNSP-11 treated animals.

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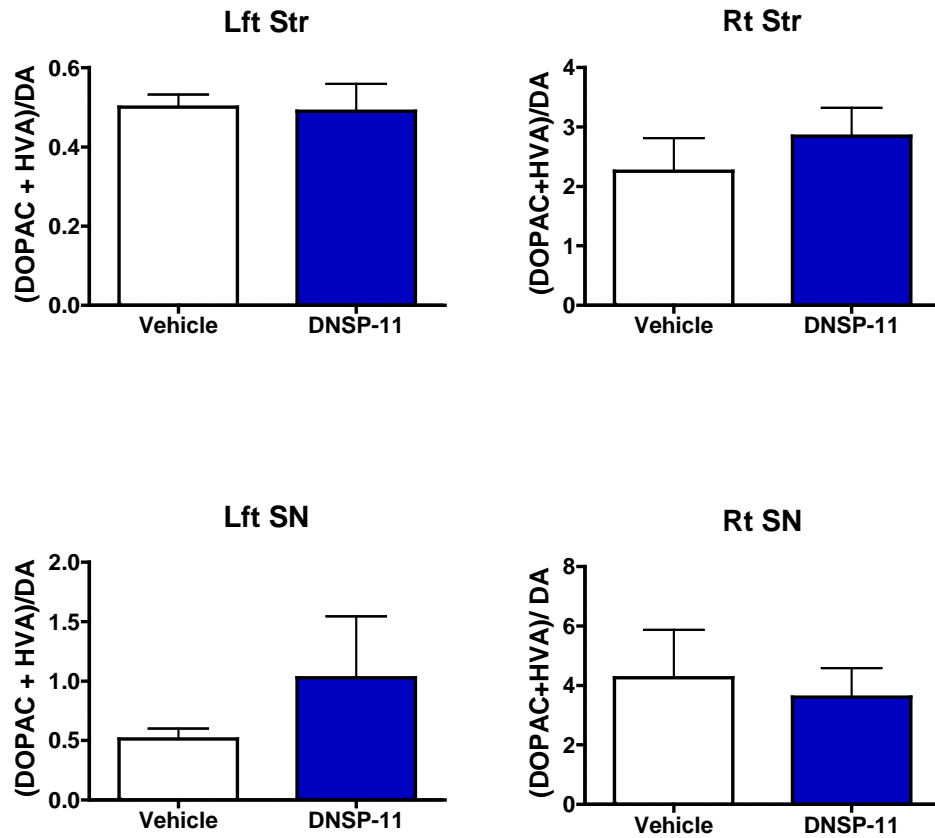
Figure 5.7 DA Metabolism in a 6-OHDA Lesioned Rat 5 Weeks after SN Infusion Treatment

The ratio of tissue DA metabolites to tissue DA 5 weeks after infusion treatment was not significantly different in any brain region. Data from each brain region was analyzed using a two-tailed Student's t-test; Rt striatum: ($t_{(12)}$ = 0.814 , p = 0.432), Rt SN: ($t_{(12)}$ = 0.356 , p = 0.729), Lft striatum: ($t_{(12)}$ = 0.752 , p = 0.467), Lft SN: ($t_{(12)}$ = 0.986 , p = 0.344). Data: Mean ± SEM, n = 8.
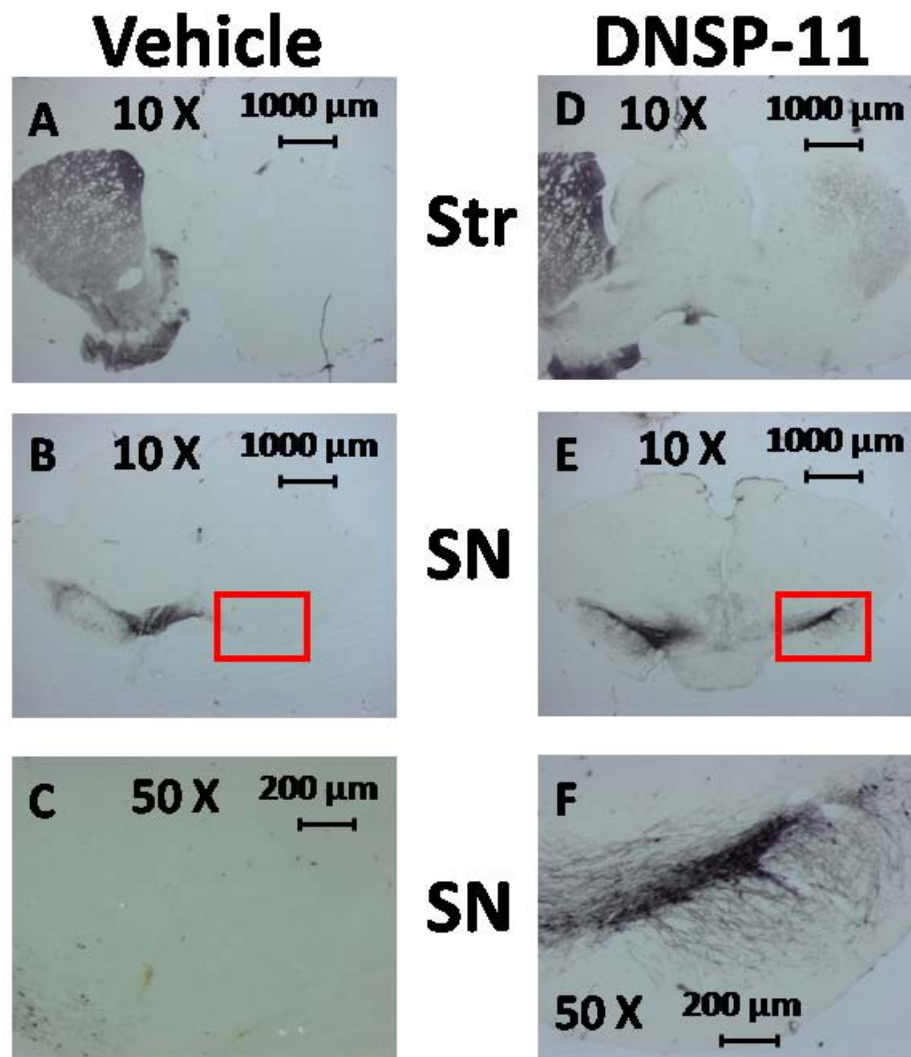
Figure 5.8 TH Immunohistochemistry 5 weeks after DNSP-11 Treatment

TH Immunohistochemistry was investigated in two animals per treatment group 5 weeks after infusion delivery. Photographs A – C and G – I were taken from the two animals treated with citrate buffer vehicle, while D – F and J – L where taken from the two animals treated with DNSP-11. TH staining showed possible increases in the cell body number and enhanced dendritic arborization within the SN. Continued on the next page.
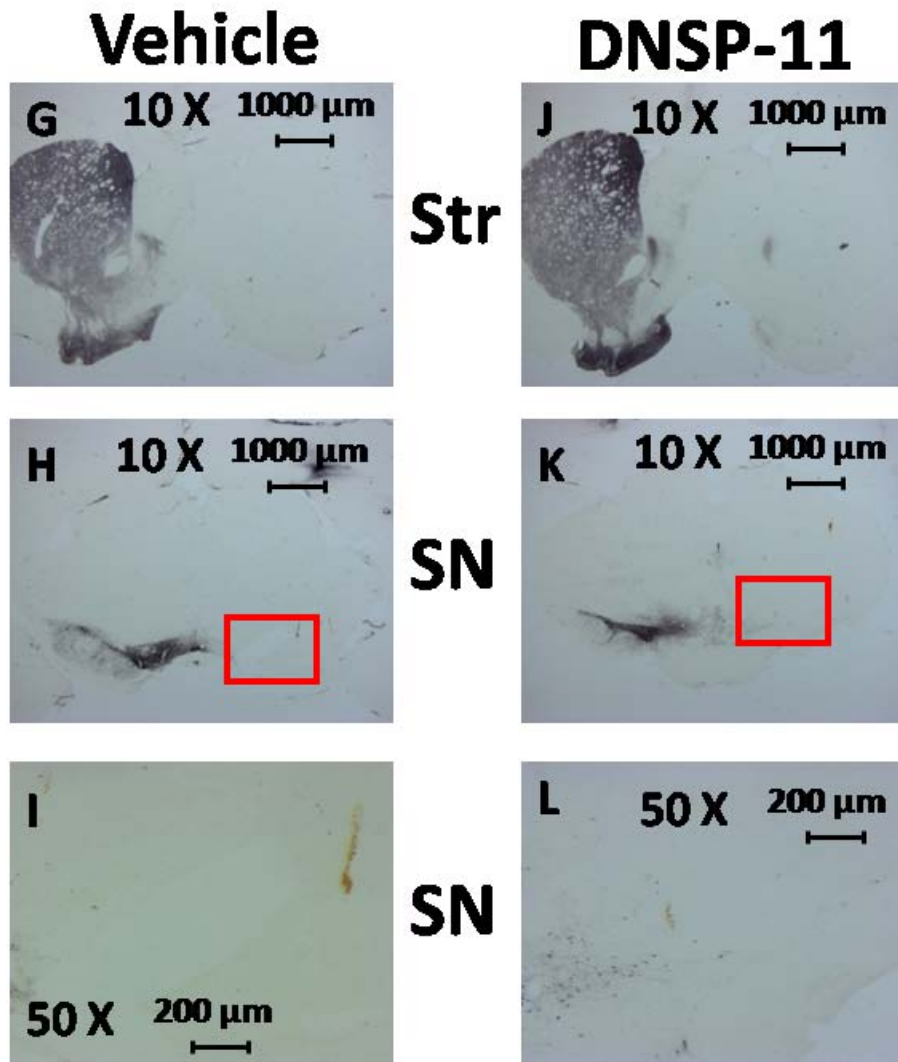
Figure 5.8 Continued

Corresponding increases in TH positive fiber density were observed in the striatum. The DNSP-11 animals do not reveal the same magnitude of TH staining both do demonstrate restoration of TH expressing neurons. Further investigation and replication of this study will be necessary to determine typical TH staining after DNSP-11 treatment. Photographs A, B, D, E, G, H, J and K were all visualized under 10 x magnification, while C, F, I and L were observed under 50 x magnification (n=2).

Discussion

In an effort to determine DNSP-11's ability to protect dopaminergic neurons from neurotoxin-induced cell loss, DNSP-11 was delivered before neurotoxin treatment. No significant alterations in behavior were observed in the DNSP-11 pretreatment group when compared to vehicle in either rotational behavior or paw placement. The lack of behavioral changes would suggest limited neurochemical differences between the DNSP-11 and vehicle treatment groups providing evidence of a similar dopaminergic loss in both treatment groups. Therefore, minimal to no protection was provided by DNSP-11 treatment and this assumption was validated using the neurochemical content of the tissue. The neurochemical content showed no significant increases in DA, DOPAC, or HVA between treatment groups providing further evidence for a lack of significant neuroprotective effects by DNSP-11.

When the tissue neurochemical analysis was examined, reductions in the DA content in the striatum were not paralleled by DA metabolite reductions of the same magnitude. DA metabolism was increased, which was demonstrated through the significant increase in turnover ratio implying increases in DA signaling or production possibly within the weeks before the tissue collection (Hudson et al., 1995). The possibility remains that there was an increase in DA synthesis before tissue dissection and the observed effects on metabolism are all that remains 7 weeks after DNSP-11 treatment. But, that would also imply the changes in DA synthesis were not robust enough to elicit behavioral changes in the DNSP-11 treated animals. The lack of a neurochemical effect could also be related to the area of administration, since all previous studies delivered DNSP-11 to the SN. The retrograde transport of DNSP-11 has not been established, and therefore if the target of action for DNSP-11 is only located in the SN, then DNSP-11 may not reach its target. From the results of the neuroprotection study, it was hypothesized that DNSP-11 is minimally transported to the SN and DSNP-11 treatment provides nominal lasting effects on DA neurochemistry. Minimal delivery of DNSP-11 to its site of action would explain the lack of observed effects on TH staining or behavior and the mild effect on DA metabolism.

To determine DNSP-11's ability to restore a damaged dopaminergic system, DNSP-11 or vehicle was infused into the SN of F344 rats exhibiting severe and stable dopaminergic cell loss (>95 %). DNSP-11 improved dopaminergic function within the severely lesioned nigrostriatal pathway; decreasing apomorphine induced rotation behavior and increasing tissue content of both DA and DOPAC. TH immunohistochemistry showed possible increases in the number of TH$^+$ cell bodies and fiber density in the SN and increases in fiber density in the striatum.

The reduction of rotational behavior in the severely lesioned rat by DNSP-11 treatment is most likely directly related to increased synthesis and release of DA within the SN (Robertson and Robertson, 1988, 1989). Increased levels of DA would decrease the post-synaptic receptor supersensitivity and reduce the activation related to the DA receptor agonist, apomorphine (Kostrzewa, 1995; Kostrzewa et al., 2008). The behavioral manifestation, though drug induced, was the first instance behavioral modification related to DNSP-11 induced neurochemical changes was observed.

Tissue neurochemical analyses were performed to confirm the presumed neurochemical changes and demonstrated significant increases in DA and DOPAC in the SN ipsilateral to the lesion. The increase in DA and DOPAC may be related to increases in DA synthesis either through the restoration of damaged dopaminergic neurons or enhancement of the remaining functional dopamine neurons. TH staining could indicate improvements in the number of damaged dopaminergic neurons because of the increased density in TH positive neurons, but this does not preclude the possibility of simultaneous enhancement of function in surviving neurons. Baseline neurochemical data from chapter 3 (Figure 3.7) showed an increase in baseline extracellular dopamine, which would be best explained by the enhancement of DA release. Therefore, DNSP-11 was able to improve and/or enhance the function of the nigrostriatal pathway five weeks after a 95 % loss of function.

In the lesioned animals, the intact hemisphere had altered DA neurochemistry after DNSP-11 treatment with increases in DOPAC tissue

content. The possibility of DNSP-11 reaching the contralateral hemisphere through diffusion is unlikely because it is removed from the extracellular space within 2 hrs of delivery (Bradley et al., 2009 (Submitted)).It is more likely that there is signaling between the two hemispheres causing compensatory changes after DNSP-11 related increases in function.

Results from studies of GDNF have previously demonstrated neuroprotective and neurorestorative effects against similar models of neurotoxin induced cell death. DNSP-11's restorative effects on the severely damaged dopaminergic system parallel GDNF's effects in all measured parameters including TH staining, neurochemistry, and rotation behavior (Hoffer et al., 1994; Bowenkamp et al., 1995). GDNF showed a similar ability to affect the contralateral hemisphere neurochemistry in both non-human primate and rodent models (Hoffer et al., 1994; Slevin et al., 2005; Stanford et al., 2007).

Neuroprotective studies demonstrate a drastic difference between GDNF and DNSP-11. GDNF delivered to the striatum was able to protect against dopamine cell loss induced by 6-OHDA in both the SN and striatum (Kirik et al., 2000). Significant increases in rotational behavior and forepaw use were observed as well as enhanced TH positive fiber density. Effects on TH positive fiber staining or behavior demonstrated by GDNF were not mimicked by DNSP-11. This provides further evidence of drastically different mechanisms between the two molecules possibly related to DNSP-11's site of action and lack of retrograde transport.

Chapter Summary

DNSP-11 did not provide neuroprotection when delivered to the striatum 6 hours before 6-OHDA. Minimal neurochemical effects were demonstrated by DNSP-11 on DA metabolism, but no neuroprotective effects were observed in TH staining, behavior, or tissue neurochemical. Minimal effects by DNSP-11 in the neuroprotection model could be due to the striatal delivery, a lack of DNSP-11 retrograde transport, and localization of DNSP-11's site of action to the SN. The lack of a neuroprotective effect could be attributed to the molecular properties of the molecule and mechanism of action. In a rat model of severe nigrostriatal

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dopaminergic cell loss, DNSP-11 was able to restore dopaminergic function, modulate behavior, and possibly increase TH fiber density in both the striatum and SN (Table 5.4). DNSP-11 demonstrated potential as a therapeutic agent to restore and/or enhance the function of a severely damaged dopaminergic system. The restorative or enhancing actions of DNSP-11 support the molecule's therapeutic potential and warrant continued study in models of dopaminergic function.

Table 5.4 Comparison of the Effects of DNSP-11 and GDNF in the Severe 6-OHDA Lesion

	DNSP-11	GDNF
Apomorphine Rotations	⬇	⬇ (Hoffer et al. 1994)
DA and DOPAC Content in the striatum	▬	▬ (Hoffer et al. 1994)
DA and DOPAC Content in the SN	⬆	⬆ (Hoffer et al. 1994)
TH Immunohistochemistry	⬆	⬆ (Bowenkamp et al. 1995)

Comparison of the effects of DNSP-11 and previous work with GDNF in the severe 6-OHDA-lesioned rat showed similar alterations in the nigrostriatal dopaminergic system. Both GDNF and DNSP-11 reduced apomorphine-induced rotations, increased DA and DOPAC content in the SN, and enhanced TH+ neuron density.

Chapter Six: Major Conclusions and Future Directions

In all rat models studied in this dissertation DNSP-11 treatment modified DA function. In the young adult F344 rat, DNSP-11 enhanced dopaminergic function (Chapter 3); in the 24 month old F344 rat, DNSP-11 reduced basal and releasable DA (Chapter 4); in severely lesioned F344 rat, DNSP-11 improved dopaminergic function (Chapter 5); and pretreatment with DNSP-11 in the F344 rat before neurotoxic insult modified DA metabolism (Table 6.1). Thus, DNSP-11 enhances dopaminergic function in the F344 rat.

Mechanisms of DNSP-11 Activity

The differential activity of DNSP-11 relative to the established effects of GDNF is evidence that peptides cleaved from the prodomain may have alternative signaling relative to their parent molecules. Bradley et al. (Submitted 2009) provided evidence of DNSP-11 signaling through a pathway other than the GFRα1 receptor, the known signaling pathway for GDNF. According to Bradley et al. (Submitted 2009), DNSP-11 directly binds the glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and could be the origin of DNSP-11's function. GAPDH has a multitude of functions beyond glycolysis such as RNA export, DNA maintenance and repair, exocytotic membrane fusion, and cytoskeletal organization (Sirover, 1999, 2005). A recently identified function of GAPDH is the initiation of apoptotic cascades by nuclear translocation (Berry and Boulton, 2000). The induction of apoptosis and malfunction of GAPDH has been implicated in the energy dysfunction and progressive cell loss associated with aging and neurodegenerative disease (Mazzola and Sirover, 2002, 2003).

Modulation of GAPDH by DNSP-11 would potentially modulate metabolism and apoptosis possibly enhancing the function and increasing survival of dopaminergic neurons. Deprenyl, also known as selegiline, a selective MAO B inhibitor, has anti-apoptotic functions through GAPDH. GAPDH binding selectively decreases synthesis of pro-apoptotic proteins and increases synthesis of anti-apoptotic proteins (Tatton et al., 1995; Tatton et al., 2003; Hara et al., 2006). DNSP-11 could have similar actions to deprenyl modifying apoptosis,

Table 6.1 Summary of DNSP-11 Related Effects on DA in Rat Model Systems

F344 Rat Model	Modulation of DA			Fiber/Cell Body Density
	Synthesis	Metabolism	Release	
Normal (3-6 Month)	+	+	+	+/?
Aged (24-26 Month)	−	−	+	−
Severe MFB Lesion (Neurorestoration)	+	+	−	+/?
Striatal Lesion (Neuroprotection)	−	+	−	−

A table summarizing the effects of DNSP-11 on DA function in each of the previous chapters using the specified rat models. + Positive, − Negative, ? Inconclusive

energy utilization, and MAO function (Berry, 2004). DNSP-11 binds GAPDH similarly to deprenyl and has parallel neurochemical effects. The potential anti-apoptotic function through GAPDH binding could explain the restorative effects demonstrated by DNSP-11 (Lamensdorf et al., 1996). Therefore, interaction with both MAO and GAPDH by DNSP-11 could explain the similarities to deprenyl's neurochemical and anti-apoptotic actions.

DNSP-11 shares functional similarities with GDNF, but Bradley et al. (Submitted 2009) provided evidence of a divergence in mechanism because DNSP-11 does not bind to the GFRα1 receptor. Induction of GDNF production by DNSP-11 would potentially explain many of the neurochemical effects observed within this dissertation. The induction of GDNF production and subsequent trophic actions could explain the enhanced neurochemical function and neurorestoration observed in the rat but would not explain the acute effects of DNSP-11. The function of DNSP-11 and GDNF were investigated by Bradley et al. (Submitted 2009) by comparing DNSP-11 and GDNF as neuroprotective agents against mitochondrial toxins. DNSP-11 showed neuroprotective effects and blocked the release of cytochrome C, while GDNF was unable to provide any neuroprotective effects against mitochondrial induced apoptosis. DNSP-11 and GDNF differ in their ability to bind GFRα1 and in their mechanisms of mitochondrial neuroprotection showing differences in function. Difference in mechanism and function between GDNF and DNSP-11 do not preclude the possibility for DNSP-11 to modulate GDNF translation. Therefore, future studies will be necessary to determine if DNSP-11 effects the translation of its parent molecule, GDNF.

DNSP-11 may induce GDNF translation, but it is also possible that GDNF and DNSP-11's distinct signaling pathways could converge on similar targets such as the ERK/MAPK pathway GDNF signals through (Sariola and Saarma, 2003). A convergence of signaling pathways could provide similarities of function, while maintaining distinct differences. As noted earlier, DNSP-11 interacts with GAPDH and could potentially alter neurochemical function through inhibition of MAO or convergence with a GDNF related signaling pathway. To allow the

development of molecules targeting specific aspects of DNSP-11 signaling, the potential signaling pathways for DNSP-11 activity need to be investigated.

The Significance of the DNSPs

Generally, the prodomains of trophic factors have been theorized to only be involved in protein folding and synthesis with no distinct function after cleavage (Ibanez, 2002). Prodomains of the trophic molecules BDNF and NGF modulate signaling activity but primarily in conjunction with the mature protein (Lee et al., 2001; Chen et al., 2005; Teng et al., 2005b). The modulation of dopaminergic function by DNSP-11 is evidence for a potential paradigm shift in the activity of peptides cleaved from the prodomain of trophic factors. Peptides from the prodomains of other neurotrophic factors need to be investigated because of the potential discovery of other peptides with signaling activity.

The primary goal of this dissertation was to determine if the DNSPs provide any physiological effects on the dopaminergic system, and if so determine the potential therapeutic value of those effects. Alteration of dopaminergic function assessed through neurochemical analysis was the primary endpoint of interest in all studies. One of the DNSPs, DNSP-11, has demonstrated positive physiological effects on neurochemical function within the nigrostriatal dopaminergic system. Effects on dopaminergic function have been seen in multiple model systems of dopaminergic dysfunction and the effects show the potential DNSP-11 has for providing the basis for future molecules targeting the enhancement or restoration of dopaminergic function. An eleven amino acid peptide providing robust modulation of the dopaminergic system is completely novel. More importantly, these studies provide the foundation for future work investigating mechanistic and therapeutic aspects of DNSP-11.

Much of the work discussed in this dissertation was compared to previous work with GDNF because of the therapeutic potential demonstrated by GDNF in the laboratory. GDNF demonstrated the most robust and lasting trophic effects on dopaminergic neurons of any known trophic factor, but failed in the clinic because of aspects related to delivery and toxicity. Results obtained during the completion of this dissertation could potentially be used in the development of a

molecule that maintains the trophic function of GDNF but lack some of the detrimental aspects. DNSP-11 has demonstrated the potential to have trophic actions like GDNF, but DNSP-11's size and molecular properties could reduce the negative aspects associated with GDNF. DNSP-11 is easily synthesized and can be readily modified to modulate function. Continued study of DNSP-11 can provide the necessary basis for a molecule that is non-toxic, easily administered, and restores dopaminergic function.

Future Work

In the studies within this dissertation, neurochemical measures were always granted precedence over all other endpoints because dopaminergic function was the primary endpoint of interest. Therefore, the TH immunohistochemical data within this dissertation is not definitive but merely supporting data for the neurochemical studies. Studies within this dissertation have established positive neurochemical effects from DNSP-11 treatment but it will be necessary in the future for TH immunohistochemistry to be the primary endpoint when studying DNSP-11. Studies definitively identifying TH positive neurons after DNSP-11 treatment will distinguish functional enhancement and restoration; thus providing more insight into the actions of DNSP-11.

To establish the value of DNSP-11 as a therapeutic, the areas GDNF was deficient in need to be explored such as the route of administration and toxicity. The small size of DNSP-11 relative to other trophic factors provides the potential for a molecule that could be delivered through intranasal or intravenous delivery making it a much more widely used and safe intervention. Also, the size could allow for larger dispersion upon delivery thus permitting DNSP-11 to affect dopaminergic function in an increased neuronal population. Exploration of DNSP-11's toxicity will also be necessary in the future to continue the progression towards use as a therapeutic.

The rat model systems used in this dissertation allowed the exploration and discovery of DNSP-11's effects but have not always been effective predictors of function in humans. Testing DNSP-11's function in animals more closely related to humans, such as non-human primates (NHP), will be

necessary to translate DNSP-11 into a therapeutic. The larger size and complexity of the NHP brain will allow aspects of distribution and delivery to be more thoroughly addressed. Future studies of DNSP-11 in NHP models of dopaminergic dysfunction will determine if DNSP-11 can modulate and restore dopaminergic function without adverse effects. Studying DNSP-11 in the NHP will provide insight into DNSP-11's action within the primate brain and its ability to modulate dopaminergic function.

Final Thoughts

The study of DNSP-11's potential as a therapeutic has the goal of developing a therapeutic for diseases related to dopaminergic dysfunction such as PD. The ambition of the author and the many collaborators studying DNSP-11 is the development of a treatment for PD that would allow the restoration and enhancement of dopaminergic function. Restoration of dopaminergic function could replace or increase the effectiveness of the chemical replacement therapies currently available. DNSP-11 has the potential to restore dopaminergic function and in turn restore the quality of life to individuals suffering from PD but reaching that potential will require continued exploration and understanding.

Appendix: Isoflurane Affects Evoked Glutamate Release

Isoflurane anesthesia was associated in the modulation of neurotransmitter function in [Chapter 4](). DA neurochemical release was potentially altered by anesthesia through interactions with the DA autoreceptor (Tsukada et al., 1999). To further explore isoflurane-mediated effects on neurochemical release, another prominent striatal neurotransmitter was examined, glutamate. Previous work in our lab has focused on anesthetic interactions with neurotransmitters in an effort to design experiments and interpret data. Urethane, an injectable anesthetic, has been extensively used in anesthetized recording preparations but recently was implicated in reducing the basal extracellular concentrations of glutamate relative to the awake animal and the isoflurane anesthetized animal (Rutherford et al., 2007; Stephens, 2009). Isoflurane was considered as the primary anesthetic to replace urethane in anesthetized preparations for neurochemical recordings. Therefore, isoflurane's effects on neurochemistry needed to be investigated because it has been implicated in neurochemical modulation *in vitro.* Isoflurane has been associated with increases in the basal extracellular concentration of glutamate in brain slices (Eilers and Bickler, 1996). Also, in brain slices, isoflurane reduces excitatory postsynaptic potentials and reduces the glutamate content of vesicles (Berg-Johnsen and Langmoen, 1992). Isoflurane's effects on glutamatergic function have been principally observed in slice and primary cell culture preparations. *In vivo* recording techniques allow for the measurement of stimulus-evoked glutamate release and basal glutamate concentrations in an isoflurane-anesthetized rat that maintains all of the glutamatergic inputs. The intact rat can provide a better understanding of the effects of isoflurane on glutamate release and, therefore, will allow insight into future study designs and interpretation of previous results.

Measurement of glutamate in isoflurane (2 - 3%) anesthetized rats used a ceramic microelectrode array (MEA) with four platinum recording sites. All four recording sites were electroplated with *m*-phenylenediamine, creating a size exclusion layer (A.1). The size exclusion layer allows only small molecules, such as hydrogen peroxide, to reach the platinum recording sites. Two of the recording

sites were also coated with glutamate oxidase (GluOx), which reacted with glutamate to produce hydrogen peroxide and α-ketoglutarate while the other two sites did not have a GluOx coating (Burmeister and Gerhardt, 2003). The hydrogen peroxide molecule acts as a reporter molecule for glutamate and is oxidized on the platinum recording sites by applying a constant potential of +0.7V versus an Ag Ag/Cl reference electrode. The hydrogen peroxide molecule releases two electrons that are measured as current and subsequently digitized and amplified by the Fast 16 Mark II Electrochemical Recording System (Quanteon, Nicholasville, KY). Therefore, the GluOx-coated sites measure glutamate release while the other two sites only measure background current. The difference between the two pairs provides a noise-free, specific glutamate signal. MEAs were calibrated before the experiment as in (Burmeister et al., 2002; Day et al., 2006). The described electrochemical recording technique was used to measure striatal glutamate basal concentrations and K^+ evoked concentrations. The isotonic 70 mM K^+ solution was delivered by a pipette affixed to the recording electrode.

Glutamate release under urethane anesthesia was reproducible every 1-2 minutes and agreed with previous results (Burmeister et al., 2002). Glutamate release peaks with amplitude of about 30 µM were observed when 100 nL of 70 mM K^+ was applied while baseline concentrations were below 1 µM (Figure A.2). Meanwhile, glutamate release under isoflurane did not produce consistent glutamate signals with a drastic reduction in glutamate release after the initial event. The initial evoked glutamate release event under isoflurane was also decreased relative to stimulated releases under urethane. Basal concentrations of glutamate were elevated under isoflurane relative to the concentration observed using urethane (Figure A.3). Additional time (~10 minutes) between evoked releases did not allow recovery of the glutamate signal, and increased K^+ delivery up to 300 nL did not return evoked glutamate signals to levels corresponding to the initial release.

The initial decline in evoked glutamate release could be related to a decrease in the excitability of glutamatergic neurons in isoflurane-anesthetized

animals. A reduction in the excitability of glutamatergic neurons could be caused by membrane hyperpolarization making fewer neurons responsive to stimulation with K+. The decline in excitatory post-synaptic potentials demonstrated by (MacIver et al., 1996; Larsen and Langmoen, 1998) is evidence of the potential modulation of presynaptic glutamate release. Previous studies showed evidence of a modulation of glutamate packaging under isoflurane, which could cause the observed lack of reproducible glutamate release (Berg-Johnsen and Langmoen, 1992).

Another component of glutamate regulation is the glutamate transporter, which is located on glial cells and is responsible for the removal of glutamate from the synapse. Isoflurane has demonstrated effects on glutamate transporters and the increased levels of basal glutamate could be related to a reduction in the function of the glutamate transporters (Do et al., 2002). All glutamatergic alterations could be the result of direct effects of isoflurane on components of glutamatergic signaling, but they could also be related to the activation of the group II metabotropic glutamate receptors (Harrison et al., 2008; Raiteri, 2008). Group II metabotropic glutamate receptors are presynaptic autoreceptors that negatively regulate glutamate signaling. Increased activation of the Group II metabotropic glutamate receptors by isoflurane could reduce the evoked glutamate signals similarly to the changes seen in release and basal concentrations (Spooren et al., 2003).

Glutamate release and basal concentrations under urethane and isoflurane depict glutamate function in different manners. Both isoflurane and urethane anesthetics potentially alter releasable and basal glutamate concentrations. The differences in glutamate release under the two anesthetics highlight the necessity for investigating glutamate neurochemical signaling in awake animals to exclude the effects of anesthesia.
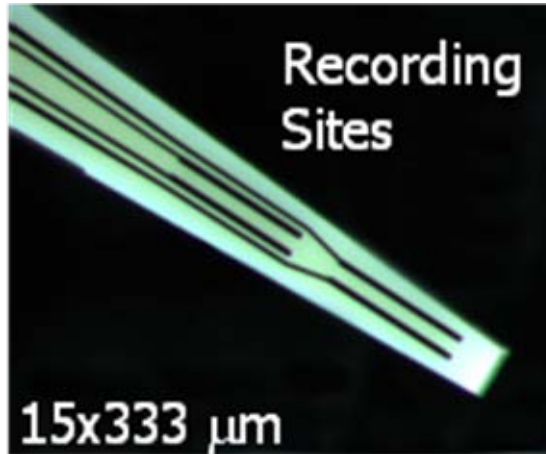
Figure A.1 Microelectrode Array

A photograph of the tip of the microelectrode array used for recording glutamate. An exclusion layer of *m*-phenylenediamine is electroplated on all four sites and the bottom pair receives a coating of glutamate oxidase. The glutamate oxidase allows the microelectrode to be sensitive to glutamate, while the *m*-phenylenediamine is size exclusion layer and increases selectivity for recording glutamate.
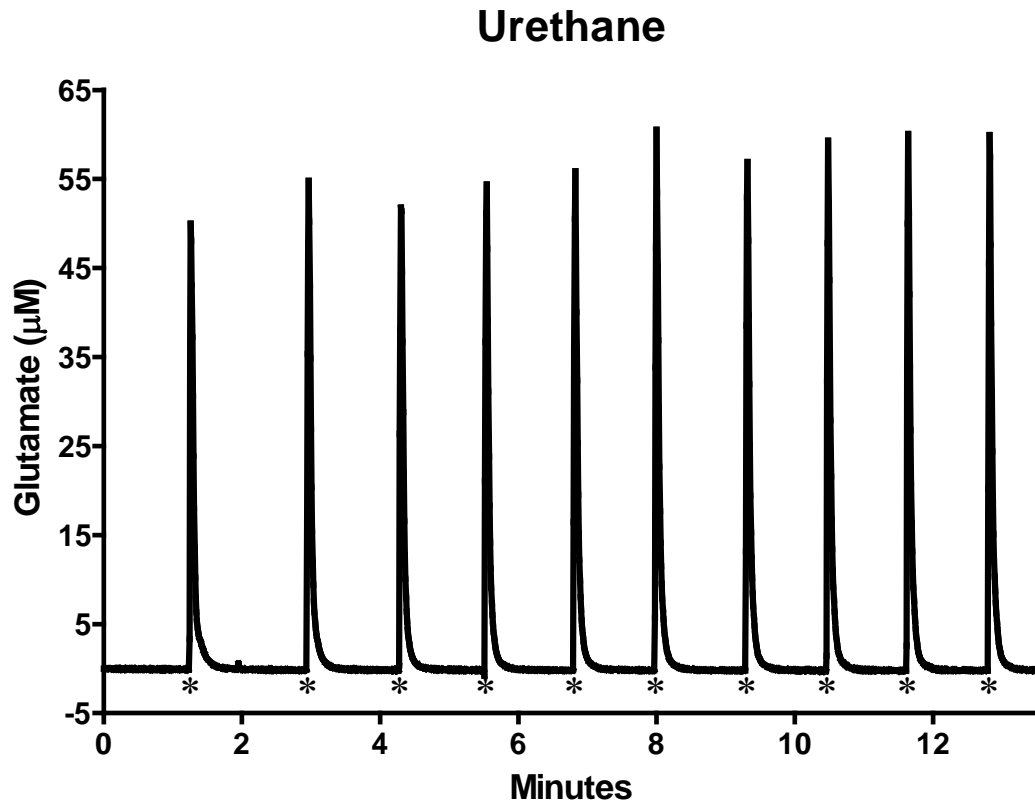
Urethane

Figure A.2 Representative Trace of K⁺-evoked Glutamate Release under Urethane Anesthesia

Representative traces of glutamate signals produced by K⁺-evoked release in the striatum of a urethane-anesthetized rat. The application of the K⁺ solution is marked by asterisks. Reproducible peaks were elicited by K⁺ with approximately 1.5 minutes between release events.

Isoflurane
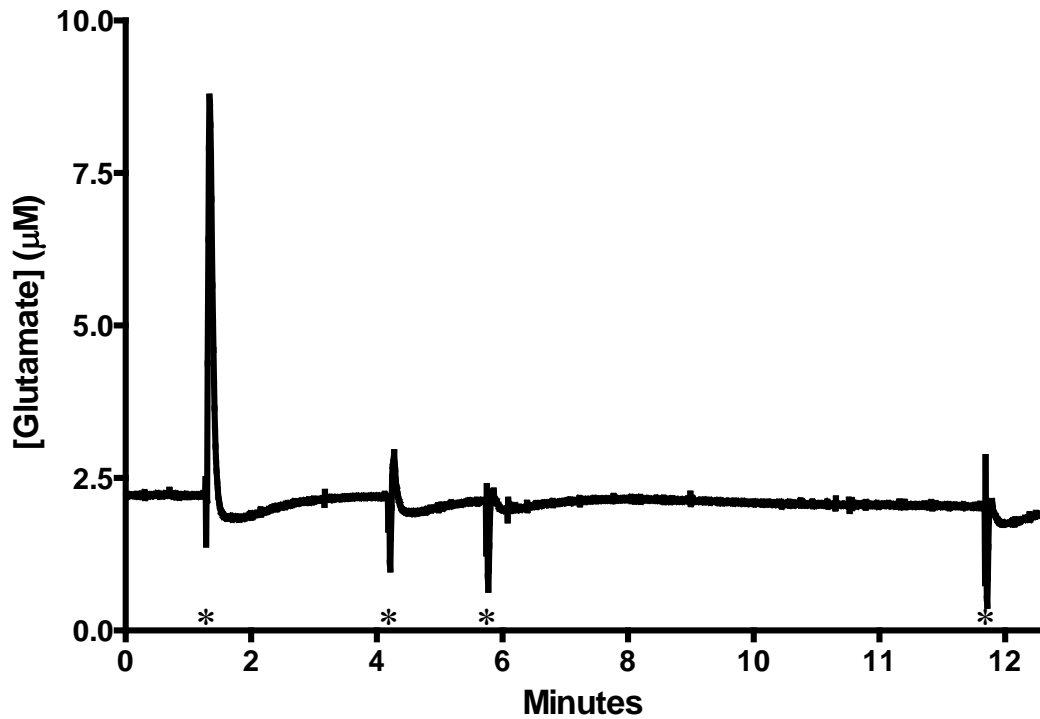
Figure A.3 Representative Trace of K⁺-evoked Glutamate Release under Isoflurane Anesthesia

Representative traces of glutamate signals produced by K^+-evoked release in the striatum of an isoflurane-anesthetized rat. The applications of the K^+ solutions are marked by asterisks. Increased basal glutamate concentrations, decreased initial response to K^+ and a lack of reproducibility was observed while using the isoflurane anesthesia.

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VITA

Name: Joshua L. Fuqua

Birthdate: February 4th, 1983

Birthplace: Louisville, KY

Education

University of Kentucky Ph.D. Candidate   May 2005 – Present

Kentucky Wesleyan College

Owensboro, KY

Bachelor of Science   May 2005

Major: Biology

Minor: Chemistry

Christian Academy of Louisville

Louisville, KY

      High School Diploma   May 2001

Professional Positions Held

Graduate Student Representative   May 2008 – May 2009

Center for Sensor Technology   May 2005 – May 2009
Training Course Instructor

Large Scale Biology Lab Assistant   August 2004 – July 2005

Kentucky Biomedical Research   September 2003 – July 2004
Infrastructure Network Fellowship   June 2003 – September 2003

Maize Database Curator   September 2003 – July 2004

Scholastic and Professional Honors

| Therapeutic Strategies for | January 2007 – June 2007, |
| Neurodegeneration Training Grant | January 2008 – January 2009 |

Lyman T. Johnson Fellowship　　　　　　August 2007 – May 2007

James Graham Brown Scholar　　　　　　August 2001 – May 2005

Professional Publications

Journal Articles

1. Antiparkinsonian Actions of a GDNF Propeptide

Public Library of Science: One　　　　(Submitted December 2009)

Luke H. Bradley, **Joshua L. Fuqua**, April Richardson, Kristen A. Kelps, Jadwiga Turchan-Cholewo, Yi Ai, John D. Glass, Xiuquan He, Zhiming Zhang, Richard Grondin, Peter Huettl, O. Meagan Littrell, Francois Pomerleau, Don M. Gash & Greg A. Gerhardt

2. Neurochemical Effects of DNSP-11 on the Nigrostriatal Pathway in Normal and Dopaminergic Depleted F344 Rats (In Preparation)

Joshua L. Fuqua, O. Meagan Littrell, Peter Huettl, Francois Pomerleau, Greg A. Gerhardt

Abstracts

1. **J.L. Fuqua**, R. Kelly Dawe (University of Georgia), and Evelyn N. Hiatt. Mapping the *smd3* Mutation of Maize Meitotic Drive. Kentucky Academy of Science, Bowling Green, KY, September 2003.

2. G.A. Gerhardt, E. Rutherford, K. Hascup, J.E. Quintero, P. Talauliker, T. Currier Thomas, M. L. Stephens, **J.L. Fuqua**, T. Coates, J. J. Burmeister, F. Pomerleau, P. Huettl. Second-by-Second Measurements of L -Glutamate and Other Neurochemicals in the CNS of Animal Models and Humans. 12th Institute of Biological Engineering Meeting. St. Louis, Missouri, March 2007.

3. **J.L. Fuqua**, M. Lundblad, P. Huettl, F. Pomerleau, G.A. Gerhardt. Behavioral and Neurochemical Effects of DNSP-11, a GDNF Related Peptide, on a Rat Model of Dopaminergic Dysfunction. Spring Neuroscience Day - Bluegrass Chapter for the Society of Neuroscience, Lexington, KY, March 12th 2008.

4. O.M. Littrell, **J.L. Fuqua**, E.R. Hascup, P. Huettl, F. Pomerleau, G.A. Gerhardt. I*n vivo* Effects of a Novel Dopamine Neuron Stimulating Peptide (DNSP-11). Spring Neuroscience Day - Bluegrass Chapter for the Society of Neuroscience. Lexington, Kentucky, March 2008.

5. **J.L. Fuqua**, M. Lundblad, S.P. Surgener, P. Huettl, F. Pomerleau, G.A. Gerhardt. *In Vivo* Neurochemical and Behavioral Effects of GDNF Related Peptides. Monitoring Molecules in Neuroscience. 12th International Conference on *In Vivo* Methods. Vancouver, British Columbia, Canada, August 2008.

6. O.M. Littrell, **J.L. Fuqua**, A. Richardson, E.R. Hascup, F. Pomerleau, P. Huettl, D.M. Gash, and G.A. Gerhardt. Characterization of the effects of Dopamine Neuron Stimulating Peptide (DNSP-11) on Dopamine Neuron Function. Monitoring Molecules in Neuroscience. 12th International Conference on *In Vivo* Methods. Vancouver, British Columbia, Canada, August 2008.

7**. J.L. Fuqua**, M. Lundblad, P. Huettl, F. Pomerleau, and G.A. Gerhardt. "Behavioral and Neurochemical Characterization of DNSP-11, a GDNF Related Peptide, on a Rat Model of Dopaminergic Dysfunction." Bluegrass Society for Neuroscience. Lexington, KY. March 2009.

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