2013

EFFECTS OF INTRANASALLY ADMINISTERED DNSP-11 ON THE CENTRAL DOPAMINE SYSTEM OF NORMAL AND PARKINSONIAN FISCHER 344 RATS

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EFFECTS OF INTRANASALLY ADMINISTERED DNSP-11
ON THE CENTRAL DOPAMINE SYSTEM
OF NORMAL AND PARKINSONIAN FISCHER 344 RATS

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
James William Hendry Sonne
Lexington, Kentucky

Director: Don Marshall Gash, Ph.D., Professor of Anatomy and Neurobiology
Lexington, Kentucky
2013

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ABSTRACT OF DISSERTATION

EFFECTS OF INTRANASALLY ADMINISTERED DNPSP-11 ON THE CENTRAL DOPAMINE SYSTEM OF NORMAL AND PARKINSONIAN FISCHER 344 RATS

Due to the blood-brain barrier, delivery of many drugs to the brain has required intracranial surgery which is prone to complication. Here we show that Dopamine Neuron Stimulating Peptide 11 (DNSP-11), following non-invasive intranasal administration, protects dopaminergic neurons from a lesion model of Parkinson's disease in the rat. A significant and dose-dependent increase in an index of dopamine turnover (the ratio of DOPAC to dopamine) was observed in the striatum of normal young adult Fischer 344 rats by whole-tissue neurochemistry compared to vehicle administered controls.

Among animals challenged with a moderate, unilateral 6-hydroxydopamine (6-OHDA) lesion of the substantia nigra, those treated repeatedly with intranasally administered DNSP-11 exhibited greater numbers of tyrosine hydroxylase (TH) positive dopaminergic neuronal cell bodies in the substantia nigra and greater TH+ fiber density in the striatum when compared to animals treated intranasally with vehicle only or a scrambled version of the DNSP-11 sequence. Lesioned animals that received intranasal DNSP-11 treatment did not exhibit abnormal, apomorphine-induced rotation behavior, contrasted with animals that received only vehicle or scrambled peptide that did exhibit significantly greater rotation behavior.

In addition, the endogenous expression of DNSP-11 from the pro-region of GDNF was investigated by immunohistochemistry with a custom, polyclonal antibody. Signal from the DNSP-11 antibody was found to be differentially localized from the mature GDNF protein both spatially and temporally. While DNSP-11-like immunoreactivity extensively colocalizes with GDNF immunoreactivity at post-natal day 10, the day of maximal GDNF expression, DNSP-11-like signal was found to be present in the 3 month old rat brain with signal in the substantia nigra, ventral thalamic nucleus, dentate gyrus of the hippocampus, with the strongest signal observed in the locus ceruleus where GDNF is not expressed. Results from immunoprecipitation of brain homogenate were not consistent with the synthetic, amidated 11 amino-acid rat DNSP-11 sequence. However, binding patterns in the literature of NPY, the only
homologous sequence present in the CNS, do not recapitulate the immunoreactive patterns observed for the DNSP-11 signal.

This study provides evidence for a potential easy-to-administer intranasal therapeutic using the DNSP-11 peptide for protection from a 6-OHDA lesion rat model of Parkinson’s disease.

KEYWORDS: Parkinson’s disease, GDNF, 6-OHDA, aging, non-invasive

James William Hendry Sonne

May 3rd, 2013
EFFECTS OF INTRANASALLY ADMINISTERED DNSP-11
ON THE CENTRAL DOPAMINE SYSTEM
OF NORMAL AND PARKINSONIAN FISCHER 344 RATS

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May 3rd, 2013
For my grandparents, Jody Tharp and Lloyd Gould Hendry
ACKNOWLEDGEMENTS

I am truly grateful for the help and invaluable assistance I have received, without which this work would not have been feasible. My success is truly not my own. First and foremost, I am indebted to my mentor, Don Marshall Gash, Ph.D., for his support, faith and confidence in me, as well as his friendship. Before beginning my endeavors, when I envisioned a translational research laboratory, it was the goals and motivations that he shares which were the core of my vision. It is difficult to imagine how I can take a step upward from his laboratory.

My committee members have provided excellent guidance, and I appreciate them providing me with resources and taking the time out of their productive schedules to support my progress. They are Michael Bardo, Ph.D., Luke H. Bradley, Ph.D., Wayne A. Cass, Ph.D., James Geddes, Ph.D., Greg A. Gerhardt, Ph.D., Zhiming Zhang, M.D., my outside examiner John T. Slevin, M.D., and Susan Barron, Ph.D. for acting as a substitute committee member for my defense. I would like to especially thank Yi Ai, M.D., Wayne Cass, Ph.D., Richard Grondin, Ph.D., and Zhiming Zhang, M.D. They are fantastic investigators and sincere and caring people who have provided technical assistance and a deep interest in my development as a scientist. I would also like to thank the teaching faculty, especially April D. Richardson-Hatcher, Ph.D. and Bruce E. Maley, Ph.D., for helping me to become a better instructor and exposing me to the joy of helping students succeed.

The members of Dr. Gash’s laboratory and his collaborating laboratories have been more than simply invaluable resources, but true friends that I know I can trust. My fellow denizens of the laboratory have been tremendous in their technical assistance and comic relief, especially Ramsey Edwards, M.B.A., Eric Forman, Hamed Haghnazar, M.P.H., April Evans, Ryan Weeks, Daisy Ramos, Ofelia Meagan Littrell, Ph.D. and Jennifer Moorehead. Most of all, Mallory J. Stenslik, M.S. has provided countless hours of laughs and teamwork. These people have kept me going during tough times.
I am indebted to my grandparents, Jody T. and Lloyd G. Hendry, J.D., for teaching me so much about life. They instilled in me the meaning of hard work, devotion, and the importance of taking joy in a job well done, and taught me that without these things success is not possible. I believe that every act they performed was directed at making life better for their grandchildren. Their way of looking forward is what made them successful, and it has influenced my life beyond measure. They are my role models in every aspect of life, while my parents, Mary C. H. Sonne, J.D., and Kenneth G. Sonne, Jr., have provided the support to allow me to pursue my dreams wherever they lead.

Above all of these, I owe my wife, Stephanie Lynn Sonne, C.P.A., everything. During my long hours and tribulations, she has stood by me, supporting me when I needed it the most. Without her love, I would be nothing.

These people have been my fuel, the oil in my engine, and the tread on my tires. My successes and accomplishments are because of them, while my missteps are purely mine own.
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<th>Definition</th>
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<tr>
<td>AP</td>
<td>antero-posterior</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BEP</td>
<td>rat DNSP-11 (see rDNSP-11), brain excitatory protein</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-o-methyltransferase</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DNSP-11</td>
<td>human dopamine neuron stimulating peptide -11 amino acids</td>
</tr>
<tr>
<td>hDNSP-11</td>
<td>human DNSP-11 sequence (see also DNSP-11)</td>
</tr>
<tr>
<td>rDNSP-11</td>
<td>rat DNSP-11 sequence</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxy-phenylacetate</td>
</tr>
<tr>
<td>DV</td>
<td>dorso-ventral</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDNF</td>
<td>glia cell-line derived neurotrophic factor</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>5-hydroxyindoleacetic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>IC</td>
<td>intracranial</td>
</tr>
<tr>
<td>IN</td>
<td>intranasal</td>
</tr>
<tr>
<td>LC</td>
<td>see HPLC</td>
</tr>
<tr>
<td>ML</td>
<td>medio-lateral</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectroscopy</td>
</tr>
<tr>
<td>3-MT</td>
<td>3-methoxytyramine</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline (see also NE)</td>
</tr>
<tr>
<td>NE</td>
<td>norepinephrine (see also NA)</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>Scr</td>
<td>scrambled version of the DNSP-11 sequence</td>
</tr>
<tr>
<td>TB</td>
<td>tooth bar</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
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Chapter One: Introduction

Parkinson’s Disease

A History of Parkinson’s Disease

Parkinson’s Disease (PD) was characterized in 1817 through the observations of James Parkinson in the field of apothecary surgery. His work, An Essay on the Shaking Palsy, characterized through six case studies a motor disorder of the extremities which today affects an estimated 1% of the population over the age of 65 (Tanner, 1992; de Lau and Breteler, 2006; NINDS, 2006), and its prevalence is expected to double every 25 years due to the increased average age of the population (Van Den Eeden et al., 2003; Dorsey et al., 2007; Wright Willis et al., 2010; Collier et al., 2011). It was not until sixty years after Parkinson produced his Essay that the disease began to carry his name when Jean Martin Charcot distinguished the shaking palsy described by Parkinson as “Parkinson’s disease”. Parkinson, in the final chapter of his Essay, describes the potential for a cure, saying that, although there …

“exists no countervailing remedie […] there appears to be sufficient reason for hoping that some remedial process may ere long be discovered, by which, at least, the progress of the disease may be stopped.”

Nearly 200 years later, a cure for the disease described by Parkinson is still not available; but, as Parkinson concludes his work, there is reason for hoping because:

“To such researches the healing art is already much indebted for the enlargement of its powers of lessening the evils of suffering humanity. Little is the public aware of the obligations it owes to those who, led by professional ardour, and the dictates of duty, have devoted themselves to these pursuits, under circumstances most unpleasant and forbidding.”
Signs and Symptoms

Although much has changed between 1817 and today with regard to research techniques, the signs and symptoms of Parkinson’s disease have remained constant. Primary complaints focus on the cardinal motor symptoms: resting tremor, bradykinesia or akinesia, “cogwheel” rigidity, shuffling gait and postural instability. Secondary symptoms can include insomnia, urinary and fecal incontinence, and orthostatic hypotension, disorders of olfaction, cognitive decline, mood disorders and dementia. Indeed, as many as 47% of Parkinson’s disease patients have symptoms of depression that arose prior to the cardinal motor signs (Kummer and Teixeira, 2009; Lohle et al., 2009). In addition, a plethora of secondary motor symptoms, although less common, can be present especially in later stages, including muffled speech, loss of facial expression, small hand-writing and difficulty swallowing (Jankovic, 2008).

Parkinson’s disease, as a progressive neurodegenerative disease, primarily affects the aged population with a mean age of onset of 60 years with the prevalence rising with age; but between 5-10% of Parkinson’s disease cases occur in individuals between the ages of 20 and 50 years (NINDS, 2006). As the average age of the population rises, it is expected that prevalence will also increase.

Some estimates of the cost of the disease to the population of the United States range from $5.6 billion (NINDS, 2006) to as high as $23 billion (Huse et al., 2005; Findley, 2007). This includes not only the estimated cost of pharmaceutical therapies of $10,000 per individual per year, but also surgical treatment options, the cost of nursing home stays and the loss of economically productive years and quality of life of both the patients and his or her family.

Pathogenesis of Parkinson’s Disease

There are two forms of Parkinson’s disease: familial and idiopathic (or sporadic). Familial Parkinson’s disease accounts for approximately 5% of all known cases of PD (NINDS, 2006) and always involves an inherited genetic mutation. Several target genes in these population samples have been identified and are the subject of study, especially the genes PINK1 (Valente et al., 2004),
PARKIN (Kitada et al., 1998), SNCA (Abou-Sleiman et al., 2006a), and LRRK2 (Mata et al., 2006). Many of these genes are translated to proteins that are associated with either mitochondrial function (Abou-Sleiman et al., 2006b) or proteasomal degradation (Dawson and Dawson, 2003; Bender et al., 2006; Mizuno et al., 2006; Bueler, 2009). Familial Parkinson’s disease is also associated with early onset of the disease, before the age of 40 years.

However, idiopathic Parkinson’s disease comprises the majority of cases. Literally meaning that the cause of the disease is unknown, “idiopathic” or sporadic Parkinson’s disease has in fact been linked to several factors, both environmental and genetic. One especially potent non-genetic inducer that has been identified is MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Langston and Ballard, 1983; Langston et al., 1984). In 1976, illicit users of MPPP (desmethylprodine), an opioid analgesic, were subjected to high levels of impurities in the form of MPTP, a potent mitochondrial toxin, resulting in the development of parkinsonian symptoms. One of these patients subsequently died of unrelated causes, and an autopsy revealed extensive loss of dopaminergic neurons of the substantia nigra (Fahn, 1996). This finding helped lend credence to earlier studies showing a deficit of the neurochemical dopamine in both the striatum and substantia nigra (Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1963). MPTP provided additional evidence for the role of mitochondrial dysfunction of dopaminergic neurons in Parkinson’s disease.

Indeed, it has been determined that a loss in excess of approximately 60% of dopaminergic neurons of the substantia nigra results in the onset of the characteristically parkinsonian motor signs (Braak et al., 2006). This loss correlates to an approximate 80% reduction in dopamine content (Gibb and Lees, 1991). As determined from familial cases of Parkinson’s disease, it is postulated that an accumulation of mitochondrial-associated mutations and degradations throughout life ultimately are the causative agent of sporadic parkinsonism (Bender et al., 2006). The cause of this accumulation can be varied and multimodal.
This life-long accumulation can be attributed to environmental elements, typically pesticides and herbicides including rotenone and paraquat (Tanner et al., 2011), and “Agent Orange” (IOM, 2009), and industrial agents such as trichloroethylene (Gash et al., 2008), but can also include traumatic damage from head injuries (Davie et al., 1995; Bower et al., 2003; Sauerbeck et al., 2011). Many of these insults induce a concomitant inflammatory response within the CNS, and in fact neuroinflammation has been implicated in many neurodegenerative diseases including Parkinson’s disease (Hunter et al., 2007; Whitton, 2007; Choi D. Y. et al., 2009). It is even thought that reactive oxygen species created during normal dopamine metabolism can be a cause of progressive dopaminergic neurodegeneration (Stokes et al., 1999).

A defining histological characteristic of the disease is the presence of “Lewy Bodies”, or protein aggregates commonly observable within the cell bodies of catecholaminergic neurons (Figure 1.1). Frederick Henry Lewey in 1912 was the first to observe these eosinophilic spheroidal inclusions encircled by fibrils in the CNS of parkinsonian patients. A major component of Lewy Bodies is the α-synuclein protein implicated in familial cases of Parkinson’s disease; but α-synuclein containing Lewy Bodies are also present in several other diseases of the CNS including Dementia with Lewy Bodies (or DLB), Multiple Systems Atrophy (MSA), and corticobasal degeneration, all of which present with some, but not all, of the symptoms of Parkinson’s disease. Some researchers believe these protein aggregates are the causative element in Parkinson’s disease due to their high correlation to areas of greatest neuronal loss (Gibb and Lees, 1989). However, because of the wide array of neurological disorders characterized by the presence of Lewy Bodies, and because Lewy Bodies are also present in asymptomatic individuals, others hypothesize that these aggregates may be a general protective mechanism against molecular damage to cellular enzymes, serving as a kind of “waste dump” when proteasomal degradation processes are insufficient (Burke et al., 2008), thus appearing as a marker for neuronal distress and not acting as the direct cause of the dysfunction and death itself.
Not all of the histological and neurochemical damage occurring in Parkinson’s disease is isolated to the substantia nigra. Other catecholaminergic centers of the brain are implicated in Parkinson’s disease as well. In particular, degeneration of the locus ceruleus (Figure 1.2) is thought to be the causative element in many of the non-motor symptoms observed in parkinsonian patients, especially insomnia and dementia. The locus ceruleus, literally the “blue spot” due to its high neuromelanin content, located in the dorsal tegmental pontine brainstem, serves as the norepinephrinergic center of the brain and acts as a “gain setting” nucleus for cognitive function. It sends projections throughout the CNS, influencing cortical, subcortical, cerebellar, and brainstem circuitry and is implicated in stress-response and attention focusing, memory, sleep-wake cycles, and even postural stability. The locus ceruleus is self-modulatory and may also play a role in compensatory mechanisms during pre-symptomatic parkinsonism (Rommelfanger and Weinshenker, 2007), possibly explaining the insidious loss of dopaminergic neurons without noticed behavioral deficits. In addition to the locus ceruleus, the dorsal nucleus of the vagus nerve is implicated in parkinsonian symptoms including incontinence. Due to the many systems typically affected in Parkinson’s disease, a “dual-hit” hypothesis of the etiology of sporadic Parkinson’s disease has been proposed, controversially suggesting possible dual environmental vectors for entry to the CNS via the olfactory system and spinal cord (Hawkes et al., 2007). The dopamine system, however, remains the single most prevalent and consistent area of degeneration observed in parkinsonism, and is responsible for the most frequent symptomatic complaints in regards to the disease. As such, the majority of research focuses on dopamine and the nigrostriatal system as the key to unlocking an intervention for Parkinson’s disease.

The Discovery of Dopamine

Dopamine (DA) was first synthesized in 1910 by George Barger and James Ewens, but this monoamine was considered merely to be a precursor in the natural synthesis of norepinephrine (NE) (Blaschko, 1942). It was not recognized to be a distinct catecholamine neurotransmitter in its own right until
1958, after Arvid Carlsson and Nils-Åke Hillarp in Sweden elucidated the role of the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA) as a reserpine antagonist (Carlsson et al., 1957; Abbott, 2007), followed closely by the discovery of dopamine in the central nervous system (CNS) of the mammalian brain (Montagu, 1957) and later in the human brain (Sano et al., 1959).

**Dopamine Biosynthesis and Signaling**

Dopamine is synthesized along the same enzymatic sequence as, and is a precursor compound for, norepinephrine (NE) (Figure 1.3). The first, and rate-limiting, step of dopamine synthesis is the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH) (Carlsson et al., 1972; Haavik and Toska, 1998). This step requires molecular oxygen (O₂), an iron atom co-factor (Fe²⁺) and tetrahydro-biopterin (BH₄ or THB) in order to add a hydroxyl group to the aromatic ring. From here, L-DOPA is converted to dopamine by the enzyme aromatic L-amino acid decarboxylase, which removes the carboxyl group from L-DOPA. Dopamine is then packaged into synaptic vesicles by vesicular monoamine transporter 2 (VMAT2) for later release. L-tyrosine, consumed in abundance in a normal diet, readily crosses the blood-brain barrier, as does L-DOPA, but dopamine cannot and thus its utility is spatially restricted. However, if L-tyrosine levels are low, L-phenylalanine may be converted into L-tyrosine by phenylalanine hydroxylase.

After release into the synaptic terminal, dopamine interacts with a range of receptors on the pre- and post-synaptic terminal, causing either neuronal excitation or inhibition in the target neuron. Two entire families of dopamine receptors have been identified, composed of as many as five different isoforms, each of which affect different intracellular signaling pathways. These two families of dopamine receptors, D₁ and D₂, are both G-protein coupled receptors, but D₁ receptors result in the depolarization of the neuron on which they are expressed, whereas D₂ receptors inhibit neuronal firing.

Once in the synaptic cleft, dopamine may either be transported back into the pre-synaptic neuron via dopamine transporters (DAT) for repackaging or remain in the extracellular space to be taken up by glial cells or metabolized on
cellular membranes. Dopamine may be metabolized extraneuronally by catechol-o-methyltransferase (COMT) to 3-methoxytyramine (3-MT) while monoamine oxidase-B (MAO-B) will rapidly metabolize 3-MT to homovanillic acid (HVA) (Tank et al., 1981; Kopin, 1985; Shih et al., 1999); or, inside the cytoplasm, MAO and aldehyde dehydrogenase (ALDH) work together to convert dopamine to DOPAC (Iversen, 2009).

As a result of this complex sequence, the modulation of dopamine can occur not only at the level of the whole neuron, its projections, and neuronal circuitry across the nervous system, but also during synthesis of dopamine (transcriptional, translational, and post-translational regulation), synaptosomal packaging (regulation of VMAT, transport of vesicle to synapse), dopamine release (neuronal depolarization, calcium signaling, vesicle fusion), and via reuptake and metabolism through regulation of the respective enzymes and their spatial localization relative to their substrate. Although the cause of parkinsonism is well known: a deficit of dopamine in the nigrostriatal pathway, the underlying mechanisms for this dysfunction can be diverse.

**Dopaminergic Pathways**

The study of dopamine has led to the discovery of its central role in reward-seeking behavior, wherein dopamine transmission becomes markedly increased. This has resulted in research of the role of dopamine in a variety of psychiatric conditions ranging from substance abuse, schizophrenia to attention deficit disorder (Iversen, 2009). These conditions all involve disorders in the mesolimbic and mesocortical pathways of dopamine innervation. The mesolimbic pathway originates in the dopaminergic ventral tegmental area (VTA) and substantia nigra (SN), which project to the nucleus accumbens and amygdala, implicated in reward-stimulation and the fear-response. The mesocortical pathway, while originating in these same anatomical nuclei, innervate the prefrontral cortex, responsible for executive functioning, attention, and complex planning. A third pathway, the tuberoinfundibular pathway, is mainly responsible for the regulation of the neuroendocrine prolactin from the anterior pituitary gland, which is well known for its role as a lactation inducer, but
also has lesser roles in water/salt homeostasis and immuno-system and cell-cycle regulation.

The nigrostriatal pathway is the pathway involved in the motor deficits observed in Parkinson’s disease. This pathway is comprised of dopaminergic neurons that originate in the substantia nigra and project to the striatum via the medial forebrain bundle, forming synapses with several neuronal populations in the putamen, caudate nucleus, GPi and STN. This forms the afferent connections of the substantia nigra to the circuitry involved in motor movement, the basal ganglia.

**Basal Ganglia**

The basal ganglia (or, more appropriately, the basal nuclei) are a collection of nuclei located in the ventral forebrain which have been implicated in voluntary motor control and habitual motor output while also playing a role in cognition and emotional functions (Mink, 2007). The basal ganglia is comprised of the striatum, globus pallidus and subthalamic nucleus, and has its dopaminergic input from the substantia nigra (Figure 1.4). Dysfunctions in this circuitry are core elements of a variety of motor disorders, including Huntington’s disease, hemiballism, Tourette’s syndrome, and obsessive-compulsive disorder (Fix, 2008). The basal ganglia also include the nucleus accumbens, ventral pallidum, and VTA, which play a central role in reward learning, and dysfunction in these nuclei have been implicated in schizophrenia and attention deficit hyperactivity disorder (ADHD).

The striatum (Str), the largest of the basal ganglia nuclei, is the major destination of the dopaminergic neurons of the substantia nigra. Named for its striated appearance due to the prevalence of bands of white matter tracts, the human striatum can be divided into two distinct sections, the caudate nucleus more dorsomedially and the putamen ventrolaterally. The internal capsule and its limbs, extensive bands of white matter axons traveling between the cortex and the pyramids of the medulla, divide these units. The striatum of the rodent differs from that of the human in that the caudate and putamen are not distinguishable due to a poorly defined internal capsule. Instead, the striatum is separated
histologically into “patch” and “matrix” (Desban et al., 1993). The patch is the predominant target for dopaminergic projections, whereas the matrix possesses acetylcholinesterase and is the site of arborization of the cortical acetylcholinergic connections to the striatum (Gerfen, 1992). Classical descriptions of the caudate and putamen suggested different functions, but modern neuroscience attributes those differences to the somatotopic organization of the striatum as a whole, so these observed neuroanatomical differences may be a false distinction (Choi et al., 2012). The more dorsolateral region of the striatum predominantly receives input from the substantia nigra and is involved in motor control, the central region of the striatum is referred to as the associative region, while the ventromedial region is involved in limbic function receiving mainly VTA projections. In fact, this continuum may result in the manifestation of some of the secondary complaints of Parkinson’s disease, including dementia and depression. Medium spiny, inhibitory GABAergic afferent neurons predominate throughout the striatum.

The pallidum, or globus pallidus (GP), is divided into two segments, the internal and external segments. Both of these segments are predominantly GABAergic, and thus inhibitory in effect, but they are constitutively active, tonically inhibiting their targets.

The subthalamic nucleus (STN) is the only excitatory nucleus of the basal ganglia complex and is one of the nuclei that receive tonic inhibition by the globus pallidus (from the external segment).

The substantia nigra can be divided into two parts: pars compacta (SNc) and pars reticulata (SNr). The pars reticulata receives glutamatergic innervation from the STN and sends GABAergic projections to the ventral anterior and ventrolateral nuclei of the thalamus. The pars reticulata also regulates the firing of the pars compacta by sending GABAergic arborizations into this neighboring region. The pars compacta is the dopaminergic center of the basal ganglia and serves as midbrain input of the striatum. The pars reticulata is capable of modulating the firing rate of the pars compacta throughout feedback from the basal ganglia circuitry. The substantia nigra pars compacta, being the source of
the primary dopamine projections to the motor region of the striatum, is the region implicated in the motor symptoms of Parkinson’s disease.

**Direct and Indirect Pathways**

Motor output is modulated by the substantia nigra indirectly, through this complex basal ganglia circuitry (Figure 1.5A) which serves as a “weigh-station” for motor-output. The classical, simplified version of this pathway is described below, but far more interconnections are present than defined here. A more in depth review can be found in the literature (Alexander *et al.*, 1986; Albin *et al.*, 1989; Mink, 2007). The dopaminergic fibers of the substantia nigra synapse in the putamen of the striatum on two different populations of neurons: one expressing the D\(_1\) family and the other expressing the D\(_2\) family of receptors. The D\(_1\)R family of neurons project to the GPi and are referred to as the “direct” pathway. When dopamine is released on to and bound by these D\(_1\)R neurons, the intraneuronal pathways activated result in the excitation of this group of striatal neurons. These “direct” pathway neurons release GABA, inhibiting the GPi (Figure 1.5B).

The population of striatal neurons expressing the D\(_2\)R family, when they bind dopamine, undergo intracellular signaling that ultimately results in the inhibition of this group of neurons. These D\(_2\)R neurons, inhibited in firing when dopamine is bound, send GABAergic projections to the GPe. The GPe in turn sends constitutively active GABAergic projections to the STN, and the STN projects excitatory glutamatergic fibers to the GPi. This is referred to as the “indirect” pathway.

Both pathways ultimately synapse on the GPi, which balances the input from the two pathways, resulting in a net output to the ventral anterior nucleus of the thalamus (VA). The GPi, like the GPe, is constitutively active, tonically releasing GABA into synapses within the VA. In a sense, motor output is normally inhibited by the GPi. As a result, when the input to the GPi from the “direct” pathway is outweighing the input from the “indirect” pathway, the net result is inhibition of the inhibitory GPi. This effectively “disinhibits” motor output.
However, when dopamine is not present or its release has been reduced, as is the case in Parkinson’s disease, the “indirect” pathway begins to outweigh the “direct” pathway (Figure 1.5C). The normal inhibition of the VA by the GPi, and thus the corresponding motor output, becomes even stronger, resulting in poor motor initiation and decreased motor speed in accordance with the observed signs of Parkinson’s disease.

**Rat Models of Parkinson’s Disease**

In order to study neurodegenerative diseases, animal models of the disease state must be developed. Many models have been developed, some using the genetic mutations or non-genetic toxins discussed above, especially rotenone, paraquat, and MPTP (Blesa *et al.*, 2012). However, not every model can perfectly replicate the human disease in the animal. For instance, due to the rat’s tolerance for the MPTP toxin, in contrast with mice and primates, MPTP does not make an effective rat model of parkinsonism.

A widely used and perhaps the oldest model of Parkinson’s disease in the rat, and the model used for the present work, is the unilateral 6-hydroxydopamine (6-OHDA) lesion model. As a catecholaminergic-neurotoxin, 6-OHDA selectively destroys dopaminergic and norepinephrinergic neurons. In order to induce parkinsonism in rats via dopaminergic cell death, 6-OHDA is injected into points along the nigrostriatal pathway in various amounts. Injection into the striatum can produce a partial lesion, while injection into the medial forebrain bundle (MFB) typically produces extensive lesions in the nigrostriatal pathway. Injection directly into the substantia nigra typically produces moderate lesions, leaving the animal with some intact functionality. This theoretically produces a disease state similar to moderate Parkinson’s disease in humans, a time at which point a therapeutic intervention may begin. Also, it is noted that the pattern of cell loss in Parkinson’s disease is mimicked most closely by lesions of the substantia nigra directly (German *et al.*, 1989; Goto *et al.*, 1989; Deumens *et al.*, 2002).

The unilateral nature of this lesion not only is easier for the animal to tolerate, but also provides some interesting behavioral consequences. Because the motor system has a deficit in only one hemisphere, the aggregate
movements of the animal will result in a net rotation. This can be quantified to provide an accurate estimate of the number of neurons lost due to the toxic insult (Perese et al., 1989; Hudson et al., 1993; Deumens et al., 2002), especially with the use of dopamine-system modulating compounds to induce elevated activity. One such compound, apomorphine, increases activity in the animal by acting as a dopamine receptor agonist. As dopaminergic innervation of the striatum is lost, the striatal neurons compensate by increasing the expression of dopamine receptors, inducing a state of super-sensitivity. Apomorphine then binds to this upregulated receptor activity in the denervated hemisphere, producing increased motor output from that hemisphere. This results in the animal rotating contralaterally, “away from” the lesioned hemisphere; or, in other words, the lesioned hemisphere is toward the outside of the imaginary circle formed by the animal’s rotation. Because apomorphine-induced rotation behavior relies on striatal super-sensitization due to a denervation of greater than 90%, it produces a more binary effect: animals tend to either rotate or not to rotate.

Some 6-OHDA lesion models may be considered a progressive model of the disease (Zigmond et al., 1990). Lesions of the substantia nigra by 6-OHDA result in cell death within a week of injection (Wright et al., 2009), but behavioral consequences tend not to stabilize until several weeks after injection (Perese et al., 1989). Although rapid in pace, this progression allows some potential for the study of preventive interventions. 6-Hydroxydopamine achieves its neurotoxicity by way of its ability to act as a dopamine analogue with catecholamine transporters, taking advantage of selective transportation into the cytoplasm. From there, 6-OHDA reacts to form reactive oxygen species including hydrogen peroxide and the quinone form of the compound. In order to selectively target dopaminergic catecholamine neurons, desipramine may be used to inhibit the uptake by norepinephrinergic neurons. Monoamine oxidase can also be administered to prolong the life, and thus enhance the effect of 6-OHDA (Zigmond et al., 1990).
Treatment Options

A variety of treatment options are currently available for Parkinson’s disease, but all are palliative and do not slow the progression of the disease or affect the loss of dopaminergic neurons of the substantia nigra. These treatments can be divided into three categories: dopamine supplementation, dopamine replacement, and surgical intervention.

Dopamine supplementation therapies attempt to increase the natural levels of dopamine in the CNS by a number of ways. The first of these and the “gold” standard of such treatment is the administration of L-DOPA, the immediate precursor in the synthesis of dopamine, commonly combined with carbidopa to reduce peripheral effects by inhibiting DOPA decarboxylase. L-DOPA has the benefit of being able to cross the blood-brain barrier (BBB) and so can be administered systemically in oral pill form. By providing a surfeit of precursor to the remaining dopaminergic neurons, this allows for the ready production of dopamine.

Dopamine levels can also be increased on the opposite end, by inhibiting the break-down of dopamine that has already been synthesized. By inhibiting monoamine oxidase, dopamine that has been released into the synapse can be more readily recycled by the pre-synaptic neuron for later reuse instead of being metabolized to DOPAC.

Elevated levels of dopamine, either in the brain or the periphery, can result in a wide array of side effects. Common neurological effects include disorientation, hallucinations, anxiety, increased libido, and somnolence or narcolepsy. Peripheral side effects can be even more severe, including cardiac arrhythmias, hypotension, respiratory disturbances, gastrointestinal bleeding, nausea and even hair loss. Chronic L-DOPA administration leads to dyskinesia, freezing during motor movement, drug resistance, serotonin depletion, loss of impulse control, deterioration of function and dopamine dysregulation syndrome (Merims and Giladi, 2008). In addition, dopamine supplementation therapies become ineffective in late stages of Parkinson’s disease, when no dopaminergic connections remain that can be supplemented.
Other compounds – such as pramipexole, ropinirole, and apomorphine – function as a dopamine receptor agonist and have the potential to be true dopamine replacement therapy in late stage patients. Due to apomorphine’s highly emetic activity, it is commonly given as a subcutaneous injection. It can enter the CNS and begin affecting the striatum within 20 minutes of injection, and its effects can last for as long as 90 minutes, providing motor function for that period. Due to apomorphine’s injection-based delivery and its relatively short time of effect, it can only provide brief periods of functional recovery and cannot fully ameliorate parkinsonian symptoms. The constant infusion of apomorphine via implantable pump is an available treatment in Europe, but apomorphine is a non-selective DA receptor agonist and produces many of the same side effects as dopamine supplementation therapy. In addition to nausea and vomiting, other effects are also present including increased sweating, agitation, behavioral and mood changes, persistent headache, cardiovascular effects and changes in vision. For these reasons, apomorphine is rarely used as a treatment in the United States, but more selective agonists, such as pramipexole or ropinirole, may be prescribed with different side effect profiles.

Surgical interventions for Parkinson’s disease are varied and some are currently in the developmental phase. Deep-brain stimulation (DBS) relies on the intracranial implantation of electrodes and is used for several disorders, including Parkinson’s disease. A procedure called subcortical lesioning can produce similar effects. These surgeries attempt to upset the balance between the “direct” and “indirect” pathways of the basal ganglia to compensate for the loss of dopaminergic innervation in the direct pathway.

Deep-brain stimulating surgery requires the implantation of the lead, placed in the target brain region, an extension that is located under the skin, and a battery-powered, implanted pulse generator. This system is similar to a pacemaker, providing electrical pulses to the lead or electrode. This electrical pulse is thought to inhibit the brain region in association with the electrode, and thus this system usually targets either the subthalamic nucleus or the internal segment of the globus pallidus for Parkinson’s disease, and the
ventrointermediate nucleus of the thalamus to treat Essential Tremor. Subcortical lesioning is similar in its approach, but its outcome is irreversible.

None of these approaches affect the disease mechanism, nor slow the progression of the disease, and most may have severe side effects or complications. An ideal treatment would be easy to administer without complication, would target the specific brain regions affected producing few side effects, and halt or reverse the progression of the disease. This goal led to the search for neurotrophic factors that could actually affect the disease progression.

**Neurotrophic Factors**

**History**

The groundwork for the discovery of neurotrophic factors was laid in 1948 by Elmer Bueker. In grafting a tumor tissue line from the mouse (known as sarcoma 180) into embryonic chicks, it was discovered that sensory nerve fibers from the nearby dorsal root ganglia, but not motor neurons, invaded the tumor (Bueker, 1948). These results were expanded upon by Rita Levi-Montalcini, who not only compared the results to non-tumorous embryonic tissue implants (Levi-Montalcini and Hamburger, 1951), but later showed that the cause of these neurogenic growths was due to a diffusible nerve growth factor (Levi-Montalcini, 1952; Levi-Montalcini et al., 1954). Stanley Cohen, a member of Levi-Montalcini’s group, identified a purification fraction that elicited this nerve growth promotion (Cohen et al., 1954), but in the process of purification, which required the use of moccasin snake venom to break down the nuclear DNA, discovered that the snake venom itself enhanced this neurogenic activity, leading to the discovery of a 20 kDa protein that would become known as Nerve Growth Factor (NGF) (Cohen and Levi-Montalcini, 1956). This discovery began a conceptual shift resulting in the search for other soluble signaling proteins with neurotrophic effects, and in 1993 a potent neurotrophic factor responsible for the protection and maintenance of dopaminergic neurons was identified (Lin et al., 1993). Purified from the B49 rat glioma cell line, it became known as Glial cell line-Derived Neurotrophic Factor (GDNF).
The Effects of GDNF

Natural cell death is an important element of an organism’s development, which ensures only properly formed and connected neurons remain. Indeed, natural or developmental cell death is responsible for the elimination of 50% or more of the neurons in the CNS (Cowan et al., 1984; Oppenheim, 1991). In order to be spared of this neuronal paring process, a neuron’s projections must interact with its target (Barde, 1989). By releasing neurochemical compounds into a synapse with a receptive neuron, the post-synaptic, target neuron releases a signal to the pre-synaptic neuron that it has received the message. This signal is the neurotrophic factor. This is true of the nigrostriatal pathway, in which the dopamine-sensitive neurons of the striatum release GDNF as confirmation of the dopamine signal from the projections of the substantia nigra. The developmental process of sparing in the nigrostriatal pathway in the rat coincides with maximal GDNF expression levels found in the first post-natal week. The potential of exogenous GDNF administration in an aged or diseased-state is in the capability of GDNF to spare and restore dopaminergic neurons.

To that end, it was demonstrated that the protective effects of GDNF extends to protection from cell toxins, including 1-methyl-4-phenylpyridinium ion (MPP+) in cell culture (Hou et al., 1996), reducing markers of cell death and dysfunction including caspase-3 activation and reactive oxygen species production (Zeng et al., 2006). Exogenous GDNF, administered directly to the substantia nigra in the rodent, also protects dopaminergic neurons from a 6-OHDA lesion (Kearns and Gash, 1995; Kearns et al., 1997), and this treatment corresponds to a preservation of evoked-release of dopamine (Cass and Manning, 1999). Glial cell-line derived neurotrophic factor, when administered to the striatum, also reduced apomorphine-induced rotation behavior four weeks after a unilateral 6-OHDA lesion of the medial forebrain bundle (Hoffer et al., 1994). These and other studies in rodents spurred pre-clinical trials in non-human primate models of Parkinson’s disease. Intracranial injections of GDNF in a MPTP-induced model of Parkinson's disease in non-human primates resulted in significant improvements in parkinsonian symptoms with improved dopamine
levels in the substantia nigra and enhancing dopaminergic fiber and cell size in these animals (Gash et al., 1996; Zhang Z. et al., 1997; Grondin et al., 2002).

**Clinical Trials of GDNF**

Glial cell-line Derived Neurotrophic Factor, delivered intracranially via pump and catheter system, proceeded to clinical trials. Two initial open-label phase I trials of GDNF resulted in a 24% average increase in “off L-DOPA” Unified Parkinson’s Disease Rating Scale (UPDRS) score, and a 39% average increase in “on” UPDRS scores (Gill et al., 2003); and in a different study a 30% bilateral increase in UPDRS “on” and “off” scores (Slevin et al., 2005). However, a double-blind placebo-controlled phase IIa clinical trial did not meet the trial requirements of 25% improvement in UPDRS “off” scores (Sherer et al., 2006).

For a variety of reasons, these results from the phase II trial were controversial. The variability in UPDRS score effect observed between trials was of concern. However, each of the three trials used different catheter designs and infusion parameters which may have contributed to differences in diffusion of GDNF, and resulted in differences in the rate and dose of delivery of GDNF between studies. All of these changes are capable of altering the volume of efficacious delivery (Salvatore et al., 2006; Sherer et al., 2006), contributing to the inter-trial variability. Supporting this explanation are studies revealing variability in the area of GDNF delivery around the catheter tip, and that this diffusion area, although adequate in rhesus macaques, only covers 2 to 9% of the human putamen (Salvatore et al., 2006). Safety concerns also arose when antibodies to GDNF were found in 10% of the patients undergoing the trial; while no adverse effects were observed in the patients (Slevin et al., 2006) the possibility of the neutralization of endogenous GDNF by these antibodies and observed toxicity in the cerebellum of non-human primates prompted the discontinuation of the trial (Gill et al., 2003; Slevin et al., 2005; Lang et al., 2006). Cerebellar Purkinje cell loss was observed in rhesus macaques, at a dose three times as high as in the clinical trials, and after being subjected to three months of withdrawal prior to histological examination (Hovland et al., 2007b). In contrast to that study in non-human primates is MRI data from patients who participated in
the GDNF clinical trials which indicated no cerebellar changes were present, in agreement with clinical motor-function examination (Chebrolu et al., 2006).

Due to these obstacles, including the volume of diffusion of GDNF, its potential side effects, the need for the implantation of the pump and catheter for delivery of GDNF and the patent issues surrounding GDNF, the search for mimetics of GDNF began.

**The Search for Mimetics, Signal and Pro-Proteins**

Mimetics for GDNF, compounds with similar function but with a different structure, are attractive areas of research for a variety of reasons. Mimetics may have the advantage of being smaller compounds with the capability of being chemically or biologically engineered for greater efficacy or more specific targeting for their effect. For this reason, such compounds could be more versatile in their delivery approach, utilizing oral or nasal administration. Mimetics can also avoid economic barriers to the pharmaceutical market by being more easily synthesized and by avoiding patent infringement. To that end, several compounds are being researched that may activate the same signaling pathway as – or stimulate the endogenous release of – GDNF for the treatment of Parkinson’s disease.

Glial cell-line derived neurotrophic factor itself is synthesized as a larger pro-protein of 211 amino acids before being cleaved to produce the mature, soluble GDNF protein targeted for clinical trials of Parkinson’s disease. However, the pro-region of many neurotrophic factors produce biological effects on their own (Lu, 2003). For this reason, attention was given to the pro-region of many neurotrophic factors, including GDNF.

**Development of DNSP-11**

John Glass, Ph.D. (Stony Brook, NY USA) predicted the proteolytic cleavage of 3 peptide sequences in the preproGDNF protein by *in silico* studies of known post-translational cleavage pathways, and these sequences were independently reported on by a team at the University of Helsinki (Immonen et al., 2008) that also predicted a fourth peptide cleavage product. Of these
theoretical cleavage products (Figure 1.6), one of them, named Dopamine Neuron Stimulation Peptide -11 (DNSP-11) for its 11 amino acid sequence length (PPEAPAEDRSL-NH₂), has been the most studied (Immonen et al., 2008; Bradley et al., 2010; Kelps et al., 2011).

Previously published data (Bradley et al., 2010) show that an antibody raised against the DNSP-11 sequence produces strong signal in the midbrain substantia nigra and ventral tegmental area that is highly colocalized with tyrosine hydroxylase positive neurons. Exogenously administered DNSP-11 is rapidly and specifically taken up by TH+ neurons after intracranial injection. In cell culture, DNSP-11 potently increases the percentage of TH+ neurons in E14 rat primary cell culture at a greater range of doses than GDNF. In these primary cell cultures, both DNSP-11 and GDNF significantly increased cell survival, combined neurite length, and average number of branches per neuron, while not affecting soma size. In normal rats, the baseline concentrations of dopamine and its metabolites DOPAC and HVA are significantly elevated in animals administered DNSP-11 up to 28 days prior to striatal microdialysis. In rats subjected to a 6-OHDA lesion of the medial forebrain bundle and their lesion allowed to stabilize for four-weeks, a single DNSP-11 treatment reduced apomorphine-induced rotation behavior and increased striatal dopamine levels as determined by HPLC-EC analysis of substantia nigra and dorsolateral striatal tissue punches.

In that same body of work (Bradley et al., 2010), it was reported that DNSP-11 is protective of MN9D cultured cells from a variety of toxins. DNSP-11 reduces TUNEL positive cells similarly to GDNF when co-administered with 6-OHDA, and it also reduces caspase-3 activity to control levels in these cultures. When co-administered with staurosporin, DNSP-11 reduces cytotoxicity beyond control levels, whereas GDNF does not protect against this toxin, in B65 cells. Similarly, DNSP-11 but not GDNF protects against gramicidin cytotoxicity in cell culture. Staurosporin triggers the release of cytochrome c and the loss of mitochondrial potential, while gramicidin is a mitochondria depolarizing substance, suggesting a mitochondrial protective role of DNSP-11, which is quite
dissimilar to the cell preservation signaling pathway of GDNF, involving GFRα1 and RET. A direct ELISA binding assay confirmed that DNSP-11 does not bind to GFRα1. A cytosolic homogenate pull-down assay identified an array of proteins (by MALDI-TOF mass spectrometry), many of which possess metabolic functions in the cell. Most notable of these is GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which has been linked to Parkinson’s disease (Tatton N. A., 2000) and apoptosis (Tatton W. G. et al., 2000; Hara et al., 2005), and is thus a target for drug development (Kragten et al., 1998; Carlile et al., 2000; Tatton W. et al., 2003; Hara et al., 2006). Later work (Kelps et al., 2011) shows that DNSP-11 protects against a 3-NP toxin that targets succinate dehydrogenase of the mitochondrial complex II, in non-neuronal HEK-293 cells, again suggesting powerful mitochondrial protection. It was also shown that DNSP-11 remains stable in citrate buffered saline at 37 °C for 31 days, a critical element of any pharmacological compound. It has also been shown that DNSP-11 increases both mitochondrial potential and the uncoupled rate of mitochondrial oxygen consumption indicating an increased respiratory reserve capacity (Turchan-Cholewo et al., 2010).

Not only do these studies characterize DNSP-11 as being potently protective of mitochondria both \textit{in vitro} and \textit{in vivo} for protection from models of Parkinson’s disease, but they comprise a body of bioassays that can be used for analysis of dosing optimization and bioavailability enhancements, as well as for engineered alterations to the sequence or structure. Through such biochemical engineering, the potency, stability, and versatility of the peptide may be further enhanced in future studies.

\textbf{Delivery of Drugs to the Central Nervous System}

\textbf{Blood-Brain Barrier}

One problem in the use of pharmacological compounds for neurodegenerative diseases is the safe delivery of the drug to the central nervous system (Pardridge, 2005). Due to extensive tight-junctions between endothelial vasculature, only very small non-polar compounds, typically less than
500 Daltons, may pass this so-called blood-brain barrier (Pardridge, 2003) and reach the CNS by systemic-vascular delivery. An alternative method of infusing drugs in the CNS requires the intracranial implantation of a catheter. This catheter is placed at the disease’s focal point and is anchored to the skull; a connecting tube runs subcutaneously to a subcutaneously implanted infusion pump. These pumps can be refilled by injection with a common needle, and recharged and reprogrammed by induction field. Such an approach provides precise targeting and control of delivered quantity of a therapeutic compound to a brain region of interest. However, this surgical implantation itself can introduce complications in addition to any side-effects a delivered compound may produce. One peer-reviewed survey of a similar surgery, deep-brain stimulation surgery, observed a 12.8% overall complication rate from the surgery itself. Complications ranged from misplacement of the lead, to intracranial infections, hemorrhaging, and even death (Umemura et al., 2003). While complication rates have improved over the past decade, not all patients are good candidates for the surgical implantation and recovery is still lengthy. For these reasons other approaches to deliver therapeutics to the CNS need to be considered.

Penetration

Although surgical penetration of the blood-brain barrier is prone to complication, there are alternative approaches that may temporarily disrupt the barrier. One such possibility is disruption by ultrasound (McDannold et al., 2008b; McDannold et al., 2008a). It is believed that certain frequency ranges of ultrasound produce microbubbles that temporarily affect the microvasculature (Reed and Leighton, 1994). With focused ultrasound, a highly targeted region of the brain can be transiently permeabilized to systemic blood-flow, allowing the transport of a systemically delivered drug to enter the CNS in bulk. This approach is still experimental and is not yet approved by the US FDA for use in human patients. As such, the safety of the technique is unknown. This also requires systemic delivery of the drug, either intravenously or intraorally, which is not suitable for the delivery of peptide or protein therapeutics which are quickly inactivated and broken down in the gastrointestinal tract and blood circulation.
Also, penetration by ultrasound requires expensive equipment and must be performed by a trained individual, so is not ideal for use at home by the patient. Compounds have been identified that can transiently enhance transport across the blood-brain barrier (Cannon et al., 2012). A major active component of the blood-brain barrier is P-glycoprotein, a membrane-bound transport protein with broad substrate specificity ranging from lipids to peptides and glucocorticoids as well as a variety of therapeutic chemical compounds. This pump is ATP-dependent and can be inhibited by sphingolipid signaling. This approach enhances intracellular trafficking of a therapeutic compound, because it does not affect the intercellular tight junctions that comprise the blood-brain barrier. Because of this the delivered compound must have blood-brain barrier crossing characteristics, such as being lipophilic and nonpolar. In effect, inhibition of P-glycoprotein prevents the efflux of compounds that have already entered the CNS.

**Circumvention**

Research is also being performed on the circumvention of the blood-brain barrier. As opposed to physically affecting the continuity of the endothelial junctions, these approaches attempt to utilize natural transports into the CNS. For instance, immune cells such as activated monocytes readily cross into the CNS (Miller, 1999; Engelhardt and Ransohoff, 2012). By masking a compound intracellularly, it can bypass the barrier and potentially be released within the CNS. In a similar vein, encapsulation of compounds within liposomes allows compounds which are otherwise too large, hydrophilic, or polar to be protected within systemic circulation and to cross the blood brain barrier (de Lima et al., 2005; Frank et al., 2011). These approaches may allow a compound to enter the CNS, but once there, it remains in this encapsulated state, masked not only from the blood-brain barrier but also from key receptors to affect their ultimate function.

Natural openings in the blood-brain barrier exist in the form of circumventricular zones. These zones lack the extensive network of tight junctions in the vasculature. However, these zones are typically in areas of
outward flow, for instance where hormones from the pituitary are released into systemic circulation. Without specific retrograde transport, these circumventricular zones provide little therapeutic use in terms of delivering drugs to the CNS.

More interesting approaches to bypassing the barrier involve conjugating a therapeutic compound to natural sequences that target compounds for entry to the CNS. One such conjugation element is the Tat-protein. Tat is a regulatory protein encoded by the Tat gene of HIV. The Tat protein consists of a protein-transduction domain which allows it to penetrate cellular membranes. By conjugating the Tat protein transduction domain to other compounds, it is possible to induce cellular penetration, and even the circumvention of the blood-brain barrier after systemic delivery (Kilic et al., 2005; Rapoport and Lorberboum-Galski, 2009). However, due to its broad functionality, Tat will deliver the conjugated compound across any and all cellular membranes, thus producing a possible increase in side effects, and even reactions to the Tat protein itself. A combined approach, liposomal-encapsulation with membrane-bound rabies viral transduction elements (rabies virus glycoprotein peptide), attempts to enhance delivery across the blood-brain barrier (Tao et al., 2012), but downsides of both techniques still exist.

An ideal technique for targeting the CNS with a therapeutic compound would not subject the patient to increased risk of infection by opening the blood-brain barrier, it would specifically target the brain and anatomical subregions of interest, and it would be easy-to-administer requiring no specialized equipment or requiring new FDA approval. Intranasal administration for delivery to the CNS may provide many of these benefits.

**Intranasal Administration Bypasses the Blood-Brain Barrier**

The concept behind a passage-way between the nasal cavity and the brain dates back to 1929 when Le Gros Clark proposed such a route for the transit of infections from the nasal epithelium to the central nervous system. Indeed, Braak’s “dual-hit” hypothesis of Parkinson’s disease suggests a nasal route as one vector for the entry of Parkinson’s-inducing factors to the brain.
This pathway relies on the patency of the perineural space around the olfactory receptor neurons and their olfactory ensheathing cells (Brierley and Field, 1948) due to a purported lack of tight-junctions (Steinke et al., 2008), the proteins that normally seal cells together especially in the formation of the blood-brain barrier. But it wasn’t until 1986 that this open pathway was visualized with electron microscopy using an intranasally administered tracer molecule (Balin et al., 1986). This process was found to be primarily extracellular in nature; however, a variety of routes may be responsible for transport to the brain. These routes are thought to include extracellular routes such as perineural routes described above, perivascular routes facilitated by pulsatile arterial flow, and intracellular routes such as specific receptor-mediated endocytosis or non-specific pinocytosis followed by retro-grade transport (see Figure 1.7) (Illum, 2003). Since that time much work has been done not only to visualize (Lochhead and Thorne, 2012) but to also quantify the delivery of compounds from the nasal cavity to the brain (Thorne et al., 2004; Dhuria et al., 2010). Evidence in the literature also implicates not only the olfactory receptor route to the olfactory bulb, but also the trigeminal nerve as providing transport to the brainstem (Thorne et al., 2004). Furthermore, administration along a number of cranial nerves has been identified, including the optic nerve via intraocular administration (Rapoport and Lorberboum-Galski, 2009) and by way of the trigeminal and facial nerves through transdermal buccal patch (Yang, 2010; Yuan et al., 2011). Traumatic forces, such as those in a motor-vehicle collision, can often tear the olfactory nerves at the cribriform plate; however, intranasal and other administrations, due to the variety of routes for transit, may still be suitable even in cases of such olfactory denervation.

Much work has been done since the pathway was first identified using molecular tracer molecules, and in 1989 William Frey II, Ph.D. patented the concept of intranasal delivery of therapeutics to the brain by intranasal delivery (Frey II, 1997; Frey II, 2002). With mounting scientific data to validate intranasal delivery (Illum, 2000; Lochhead and Thorne, 2012), phase II United States Food and Drug Administration clinical trials began with the goal of delivering insulin to
the brain to prevent Alzheimer’s Disease in humans (Craft, 2006; Craft et al., 2012). Data from this trial suggest that intranasal insulin slows the progression in patients from mild cognitive impairment to entering full-on dementia (Craft et al., 2012), improves verbal memory (Reger et al., 2008a) and modulates β-amyloid in early stage Alzheimer’s disease, without affecting systemic blood-glucose and insulin levels (Reger et al., 2008b). Due in part to the successes of this trial, other trials that utilize the nose-to-brain route for delivery of therapeutics to the CNS have entered pilot stages (Liu, 2011). These include the use of NAP (or AL-108) for schizophrenia and dementia (Javitt, 2007) to oxytocin for treatment of autism (Anagnostou, 2010).

This pathway between the olfactory epithelium and the deeper regions of the brain parenchyma may serve a developmental role in the organism. Neurons that secrete gonadotropin-releasing hormone (GnRH) develop in the olfactory placode, or epithelium, and migrate along the olfactory receptor neurons to enter deeper regions of the brain, especially the pre-optic area of the hypothalamus (Hilal et al., 1996; Wray, 2010). In addition, olfactory receptor neurons themselves constantly turn-over. As an olfactory receptor neuron dies, another takes its place through the constant replenishment from both the rostral migratory stream, which replaces and restructures granule and glomeruli layers in the bulb, and differentiation of olfactory precursor neurons in the olfactory epithelium to replace peripheral, olfactory receptor neurons. This constant turn-over may result in the open extracellular pathways that allow the passage of compounds to the brain.

The mucociliary clearance mechanisms in the nasal epithelium are extensive and rapid (Illum, 2003). The mechanical action of ciliated cells in the epithelium ensure the rapid movement of substances to the posterior nasal cavity in as little as 20 minutes (Soane et al., 1999) where it is swallowed and degraded in the gastro-intestinal tract. In addition, the nasal epithelium contains high concentrations of oxidizing enzymes and aminopeptidases that will quickly metabolize compounds and peptide drugs. In fact, the concentrations of degradation enzymes in the nasal epithelium are comparable to that found in the
liver (Sarkar, 1992) and serve as a potentially major roadblock to administration via this route. A range of methods to maximize the transport of nasally applied compounds to the CNS is an area of burgeoning research (Patel et al., 2009), and many of the techniques used in the circumvention of the blood-brain barrier during systemic delivery are being applied with intranasal delivery. For instance, the conjugation of the Tat-protein to FGF significantly increased concentrations of the factor in the brain compared to non-conjugated FGF, and increased the efficacy of the compound at the same dose-concentration (Lou et al., 2012). Liposomal encapsulation of intranasally delivered compounds seems to increase transport to the CNS (Migliore et al., 2010) by increasing paracellular transport and protecting the loaded compound from degradation. The co-administration of short peptide sequences has been shown to enhance transport by saturating aminopeptidases in the epithelium (Sarkar, 1992). Chitosan, a mucopolysaccharide, has also shown promise in increasing bioadhesion and enhancing absorption perhaps through the modulation of tight-junctions (Artursson et al., 1994; Dodane et al., 1999), leading to an increased bioavailability of nerve growth factor (NGF) by as much as 14-fold over the same concentration without chitosan (Vaka S. R. et al., 2009) and BDNF by 12-fold (Vaka S. et al., 2010). However, bioavailability of cefotaxime, a cephalosporin antibiotic of 455 Daltons, was not found to be increased intranasally with chitosan compared to intravenous injection with chitosan (Manda et al., 2011). Questions remain as to whether such enhancers result in greater biological efficacy than a simple aqueous solution formulated at an optimized concentration, pH and osmolarity (Illum, 2007). The advantages of a simpler, saline solution include a more expedited FDA-approval process for clinical trials potentially with the need for fewer safety studies, and a more stable drug solution for longer life and versatility in storage conditions for improved patient compliance and outcomes.

The low-cost and ease of administration of this route has the potential to open up a new range of brain-targeting treatments for neuropsychiatric disorders that would otherwise go untreated or be treated with poorly targeted compounds. With the advent of a greater understanding of neurotrophic factors, their
receptors and signaling cascades, and their role in development and cellular maintenance, a wide range of intracellular signaling processes and receptors are available for targeting through biopharmacological engineering. One of the main issues that remain is the ability to effectively deliver these compounds to the regions of the brain sensitive to such therapies. Intranasal administration may, with the right compound, be capable of transporting these new classes of drugs to the CNS.

**Goal**

To determine if the DNSP-11 peptide can be administered to the nasal cavity and be transported to the brain in sufficient quantity to produce protective effects from neurodegenerative diseases such as Parkinson’s disease.

**Study Outline**

In order to test the hypothesis that intranasally administered DNSP-11 enters the CNS and protects the nigrostriatal dopamine system from a unilaterally injected 6-hydroxydopamine lesion of the substantia nigra in Fischer 344 rats, three endpoints for analysis were performed: histological, neurochemical, and behavioral. In addition, some measures of the safety of the drug were recorded, and questions regarding the presence of the DNSP-11 sequence as an endogenous peptide were addressed. All studies and analyses were performed in a blinded, randomized controlled manner.

The aim of the histological endpoint was to quantify the number of tyrosine hydroxylase (TH) positive cells in the lesioned substantia nigra as a percentage of the unlesioned, contralateral substantia nigra. Tyrosine hydroxylase positive striatal fiber density was also quantified by computerized threshold analysis as a percentage of the unlesioned hemisphere. Tissue sections of 30 μm in thickness were immunoreacted with a monoclonal antibody against TH and visualized by nickel-enhanced DAB chromogen. The presence of DNSP-11 in the CNS was also examined by quantification of an iodine-125 radiolabelled version of the
modified peptide sequence. These studies provided information about the quantity of this neuronal population and their projections to the striatum, and this work comprises the whole of Chapter Three.

Neurochemical quantification was performed by two methods: whole striatal tissue neurochemical content, and functional release by striatal microdialysis. Baseline, tonic release of dopamine in the striatum was collected by microdialysis probe and analyzed by HPLC-EC. Potassium-evoked release of dopamine and outflow of dopamine by d-amphetamine were also studied during striatal microdialysis. The whole striatum was excised post-mortem and its neurochemical content analyzed by HPLC-EC. Acute effects of DNSP-11 on striatal dopamine release were also performed by reverse microdialysis. These studies can be found in Chapter Four.

The unilateral 6-OHDA rodent lesion model of Parkinson’s disease produces a rotation behavior due to asymmetric motor output that can be accentuated by the injection of apomorphine or other compounds. This provides an interesting quantification of possible protection from the motor symptoms of parkinsonism. Animals were administered apomorphine and placed in a standardized rotation apparatus in which their rotations were unbiasedly quantified by computer. In addition, to continuously measure changes in activity levels and body temperature as a quantification of treatment safety, animals were implanted with a wireless, battery free device that transmitted activity levels and body temperature data to a computer by induction field located under their homecage. The animals’ body weight was also measured daily as a measure of rate of recovery from lesion induction. These data are provided in Chapter Five.

Finally, it is hypothesized that the DNSP-11 sequence is endogenously processed from the pro-region of its parent protein, GDNF. A custom, polyclonal antibody to DNSP-11 was acquired and differential immunoreactivity patterns in naïve Fischer 344 brain tissue was characterized both at post-natal day 10 and at 3 months of age. Finally, tissue sections were homogenized and reacted for the DNSP-11 antigen in immunoprecipitation, and the eluent sequenced. This study is contained within Chapter Six.
Figure 1.1 – Images of α-synuclein containing Lewy Bodies.

Lewy Bodies, visualized here as dark brown intracellular inclusions, are protein aggregates located within neurons of the central nervous system, especially the substantia nigra and are characteristic of Parkinson’s disease and other disorders of the CNS. Lewy Bodies typically are spherical in shape with a dense central core surrounded by small fibrils comprising a shell or corona. Image from (Spillantini et al., 1997).
Figure 1.2 – Sectional anatomy of the human brain comparing parkinsonian and normal patients.

Shown are the substantia nigra (A) dorsal to the cerebral peduncles in the midbrain, and locus ceruleus (B) located in the dorsal tegmental pontine brainstem. Both the substantia nigra and locus ceruleus are catecholaminergic neurons and contain high concentrations of neuromelanin. In each inset image, the left section shows an example of a normal human brain, and the right section is an example of a parkinsonian brain. Notice the loss of the darkly colored nuclei in both sections, indicated by the red arrows. (Images modified from: MedScape <http://emedicine.medscape.com/>; Rosenblum, W. I., VCU <http://www.pathology.vcu.edu>)}
Figure 1.3 – The biosynthetic pathway of dopamine and norepinephrine from L-tyrosine.

This pathway is utilized by catecholaminergic neurons, such as those of the substantia nigra pars compacta and locus ceruleus. (Image from Wikimedia: <http://en.wikipedia.org/wiki/File:Catecholamines_biosynthesis.svg>)
**Figure 1.4** – Drawings from sagittal sections of the human brain showing the nuclei of the basal ganglia.

The striatum is comprised of both the caudate nucleus dorsomedially and the putamen ventrolaterally, which are separated by the white matter tract known as the internal capsule. The globus pallidus contains both internal (GPi) and external (GPe) segments. The subthalamic nucleus (STN) is proximal to the substantia nigra (SN) of which the pars reticulata segment is innervated by the STN. The substantia nigra pars compacta is the dopaminergic input to the striatum. (Images modified from Shuenke et al, Atlas of Anatomy, 2007, pp. 312-313. Illustrated by Markus Voll).
Figure 1.5 – Simplified version of the basal ganglia circuitry.

Arrow color indicates the type of projection. **Blue** is dopaminergic projections, which can either be stimulatory of the D₁R “direct” pathway or inhibitory of the D₂R “indirect” pathway. **Red** is inhibitory, GABAergic projections, for example from the striatum, the GPe, and GPI. **Green** is excitatory, glutamatergic projections, for instance from the STN, VA, and motor cortex. Note that this circuit diagram does not represent all of the known connections, interconnections, or nuclei of this pathway, but it does serve to illustrate the functional roles of the direct and indirect pathways. A) The first illustration shows the complete connections of this simplified circuit diagram. B) The second illustrates an example of a normally functioning individual during motor output, while C) shows the balance in the pathways when the putamen is not receiving dopaminergic input from the substantia nigra. (GPI, globus pallidus internal segment; GPe, external segment; STN, subthalamic nucleus; VA, ventral anterior nucleus of the thalamus; SNc, substantia nigra pars compacta.)
Figure 1.6 – The immature or prepro-form of GDNF.

Represented here in single letter amino acid sequence and block diagram form is the location of the DNSP-11 sequence within the pro-region of GDNF as determined by \textit{in silico} examination of purported dibasic peptidase cleavage sites (Bradley \textit{et al.}, 2010), including two other potentially cleaved “DNSP” sequences with identified biological activity (Kelps \textit{et al.}, 2011; Littrell \textit{et al.}, 2012), DNSP-5 between the signal peptide and DNSP-11, and DNSP-17 within the mature GDNF sequence. It is theorized that DNSP-11 is cleaved from proGDNF by a combination of enzymes including prohormone convertase, endopeptidases and carboxypeptidase H, followed by amidation by peptidylglycine amidating monooxygenase (PAM). (Modified from Bradley 2010).
Figure 1.7 – Diagram of the olfactory epithelium.

Olfactory epithelium at the top of the image contains the olfactory receptor neuron cell bodies which project as a nerve bundle into the lamina propria then through the foramen of the cribriform plate to synapse in the glomeruli layer of the olfactory bulb. Three possible means of transit from the nasal cavity to the brain exist: a) intracellular, b) paracellular, and c) transcellular transport as illustrated starting at the top of the diagram. Intracellular transport through the projections of the olfactory epithelium could occur slowly by non-specific mechanisms. A purported lack of tight junctions between the olfactory receptor neuron and the olfactory ensheathing cell surrounding the neuron facilitates extracellular pathways referred to here as paracellular and transcellular, resulting in access to the perivascular and perineural spaces. Modified from (Lochhead and Thorne, 2012).
Chapter Two: Materials and Methods

Reagents
All reagents used for surgical procedures were U.S. Pharmacopeia (USP) purity certified when available, or of the highest purity available if not regulated by the USP. Reagents used for histology were procured from Fisher Scientific (Fisher Chemical, Fairlawn, NJ) and reagents for neurochemistry were obtained from Sigma Aldrich (St. Louis, MO), unless otherwise noted. The 6-hydroxydopamine was obtained from Sigma Aldrich at 99.9% purity.

Animals

Ethics Statement
All procedures were conducted at the University of Kentucky, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and approved by Institutional Animal Care and Use Committee.

Animal Care and Housing
Three to four month old male Fischer 344 rats were used in this study, housed in an environmentally controlled vivarium and kept under a standard 12-hr light/dark cycle and provided with food and water ad libitum.

Surgical Procedures
Prior to surgery, animals were visually assessed for normal activity, inquisitiveness, condition of hair coat, eating, drinking, evidence of defecation and urination in the animal's homecage, appearance of eyes, breathing rate, gait, and bodyweight. If any abnormal conditions were observed, the animal was not used and the Department of Laboratory Animal Research veterinary services were consulted.

All surgical procedures were performed under aseptic conditions and sterile field, using ethylene-oxide or autoclave sterilized tools and devices. The surgical incision site was scrubbed three times alternating between both isopropyl alcohol and betadine (iodine) disinfectant. The anesthetic primarily used was isoflurane at 5% in an anesthetic chamber to ensure anesthetic depth.
then moving to a nose-cone, typically at 2-3% isoflurane, to maintain anesthetic plane. However, when noted, urethane and pentobarbital anesthesia were also used. Anesthetic depth was determined by toe-pinch or corneal reflex response. Incisions were closed with 4-0 vicryl sutures (Ethicon, Johnson & Johnson) placed in a simple, interrupted pattern approximately 2 to 4 mm apart. Animals were monitored post-operatively until awake and ambulatory in a fresh cage and then returned to the vivarium. Animals were monitored daily post-operatively for 3 to 5 days for signs of discomfort or infection and observations noted in a post-operative chart. If signs of distress were observed, Department of Laboratory Animal Research veterinary services was consulted.

**Unilateral 6-OHDA lesion of the substantia nigra**

Animals were anesthetized with isoflurane and placed in a stereotactic frame. A hole over the area of injection was created and a Hamilton syringe (26 gauge, 10 µL, tip style #3) was inserted at the following coordinates (with respect to bregma): AP: -5.4 mm, ML: -2.2 mm, DV: -7.5 mm (from dural surface), TB: -3.3 mm (Figure 2.1) (Paxinos and Watson, 2005). A fresh solution of 6-hydroxy-dopamine HCl (6-OHDA·HCl, 99.9% purity, 4.0 mg/mL in 0.9% saline with 0.02% ascorbic acid) was injected at a total volume of 2.0 µL delivered over a 2 minute period. The syringe was removed 2 minutes following injection. Lesion site was verified histologically post-mortem on a subset of animals. (AP: anterioposterior; ML: mediolateral; DV: dorsoventral; TB: tooth bar.)

**Blinded intranasal administration**

Solutions of vehicle only, scrambled peptide, or DNSP-11 were prepared by Luke H. Bradley, Ph.D. and provided as coded vials randomly assigned to subjects. Animals were anesthetized via isoflurane nose-cone and placed supine. Coded solutions were administered in 12.5 µL boluses by micropipetter at the opening of each nostril and allowed to be naturally drawn into the nasal cavity by inhalation. The administration was repeated after a 2 to 5 minute period to allow nasal clearance. Administration of the solutions, endpoint
processes, quantification and analyses were performed by a researcher blind to the solution contents.

Solution containing either DNSP-11, scrambled peptide, or vehicle only was administered intranasally five consecutive days a week for three weeks (two consecutive days of rest from administration for every 5 days of administration) by a researcher blind to the solutions. Animals were administered as above, alternately administering 12.5 µL to each nostril with 2 to 5 minute intervals between administrations for a total of 50 µL of solution per animal per day distributed between both nostrils (Figure 2.2). On the 21st day after lesion induction, after two days off-treatment, the animals were subjected to endpoint analysis. (Figure 2.3)

Peptide Synthesis and Dilution

As reported previously (Bradley et al., 2010), peptide synthesis and purification were performed by GenScript (Piscataway, NJ USA). The following peptide validations, preparations, and dilutions, as described in this section, were all performed by the laboratory of Luke H. Bradley, Ph.D. Peptides were verified as >98% purity upon receipt via reverse-phase HPLC, LC-MS, and amino acid sequencing; and to be stable at physiologically relevant conditions by in vitro assay. DNSP-11 peptide (PPEAPAEDRSL-NH₂) was diluted to 2 µg/µL, 6 µg/µL, or 20 µg/µL in normal saline (0.9% NaCl vehicle) and the researchers were blind to the solution contents. Controls included saline vehicle only and a scrambled DNSP-11 peptide sequence (LPSREDAEPA-NH₂) used at the same concentrations. For studies involving iodinated, radiolabelled DNSP-11 peptide, the sequence was modified to include a lysine residue (R9K), synthesized by AAPPTEC (Advanced Automated Peptide Protein Technologies, Louisville, KY) and contract iodinated by the Bolton-Hunter method to a specific activity of 50 µCi/g of DNSP-11 (Perkin Elmer NEN, Waltham, MA) and verified to be 95% pure by HPLC. The bioactivity of these peptides was confirmed by in vitro assay, and purity by TLC. For a list of these peptide sequences, see Table 2.1
MiniMitter Automated Monitoring System

MiniMitter E-Mitter (Philips Respironics, Bend, OR, USA) devices are wireless, implantable, battery-free devices that continuously records an animal’s activity levels and body temperature. The animal’s home cage is then placed on top of a specialized induction-field pad (Figure 2.4) connected to a computer with specialized MiniMitter software that translates the data into “Activity Units” or “Counts” and temperature. Activity units are an arbitrary unit calculated by a change in the signal strength of the E-Mitter transmitter in the induction field. Any change in signal is counted as an activity unit and the total number of these changes during the designated time interval is given for that data point. These devices were used to determine the activity levels and body temperature of the animals in the study.

Surgical implantation of the MiniMitter device was performed at the same time as lesion induction. Up to 24 hours prior to surgical implantation, and daily for three days post-operatively, the animal received carprofen (5 mg/kg, s.c. or p.o.) analgesic. The animals, anesthetized via isoflurane, were weighed and placed in the surgical prep area. The hair covering the surgical site, between the scapulae on the back, was clipped and the site prepared with anesthetic scrubs. Ocular lubricant (“artificial tears”) was applied to the eyes. A small medio-lateral incision was made and a subcutaneous pocket was created in the fascial plane for insertion of the ethylene-oxide sterilized MiniMitter device.

Statistical analysis

Unless otherwise stated, column analysis was performed using unpaired Student’s t test or one-way ANOVA and grouped analysis was performed using two-way ANOVA. Multiple comparisons were corrected for by Bonferroni’s post-hoc test. Due to the observed protective effects from previous studies of DNSP-11 (Bradley et al., 2010), Student’s t tests were one-tailed unless otherwise stated, with 95% confidence levels, $\alpha=0.05$, $\beta=0.80$ with significance defined as $P<0.05$. Error bars are mean ± SEM. Statistical analyses were performed on GraphPad Prism 6.01. (*$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.)
Table 2.1 – Amino acid sequences of peptides administered in this study.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Human DNSP-11</td>
<td>PPEAPAEDRSL-NH₂</td>
</tr>
<tr>
<td>Modified DNSP-11 for ^125^I</td>
<td>PPEAPAEDKSL-NH₂</td>
</tr>
<tr>
<td>Scrambled peptide</td>
<td>LPSPREDAEPA-NH₂</td>
</tr>
</tbody>
</table>

Three different synthetic peptides were administered in the present studies, including the human DNSP-11 sequence, a version of the DNSP-11 sequence modified to accept iodination (R9K), and a scrambled version of the DNSP-11 sequence as a negative control. All peptides were C-terminal amidated.
Figure 2.1 – Representation of the 6-OHDA lesion injection of the SN. The vertical black line represents the needle at its final position at the following coordinates (with respect to bregma): AP: -5.4 mm, ML: -2.2 mm, DV: -7.5 mm (from dural surface), TB: -3.3 mm. Modified from (Paxinos and Watson, 2005). (SNc, substantia nigra pars compact; SNr, substantia nigra pars reticulate; VTA, ventral tegmental area; cp, cerebral peduncle; III, root of optic nerve or cranial nerve III)
Figure 2.2 – Illustration of intranasal administration in the rat.
Intranasal administration was performed as follows. Animals were anesthetized via isoflurane nose cone and laid supine. Using a micropipette, a bolus was ejected into the opening of the nostril, here shown is a 12.5 µL ejection first performed on the right nostril. The animal was given 2 to 5 minutes for the solution to be cleared from the nasal cavity, and then a bolus was ejected into the opposite nostril, followed by a 2 to 5 minute clearance period. This was repeated again for a total of 50 µL over approximately 10 minutes as shown here.
Outline above is the overall study timeline with the time of lesion induction defined as “Day 0”. The day prior to lesion induction, animals underwent baseline rotation behavior testing. The next morning, the animals received their first intranasal administration followed approximately 30 minutes later by 6-OHDA lesion induction of the substantia nigra (shown with red background). The animals received intranasal treatment (shown with blue background) for five of seven days a week for three weeks (two days “off” treatment every seven), with weekly rotation behavior. On Day 21, the animals were taken for endpoint analysis, either for histology or neurochemical studies (data collection points shown with orange background). Animal body weight was recorded daily.
Figure 2.4 – Example images of MiniMitter device and setup.

The small E-Mitter probe, shown in (A), is the approximate size of an osmotic mini-pump and can be implanted in the back or abdomen. The animal, with E-Mitter implanted, is then placed in their homecage which is set atop an induction field pad, the black box shown in (B), which continuously monitors changes in the signal from the E-Mitter, and changes in temperature recorded by the E-Mitter thermistor.
Chapter Three: A study of the effects of intranasally administered DNSP-11 on tyrosine hydroxylase positive dopaminergic neurons and their projections in a 6-hydroxydopamine lesion rat model of Parkinson’s disease

Hypothesis: Efficacious doses of DNSP-11 can be delivered intranasally to enter the CNS in quantities sufficient to protect tyrosine hydroxylase positive neurons from a moderate 6-hydroxydopamine lesion of the substantia nigra.

Introduction

In order to test the hypothesis, young adult male Fischer 344 rats were subjected to a unilateral 6-OHDA lesion directly to the substantia nigra following an initial intranasal treatment thirty minutes prior to lesion induction (see Chapter Two). The animals were intranasally administered solutions, by a researcher blind to treatment, five of seven consecutive days a week for three weeks (see Figure 2.3). On the 21st day the animals were euthanized by transcardiac perfusion for immunohistochemical analysis of tyrosine hydroxylase positive neurons of the substantia nigra and fibers of the striatum. In a separate cohort of animals, a 125I-radiolabeled version of the DNSP-11 peptide was administered intranasally and thirty minutes after administration the animal was euthanized by transcardiac perfusion its brain was sectioned and quantified for signal by gamma counter.

Tyrosine Hydroxylase Enzyme

Tyrosine hydroxylase (TH) is the enzyme involved in the rate-limiting step in the dopamine synthetic pathway and is responsible for converting L-tyrosine to L-DOPA (see Figure 1.3). The activity of the TH enzyme is dependent on phosphorylation of a number of serine sites, primarily serine-40 by the cAMP-dependent protein kinase A (PKA) (Fitzpatrick, 1999). The activity of TH is subject to feedback inhibition by the products of the enzyme, namely catecholamines including dopamine (Daubner et al., 1992). Once the enzyme is
active, it uses diatomic oxygen (O₂) and tetrahydrobiopterin (BH₄) in conjunction with an active site iron atom to hydroxylate the tyrosine substrate.

In the human, nine transcripts for TH have been identified, but only four of these isoforms are translated to functioning enzymes. Of those four, only two are prevalent in the human brain and they differ by the number of phosphorylation sites present on the enzyme. Transcriptional regulation is responsible for long-term regulation of the TH enzyme. Because TH is necessary for catecholamine synthesis, its presence in a neuron can be used as a marker for catecholaminergic cells such as the dopaminergic neurons of the substantia nigra.

Aside from phosphorylation, evidence in the literature suggests that TH is regulated by enzymatic complexing. It has been reported that the protein 14-3-3, after phosphorylation by CaMKII, complexes with TH to enhance its activity (Ichimura et al., 1987; Wang J. et al., 2009); while α-synuclein has been shown to bind to TH and decrease its activity through the dephosphorylation of serine-40 and the increase in activity of protein phosphatase 2A (Peng et al., 2005).

While the quantification of tyrosine hydroxylase positive (TH+) cells in the substantia nigra has long been a key component of pre-clinical studies of potential therapies for Parkinson’s disease or dopaminergic neurodegeneration (Kearns and Gash, 1995; Kearns et al., 1997; Ai et al., 2003), it cannot be the only metric for efficacy due to its vast array of post-translational regulatory possibilities. It does serve as a powerful molecular marker of neuronal phenotype and morphology. For this reason, TH+ cell counts of the substantia nigra and the quantification of their fiber projections to the striatum have long been powerful endpoints as one element of the analysis of potential therapies.

Materials and Methods

Animals were dosed intranasally while supine under isoflurane anesthesia, for five of seven consecutive days a week (see Chapter Two) by a researcher blind to treatment. The first intranasal dose was given approximately 30 minutes prior to unilateral 6-OHDA lesion induction (see Chapter Two). On the 21st day
after lesion induction (see Figure 2.3) animals were euthanized for immunohistochemical analysis.

**Tissue preparation and immunohistochemical processing**

Animals were perfused transcardially with cold saline and 4% paraformaldehyde fixative, the brain or whole skull was immersed in fixative for 24 to 48 hours, followed by submersion in 30% sucrose. Tissue was then sectioned coronally (30 µm) and stored in cryoprotectant solution (30.0% sucrose, 30.0% ethylene glycol in 100 mM NaPB pH 7.2) at -20 °C until immunostaining procedure.

Every 12th section underwent 10 mM sodium citrate buffered antigen retrieval (with 0.05% Tween 20) at 80 °C for 10 minutes, then blocked with 4% normal goat serum for 1 hour at room temperature. The sections were then immunolabeled with a monoclonal anti-tyrosine hydroxylase antibody (1:1000, Chemicon Millipore, Temecula, CA USA) and incubated overnight at 4°C. The sections were repeatedly washed and then incubated with peroxidase-conjugated secondary antibody (1:1000, Vector Labs, Burlingame, CA USA). Immunoreactivity was visualized with NiDAB chromogen. This procedure is modified from one previously described (Ai et al., 2003).

**Substantia nigra TH+ cell counting**

Tyrosine hydroxylase positive cells of the substantia nigra were quantified in three 30 µm thick sections per animal according to the following criteria: only TH+ cells with clear nuclei and lying lateral to the oculomotor nerve root were included in the quantification. Three sections per animal were chosen in relation to the 6-OHDA injection site: the section containing the injection point (-5.4 mm with respect to bregma), the section 360 µm rostral to the injection site (approximately -5.0 mm) and the section 360 µm caudal to the injection site (approximately -5.8 mm). Images were captured at 5x magnification and quantified by a researcher blind to treatment. The contralateral unlesioned hemisphere was also quantified and the proportion of TH+ cells in the lesioned
versus the unlesioned hemispheres is shown. A total of 18 animals were used for this analysis (Table 3.1).

**Striatal fiber density quantification**

Striatal fiber density measures were quantified using contrast threshold analysis software (BioQuant, Nashville, TN USA). Again, three striatal sections were analyzed per animal. The unlesioned hemispheres were standardized to approximately 90% TH+ striatal area and the lesioned hemispheres compared as a percentage of unlesioned hemispheres. Quantification was performed by a researcher blind to treatment.

**Tracking of radiolabeled peptide to the CNS**

The DNSP-11 sequence was conservatively modified to contain a lysine residue (R9K, see Table 2.1), and this sequence was confirmed by previously defined *in vitro* bioassay in the laboratory of Luke H. Bradley, Ph.D. (Bradley *et al.*, 2010) to produce similar biological effect as DNSP-11. Modified DNSP-11 was radiolabeled by contract (Perkin Elmer NEN, Boston, MA) with iodine-125 by the Bolton Hunter method.

A total of 50 µCi (300 µg) was administered intranasally in a total of 50 µL as previously described. Thirty minutes after the first intranasal dose was administered, a sample of CSF was removed by cisternal puncture, the animal was euthanized by thoracotomy and a blood sample was taken from the left ventricle. Transcardiac perfusion with cold saline was performed followed by decapitation. The brain was removed and 2 mm thick sections were serially collected using a brain mold. Sample weight was determined and gamma counting was performed to quantify the amount of radioactivity present in the CNS. A total of seven animals was used in this study (Table 3.2), four given radiolabelled I$_{125}$-DNSP-11, three given saline only. One of the four I$_{125}$-DNSP-11 treated animal was excluded from this group due to poor perfusion quality. This study was performed by Mallory J. Stenslik.
Results

Scrambled DNSP-11 as a negative control

Concerns existed that the constituent amino acids in the DNSP-11 sequence may be cleaved by the numerous aminopeptidases and oxidative enzymes in the olfactory epithelium (Sarkar, 1992) to cross the blood-brain barrier via amino acid transporters, entering the CNS and producing a stimulatory effect on the dopamine system. To control for this possible effect in protection bioassays, a scrambled version of the DNSP-11 sequence (6 \( \mu \text{g/\muL} \), see Table 2.1) was administered intranasally along with DNSP-11 or saline vehicle only, all of which were assigned randomly and administered in a blinded manner. Scrambled peptide was then evaluated against the saline vehicle control treated animals for tyrosine-hydroxylase protection determined by TH+ positive substantia nigra neuronal cell counts. It was observed that scrambled peptide and vehicle treated animals resulted in the same TH+ neuronal cell loss in the substantia nigra (P=0.88. Vehicle treated cell loss: 91.4% ±4.1%, n=6. Scramble treated cell loss: 92.2% ±3.5%, n=6.). Comparisons between Vehicle only and Scrambled peptide treated animals were performed for all measurements with no significant differences observed (P > 0.05). As such, vehicle-only and scramble treated animals were combined as one negative control group.

Cell counts of the substantia nigra

In order to determine if intranasally administered DNSP-11 peptide could result in protection of tyrosine hydroxylase positive neurons and fibers, Fischer 344 rats received the first intranasal treatment approximately 30 minutes prior to a unilateral 6-OHDA (4.0 mg/kg) lesion of the substantia nigra (see Chapter Two). The animals were given intranasal treatment 5 days a week for 3 weeks, and euthanized by perfusion fixation on day 21 post-lesion. The animals brains were removed and processed for immunohistochemical analysis of tyrosine hydroxylase (TH) reactivity compared with the unlesioned hemisphere. When those animals treated intranasally with scrambled peptide or vehicle only were combined in one negative control group, animals treated intranasally with DNSP-
11 exhibit significantly greater TH-positive neuronal cell bodies in the substantia nigra (Figure 3.1, P=0.0192, n=18). Negative control treated animals exhibited a 92.1% (±2.6%, n=12) TH+ nigral cell loss 21 days after 6-OHDA lesion, while DNSP-11 treated animals exhibited a 75.2% (±9.5%, n=6) cell loss. Animals treated with DNSP-11, unlike negative control animals, closely resembled unlesioned substantia nigra tissue (Figure 3.2). There were no statistically significant differences observed in the number of TH+ cell counts of the intact, unlesioned substantia nigra (data not shown, P=0.9223, n=18). No statistical outliers by Grubb’s test were present, and the lesion site was histologically verified prior to analysis. All procedures and analyses were performed by a researcher blind to the treatment group identities.

**Fiber density of the striatum**

In this same cohort, TH+ fiber density in the striatum was quantified by computerized threshold analysis. Animals that received intranasal DNSP-11 treatment exhibited significantly greater striatal densities compared to control treated animals (Figure 3.3, P=0.047, n=18). DNSP-11 treated animals exhibited 76.2% (±9.8%, n=6) striatal TH+ density loss, compared with 90.7% (±3.1%, n=12) loss in control treated animals. Animals treated with DNSP-11, unlike negative control animals, more closely resembled unlesioned striatal tissue (Figure 3.4). A linear regression of TH reactivity of nigral cell bodies to striatal fiber density resulted in a significantly non-zero slope (Figure 3.5, m=0.716 ±0.158, P=0.0003, R²=0.561), indicating intra-animal TH+ immunoreactivity levels are reflected in both critical regions of the dopaminergic motor system. No statistical outliers by Grubb’s test were present. All procedures and analyses were performed by a researcher blind to the treatment group identities.

**Quantification of peptide by radiolabel**

Presence of the radiolabel was confirmed in the CNS 30 minutes after intranasal administration. Mean radiolabel signal corresponded to approximately 0.29% of the 300 µg administered to the nose. (Table 3.3)(Figure 3.6)
Discussion

Overview and Interpretation

These results support the hypothesis that intranasally administered DNSP-11 enters the CNS and protects TH+ neurons from the 6-OHDA toxin in the Fischer 344 male young adult rat. This provides important data to support the overall hypothesis that DNSP-11 can be administered intranasally, enter the CNS and protect TH+ neurons from degeneration.

Lesion accuracy and quality as a potential variable

The design of this study relied on a concomitant delivery of the lesion-inducing toxin (6-OHDA) immediately after delivery of a protective agent (DNSP-11) against the toxin. This design can elicit questions as to the causative variable in the quantity of cell loss observed as the endpoint. However, because the treatments were performed in a manner blind to the treatment contents, this protects the results of the study from bias. Lesion variability among the negative control groups was low (92.1% cell loss ±2.6%, n=12), indicating high precision of the standard operating procedure used for lesion induction among this cohort. Finally, a higher resolution technique for more precise quantification of TH+ cells of the substantia nigra, to include cell body size, may identify additional differences between groups.

Permeabilization of the blood-brain barrier from 6-OHDA lesion

It has been shown that a 6-OHDA lesion can transiently permeabilize the blood-brain barrier (Cooper et al., 1982), which may allow systemic delivery during the timeframe of this study. It was not felt that this was a major concern, as an increased permeability of the blood-brain barrier has been described in Parkinson’s disease (Hunot and Hirsch, 2003; Kortekaas et al., 2005; Rite et al., 2007), thus a degree of blood-brain barrier permeability may model the disease state. Due to this observation in the literature, it was not a specific concern to determine whether or not protection resulting from intranasally administered DNSP-11 occurs through a direct transit along cranial nerve routes or through systemic vascular delivery. However, studies of the radiolabeled DNSP-11
peptide (Figure 3.6) support the hypothesis that intranasal administration of this peptide may result in direct transit to the CNS through the olfactory system but does not rule out vascular delivery (see Figure 1.7 for a description of transport across the olfactory epithelium). And although it is assumed that the peptide is excluded from the CNS by the blood-brain barrier, the possibility of the peptide’s active transport across the blood-brain barrier is not addressed in this study.

**Quantification of radiolabeled peptide**

The observed distribution of radioactivity after intranasal administration of the radiolabelled peptide supports the hypothesis of transit of the peptide from the nasal cavity to the brain. Radioactivity was found throughout the brain rostro-caudally at the thirty minute time point.

Variability in the perfusion quality may affect the overall quantity of radiolabel found in the brain. For this reason, it is hypothesized that quantities of the radiolabel within the vascular system contributed to the total quantity observed in the brain, increasing the level of noise. In order to confirm the direct nose to brain route, future studies may be designed to compare the pharmacokinetics of intranasal and intravascular delivery using the radiolabelled peptide in a time-response study. If intranasal administration results in a peak concentration in the brain prior to a peak observed after intravascular administration then this suggests a direct route from the nasal cavity. In addition, such a time-response study would provide more precise data on the total quantity that enters the brain after a single administration. Finally, to confirm that the intact DNSP-11 transits to the CNS, an immunoprecipitation for DNSP-11 after intranasal administration of the $^{125}$I-labeled peptide followed by quantification by gamma counting would be strong evidence to support the observed biological effects. A highly sensitive, monoclonal antibody would benefit such a study.

The present study, while not addressing these issues, does support the transport of intranasal DNSP-11 to the brain; however, the possibility remains that the peptide is being degraded within the nasal cavity or vasculature and that the constituent amino acids (of which the lysine residue is radiolabeled in this study) would be able to enter the CNS by the cationic amino acid transporter,
producing a false positive. Taken in conjunction with the biological activity of the DNSP-11 peptide, the similarity of that effect to the intracranially injected DNSP-11 peptide, and the lack of effect observed in the scrambled peptide treated animals, this suggests that the intact peptide is directly entering the CNS in biologically efficacious quantities. Indeed, the preponderance of evidence in the literature indicates that this direct route into the CNS is sufficient for compounds as small as dopamine and as large as entire mesenchymal stem cells to enter the CNS.

Finally, it is important to keep in mind the translational nature of this work. While intranasal delivery of DNSP-11 in rats is an important step, the ultimate goals are intranasal administration in non-human primates and eventually humans. The percentage of transit to the CNS from the nasal cavity may be quite different between species. Changes in formulation aimed at increasing intranasal delivery to the CNS in rats may not translate to improvements in primates. While the quantification of radiolabeled DNSP-11 may be a satisfactory proof of concept experiment, it may be an ineffective assay for a translationally aimed study.

**Implications of post-transcriptional regulation of the tyrosine hydroxylase enzyme**

Although the hypothesis is supported and TH+ neurons and their fiber projections are higher in the animals treated with intranasal DNSP-11, because TH can be post-translationally regulated these results are insufficient to show efficacy for the treatment of Parkinson’s disease and the resultant behavioral or motor deficits of the disease. These neurons may maintain their TH+ phenotype but may not produce or release dopamine in the striatum. For this reason, an evaluation of dopamine content and its functional release in the striatum, including an evaluation of the animal’s behavior, must follow.
A total of 18 young adult male Fischer 344 rats were used for the histological quantification of TH+ cells of the substantia nigra and fiber density of the striatum. Due to similarities in response between the Vehicle and Scrambled peptide treated groups, these two groups were combined to one “Negative Control” group. The DNSP-11 treated group received 300 µg in 50 µL per treatment. No animals were excluded from this study.

<table>
<thead>
<tr>
<th></th>
<th>DNSP-11 Treated</th>
<th>Vehicle-only Treated</th>
<th>Scrambled Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Numbers:</strong></td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3.1 – Animal groups for TH+ histological study
Figure 3.1 – Percentage of intact TH+ cells of the substantia nigra of animals 21 days after 6-OHDA lesion induction.

Of animals with a 6-OHDA lesion of the substantia nigra, those that received intranasally administered DNSP-11 at 300 µg/day (75.2% cell loss ±9.5%, n=6) exhibited significantly more TH+ cells in the substantia nigra (*P=0.0192, t_{16}=2.257) 21 days after lesion induction when contrasted with the cell counts of those that received negative control treatment (92.1% cell loss ±2.6%, n=12; either saline vehicle only (n=6), or a scrambled version of the peptide sequence (n=6)). As predicted, animals treated with scrambled peptide or vehicle only were not significantly different from each other (P=0.806) and were grouped as one “Negative Control” group. Numbers are represented as the percent of unlesioned hemisphere. Analysis by t-test.
Figure 3.2 – Representative sections showing TH+ immunoreactivity of the substantia nigra in treated and negative control animals 21 days after 6-OHDA lesion induction. Images contrast enhanced for clarity. Measurement bar represents 200 µm.
Figure 3.3 – Percentage of intact TH+ fiber density in the striatum of animals 21 days after 6-OHDA lesion induction.

Of animals with a 6-OHDA lesion of the substantia nigra, those that received intranasally administered DNSP-11 at 300 $\mu$g/day ($76.2\%$ density loss $\pm 9.8\%$, $n=6$) exhibited significantly greater TH+ striatal fiber density (*$P=0.0468$, $t_{(16)}=1.782$) 21 days after lesion induction when contrasted with the fiber density of those that received negative control treatment ($90.7\%$ density loss $\pm 3.1\%$, $n=12$; either saline vehicle only ($n=6$), or a scrambled version of the peptide sequence ($n=6$)). As predicted, animals treated with scrambled peptide or vehicle only were not significantly different from each other ($P=0.654$) and were grouped as one “Negative Control” group. Numbers are represented as the percent of unlesioned hemisphere. Analysis by t-test.
Figure 3.4 – Representative sections showing TH+ immunoreactivity of the striatum in treated and negative control animals 21 days after 6-OHDA lesion induction. Images contrast enhanced for clarity. Measurement bar represents 500 μm.
Figure 3.5 – Correlation of TH+ nigral cell counts and striatal fiber density in 6-OHDA lesioned animals.

Animals that exhibited the greatest number of remaining TH+ cells in the substantia nigra also tended to have the greatest TH+ striatal fiber density, as illustrated by the significantly non-zero (P=0.0003, F(1,16)=20.45) linear regression slope (m=0.716). Dotted curves indicated 95% confidence interval.
To determine the quantity of radioactivity in the CNS, blood, and CSF after intranasal administration, three groups of animals were used: four animals received intranasal $^{125}\text{I}$-DNSP-11 and three animals received intranasal saline only. One $^{125}\text{I}$-DNSP-11 treated animal was excluded from this group due to poor perfusion quality. This study was performed by Mallory J. Stenslik.

**Table 3.2 – Animal groups for radiolabel tracking study.**

<table>
<thead>
<tr>
<th></th>
<th><strong>Intranasal $^{125}\text{I}$-DNSP-11</strong></th>
<th><strong>Intranasal Saline</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Perfused with:</strong></td>
<td>Normal saline</td>
<td>Normal saline</td>
</tr>
<tr>
<td><strong>CSF collected?</strong></td>
<td>Cisternal puncture</td>
<td>Cisternal puncture</td>
</tr>
<tr>
<td><strong>Numbers:</strong></td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3.3 – Quantification of radioactivity in the CNS after administration of I^{125} labeled, modified DNSP-11 peptide.

<table>
<thead>
<tr>
<th></th>
<th><strong>Intranasal I^{125}-DNSP-11 (ng/mg ± SEM)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>1.46±0.18</td>
</tr>
<tr>
<td>CSF</td>
<td>0.22±0.047</td>
</tr>
<tr>
<td>Olfactory Bulb</td>
<td>0.086±0.005</td>
</tr>
<tr>
<td>Section 1</td>
<td>0.051±0.011</td>
</tr>
<tr>
<td>Section 2</td>
<td>0.054±0.007</td>
</tr>
<tr>
<td>Section 3</td>
<td>0.027±0.023</td>
</tr>
<tr>
<td>Section 4</td>
<td>0.057±0.011</td>
</tr>
<tr>
<td>Section 5</td>
<td>0.061±0.008</td>
</tr>
<tr>
<td>Section 6</td>
<td>0.059±0.011</td>
</tr>
<tr>
<td>Section 7</td>
<td>0.059±0.006</td>
</tr>
<tr>
<td>Section 8</td>
<td>0.057±0.003</td>
</tr>
<tr>
<td>Section 9</td>
<td>0.058±0.010</td>
</tr>
<tr>
<td>Section 10</td>
<td>0.064±0.013</td>
</tr>
<tr>
<td>Section 11</td>
<td>0.080±0.002</td>
</tr>
</tbody>
</table>

Tabular quantification of radioactivity (calculated nanograms of I^{125}-DNSP-11 per milligram of tissue). Brain tissue was sectioned in 2 mm thick serial coronal sections using a rat brain mold. Animals treated with radiolabeled DNSP-11 (n=3) produced significant signal, while control animals treated with normal saline (n=3) were observed to have CPM values at or below background levels (<100 CPM). This study was performed by Mallory J. Stenslik.
Figure 3.6 – Normalized DNSP-11 Concentrations (ng/mg) at 30 Minutes in Blood, CSF and Brain.

Calculated concentrations of DNSP-11 after administration of a radiolabeled version of the peptide support the hypothesis that DNSP-11 enters the CNS after intranasal administration. Male Fischer 344 rats (n=3 per group) were either given a total bilateral dose (50 µCi/300 µg) of ¹²⁵I-labeled DNSP-11 in vehicle (50 µL of normal saline) or vehicle only. At 30 minutes post administration, aortic blood (Blood) and cerebrospinal fluid (CSF) were taken. The animals were euthanized, perfused with 200 mL of normal saline and serial sections of the animals' brains were removed rostro-caudally in 2 mm thick sections (OB: olfactory bulb, 1-11: brain sections). The percentage of ¹²⁵I-labeled-DNSP-11 found in blood, CSF and total brain were 0.27%, 0.0084% and 0.29% respectively. Animals treated with normal saline were found to have CPM values below background levels (100 CPM). This study was performed by Mallory J. Stenslik.
Chapter Four: A study of the effects of DNSP-11 on monoamine neurotransmitter tissue content and functional dopamine release in normal and 6-hydroxydopamine lesioned rats

Hypothesis: Efficacious doses of DNSP-11 can be delivered intranasally to modulate dopamine tissue content and metabolites in normal rats and to protect dopamine tissue content and functional release from a moderate 6-hydroxydopamine lesion of the substantia nigra.

Introduction

In order to test the hypothesis, normal unlesioned young adult male Fischer 344 rats were intranasally administered a range of doses of DNSP-11 for five of seven consecutive days a week for three weeks (Chapter Two). On the 18th day, approximately 30 minutes following the final intranasal administration, animals were euthanized and the olfactory bulb, striatum, and substantia nigra were removed for analysis of whole tissue neurochemical content.

Then, a separate cohort of young adult male Fischer 344 rats was subjected to a unilateral 6-OHDA lesion of the substantia nigra directly (see Chapter Two) following an initial intranasal protective treatment 30 minutes prior to lesion induction. The animals were intranasally administered DNSP-11, a scrambled version of the peptide, or vehicle only five consecutive days a week for three weeks (see Figure 2.3). On the 21st day the animals were subjected to striatal microdialysis under urethane anesthesia to measure baseline, K+ and d-amphetamine evoked release of dopamine, then euthanized and a 2 mm thick section of the striatum was collected at the level of the optic chiasm for whole tissue neurochemical content analysis.

Finally, unlesioned rats with no prior treatment were subjected to microdialysis during which potassium-evoked release of dopamine was initiated, followed by perfusion with a solution of DNSP-11 through the microdialysis probe. Then a second potassium-evoked release of dopamine was initiated to determine intra-animal changes after DNSP-11 perfusion.
Increases in dopamine tissue content ameliorate parkinsonian symptoms

The loss of dopamine in the striatum of both humans and in animal models of Parkinson’s disease is the hallmark sign correlated to motor function deficit. It has been observed that a loss of 80% of striatal dopamine content corresponds to the onset of motor symptoms (Gibb and Lees, 1991) as a result of an approximate 60% loss of dopaminergic neurons (Braak et al., 2006). The biosynthesis of dopamine occurs in the cytoplasm of tyrosine hydroxylase positive neurons from the precursor L-DOPA, so an elevated presence of TH+ nigral neurons and their striatal fibers may allow for the production of elevated dopamine content in the target striatum. For this reason, the determination of dopamine content in the putamen of the striatum has been an important endpoint in studies of Parkinson’s disease treatment models.

Contrasting neurochemical content and functional release

Dopamine content on its own is only a snap-shot of the absolute quantity of dopamine present, and does not reveal functional characteristics regarding the release and metabolism of dopamine. The use of an intra-striatal microdialysis probe (CMA 11, 6000 MW cut-off), allows for the collection of dopamine and its metabolites from the interstitial space. By simultaneously perfusing through the probe solutions designed to stimulate the release of dopamine, the functional characteristics of the circuitry may be elucidated. For instance, perfusion with a 100 mM K+ solution in artificial cerebrospinal fluid will induce a change in the ionic membrane potential and the synaptic release of dopamine by a mechanism dependent on intracellular Ca2+ stores similar to physiological release. Also, d-amphetamine (d-amp) increases extracellular dopamine by increasing the net efflux of dopamine through the dopamine transporter, which can provide information about total intracellular dopamine and the function of the dopamine transporter. d-Amphetamine acts through a variety of mechanisms. One such mechanism is by acting as a substrate for the dopamine transporter (DAT) effectively inhibiting the reuptake of extracellular dopamine by binding to the normal “outward-facing” or “inward-flowing” conformity of DAT thus competitively
blocking the influx of extracellular dopamine in a calcium-independent manner (Rothman et al., 2012). Furthermore, d-amphetamine forces dopamine out of synaptic vesicles by altering ionic gradients across lipid membranes (Tsai et al., 2000). These intracellular ionic changes have also been shown to alter the conformity of the dopamine transporter to its inward-facing, outward-flowing conformity (Zhen et al., 2005) effectively reversing its flow to cause the efflux, or carrier-mediate release, of cytoplasmic dopamine (Liang et al., 2009; Steinkellner et al., 2012). Data from studies of the functional release of dopamine, in conjunction with tissue dopamine content, can provide information on the functioning of the circuitry in ways that dopamine content alone cannot provide.

Materials and Methods

Dose response study in normal animals

In order to determine the appropriate intranasal dose required to elicit a biological response, 100 µg, 300 µg, 1000 µg of DNSP-11, or vehicle only was administered intranasally using the administration protocol described in Chapter Two. In short, animals were administered intranasal DNSP-11 or vehicle (Table 4.1) by a researcher blind to treatment for five of seven consecutive days a week while under isoflurane anesthesia and laid supine. With the day of the first administration defined as day 0, animals were euthanized by isoflurane overdose on the 18th day immediately following intranasal administration, their brains removed and the neurochemical tissue content of the olfactory bulb, striatum, and substantia nigra were investigated, as described below.

Neurochemical protection studies in lesioned animals

A target dose of 300 µg DNSP-11 was administered intranasally (using the same regimen as in Chapter Three and as described by Figure 2.3) in order to determine the protective effects of this dose on dopamine neurochemical tissue content and functional release of dopamine by striatal microdialysis.
Tissue preparation for neurochemical quantification

In order to acquire samples of brain tissue, animals were overdosed with isoflurane and euthanized by decapitation. The brain was removed, submerged in ice-cold saline, and then a 2 mm section of the whole striatum was removed at the level of the optic chiasm. The sections were weighed, flash-frozen in dry ice and stored at approximately -74 °C. Immediately prior to analysis by HPLC-EC, tissue samples were sonicated in 300 μl of cold 0.1 M perchloric acid containing dihydroxybenzylamine as an internal standard. The samples were centrifuged for 5 min at 12,000 g and the supernatant transferred to 0.22 μm pore size Millipore Ultrafree centrifugal filters and spun at 12,000 g for 1 min. The filtrate was diluted with HPLC mobile phase and 50 μL injected onto the HPLC column. For dialysis samples, 20 μL of the dialysate was injected directly onto the column.

Neurochemical quantification by HPLC-EC

All neurochemical assays were performed by the laboratory of Wayne A. Cass, Ph.D. as previously described (Cass et al., 2003). The HPLC system consisted of a Beckman Model 118 pump, a Beckman Model 507 autoinjector and an ESA Model 5200A Coulochem II electrochemical detector with a Model 5011 dual-detector analytical cell (detector 1 set at +350 mV and detector 2 set at −300 mV). An ESA Hypersil ODS 3 μm particle C18 column (80Χ4.6 mm) was used for separations. Flow rate was 1.4 mL/min and the mobile phase was a pH 4.1, 0.17 M citrate–acetate buffer (containing 5 mg/L EDTA, 70–80 mg/L octanesulfonic acid, and 7–8% methanol). Chromatograms were recorded from both detectors using two dual-channel strip chart recorders. Retention times of standards were used to identify peaks, and peak heights were used to calculate recovery of internal standard and amount of DA and metabolites.

Striatal microdialysis

The microdialysis technique allows for collection of extracellular compounds, especially monoamine neurotransmitters of interest in this study, by passive diffusion across the semipermeable probe membrane. Microdialysis is
performed as a non-recovery surgery in this study. Prior to *in vivo* microdialysis, probes were prepared by perfusion with 70% ethanol at 3 µL/min for approximately 20 minutes followed by reverse-osmosis water at 0.2 µL/min until *in vitro* probe recovery collection was performed. Probes were then submerged in a solution containing known quantities of the analytes of interest (see p. 68) and perfused with artificial cerebrospinal fluid (see p. 68). The animals were anesthetized with urethane (1.25 g/kg in 0.9% NaCl, i.p.) and placed in a stereotactic frame. A rostro-caudal incision was made in the skin above the calvarium and a craniotomy was performed allowing the microdialysis probe (CMA 11 probe with 4 mm Cuprophane membrane length and 6000 Da MW cut-off. CMA Harvard Apparatus Co., Kista, SE) to be placed in the whole striatum using stereotactic coordinates with respect to bregma: AP: +1.5 mm, ML: -2.3 mm, DV: -8.0 mm (from dural surface), TB: -3.3 mm (Figure 4.1). The probe tract was visualized post-mortem. Artificial cerebrospinal fluid (aCSF) was then perfused at a rate of 1 µL/min and the probe was submerged in a standard solution containing known concentrations of the analytes of interest and a 20 min (20 µL) sample was collected for determination of probe recovery. Then the probe was placed in the striatum using the above coordinates and samples collected after each 20 min period (totaling 20 µL per sample). Six samples (0-120 minutes) were collected and then the perfusate solution was switched to a 100 mM potassium (K+) aCSF solution using a CMA 110 liquid switch for the seventh sample. The seventh sample was collected over a 20 minute period and then perfusate solution was switched back to aCSF for the collection of the next four samples (140-220 minutes). After the eleventh sample was collected, the perfusate was switched to a d-amphetamine containing aCSF solution. Sample 12 was collected over a 20 minute period and the perfusate switched back to aCSF for the following five samples (240-340 minutes). The animals were then euthanized via anesthetic overdose and their brains removed for determination of neurochemical tissue content. Sections of the substantia nigra were retained and submersion fixed, and a subset was processed histologically for TH
imunoreactivity to confirm lesion induction. Solutions for microdialysis perfusion were prepared as described below.

**Solutions for microdialysis**

Microdialysis perfusates include three separate solutions: artificial cerebrospinal fluid (or aCSF, 124 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM NaH₂PO₄, 25 mM NaHCO₃, 5.9 mM d-glucose), 100 mM K⁺ aCSF (26 mM NaCl, 100 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM NaH₂PO₄, 25 mM NaHCO₃, 5.9 mM d-glucose), and 250 µM d-amphetamine aCSF (250 µM d-amphetamine sulfate, 124 mM NaCl, 3 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM NaH₂PO₄, 25 mM NaHCO₃, 5.9 mM d-glucose), and all were pH adjusted with sulfuric acid and/or sodium hydroxide to within the 7.2-7.4 range.

A standard solution of analytes for probe recovery contained the following concentrations (M) of neurochemicals: 2 x 10⁻⁷ norepinephrine (NE), 1 x 10⁻⁷ 3,4-dihydroxyphenylacetic acid (DOPAC), 2 x 10⁻⁷ dopamine (DA), 1 x 10⁻⁷ homovanillic acid (HVA), 2 x 10⁻⁷ serotonin (5-hydroxytryptamine, 5-HT), 4 x 10⁻⁷ 5-hydroxyindoleacetic acid (5-HIAA).

**Perfusion of DNSP-11 through microdialysis probe**

In order to determine the percentage of DNSP-11 that perfuses through the CMA 11, 6000 Da MW cut-off, microdialysis probe, probes were submerged in solutions containing a range of concentrations of DNSP-11 between 0.1 µM and 1000 µM in aCSF that was kept at approximately 37°C via water bath. Probes were perfused as described above, with aCSF at 1 µL/min, and samples were collected every 20 minutes. The concentration of DNSP-11 was quantified by enzyme-linked immunosorbent assay (ELISA).

**Enzyme-linked immunosorbent assay**

A 1:1 serial dilution of the DNSP-11 peptide provided a detection range of 5 ng/mL to 2000 ng/mL. Sample was placed in wells with sodium carbonate/bicarbonate coating buffer (pH 9.6) overnight at 4°C, followed by blocking (0.1% purified BSA in PBS) at room temperature for 1 hour. The
custom, polyclonal antibody to DNSP-11 (1:2000, α-Diagnostic, San Antonio, Texas USA) was added and allowed to incubate for 2 hours at room temperature, followed by a 1 hour incubation at room temperature with secondary, peroxidase-labeled antibody (1:10,000). The chromogen (R&D Systems, Reagent Kit DY999) was allowed to develop for 20 minutes at room temperature at which time the reaction was quenched (R&D Systems, Stop Solution DY994). The optical density of the wells was recorded at 450 nm immediately after quenching.

**Acute perfusion of DNSP-11 during microdialysis**

In order to determine possible acute effects of DNSP-11 on the release of dopamine, a 50 µM DNSP-11 solution in aCSF was perfused through the CMA 11 microdialysis probe. Microdialysis was performed as above with modifications. The probe was perfused with aCSF for the 1st through 5th 20 minute samples, then the perfusate was switched to the 100 mM K+ aCSF solution for the 6th sample collection. For the 7th through 9th sample collections, the perfusate was switched back to aCSF; then the perfusate was switched to an aCSF solution or a solution of 50 µM DNSP-11 in aCSF which was perfused for the 10th to 14th samples. For the 15th sample the perfusate was switched back to the 100 mM K+ aCSF, followed by perfusion with aCSF for the 16th through 18th samples. A total of 8 animals were used in this study, four controls and four DNSP-11. Two animals in the DNSP-11 group were excluded due to non-detectable baselines.

**Results**

**Dose response in normal animals**

Normal, unlesioned animals given DNSP-11 intranasally for five of seven days a week for three weeks showed significant changes in monoamine neurochemicals at different doses (Table 4.2). While dopamine levels significantly decreased at 100 and 1000 µg doses, DOPAC significantly increased at 300 µg (Figure 4.2). This resulted in a significantly higher dopamine turnover (here defined as the ratio of all dopamine metabolites (HVA+DOPAC) to
dopamine) in animals that received 300 µg per treatment of DNSP-11 (Figure 4.3, **P=0.0014, 24.3% increase).

Significant changes the monoamine in the substantia nigra were also observed (Table 4.3). Both DOPAC and HVA were significantly elevated in the animals that received 300 mg of DNSP-11 per treatment (Figure 4.4). Dopamine turnover in the substantia nigra of these animals was also significantly elevated at this dose (Figure 4.5, ****P<0.0001, 33.7%, n=12), and at the 1000 µg per treatment dose (31.6% increase, n=12).  Also of note, the level of serotonin in the substantia nigra of animals given 1000 µg per treatment was significantly decreased (Figure 4.6, ***P=0.0003, 31.3% decrease, n=12) with a significant increase in serotonin turnover (Figure 4.7, ***P=0.0006, 21.3% increase, n=12). Finally, in the olfactory bulb (Table 4.4), dopamine levels were significantly affected by DNSP-11 treatment, but there was no observed effect on dopamine metabolites (Figure 4.8) and turnover was not significantly affected (Figure 4.9).

**Whole striatum neurochemical content in lesioned animals**

Of the lesioned animals (Table 4.5) treated with DNSP-11, scrambled peptide, or vehicle-only, no changes to striatal monoamine neurochemical tissue content was observed (Table 4.6) when comparing the negative control treated animals and the DNSP-11 treated animals (Figure 4.10, P=0.3727). The unlesioned hemispheres also were not significantly different between groups (P=0.8437). However, the animals in this study were present in two distinct populations within each group: those with a partial lesion of striatal dopamine content, and those with a severe lesion (<1000 ng/g DA, see Figure 4.10). These populations were separated for further analysis of the effects of intranasal DNSP-11 treatment.

When analyzing only the partially lesioned animals, a significant difference was observed with respect to treatment. Firstly, animals treated with vehicle only or scrambled peptide were not significantly different from each other (P=0.7905, see Table 4.7), as predicted, and were combined as one “Negative Controls” group. When comparing DNSP-11 and negative control treated animals, the effect of
treatment was significant, and the DNSP-11 treated animals contained 56.4% more striatal dopamine content than the negative control animals (Figure 4.11. ###P=0.0006, DNSP-11: 10037 ± 1672 ng/g DA; Negative Controls: 6417 ± 835.6 ng/g DA). This effect in the DNSP-11 treated animals resulted in dopamine content in the lesioned hemisphere that was not significantly different from the unlesioned hemispheres (P=0.6017). Also of note, the unlesioned hemispheres of DNSP-11 treated animals contains significantly greater dopamine by 18.2% than that of the unlesioned hemispheres of negative control treated animals (Figure 4.11, P=0.0301. DNSP-11 unlesioned hemispheres: 12101 ± 783.9; Negative Controls, unlesioned hemispheres: 10235 ± 522.9). However, in the severely lesioned animals, there was no significant difference in monoamine neurotransmitter levels (Table 4.8) between treatment groups (Figure 4.12).

Functional neurochemical release by microdialysis in lesioned animals

Of the animals subjected to striatal microdialysis (Table 4.9), significance changes with respect to potassium-evoked dopamine overflow and DOPAC levels were observed (Table 4.10). It was observed that treatment with DNSP-11 resulted in a significant decrease in the potassium-evoked overflow of dopamine (**K+ P<0.0001, 45.8% less than the combined negative controls. Figure 4.13). The vehicle and scrambled peptide treated groups were not significantly different (P=0.9573). However, baseline dopamine and metabolite overflows were not significantly different between the negative control treated and DNSP-11 treated groups (Figure 4.13), nor were baseline dopamine metabolite concentrations (Figure 4.14). The effect of decreased evoked dopamine overflow was driven primarily by animals determined to be in the severely lesioned subgroup (<1000 ng/g striatal dopamine: DNSP-11 n=3, Vehicle n=4, Scrambled n=1). When those severely lesioned animals were removed from the analysis, no significant differences between groups of partially lesioned animals was observed (P=0.9929, Figure 4.15).
Perfusion of DNSP-11 through microdialysis probe *in vitro*

By comparing the quantity of DNSP-11 collected during microdialysis to a sample of the quantity in the vial in which the probe was submerged, it was determined that an average of 1.6% ± 0.66 DNSP-11 is recovered by the microdialysis probe *in vitro* at 37°C. It was determined that 50 µM concentration of DNSP-11 in aCSF solution would result in approximately 0.8 µM perfused in the brain every 20 minute sampling period. This concentration is within an order of magnitude of the concentration in the brain after intranasal administration of 300 µg of DNSP-11 and after intracranial injection of 30 µg of DNSP-11, which is known to produce biological effects at the two-week time point (Littrell, 2011).

**Acute effects of DNSP-11 on potassium evoked release of dopamine**

After perfusion of 50 µM DNSP-11 in aCSF through the microdialysis probe for 100 minutes, dopamine overflow during potassium-evoked release was significantly lower than the normal potassium-evoked release recorded in the same animals prior to infusion with DNSP-11 (Figure 4.16; Decrease of 40.8%, ****P<0.0001). No significant differences in baseline dopamine, DOPAC (Figure 4.17) or HVA (Figure 4.18) levels were observed.

**Discussion**

**Overview and interpretation**

These studies provide data on the possible long-term and immediate effects of DNSP-11, in both normal and lesioned animals, while providing important dosing information for future studies. The dose response study in normal animals resulted in observed changes in monoamine neurochemical levels of the striatum and substantia nigra, with especially strong changes to dopamine metabolism in the substantia nigra. Due to these results, the 300 µg dose became the focus for protection studies of intranasal DNSP-11 in 6-OHDA lesioned animals.

When considering all of the 6-OHDA lesioned animals in the protection study, treatment with DNSP-11 did not have an effect on striatal dopamine levels.
content; however, due to variability of the lesion within this cohort the animals were present in two subgroups: those with severe lesions (<1000 ng/g striatal DA), and those with partial lesions. Of the animals with partial lesions, those that were treated with DNSP-11 had significantly higher levels of striatal dopamine (Figure 4.11). Indeed, the partially lesioned animals that received DNSP-11 treatment possessed striatal dopamine levels not significantly different from their unlesioned hemispheres. It is hypothesized that natural and uncontrollable variations in the 6-OHDA lesion severity resulted in a subgroup of severely lesioned animals that could not be rescued by DNSP-11 treatment at the dose provided. Furthermore, the significant, negative effects observed in the microdialysis studies were driven primarily by the three severely lesioned animals of the DNSP-11 group. This variability may be overcome in restoration studies in which animals can be prescreened for lesion severity. In addition, due to the inherent variability of intranasal delivery, reduction of lesion variability becomes important. As such, other toxic lesion or genetic models of Parkinson’s disease may prove to be better models to quantify the effects of intranasal treatment with DNSP-11.

**Dose-response curve**

The dose response in normal, unlesioned animals shows an increase in striatal dopamine turnover at the 300 µg per day dose, but no significant difference from vehicle-only treated controls at the 1000 µg per day dose, suggesting a possible bimodal response curve. When considering the effect of DNSP-11 on neurochemical content aside from dopamine it may be hypothesized that this bimodal response was due to regulatory or compensatory mechanisms. At the 1000 µg dose, serotonin content in the nigra was significantly lower (Figure 4.6) than the vehicle treated animals while serotonin turnover was significantly higher (Figure 4.7). This may indicate a regulatory or compensatory mechanism, or it may indicate differential efficacy at a range of concentrations on other monoamine systems compared to the dopamine system.
The exact roles of serotonin on the dopaminergic motor system are complex, but it is observed that these and other monoamine systems are subject to profound loss in patients suffering from Parkinson’s disease (Scatton et al., 1983; Miyawaki et al., 1997). In fact, in many patients, the symptoms of mood disorder and autonomic dysfunction precede the motor signs of Parkinson’s disease (Kummer and Teixeira, 2009; Lohle et al., 2009). Outstanding questions remain in the literature surrounding the effects of serotonin interaction in the dopaminergic system, but it has been shown that 5-HT receptor subtypes differentially affect the dopaminergic system, some antagonizing dopamine and others agonizing dopamine release in the striatum (Lucas et al., 2000a; Lucas et al., 2000b; Lucas and Spampinato, 2000; Di Matteo et al., 2008).

While norepinephrine levels were not significantly altered with DNSP-11 treatment, levels of the metabolites of norepinephrine were not measured. Furthermore, small changes in neurochemical levels may have appreciable regulatory impacts. Similar to serotonin, the effects of changes in NE levels remain to be fully described, but it is known that lesions of the locus ceruleus increase the pathology and behavior deficits in Parkinson’s disease animal models (Mavridis et al., 1991; Srinivasan and Schmidt, 2003) suggesting that NE is sufficient, at some stages of the disease, to attenuate the behavioral output and neuronal loss associated with the disease. The locus ceruleus directly innervates the SN and striatum, stimulating burst firing of the dopaminergic system (Grenhoff and Svensson, 1993). However, like the serotonin receptors, norepinephrine is bound by a multitude of receptors, all with different functions and localizations, complicating the explanation of circuit interactions (Hein et al., 1999). Norepinephrinergic projections in the striatum have been shown to modulate dopamine turnover by the α2CAR NE receptor (Sallinen et al., 1997), and may do so indirectly by modulating glia and astrocyte activity by the whole range of membrane bound NE receptor families (Mori et al., 2002). Furthermore, the α2CAR norepinephrine receptors in the striatum are known to be sensitive to dopamine at low synaptic concentrations (Hein et al., 1999; Zhang W. et al., 2008).
1999) while not being activated by apomorphine (Fox et al., 2001), indicating complex cross-sensitivities to these various neurotransmitters.

As such, the dose response to DNSP-11 may be best described under the hypothesis of differential effects to the monoamine systems both pre- and post-synaptically. Such effects may be elucidated by primary cell culture studies or by highly localized analysis in animal models, but are beyond the scope of the present study. Due to the powerful effect on the striatal dopamine system and the relative paucity of changes to other systems and anatomical regions at the 300 µg per treatment dose, it was determined that the protection studies should focus on this concentration of administered DNSP-11.

The use of isoflurane anesthesia in this study may in itself alter neurochemical production, however no significant correlation between duration of isoflurane anesthesia and striatal dopamine turnover was found (m=0.0004, R²=0.4, P=0.0959, data not shown). Furthermore, the anesthetic used during intranasal administration may alter respiration and blood-flow, changing the dynamic of intranasal delivery. Indeed, reports differ on the use of anesthesia in rodent models of intranasal delivery. Some suggest that isoflurane anesthesia adversely affects intranasal delivery by drying the nasal mucosa, while other reports indicate anesthetics may open the blood-brain barrier (Tetrault et al., 2008). However, these effects appear to be minimal in scope. Ideally, future studies would move to self-administration, which is feasible in non-human primate models.

**Effects of intranasal DNSP-11 in 6-OHDA lesioned animals**

While DNSP-11 produced effects on the monoamine systems in the normal, unlesioned animal, the protective effects of DNSP-11 on dopamine neurochemical content were only observed in a subset of animals with partial lesions (Figure 4.11). Lesion variability among this cohort resulted in a subgroup of severely lesioned animals (Figure 4.12) upon which DNSP-11 was insufficient, at the dose given, to produce the same protective effect seen in the partially lesioned subgroup of animals.
It is important to note the differences between the location of lesion induction and the location of the measurements of both neurochemical content and functional release by microdialysis. In contrast with previous DNSP-11 studies (Bradley et al., 2010) which utilized a severe lesion of the medial forebrain bundle (MFB) and neurochemical content analysis targeted specifically to the dorsolateral rat striatum, the present study used a lesion of the substantia nigra and analysis of the whole striatum. A targeted analysis of the dorsolateral striatum, or the use of other models including the MFB lesion model, may result in reduced noise and variability. In addition, the repeated intranasal dosing regimen in which the animals were off-treatment for the two days prior to neurochemical endpoint may have created unintended consequences. The intent of this timeline was to emphasize long-term protective effects of DNSP-11 treatment; however, this may also have introduced a negative rebound or a “wash-out” effect due to being off of treatment, perhaps complicating the neurochemical data.

The effects of DNSP-11 on functional neurochemistry, as collected by microdialysis, most closely resemble the data obtained from aged Fischer 344 rats administered DNSP-11 in a single intracranial injection (Fuqua, 2010), in which potassium-evoked and baseline release of dopamine were found to be significantly decreased in DNSP-11 treated animals. In the present study, the DNSP-11 treated animals exhibited a significant decrease in potassium evoked dopamine (Figure 4.13) and a non-significant trend toward decreased baseline levels of dopamine and its metabolites, while d-amphetamine evoked release was unchanged. This decrease was driven primarily by the small (n=3 DNSP-11 treated) subgroup of severely lesioned animals suggesting these changes may be occurring only in the severely depleted striatum or may be a statistical artifact. If this effect is confirmed, one hypothesis is that DNSP-11 may have pleiotropic effects aside from long-term protection against neurotoxic lesions, all while not affecting dopamine content or d-amphetamine evoked release of dopamine. The decrease specifically in potassium-evoked, calcium-dependent release may indicate that DNSP-11 affected dopamine release pre-synaptically through a
calcium-dependent mechanism. Other possibilities include post-synaptic sensitization or an increase in auto-inhibition of the pre-synaptic neuron. Indeed, 5-HT has been shown to increase auto-inhibition of the substantia nigra and VTA by DA (Olijslagers et al., 2004). This may be an important area for future studies of long and short term effects of DNSP-11.

**Acute versus long-term effects of DNSP-11**

It was observed in the protection studies that a subgroup of severely lesioned animals exhibited greatly reduced potassium-evoked, calcium-dependent release of dopamine after three weeks of intranasal DNSP-11 treatment, and this led to questions about possible pleiotropic effects of DNSP-11. Here, naïve animals were taken for striatal microdialysis, during which they were perfused with a 50 µM solution of DNSP-11 in aCSF, or aCSF-only without peptide, through the microdialysis probe. Potassium-evoked dopamine release was performed before and after the perfusion of DNSP-11 or aCSF-only, and was found to be decreased after perfusion with DNSP-11 and significantly lower than in aCSF-only perfused animals. Data from this study supports the hypothesis that DNSP-11 has acute effects on calcium-dependent, potassium-evoked dopamine release within two hours of introduction to the striatum via microdialysis probe. Additionally, no significant intra-animal variability in potassium-evoked DA release was observed in the control animals, indicating the analysis of the second potassium-evoked release as a percentage of the first potassium-evoked release is valid. This finding of the equality of multiple potassium-evoked releases is consistent with one report of microdialysis in awake animals (Ripley et al., 1997) but contradicts another in anesthetized animals (Kametani et al., 1995).

In summary, while DNSP-11 significantly affected the ratio of dopamine to its metabolites in the striatum and substantia nigra of normal animals, the dose of DNSP-11 provided to lesioned animals may be insufficient to protect against a severe, acute toxic loss of neurons in a subset of animals. The data supports the overall hypothesis that intranasal DNSP-11 produces biological effects on the central nervous system.
Figure 4.1 – Representative placement of the microdialysis probe in the striatum. The vertical black line with blue end represents the CMA 11 microdialysis probe with 4 mm long 6,000 MW cut-off membrane at its final position at the following coordinates (with respect to bregma): AP: +1.5 mm, ML: -2.3 mm, DV: -8.0 mm (from dural surface), TB: -3.3 mm. Modified from (Paxinos and Watson, 2005). (ac, anterior commissure; cc, corpus callosum; LV, lateral ventricle.)
### Table 4.1 – Animal groups for dose response study in normal, unlesioned rats.

<table>
<thead>
<tr>
<th>Numbers:</th>
<th>9</th>
<th>6</th>
<th>6</th>
<th>6</th>
</tr>
</thead>
</table>

A total of 27 animals were used in this study. For every 6 animals in each dosing group, 3 animals were concomitantly administered and evaluated vehicle only (0 µg), and the neurochemical content of the DNSP-11 administered animals were compared as a percentage of the concomitantly administered vehicle only animals. Due to the bilateral treatment, each hemisphere was used as an independent data point. No animals were excluded from this study. Treatment was performed in a blinded manner.
Table 4.2 – Striatal neurochemical levels in normal rats given a range of intranasal doses of DNSP-11.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 µg DNSP-11 per day (Vehicle only)</th>
<th>100 µg DNSP-11 per day</th>
<th>300 µg DNSP-11 per day</th>
<th>1000 µg DNSP-11 per day</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA (ng/g)</td>
<td>12427 ± 1693</td>
<td>9043 ± 3496</td>
<td>11923 ± 2213</td>
<td>9910 ± 2428</td>
</tr>
<tr>
<td></td>
<td>t(50)=3.695</td>
<td>t(50)=2.820</td>
<td>t(50)=0.5494</td>
<td>t(50)=2.748</td>
</tr>
<tr>
<td>DOPAC (ng/g)</td>
<td>1919 ± 400</td>
<td>1257 ± 425</td>
<td>2422 ± 635</td>
<td>1574 ± 455</td>
</tr>
<tr>
<td></td>
<td>t(50)=3.722</td>
<td>t(50)=2.040</td>
<td>t(50)=1.938</td>
<td>t(50)=2.366</td>
</tr>
<tr>
<td>HVA (ng/g)</td>
<td>832 ± 254</td>
<td>570 ± 192</td>
<td>843 ± 154</td>
<td>658 ± 147</td>
</tr>
<tr>
<td></td>
<td>t(50)=3.515</td>
<td>t(50)=0.1406</td>
<td>t(50)=2.366</td>
<td>t(50)=0.4492</td>
</tr>
<tr>
<td>(HVA+DOPAC)/DA</td>
<td>0.221 ± 0.032</td>
<td>0.215 ± 0.051</td>
<td>0.274 ± 0.040</td>
<td>0.227 ± 0.032</td>
</tr>
<tr>
<td></td>
<td>t(50)=0.3571</td>
<td>t(50)=0.1406</td>
<td>t(50)=3.709</td>
<td>t(50)=0.4492</td>
</tr>
<tr>
<td>5-HT (ng/g)</td>
<td>576 ± 104</td>
<td>625 ± 110</td>
<td>555 ± 94</td>
<td>582 ± 93</td>
</tr>
<tr>
<td></td>
<td>t(50)=1.317</td>
<td>t(50)=0.5423</td>
<td>t(50)=0.1594</td>
<td>t(50)=1.033</td>
</tr>
<tr>
<td>5-HIAA (ng/g)</td>
<td>653 ± 126</td>
<td>605 ± 74</td>
<td>739 ± 61</td>
<td>640 ± 78</td>
</tr>
<tr>
<td></td>
<td>t(50)=1.364</td>
<td>t(50)=2.474</td>
<td>t(50)=0.3618</td>
<td>t(50)=0.3678</td>
</tr>
<tr>
<td>(5-HIAA/5-HT)</td>
<td>1.14 ± 0.17</td>
<td>0.980 ± 0.099</td>
<td>1.35 ± 0.17</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>t(50)=3.028</td>
<td>t(50)=3.869</td>
<td>t(50)=0.6256</td>
<td>t(50)=3.869</td>
</tr>
<tr>
<td>NE (ng/g)</td>
<td>100 ± 31</td>
<td>139 ± 83</td>
<td>92 ± 28</td>
<td>99 ± 50</td>
</tr>
<tr>
<td></td>
<td>t(50)=2.064</td>
<td>t(50)=0.3348</td>
<td>t(50)=0.06490</td>
<td>t(50)=2.089</td>
</tr>
<tr>
<td>n = (hemispheres)</td>
<td>18</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>

Animals treated intranasally with DNSP-11 exhibited significant changes in the levels of striatal dopamine and its metabolites. (Values are displayed as “analyte mean ng/g of analyte ± standard deviation”. Analysis by one-way ANOVA with Bonferroni’s post hoc test. With respect to 0 µg vehicle-only treated group, *P<0.05; **P<0.01; ***P<0.001.)
Figure 4.2 – Striatal dopamine and metabolites in normal rats given intranasal DNSP-11.

A significant decrease in dopamine content was observed in the 100 µg/day and 1000 µg/day treated groups, DOPAC was significantly different at the 100 µg/day and 300 µg/day groups, and HVA was significantly decreased in the 100 µg/day. (DA: ##Treatment P=0.0016; DOPAC: ####Treatment P=0.0001; HVA: ##Treatment P=0.0016 by one-way ANOVA. 0 µg, n=18 hemispheres; 100 µg, n=12 hemispheres; 300 µg, n=12 hemispheres; 1000 µg, n=12 hemispheres. Analysis by one-way ANOVA with Bonferroni’s post hoc test. With respect to 0 µg vehicle-only treated group, *P<0.05; **P<0.01. Error bars displayed as SEM.)
Figure 4.3 – Striatal total dopamine turnover in normal rats given intranasal DNSP-11.

A significant increase in total dopamine turnover was observed in the striatum of normal rats treated with 300 µg/day DNSP-11 for five of seven days a week for three weeks. (**Treatment, P<0.01 with respect to 0 µg vehicle-only treated group. Error bars displayed as SEM.)
Table 4.3 – Nigral neurochemical levels in normal rats given a range of intranasal doses of DNSP-11.

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>Treatment</th>
<th>Treatment</th>
<th>Treatment</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µg DNSP-11 per day</td>
<td>100 µg DNSP-11 per day</td>
<td>300 µg DNSP-11 per day</td>
<td>1000 µg DNSP-11 per day</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA (ng/g)</td>
<td>620 ± 250</td>
<td>481 ± 203</td>
<td>815 ± 204</td>
<td>416 * ± 173</td>
<td>P=0.0002</td>
</tr>
<tr>
<td></td>
<td>t(50) = 1.749</td>
<td>t(50) = 2.439</td>
<td>t(50) = 2.559</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOPAC (ng/g)</td>
<td>151 ± 69</td>
<td>103 ± 50</td>
<td>269 ****</td>
<td>124 ± 66</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>t(50) = 1.977</td>
<td>t(50) = 4.872</td>
<td>t(50) = 1.099</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HVA (ng/g)</td>
<td>55 ± 18</td>
<td>37 * ± 17</td>
<td>81 ***</td>
<td>51 ± 11</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>t(50) = 3.057</td>
<td>t(50) = 4.198</td>
<td>t(50) = 0.7712</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(HVA+DOPAC)/DA</td>
<td>0.332 ± 0.047</td>
<td>0.289 ± 0.066</td>
<td>0.443 **</td>
<td>0.436 **</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>t(50) = 1.340</td>
<td>t(50) = 0.119</td>
<td>t(50) = 3.556</td>
<td>t(50) = 3.331</td>
<td></td>
</tr>
<tr>
<td>5-HT (ng/g)</td>
<td>882 ± 252</td>
<td>767 ± 161</td>
<td>928 ± 107</td>
<td>606 ***</td>
<td>P=0.0003</td>
</tr>
<tr>
<td></td>
<td>t(50) = 1.637</td>
<td>t(50) = 0.6653</td>
<td>t(50) = 3.940</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HIAA (ng/g)</td>
<td>685 ± 152</td>
<td>572 ± 109</td>
<td>812 *</td>
<td>582 ± 95</td>
<td>P&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>t(50) = 2.606</td>
<td>t(50) = 2.898</td>
<td>t(50) = 2.375</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5-HIAA/5-HT)</td>
<td>0.796 ± 0.105</td>
<td>0.757 ± 0.135</td>
<td>0.882 *</td>
<td>1.011 **</td>
<td>P=0.0006</td>
</tr>
<tr>
<td></td>
<td>t(50) = 0.6823</td>
<td>t(50) = 1.508</td>
<td>t(50) = 3.763</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NE (ng/g)</td>
<td>303 ± 75</td>
<td>293 ± 86</td>
<td>299 ± 82</td>
<td>334 ± 191</td>
<td>P=0.8191</td>
</tr>
<tr>
<td></td>
<td>t(50) = 0.2434</td>
<td>t(50) = 0.1009</td>
<td>t(50) = 0.7191</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n = (hemispheres)</td>
<td>18</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Animals treated intranasally with DNSP-11 exhibited significant changes in the levels of nigral dopamine and its metabolites. Significance is shown with respect to the 0 µg vehicle-only treated group. (Values are displayed as “analyte mean ng/g ± standard deviation”. Analyses by one-way ANOVA with Bonferroni’s post hoc test. With respect to 0 µg vehicle-only treated group, *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.)
A significant change in the levels of dopamine and its metabolites were observed in the substantia nigra after treatment with intranasal DNSP-11. (DA: ###Treatment P=0.0002; DOPAC: ####Treatment P<0.0001; HVA: ####Treatment P<0.0001. 0 μg, n=18 hemispheres; 100 μg, n=12 hemispheres; 300 μg, n=12 hemispheres; 1000 μg, n=12 hemispheres. Analyses by one-way ANOVA with Bonferroni’s post hoc test. With respect to 0 μg vehicle-only treated group, *P<0.05; ***P<0.001; ****P<0.0001. Error bars displayed as SEM.)
Figure 4.5 – Nigral total dopamine turnover in normal rats given intranasal DNSP-11.

The ratio of HVA+DOPAC to DA was significantly different between vehicle only and DNSP-11 treated groups (####Treatment P<0.0001; 0 µg, n=18 hemispheres. 100 µg, n=12 hemispheres; **300 µg, P<0.01, n=12 hemispheres; **1000 µg, P<0.01, n=12 hemispheres. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Significance is shown with respect to 0 µg vehicle-only treated group. Error bars displayed as SEM.)
Figure 4.6 – Serotonin levels in the substantia nigra of normal rats given intranasal DNSP-11.

Serotonin levels were significantly decreased in animals treated intranasally with DNSP-11 for three weeks when compared with concomitant vehicle-only negative control animals. (###Treatment P=0.0003; **1000 µg, P<0.01, n=12 hemispheres; 0 µg, n=18 hemispheres; 100 µg, n=12 hemispheres; 300 µg, n=12 hemispheres. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Significance is shown with respect to 0 µg vehicle-only treated group. Error bars displayed as SEM.)
Figure 4.7 – Serotonin turnover in the substantia nigra of normal rats treated with DNSP-11.

Serotonin turnover was significantly elevated in animals treated intranasally with DNSP-11 for three weeks when compared with concomitant vehicle-only negative control animals. (###Treatment $P=0.0006$; **$1000 \mu g$, $P<0.01$, n=12 hemispheres; 0 $\mu g$, n=18 hemispheres; 100 $\mu g$, n=12 hemispheres; 300 $\mu g$, n=12 hemispheres. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Significance is shown with respect to 0 $\mu g$ vehicle-only treated group. Error bars displayed as SEM.)
Table 4.4 – Neurochemical levels in the olfactory bulb of normal rats given a range of intranasal doses of DNSP-11.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>0 µg DNSP-11 per day (Vehicle only)</th>
<th>100 µg DNSP-11 per day</th>
<th>300 µg DNSP-11 per day</th>
<th>1000 µg DNSP-11 per day</th>
<th>Treatment Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA (ng/g)</td>
<td>30 ± 14</td>
<td>16 ** ± 7 t_{(50)}=3.426</td>
<td>38 ± 5 t_{(50)}=1.865</td>
<td>43 ** ± 13 t_{(50)}=3.223</td>
<td>P&lt;0.0001 #</td>
</tr>
<tr>
<td>DOPAC (ng/g)</td>
<td>15 ± 5</td>
<td>16 ± 7 t_{(50)}=0.5192</td>
<td>15 ± 4 t_{(50)}=0.02533</td>
<td>14 ± 7 t_{(50)}=0.3166</td>
<td>P=0.8963</td>
</tr>
<tr>
<td>HVA (ng/g)</td>
<td>46 ± 12</td>
<td>42 ± 13 t_{(50)}=0.7615</td>
<td>39 ± 12 t_{(50)}=1.316</td>
<td>38 ± 16 t_{(50)}=1.534</td>
<td>P=0.4091</td>
</tr>
<tr>
<td>(HVA+DOPAC)/DA</td>
<td>1.63 ± 0.51</td>
<td>1.42 ± 0.32 t_{(50)}=1.331</td>
<td>1.42 ± 0.36 t_{(50)}=1.308</td>
<td>1.20 ± 0.39 t_{(50)}=2.743</td>
<td>* P=0.0671</td>
</tr>
<tr>
<td>5-HT (ng/g)</td>
<td>176 ± 34</td>
<td>207 ± 49 t_{(50)}=1.152</td>
<td>219 ± 105 t_{(50)}=1.605</td>
<td>210 ± 88 t_{(50)}=1.287</td>
<td>P=0.3703</td>
</tr>
<tr>
<td>5-HIAA (ng/g)</td>
<td>133 ± 21</td>
<td>141 ± 25 t_{(50)}=0.6271</td>
<td>157 ± 40 t_{(50)}=1.830</td>
<td>148 ± 50 t_{(50)}=1.131</td>
<td>P=0.3194</td>
</tr>
<tr>
<td>(5-HIAA/5-HT)</td>
<td>0.771 ± 0.138</td>
<td>0.699 ± 0.144 t_{(50)}=1.313</td>
<td>0.779 ± 0.175 t_{(50)}=0.1541</td>
<td>0.724 ± 0.130 t_{(50)}=0.8603</td>
<td>P=0.4583</td>
</tr>
<tr>
<td>NE (ng/g)</td>
<td>183 ± 62</td>
<td>204 ± 39 t_{(50)}=1.145</td>
<td>209 ± 34 t_{(50)}=1.376</td>
<td>121 ** ± 51 t_{(50)}=3.371</td>
<td>P=0.0002 #</td>
</tr>
<tr>
<td>n = (hemispheres)</td>
<td>18</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

Animals treated intranasally with DNSP-11 exhibited significant changes in the levels of some neurochemicals in the olfactory bulb. Significance is shown with respect to the 0 µg vehicle-only treated group. (Values are displayed as “analyte mean ng/g ± standard deviation”. Analyses by one-way ANOVA with Bonferroni’s post hoc test. With respect to 0 µg vehicle-only treated group, *P<0.05; **P<0.01.)
Figure 4.8 – Olfactory bulb dopamine and metabolites in normal rats given intranasal DSNP-11.

Significant differences in the levels of dopamine were observed in the olfactory bulb of normal rats given intranasal DSNP-11 daily for three weeks. (Dopamine: Treatment P<0.0001. 0 µg, n=18 hemispheres; 100 µg, n=12 hemispheres; 300 µg, n=12 hemispheres; 1000 µg, n=12 hemispheres. Analyses by one-way ANOVA with Bonferroni’s post hoc test. With respect to 0 µg vehicle-only treated group, *P<0.05; **P<0.01. Error bars displayed as SEM.)
Figure 4.9 – Olfactory bulb total dopamine turnover in normal rats given intranasal DNSP-11.

Total dopamine turnover in the olfactory bulb was not significantly different between groups. (Overall treatment $P=0.0671$; $*1000 \, \mu g \, P=0.0253$, $n=12$ hemispheres $0 \, \mu g$, $n=18$ hemispheres; $100 \, \mu g$, $n=12$ hemispheres; $300 \, \mu g$, $n=12$ hemispheres. Analyses by one-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM.)
Table 4.5 – Animal groups for neurochemical content study in 6-OHDA lesioned animals.

<table>
<thead>
<tr>
<th>Numbers</th>
<th>Vehicle-only Treated</th>
<th>Scrambled Treated</th>
<th>DNSP-11 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
<td>9</td>
<td>14</td>
</tr>
</tbody>
</table>

Scrambled and vehicle (saline only) were combined into one group for analysis due to similarities in response. The DNSP-11 and Scrambled peptide treated groups received 300 µg in 50 µL per treatment of each compound respectively. Due to similarities in response, vehicle-only and scrambled peptide treated groups were combined into one “Negative Control” group. Of the animals above, one DNSP-11 treated and one scrambled treated animal were excluded due to complications during endpoint collection. Three animals not included in the above numbers died due to anesthetic complications during the lesion induction surgery: two were in the DNSP-11 group and one was in the scrambled treated group. Treatment was performed in a blinded manner.
Table 4.6 – Striatal neurochemical content of 6-OHDA lesioned animals.

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>One-way ANOVA Treatment Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle Only</td>
<td>Scrambled Peptide</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA (ng/g)</td>
<td>3738 ± 3577</td>
<td>5022 ± 4391</td>
</tr>
<tr>
<td>DOPAC (ng/g)</td>
<td>677 ± 552</td>
<td>1062 ± 790</td>
</tr>
<tr>
<td>HVA (ng/g)</td>
<td>433 ± 390</td>
<td>453 ± 446</td>
</tr>
<tr>
<td>(HVA+DOPAC)/DA</td>
<td>0.527 ± 0.456</td>
<td>0.421 ± 0.262</td>
</tr>
<tr>
<td>5-HT (ng/g)</td>
<td>613 ± 182</td>
<td>640 ± 120</td>
</tr>
<tr>
<td>5-HIAA (ng/g)</td>
<td>1198 ± 209</td>
<td>1174 ± 286</td>
</tr>
<tr>
<td>(5-HIAA/5-HT)</td>
<td>2.10 ± 0.66</td>
<td>1.83 ± 0.26</td>
</tr>
<tr>
<td>NE (ng/g)</td>
<td>75 ± 65</td>
<td>92 ± 49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA (ng/g)</td>
<td>10621 ± 1847</td>
<td>9772 ± 1829</td>
</tr>
<tr>
<td>DOPAC (ng/g)</td>
<td>1777 ± 474</td>
<td>1591 ± 333</td>
</tr>
<tr>
<td>HVA (ng/g)</td>
<td>880 ± 201</td>
<td>779 ± 166</td>
</tr>
<tr>
<td>(HVA+DOPAC)/DA</td>
<td>0.258 ± 0.047</td>
<td>0.247 ± 0.049</td>
</tr>
<tr>
<td>5-HT (ng/g)</td>
<td>650 ± 152</td>
<td>671 ± 126</td>
</tr>
<tr>
<td>5-HIAA (ng/g)</td>
<td>1154 ± 225</td>
<td>1109 ± 245</td>
</tr>
<tr>
<td>(5-HIAA/5-HT)</td>
<td>1.84 ± 0.45</td>
<td>1.66 ± 0.24</td>
</tr>
<tr>
<td>NE (ng/g)</td>
<td>143 ± 34</td>
<td>150 ± 42</td>
</tr>
<tr>
<td>n =</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

The striatal neurochemical profile of rats subjected to a 6-OHDA lesion is provided above. While this data represents the mean values (± standard deviation) of the animals in each group, the animals were present in two distinct populations within these groups: those with severe lesions and those with partial lesions. These populations were separated for further analysis below.
Striatal dopamine content in the lesioned hemisphere was not significantly higher in animals given DNSP-11 compared with vehicle-only and scrambled-peptide treated animals (P=0.3727, t-test). Note the severely lesioned population in both the DNSP-11 and Negative Control treated groups below 1000 ng/g DA. As predicted, the lesioned hemispheres of Vehicle only and Scrambled peptide treated animals were not significantly different (P=0.4815) and were combined as one “Negative Control” group. Dopamine content was also not significantly different in the intact hemispheres (P=0.8437, two-tailed t-test). (DNSP-11, n=13; Negative Controls: Vehicle-only, n=11, Scrambled n=9.) (One vehicle-only, intact hemisphere data point is outside the plot at 35877 ng/g DA and was found to be a significant outlier by Grubb’s test. Displayed as mean ± SEM.)
Table 4.7 – Striatal neurochemical content of partially lesioned animals.

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>Vehicle Only</th>
<th>Scrambled Peptide</th>
<th>DNSP-11 Peptide</th>
<th>One-way ANOVA Treatment Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P=0.1354</td>
</tr>
<tr>
<td>Lesioned</td>
<td>DA (ng/g)</td>
<td>6198 ± 2539</td>
<td>6673 ± 3728</td>
<td>10037 ± 4095</td>
<td></td>
</tr>
<tr>
<td>hemisphere</td>
<td>DOPAC (ng/g)</td>
<td>1083 ± 305</td>
<td>1350 ± 629</td>
<td>1651 ± 488</td>
<td>P=0.1470</td>
</tr>
<tr>
<td></td>
<td>HVA (ng/g)</td>
<td>714 ± 236</td>
<td>604 ± 411</td>
<td>953 ± 419</td>
<td>P=0.1579</td>
</tr>
<tr>
<td></td>
<td>(HVA+DOPAC)/DA</td>
<td>0.308 ± 0.073</td>
<td>0.284 ± 0.049</td>
<td>0.276 ± 0.073</td>
<td>P=0.6677</td>
</tr>
<tr>
<td></td>
<td>5-HT (ng/g)</td>
<td>697 ± 174</td>
<td>641 ± 132</td>
<td>658 ± 132</td>
<td>P=0.7771</td>
</tr>
<tr>
<td></td>
<td>5-HIAA (ng/g)</td>
<td>1266 ± 251</td>
<td>1162 ± 328</td>
<td>1287 ± 260</td>
<td>P=0.6908</td>
</tr>
<tr>
<td></td>
<td>(5-HIAA/5-HT)</td>
<td>1.91 ± 0.57</td>
<td>1.80 ± 0.23</td>
<td>1.99 ± 0.40</td>
<td>P=0.7176</td>
</tr>
<tr>
<td></td>
<td>NE (ng/g)</td>
<td>116 ± 53</td>
<td>113 ± 29</td>
<td>122 ± 37</td>
<td>P=0.9237</td>
</tr>
<tr>
<td>Intact</td>
<td>DA (ng/g)</td>
<td>10090 ± 1913</td>
<td>9959 ± 2042</td>
<td>1210 ± 1920</td>
<td>P=0.1311</td>
</tr>
<tr>
<td>hemisphere</td>
<td>DOPAC (ng/g)</td>
<td>1629 ± 504</td>
<td>1644 ± 340</td>
<td>1845 ± 321</td>
<td>P=0.5766</td>
</tr>
<tr>
<td></td>
<td>HVA (ng/g)</td>
<td>919 ± 163</td>
<td>813 ± 175</td>
<td>993 ± 136</td>
<td>P=0.1537</td>
</tr>
<tr>
<td></td>
<td>(HVA+DOPAC)/DA</td>
<td>0.268 ± 0.060</td>
<td>0.253 ± 0.054</td>
<td>0.238 ± 0.038</td>
<td>P=0.6165</td>
</tr>
<tr>
<td></td>
<td>5-HT (ng/g)</td>
<td>687 ± 185</td>
<td>689 ± 139</td>
<td>660 ± 108</td>
<td>P=0.9304</td>
</tr>
<tr>
<td></td>
<td>5-HIAA (ng/g)</td>
<td>1193 ± 281</td>
<td>1134 ± 275</td>
<td>1155 ± 237</td>
<td>P=0.9144</td>
</tr>
<tr>
<td></td>
<td>(5-HIAA/5-HT)</td>
<td>1.82 ± 0.50</td>
<td>1.66 ± 0.27</td>
<td>1.77 ± 0.31</td>
<td>P=0.7156</td>
</tr>
<tr>
<td></td>
<td>NE (ng/g)</td>
<td>146 ± 41</td>
<td>161 ± 40</td>
<td>143 ± 40</td>
<td>P=0.6671</td>
</tr>
</tbody>
</table>

As predicted, Vehicle only and Scrambled peptide treated animals were not significantly different with regards to dopamine (P=0.7905, two-tailed t-test) and were combined as one “Negative Controls” group for further analysis below.

Analysis by one-way ANOVA with Bonferroni’s post hoc. Values are mean ± SD.
Figure 4.11 – Striatal dopamine content in partially lesioned animals after 21 days with intranasal treatment.

Among the animals with a partial lesion, those animals treated with intranasal DNSP-11 exhibited significantly greater dopamine content in the lesioned hemisphere when compared with animals treated with Vehicle only or Scrambled peptide (Overall effect of treatment ###P<0.0006, F(3,33)=7.481. Lesioned hemispheres *P=0.0343, t(33)=2.679). The lesioned hemispheres of the DNSP-11 treated animals was not significantly different than their unlesioned hemispheres (P=0.6017, t(33)=0.1448). Analysis by one-way ANOVA with Bonferroni’s post hoc test. As predicted, the lesioned hemispheres of the Vehicle only and Scrambled peptide treated animals were not significantly different (P=0.7905 by two-tailed t-test) and were combined as one “Negative Control” group. The intact hemispheres were significantly different between groups (*P=0.0301 by t-test, t(16)=2.022). Displayed as mean ± SEM.
Table 4.8 – Striatal neurochemical content of severely lesioned animals.

<table>
<thead>
<tr>
<th>Lesioned hemisphere</th>
<th>Treatment</th>
<th>One-way ANOVA Treatment Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vehicle Only</td>
</tr>
<tr>
<td></td>
<td>DA (ng/g)</td>
<td>293 ± 279</td>
</tr>
<tr>
<td></td>
<td>DOPAC (ng/g)</td>
<td>109 ± 84</td>
</tr>
<tr>
<td></td>
<td>HVA (ng/g)</td>
<td>39 ± 60</td>
</tr>
<tr>
<td></td>
<td>(HVA+DOPAC)/DA</td>
<td>0.832 ± 0.602</td>
</tr>
<tr>
<td></td>
<td>5-HT (ng/g)</td>
<td>495 ± 124</td>
</tr>
<tr>
<td></td>
<td>5-HIAA (ng/g)</td>
<td>1102 ± 79</td>
</tr>
<tr>
<td></td>
<td>(5-HIAA/5-HT)</td>
<td>2.36 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>NE (ng/g)</td>
<td>18 ± 21</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Intact hemisphere</th>
<th>Treatment</th>
<th>One-way ANOVA Treatment Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DA (ng/g)</td>
<td>11258 ± 1739</td>
</tr>
<tr>
<td></td>
<td>DOPAC (ng/g)</td>
<td>1984 ± 382</td>
</tr>
<tr>
<td></td>
<td>HVA (ng/g)</td>
<td>791 ± 236</td>
</tr>
<tr>
<td></td>
<td>(HVA+DOPAC)/DA</td>
<td>0.246 ± 0.028</td>
</tr>
<tr>
<td></td>
<td>5-HT (ng/g)</td>
<td>598 ± 81</td>
</tr>
<tr>
<td></td>
<td>5-HIAA (ng/g)</td>
<td>1098 ± 120</td>
</tr>
<tr>
<td></td>
<td>(5-HIAA/5-HT)</td>
<td>1.87 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>NE (ng/g)</td>
<td>139 ± 27</td>
</tr>
<tr>
<td>n = 5</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>

As predicted, Vehicle only and Scambled peptide treated animals were not significantly different (P=0.3300, two-tailed t-test) and were combined as one “Negative Control” group for further analysis. Analysis by one-way ANOVA with Bonferroni’s post hoc. Values are mean ± SD.
Figure 4.12 – Striatal dopamine content in severely lesioned animals after 21 days with intranasal treatment.

The lesioned hemispheres of animals that exhibited a severe lesion of striatal dopamine (<1000 ng/g DA, see Figure 4.10) were not significantly different between groups (P=0.2427, t-test). As predicted, Vehicle only and Scrambled peptide treated animals were not significantly different (P=0.3300, two-tailed t-test) and were combined as one “Negative Control” group. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Displayed as mean ± SEM.
Table 4.9 – Animal groups for functional neurochemical study in 6-OHDA lesioned animals.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-only Treated</th>
<th>Scrambled Treated</th>
<th>DNSP-11 Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers:</td>
<td>12</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

Scrambled and vehicle treated animals were combined into one group for analysis due to similarities in response. The DNSP-11 and scrambled treated group received 300 µg in 50 µL per treatment of each compound respectively. Due to similarities in response (Chapter Three), vehicle-only and scrambled peptide treated groups were combined into one “Negative Control” group. Of the DNSP-11 treated animals, one was excluded due to complications during microdialysis resulting in premature death and one was excluded due to a bad microdialysis probe or poor collection. Two vehicle treated animals were excluded due to a bad microdialysis probes or poor collection. Two scrambled treated animals were excluded due to bad microdialysis probes or poor collection. Three animals not included in the above numbers died due to anesthetic complications during the lesion induction surgery: two were in the DNSP-11 group and one was in the scrambled treated group. Treatment was performed in a blinded manner.
**Table 4.10** – Neurochemical concentrations of microdialysis collected samples.

<table>
<thead>
<tr>
<th>Perfusate solution and Sample #</th>
<th>Analyte</th>
<th>Vehicle Only group [nM]</th>
<th>Scrambled Peptide group [nM]</th>
<th>DNSP-11 Peptide group [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>aCSF Samples 4 - 6 (Baseline)</td>
<td>DA</td>
<td>7.79 ± 4.00</td>
<td>5.61 ± 5.49</td>
<td>4.88 ± 3.32</td>
</tr>
<tr>
<td></td>
<td>DOPAC</td>
<td>2360 ± 1276</td>
<td>2052 ± 1447</td>
<td>1328 ± 873</td>
</tr>
<tr>
<td></td>
<td>HVA</td>
<td>1504 ± 826</td>
<td>1394 ± 882</td>
<td>1673 ± 2139</td>
</tr>
<tr>
<td>100 mM K+ Sample 7</td>
<td>DA</td>
<td>413 ± 189</td>
<td>331 ± 283</td>
<td>191 ± 184 <strong>$$</strong></td>
</tr>
<tr>
<td>250 µM d-Amp Sample 11</td>
<td>DA</td>
<td>335 ± 163</td>
<td>408 ± 230</td>
<td>288 ± 200 $$</td>
</tr>
</tbody>
</table>

Neurochemical concentrations collected during microdialysis were significantly affected by treatment with DNSP-11. Significantly lower potassium (K+) evoked release of dopamine was observed in animals treated intranasally with DNSP-11 daily for three weeks following 6-OHDA lesion induction of the substantia nigra compared with negative control treated animals. The overall effect of treatment on dopamine was significant (\#P=0.0184, F\(F_{(2,436)}=5.073\)), the effect of treatment on 100 mM K+ evoked DA was significant (****P<0.0001, t\(t_{(436)}=6.838\) with respect to vehicle; $$$P=0.0001, t_{(436)}=4.149\) with respect to scrambled). Effect of treatment on 250 µM d-Amp evoked DA was significant ($$P=0.0026, t_{(436)}=3.334\) with respect to scrambled). Overall effect of treatment on DOPAC was significant (####P<0.0001), but no significant difference in baseline, K+ evoked, or d-amp evoked DOPAC levels were observed. Overall effect of treatment on HVA was not significant (P=0.4251). As predicted, no significant difference in dopamine was observed between scrambled and vehicle treated groups (overall effect of treatment: P=0.7244). Analysis by two-way ANOVA with Bonferroni’s post hoc test. Values shown are “mean nM of analyte ± SD.”
Figure 4.13 – Functional release of dopamine collected by microdialysis in the striatum of lesioned animals.
Significantly lower potassium (K+) evoked release of dopamine was observed in animals treated intranasally with DNSP-11 daily for three weeks following 6-OHDA lesion induction of the substantia nigra compared with negative control treated animals. No changes in baseline dopamine release were observed. (#Treatment P=0.0184, F(2,436)=5.073. ****K+ P<0.0001, t(436)=6.838 with respect to Vehicle, $$$K+ P=0.0001, t(436)=4.149 with respect to Scrambled. $$d-amp P=0.0026, t(436)=3.334 with respect to Scrambled.) DNSP-11 group, n=12. Negative controls: vehicle-only treated, n=10; scrambled-sequence treated n=8. Analysis by two-way ANOVA with Bonferroni’s post hoc test. These observed effects were driven by severely lesioned animals of striatal dopamine (<1000 ng/g DA striatal tissue content, see Figure 4.10). In animals with only a partial lesion, no significant differences between groups were observed with regards to striatal microdialysis (see Figure 4.15). Data points are mean nM ± SEM.
Baseline dopamine release and metabolites in the lesioned striatum collected by microdialysis.

Baseline levels of dopamine and its metabolites, as determined by microdialysis, were not significantly different between groups. For each data point, the levels of each compound were averaged over the fourth through sixth samples.

Dopamine P=0.1926, t(27)=1.336; DOPAC P=0.0552, t(27)=2.004; HVA P=0.2841, t(26)=1.094.

(DNSP-11, n=12. Negative controls: vehicle-only treated, n=10, scrambled-sequence treated n=8.) The HVA concentration from one animal (at 8088 nM) was found to be a significant outlier by Grubb’s test and was removed. Analyses by two-tailed t-test. Displayed as mean ± SEM.
Figure 4.15 – Functional release of dopamine collected by microdialysis in the striatum of animals that exhibited a partial lesion.

Of the animals that exhibited only a partial lesion of the striatum, as defined in by a striatal dopamine content of under 1000 ng/g (see Figure 4.10), there was no statistically significant difference of functional dopamine release between groups (P=0.9929 with respect to overall treatment effect). DNSP-11 group, n=4.

Negative controls: vehicle-only treated, n=6; scrambled-sequence treated n=7. Analysis by repeated measures two-way ANOVA with Bonferroni's post hoc test. Data points are mean nM ± SEM.
Figure 4.16 – Acute effects of DNSP-11 during microdialysis infusion on dopamine overflow. Naïve 3 month old Fischer 344 male rats were challenged with a 100 mM K+ aCSF solution and allowed to time to return to baseline. After infusion with DNSP-11 during samples 10-14, K+ evoked release of dopamine was decreased by 40.8% from previous K+ evoked release (Figure 4.16A: $F_{(1,4)}=2.112 \text{****K+}, P<0.0001, t_{(72)}=5.780$. n=4 aCSF, n=2 DNSP-11. Analysis by two-way repeated measures ANOVA with Bonferroni’s post hoc test; Figure 4.16B: $**P=0.0014, t_{(6)}=5.620$. 37.1% decrease, n=4 aCSF, n=4 DNSP-11 perfused. Analysis by two-tailed t-test). No differences between groups were observed in baseline dopamine. Of four DNSP-11 perfused animals, two were excluded from (A) due to undetectable baseline concentrations of dopamine. Data points are mean nM ± SEM.
Figure 4.17 – Acute effects of DNSTP-11 during microdialysis infusion on DOPAC overflow.

No significant differences were observed in DOPAC overflow with acute microdialysis infusion. (P=0.8256. Analysis by two-way repeated measures ANOVA with Bonferroni’s post hoc test; n=4 Controls, n=2 DNSTP-11. Of four DNSTP-11 perfused animals, two were excluded due to undetectable baseline concentrations of dopamine. Data points are mean nM ± SEM.)
Figure 4.18 – Acute effects of DNSP-11 during microdialysis infusion of HVA overflow.

No significant differences were observed in HVA overflow with acute microdialysis infusion. (P=0.4906. Analysis by two-way repeated measures ANOVA with Bonferroni’s post hoc test; n=4 Controls, n=2 DNSP-11. Of four DNSP-11 perfused animals, two were excluded due to undetectable baseline concentrations of dopamine. Data points are mean nM ± SEM.)
Chapter Five: A study of the behavioral and physiological effects of intranasally administered DNSP-11 in a 6-hydroxydopamine lesion model of the substantia nigra

**Hypothesis**: Efficacious doses of DNSP-11 can be delivered intranasally to enter the CNS in quantities sufficient to prevent the development of apomorphine-induced rotation behavior from a moderate 6-hydroxydopamine lesion of the substantia nigra.

**Introduction**

Behavioral metrics are the ultimate measure in the study of interventions for neurological disorders, especially in a neurodegenerative disease primarily concerning motor function such as Parkinson’s disease. The complexity of the neurological pathways can mean that molecular measures such as the presence of tyrosine hydroxylase or dopamine may not indicate a symptomatic relief in the organism. Feedback circuitry, compensatory sensitization, secondary pathways, and molecular regulators may all stymie the translation of molecular measures to the ultimate amelioration of motor-output. As such, neuroscience has often relied on behavioral measures. The well-characterized unilateral 6-OHDA lesion model of parkinsonism in rodents provides an excellent foundation for the quantification of a variety of behavioral functions (Woodlee and Schallert, 2004; Ciucci *et al.*, 2008; Fleming *et al.*, 2012). One behavioral metric of particular importance is drug-induced rotation behavior in the unilateral 6-OHDA lesion model used in the present studies.

The hypothesis that DNSP-11 will prevent the development of apomorphine-induced rotation behavior was tested in all animals in this study that were subjected to a unilateral 6-OHDA lesion (n=42). Animals were assessed weekly for apomorphine-induced rotation behavior according to the study timeline (Figure 2.3). Animals were weighed daily; and body temperature and activity level was recorded continuously using an implanted “MiniMitter” device (Chapter Two).
Drug-induced rotation behavior in unilateral lesion models of parkinsonism

Ungerstedt and Arbuthnott first characterized rotation behavior as a method for quantifying nigrostriatal pathway function in rodents in 1970, and they hypothesized that rotation behavior would result from an imbalance between the pathways of the two hemispheres (Ungerstedt and Arbuthnott, 1970). This asymmetry was validated in 1988 when Sonsalla, et al demonstrated changes in dopamine receptor function in rotating animals with an induced lesion (Sonsalla et al., 1988).

Two drugs are used to induce rotation behavior in rodent models of Parkinson’s disease: apomorphine and d-amphetamine, and they have different modes of action, producing rotations in different directions relative to the lesion. Apomorphine has been shown to induce rotation behavior when lesion severity in the substantia nigra is greater than 90% cell loss, providing a relatively high-fidelity, “binary” measure of animals with severe loss in the nigrostriatal pathway (Hudson et al., 1993) stabilizing by the 4th week. The mechanism of action of apomorphine is due to its role as a dopamine receptor agonist. In a highly denervated striatum a compensatory phenomenon referred to as “dopamine receptor super-sensitization” results in a high rate of activation of the striatum ipsilateral to the lesioned substantia nigra by dopamine analogues such as apomorphine (Kostrzewa, 1995). This results in rotation behavior toward the contralateral hemisphere. In other words, the animal rotates “away from” the lesion, or the circle formed by the animal’s rotations places the unlesioned hemisphere closest to the center. d-Amphetamine causes the net efflux of intracellular dopamine into the extracellular space (see Chapter Four). As such, amphetamine asymmetrically activates the unlesioned, innervated hemisphere, producing rotations in the ipsilateral direction, “toward” the lesion, thereby placing the lesioned hemisphere closer to the center of the rotation’s circle. Amphetamine’s mechanism of action also produces a relatively gradual increase in rotations as the nigrostriatal pathways become less symmetrical as the lesion becomes progressively worse (Hudson et al., 1993). In these studies,
Apomorphine based rotations were used, primarily to minimize variability in the behavioral measure. It has been shown that a portion of normal, unlesioned rats can elicit rotation behavior after d-amphetamine administration (Hudson et al., 1993). In order to reduce the possibility of false negatives of DNSP-11 treatment effect, apomorphine was used.

**Total activity levels and body temperature as a sign of general health**

Efficacy of a compound is not the only important metric when evaluating a drug for ultimate translation to human subjects. The safety of a drug on the organism is also of importance. As such, the use of the MiniMitter device (described in Chapter Two) allowed the continual recording of body temperature and activity levels, providing indicators of the overall health of the animals in these trials. The monitoring of body temperature provided a surrogate measure of inflammatory response to the nasal administration of the test subject, and overall activity levels similarly provided information regarding any overall neurophysiological effect the test subject may have on the animal both in the short- and long-term. In conjunction with measurements of body-weight, these studies provide insight into the overall health of the animals and their reactions to the treatment regimen relative to the control treated groups.

**Materials and Methods**

**Semi-quantitative, subjective behavior observations in unlesioned animals**

Animals were observed for the five minute period immediately following righting as a sign of recovery from the isoflurane anesthetic session. The extent of their activity was recorded on a scale ranging from 0, no movement after righting from the supine position, to 5, constant movement after righting. For instance, a score of “3” would indicate the animal was moving throughout the cage for approximately 3 of the 5 observed minutes. These observations were only recorded in unlesioned animals in the dose-response study, and were done so by a researcher blind to the treatment groups.
Rotation behavior procedure

To confirm that these effects correlate with the prevention of transition to the rotation behavior phenotype present in animals challenged with a unilateral 6-OHDA lesion, all animals in the study (n=42) were tested every seventh day for apomorphine-induced rotation behavior, quantified by an automated rotometer system. Animals were administered apomorphine (0.05 mg/kg, s.c.) and tests carried out in rotometer bowls with automated unbiased rotation measurement utilizing a thoracic harness connected to a counting head that interfaces with computer software (SDI Rotation, San Diego, CA USA) (Hudson et al., 1993; Hoffer et al., 1994). The numbers of contralateral and ipsilateral rotations (in relation to 6-OHDA lesion hemisphere) were recorded for 2 hours in 5 minute intervals. Rotation behavior was collected the day prior to 6-OHDA lesion induction and weekly thereafter until euthanasia (Figure 2.3). Total ipsilateral rotations for the first 60 minutes of testing was subtracted from total contralateral rotations for the first 60 minutes of testing and that number used in analysis. No animals were excluded from this study. The rotometer bowls were cleaned with disinfectant prior to use.

MiniMitter recording of activity and body temperature

MiniMitter E-Mitter probe was surgically implanted subcutaneously in the back concurrently with 6-OHDA lesion induction surgery, as described in Chapter 2. The animal’s activity and body temperature was recorded at one minute intervals until the end of the experimental timeline. These values were averaged over the 12 hour long day (light) and night (dark) cycles and compared across groups. Additionally, the total activity for the 5 minutes following recovery from the anesthetic session used for daily intranasal dosing was summed and compared across groups.

Body weight measurements

The animals’ body weights were measured daily through the entirety of the study, beginning the day prior to lesion induction and concluding on the day of
endpoint analysis. If the animal underwent anesthesia on a specified day, body weight was measured while the animal was anesthetized.

**Results**

**Animal health and comfort**

Animals tolerated the treatment and daily anesthetic sessions well. They did not exhibit signs of distress or discomfort. All animals exhibited a smooth, well groomed coat, and none displayed signs of agitation, guarding, or audible vocalization. There were no signs of nasal irritation such as edema or erythema.

**Drug-induced rotation behavior**

Animals challenged with a 6-OHDA lesion were tested weekly for apomorphine (0.05 mg/kg, s.c.) induced rotation behavior, beginning with baseline rotation the day prior to lesioning (day -1). Rotations, both contralateral and ipsilateral to the lesioned hemisphere, were quantified by an automated rotometer system (SDI Rotation, San Diego, CA USA). As predicted, Vehicle Only and Scrambled Peptide treated animals was not significant (Table 5.1, P=0.4201) and were combined in to one negative control group for further analysis. A small set of animals began to exhibit rotation behavior during the second week (day 13), but during the third week (day 20) this level rose to significance. Animals treated intranasally with DNSP-11 showed mean contralateral rotations of 0.25 (± 2.0, n=16), compared with animals treated with scrambled peptide or vehicle-only that exhibited a combined mean of 89.9 (± 35.5, n=26), resulting in significance (Figure 5.1). Furthermore, none of the animals treated intranasally with DNSP-11 exhibited rotation behavior at any timepoint during the study, either from the histological (Figure 5.2) or neurochemical (Figure 5.3) cohorts. The control treated animals were present in two populations, those that exhibited rotation behavior (≥50 rotations/hour, n=7, range of 88 to 669 rotations), and those that did not rotate (<50 rotations/hour , n=19, range of -23 to 38 rotations).
**Subjective behavior**

Of the unlesioned animals in the dose response study, animals treated daily with 1000 µg of DNSP-11 exhibited elevated mean subjective activity (Figure 5.4) or daily levels of subjective activity (Figure 5.5) immediately following intranasal treatment when contrasted with vehicle-only treated animals. Expository notes also suggest the 300 µg per day DNSP-11 treated animals were more active. The animals given 1000 µg of DNSP-11 per day exhibited a mean daily post-treatment activity level of 2.9 ± 0.31 with a range of 2.5 to 3.4, compared with 1.8 ± 0.46 with a range of 1.4 to 2.6 for the saline-only treated animals (P<0.01, Figure 5.4), on a subjective scale of 0 to 5, with 5 being the most active and 0 being not active. The animals given 1000 µg of DNSP-11 per day were significantly more active during the first and second week of treatment, but by the third week their activity levels had decreased in line with vehicle only treated animals (Figure 5.5).

**MiniMitter activity levels and body temperature**

There was no observed significant difference between groups of the 6-OHDA lesioned animals in the measure of mean post-treatment activity (Figure 5.6), nor did it show a significant difference in daily post-treatment activity (Figure 5.7). In addition, there was no difference between groups in mean or daily day-time or night-time activity levels (Figure 5.8 through Figure 5.11) or body temperature (Figure 5.12 through Figure 5.15).

**Body weight**

Body weight was not significantly different between the DNSP-11 or negative control treated groups (Figure 5.16).

**Discussion**

These results support the hypothesis that intranasal DNSP-11 treatment would prevent the development of apomorphine-induced rotation behavior in 6-hydroxydopamine lesioned rats. Furthermore, the animals treated with DNSP-11 did not show signs of distress, discomfort or nasal irritation. Neither their activity
levels nor body weight were negatively impacted by DNSP-11 treated, providing some broad data on the safety of intranasal DNSP-11 treatment.

**Subjective behavior and activity**

None of the animals in the study exhibited signs of distress or discomforting, including edema or erythema of the nasal region, suggesting the treatment itself did not produce irritation. The animals did not display guarding or unusual vocalization, suggesting the treatment regimen was well tolerated.

Among the unlesioned animals in the dose-response study, those treated with DNSP-11 exhibited higher levels of subjective activity immediately following intranasal treatment, subjectively recorded by a researcher blind to the treatment groups. This could suggest an acute and direct neuropsychological effect of DNSP-11 on the motor system, or olfactory stimulation leading to normal searching behavior. To attempt to control for this and the observed neurochemical changes, later studies included a scrambled version of the DNSP-11 peptide sequence (Table 2.1) as a negative control.

**Apomorphine-induced rotation behavior**

While apomorphine induced rotation behavior stabilizes by the 4th week after lesion, this study only evaluated rotation behavior to the 3 week timepoint. At that time point, a significant difference arose between those treated intranasally with DNSP-11 or with negative controls (Figure 5.1). None of the DNSP-11 treated animals exhibited rotation behavior, indicating DNSP-11 may be able to prevent the progression of super-sensitization caused by striatal denervation within the 3 weeks observed in this study. The negative control treated animals were present in two populations: those that did exhibit rotation behavior (88 to 669 rotations, n=7), and those that did not rotate (negative 23 to 38 rotations, n=19), defined as <50 rotations per hour (Hudson et al., 1993), due to lesion variability. All of the animals that exhibited rotation behavior were among the severely lesioned subgroup of animals as defined by striatal dopamine content of <1000 ng/g (see Figure 4.10). This behavior data supports the histological protection observed while suggesting that longer-term studies in
the 4 to 6 week time period may provide stronger efficacy data regarding the potential of intranasal DNSP-11 for protection against dopaminergic loss and the behavioral consequences. In addition, the use of d-amphetamine induced rotation behavior, after pre-screening against animals that rotate prior to lesioning, may identify significant results at earlier time points.

**MiniMitter activity levels and body temperature**

MiniMitter data did not reveal any differences between treatment groups with regard to post-treatment activity (Figure 5.6 and Figure 5.7) average daytime or nighttime activity levels or body temperature (Figure 5.8 through Figure 5.15).

The objective MiniMitter activity data does not mirror the subjective, blinded activity data. This disparity may be due to a number of reasons. The MiniMitter device, implanted in the back of the animal, may have emphasized activities that the subjective observer disregarded, such as shaking, shivering, or grooming behavior, resulting in relatively higher “noise” in the MiniMitter data. The animals implanted with the MiniMitter device were also challenged with a 6-OHDA lesion of the substantia nigra while the subjectively observed animals were unlesioned, possibly altering this subjectively observed effect.

Animals treated with DNSP-11 did not exhibit elevated body temperature values compared with saline or scrambled-peptide treated controls. This may indicate that the peptide does not produce an inflammatory response that could be detected by the MiniMitter device, providing some broad and preliminary data on the safety of the DNSP-11 peptide when delivered intranasally.

A further element of the MiniMitter studies was to determine if their use would equate to open-field testing and the Actical (Philips Respironics, Bend, OR USA) monitoring devices used in human and non-human primate studies of Parkinson’s disease and its models. Significant differences between groups in this study were not observed, however unlesioned animals were not tested with the MiniMitter system providing no basis for comparison against normal rodents. Average activity over the 12 hour light or dark periods did not indicate significant differences in activity, but these values are during an extended length of time and are not similar to one hour open-field behavior testing. The total activity
immediately after intranasal administration was not significantly different between groups. Testing the device in normal unlesioned animals for comparison and using the device for before/after treatment measurements may allow for the discernment of effects elicited by treatments and the 6-OHDA lesion model.

**Body weight**

Subject body weight can be an indicator of the overall health and speed of recovery in animal studies. The fact that the animals in this study exhibited statistically similar body weights throughout the experiment (Figure 5.16) may be an important finding. A side-effect of GDNF treatment is weight loss (Hovland et al., 2007a). Although the timeframes and means of delivery are different between the cited study and the present data, the present study suggests DNSP-11 does not produce significant weight loss as a side effect. This provides some important safety information regarding the repeated use of DNSP-11.
Table 5.1 – Number of apomorphine-induced turns per hour in control and DNSP-11 treated animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Day -1</th>
<th>Day 6</th>
<th>Day 13</th>
<th>Day 20</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrambled Peptide</td>
<td>1.167 ± 5.606</td>
<td>3.500 ± 10.318</td>
<td>33.000 ± 63.356</td>
<td>114.083 ± 187.615</td>
<td>12</td>
</tr>
<tr>
<td>DNSP-11 Peptide</td>
<td>-0.438 ± 6.750</td>
<td>6.750 ± 12.902</td>
<td>-1.063 ± 10.299</td>
<td>0.250 ± 8.153 *</td>
<td>16</td>
</tr>
</tbody>
</table>

None of the animals treated with intranasal DNSP-11 exhibited rotation behavior at any time point. On day 13, one animal in the Vehicle Only group and two animals in the Scrambled Peptide group exhibited rotation behavior, defined as >50 turns per hour (Hudson et al., 1993). By the 20th day after 6-OHDA lesion induction, three Vehicle Only and four Scrambled Peptide treated animals exhibited rotation behavior. This resulted in a significant difference between control and DNSP-11 treatment groups (overall effect of treatment P=0.0782, F(2,39)=2.722. *P=0.0248, t(156)=2.530 with respect to Vehicle, and $$$P=0.0002, t(156)=4.003 with respect to Scrambled). As predicted, the overall effect of treatment between the Vehicle Only and Scrambled Peptide treated animals was not significant (P=0.4201, F(1,24)=0.6730) at any time point (Day -1 through Day 13: P>0.9999, Day 20: P=0.9246). No animals were excluded from this analysis. Data displayed as “mean rotations per hour ± standard deviation”. Analysis performed by two-way repeated measures ANOVA with Bonferroni’s post hoc test.
Animals treated with DNSP-11 intranasally exhibited no abnormal rotation behavior on the 20th day after 6-OHDA lesioning of the substantia nigra, contrasted with negative control treated animals. As predicted, vehicle and scrambled peptide treated animals did not exhibit a significant difference in effect ($P=0.4201$). (Overall effect of treatment $P=0.0782$. $^*P=0.0248$ with respect to Vehicle, and $^{$$$}P=0.0002$ with respect to Scrambled. Vehicle treated, $n=14$; Scrambled peptide treated, $n=12$; DNSP-11 treated, $n=16$.) No animals were excluded from this analysis. Data displayed as “mean rotations per hour ± SEM”. Analysis by two-way repeated measures ANOVA with Bonferroni’s post hoc.
Figure 5.2 – Individual rotation behavior of the histological cohort.

Of animals in the histology cohort, none of the animals treated with DNSP-11 exhibited apomorphine-induced rotation behavior on day 20.
Figure 5.3  -- Individual rotation behavior of the neurochemistry cohort.

Of animals in the neurochemistry cohort, none of the animals treated with DNSP-11 exhibited apomorphine-induced rotation behavior on day 20.
Figure 5.4 – Average subjective activity levels recorded by observer blind to treatment immediately following intranasal administration.

Average subjective activity levels immediately following intranasal administration were significantly different in the group of animals administered 1000 µg per day of DNSP-11 for three weeks (##P=0.0038 overall; **1000 µg P<0.01). Activity levels in the 300 µg daily dose cohort were not recorded, but expository notes suggest elevated activity compared to the concomitantly treated 0 µg animals. Subjective behavior was assessed by a research blind to treatment. (n=6 animals per group. Analysis by one-way ANOVA with Bonferroni’s post hoc. Shown as mean ± SEM.)
Figure 5.5 – Daily subjective activity levels recorded by observer blind to treatment immediately following intranasal administration.

Animals given 1000 µg of DNSP-11 intranasally began the treatment regimen with significantly elevated subjective activity levels, especially on day 2; and these activity levels decreased as the treatment progressed to the final week. Vehicle-only treated animals exhibited significantly lower activity levels, and that subjective activity level remained low throughout the treatment. The 100 µg treated animals were not significantly different from saline vehicle only treated animals. Subjective behavior was assessed by a research blind to treatment. (**P=0.0043 overall; ****Day 2 activity levels, P<0.0001 between 1000 µg and 0 µg vehicle-only treated groups. **Day 8 and 9, P<0.01 between 1000 µg and 0 µg groups. Analysis by repeated measures two-way ANOVA with Bonferroni’s post hoc. Shown as mean ± SEM.)
Figure 5.6 – Average post-treatment activity levels recorded by MiniMitter. The animals' activity levels for the five minutes immediately following recovery from isoflurane anesthetic used during the intranasal treatment session shows no significant differences between negative control or DNSP-11 treated animals (P=0.7168 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by one-way ANOVA with Bonferroni's post hoc test. Shown as mean ± SEM).
Figure 5.7 – Daily post-treatment activity levels recorded by MiniMitter. The animals' activity levels for the five minutes immediately following recovery from isoflurane anesthetic used during the intranasal treatment session shows no significant differences between negative control or DNSP-11 treated animals (P=0.6466 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by repeated measures two-way ANOVA with Bonferroni's post hoc test. Shown as mean ± SEM).
Figure 5.8 – Average daytime activity levels recorded by MiniMitter. The 12-hour “light” period activity levels in the animals were not significantly different between groups (P=0.5398 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
**Figure 5.9** – Daily daytime activity levels in animals as recorded by MiniMitter. Daily daytime “light” activity levels were not significantly different between groups (P=0.5414 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by repeated measures two-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM). Note that Days 5, 12 and 19 the animals were off-treatment and only body weight measurements were taken.
Figure 5.10 – Average nighttime activity levels recorded by MiniMitter.

The 12-hour “dark” period activity levels in the animals were not significantly different between groups (P=0.0973 overall; Vehicle, n=8; Scrambled, n=6; DNSSP-11 n=10. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
Figure 5.11 – Daily nighttime activity levels recorded by MiniMitter.

Daily nighttime “dark” activity levels were not significantly different between groups (P=0.0927 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by repeated measures two-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
Figure 5.12 – Average daytime body temperature recorded by MiniMitter. The 12-hour “light” period body temperature in the animals was not significantly different between groups (P = 0.1449 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
Figure 5.13 – Daily daytime body temperature recorded by MiniMitter. Daily daytime light-period body temperature was not significantly different between groups (P=0.1456 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by repeated measures two-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
Figure 5.14 – Average nighttime body temperature recorded by MiniMitter. The 12-hour night-period body temperature in the animals was not significantly different between groups (P=0.2454 overall; Vehicle, n=8; Scrambled, n=6; DNPSP-11 n=10. Analysis by one-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
Figure 5.15 – Daily nighttime body temperature recorded by MiniMitter. Daily nighttime dark-period body temperature was not significantly different between groups (P=0.2454 overall; Vehicle, n=8; Scrambled, n=6; DNSP-11 n=10. Analysis by repeated measures repeated measures two-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
Figure 5.16 – Daily animal body weight post-lesion.

Animals were weighed daily before and after lesion induction no significant difference between groups was observed (P=0.2225 overall, n=42. Negative Controls, n=14 vehicle and 12 scrambled treated; DNSP-11 (300 µg), n=16. Analysis by repeated measures two-way ANOVA with Bonferroni’s post hoc test. Shown as mean ± SEM).
Chapter Six: An immunohistochemical study of the endogenous DNSP-11 sequence in the rat brain

Hypothesis: The DNSP-11 sequence is endogenously present in the rat brain as a peptide that has differential patterns of spatial and temporal localization from that of mature glia cell line-derived neurotrophic factor (GDNF).

Introduction

The dopamine neuron stimulating peptide-11 (DNSP-11) peptide was identified as a theoretical cleavage product from the prosequence of GDNF, and we and others have shown that the sequence is biologically active in the present study and in the literature, both in vitro and in vivo (Immonen et al., 2008; Bradley et al., 2010; Kelps et al., 2011), but questions remain regarding its endogenous cleavage and localization in the brain. Lending further support to a biological role for DNSP-11, the peptide sequence shares homology across species: *homo sapiens* and *macaca mulatta* share 100% homology, and *homo sapiens* shares 73% homology with both *mus musculus* and *rattus norvegicus* (P1L, P2L, R9H). Furthermore, DNSP-11 immunoreactivity has been observed to colocalize with TH+ cells of the substantia nigra in the post-natal day 10 rat brain (Bradley et al., 2010). While DNSP-11 and GDNF are likely co-expressed and processed, their target cell populations and trafficking may be quite different.

Roles of pro-protein and signal peptides in the CNS

Putative peptides have been identified in the pro-region of other neurotrophic factors expressed in the CNS. These peptides are, like DNSP-11, flanked by dibasic amino acids that are purported to be cleavage sites. Two of the most studied pro-region peptides are located within the proNGF protein, referred to as LIP1 and LIP2, and have defined biological activities through specific signaling cascades (Clos and Dicou, 1997; Dicou et al., 1997; Dicou, 2006), and their endogenous identification has been linked to conditions of aging (Dicou, 2008).
Pro-forms of other proteins have differential effects and distinct binding from their mature forms. A well studied example of this is brain-derived neurotrophic factor (BDNF), which can bind to either p75NTR or the Trk-family of receptors, if it is secreted as proBDNF or mature BDNF (Buttigieg et al., 2007). Many members of the TGFβ family of proteins can be secreted as pro-proteins, especially the bone morphogenetic proteins (BMPs), and this affects the targeting of the protein (Gregory et al., 2005). The GDNF protein is a member of the TGFβ family of proteins and its pro-region contains the DNSP-11 sequence flanked by consensus sites for cleavage by prohormone convertase (K26R27 and H/R40R41R42). Based on these consensus sites and the evidence of biological effects of DNSP-11, it is reasonable to suggest that the DNSP-11 sequence is endogenously processed and has a native function. Similar to other proteins, many possibilities exist for the proGDNF molecule: a) the DNSP-11 sequence itself may be cleaved to form a bioactive peptide in its own right, b) the proGDNF protein may be secreted whole with the DNSP-11 sequence representing the smallest necessary binding sequence, c) the whole pro-region may be cleaved as a signal peptide containing the active DNSP-11 sequence, or d) any combination of cleavage patterns may produce a biologically active derivative of proGDNF. Of note, the consensus site for cleavage contains minor differences between rats (H40) and humans (R40), possibly resulting in a difference in processing between species (Duckert et al., 2004). Based on the wide array of effects elicited by the DNSP-11 sequence both in animal models and a range of cell culture lines (Bradley et al., 2010; Turchan-Cholewo et al., 2010; Kelps et al., 2011; Bradley et al., 2012), it seems possible that the conserved DNSP-11 sequence acts as a natural ligand for a receptor, perhaps involved in metabolic homeostasis and developmental cell sparing.

**Homologous sequences in the CNS**

Neuropeptide Y (NPY) is a 36 amino acid peptide that shares five consecutive amino acids beginning with amino acid 12 (NPY sequence: YPSKPDPNPGEDAPAE MARYYSALRHYINLITRQRY; compared to the DNSP-11 sequence: PPEAPAE DRS). For full proGDNF precursor, see Figure
This five amino acid sequence homologue is found in the rat and human CNS, as determined by protein BLAST *in silico* analysis (Altschul *et al*., 1997). NPY is typically observed in the hypothalamus, amygdala and cortex, with little to no immunoreactivity detected in the midbrain (Dawbarn *et al*., 1984; de Quindt and Emson, 1986b) nor in tyrosine hydroxylase positive neurons (Everitt *et al*., 1984).

In addition to NPY, there also exists an approximately 42.9 kDa protein referred to as the TRK-Fused Gene product (Tfg) which contains a homologous sequence to DNSP-11 (Tfg amino acids 194-206 sequence: **PPSAPTEDRSG**, DNSP-11 sequence: **PPEAPAEEDRSL**) and which is also expressed in the rat brain (Maebayashi *et al*., 2012). Tfg immunoreactivity in the CNS is prevalent in regions including the mesencephalic nucleus of the trigeminal nerve and dorsal raphe nucleus, as well as the cytoplasm of Purkinje cells of the cerebellum and vestibular and cochlear nuclei of the pons.

The use of expression patterns to target DNSP-11 for increased efficacy and decreased off-target effects

While the endogenous developmental and maintenance roles of DNSP-11 are interesting in their own right, the localization of DNSP-11 within the CNS may provide insight into its effectiveness as an exogenously applied therapy. Any observed anatomical localization of an endogenous, or exogenously applied, DNSP-11 signal may indicate a reactive cell population, possibly utilizing unique receptors and signaling cascades. This information can provide insights into the possible functions of DNSP-11 and may help explain potential effects of exogenously administered DNSP-11. With this information, modifications to the peptide sequence, delivery approach or formulation may help increase on-target effects while minimizing side effects.

**Materials and Methods**

**Animals**

A combination of both young adult and post-natal day 10 Fischer 344 rats naïve to treatment were euthanized and processed for immunohistochemical visualization of brain tissue. A total of 9 young adult (3 to 6 month old) male rats
were euthanized and their brains sectioned either sagittally (n=6) or coronally (n=3) for anatomical localization of signal. Post-natal day 10 animals (n=4) of undetermined sex and one young adult female rat sectioned sagittally (n=1) were also used.

**Equipment and Reagents**

The immunoprecipitation kit by Life Sciences (Cat. No. 143.21D, Invitrogen, Oslo, Norway) was used. A custom DNPSP-11 polyclonal antibody (see below) was used for immunohistochemistry, immunoprecipitation, and ELISA. Tyrosine hydroxylase antibody was purchased from Millipore Chemicon (MAB318, Millipore Chemicon, Temecula, CA). GDNF antibody was purchased from R&D Systems (AF-212-NA, R&D Systems, Minneapolis, MN). Secondary antibodies include peroxidase-conjugated anti-rabbit IgG (PI-1000, Vector Laboratories, Burlingame, CA), Alexafluor anti-goat 568 and Alexafluor anti-rabbit 488 fluorescent (Life Sci. Invitrogen Molecular Probes). VectaShield H-1400 Hard-Set Mounting medium for coverslipping dual-fluorescent specimens was used (Vector Laboratories, Burlingame, CA). Fluorescent images acquired on a Nikon microscope. Refrigerators and freezers were continuously monitored for temperature variations. Other reagents were purchased from Fisher Scientific unless otherwise specified.

**Custom antibody to DNPSP-11**

The custom polyclonal antibody to DNPSP-11 (PPEAEPDRSL-NH₂) used in these experiments was obtained under contract from α-Diagnostics laboratory, was raised in rabbit and affinity purified against the synthesized, amidated DNPSP-11 peptide in a CN-Br activated agarose matrix and eluted in 0.1M glycine-HCl and neutralized to pH 8.0 with 1M Tris and 0.1% BSA added for stabilization. Titer by ELISA was quantified at 1:10K-100K. Pre-absorption of the antibody with 10:1 (mass:mass) human or rat DNPSP-11 peptide completely prevented reactivity with tissue. A custom antibody to the rat DNPSP-11 sequence (LLEAEPDHSL-NH₂) was obtained under the same contract conditions, resulting in a similar titer, produced indistinguishable tissue...
localization, and pre-absorption with peptide similarly prevented tissue reactivity. However, antibodies raised against the human sequence produced more intense visual signal in rat brain tissue and was used for visualization.

**Tissue preparation for immunohistochemistry**

Adult rats were perfused with approximately 200 mL ice cold saline followed by ice cold 4% paraformaldehyde, while post natal rats were not perfused. The animals’ brains were removed whole and placed in ice cold 4% paraformaldehyde fixative overnight for immunohistochemical analysis. Rat brains were sectioned to 30 µm thickness and stored in cryoprotectant solution (30% sucrose, 30% ethylene glycol in 100 mM KPBS) at -20°C until histochemical processing. I would like to acknowledge April Dawn Richardson-Hatcher, Ph.D. for the preparation of tissue from four post-natal day 10 rats.

**Immunohistochemical processing**

Every 12th section was blocked with 4% normal goat serum for 1 hour at room temperature. The sections were then immunolabeled with antibody to a custom polyclonal human DNSP-11 (1:1000, α-Diagnostic, San Antonio, Texas USA) raised in rabbit and incubated overnight at 4°C. The sections were incubated with peroxidase-conjugated secondary antibody (1:1000, Vector Labs, Burlingame, CA USA) and visualized with nickel enhanced 3,3’-diaminobenzidine (NiDAB) chromogen.

**Dual-fluorescence immunohistochemical processing**

Sections were incubated with the polyclonal primary antibody against DNSP-11 (α-Diagnostic, 1:1000) overnight at 4 °C, then incubated in Alexa Fluor 488 goat anti-rabbit secondary antibody (1:1000; Molecular Probes Inc., Eugene, OR) for 1 hour at room temperature. The same sections were subsequently incubated in an anti-GDNF antibody (1:500, Chemicon Millipore, Temecula, CA USA) overnight at 4 °C, followed by 1 hour at room temperature in Alexa Fluor 568 goat anti-mouse IgG secondary antibody (1:1000; Molecular Probes Inc.).
**Tissue homogenization**

Rat brain tissue from 3 month old male Fischer 344 rats was dissected, placed in cold lysis buffer (10mM TrisHCl, pH 7.4, 150mM NaCl, 0.5% Triton X-100 and 0.1% Proteinase Inhibitor Cocktail (Sigma P8340)), and homogenized by sonication. The homogenate was allowed to incubate on ice for one hour, followed by the removal of cell debris by centrifugation at 14,000 g at 4°C for 5 minutes.

**Immunoprecipitation**

Immunoprecipitation kit was purchased from Life Sciences (143.21D, Invitrogen, Oslo, Norway) and was performed as follows. A total of 10 mg of Dynabeads were coupled with 70 µg of polyclonal, custom DNSP-11 antibody by overnight incubation at 37°C under constant movement. Beads were washed, and then tissue homogenate was incubated with the antibody-conjugated beads for 30 minutes at 4°C under constant movement. Immunoreactive compounds were then eluted and retained at -70°C. A total of 3 animals were used per immunoprecipitation resulting in an average 0.1 to 0.2 g of tissue per immunoprecipitation. The resultant fractions containing the immunoprecipitated contents were concentrated by lyophilization, and verified by an SDS-PAGE gel to confirm the purity of the immunoprecipitation eluent by Coomassie blue and silver-stain visualization. I would like to acknowledge Kristen A. Kelps in the laboratory of Luke H. Bradley, Ph.D. for performing immunoprecipitations.

**Peptide identification**

Immunoprecipitated fractions from brain tissue homogenate underwent protein identification, compared to the synthetic amidated 11-amino acid rat sequence of DNSP-11, as performed by LC-MS/MS in the University of Kentucky Proteomics Core Facility (Lexington, KY).
Results

Differential immunostaining patterns for GDNF and DNSP-11

To characterize the location of the endogenous DNSP-11 peptide within the rat brain (Table 6.1, Figure 6.1), immunohistochemical staining of sagittal and coronal sections of the rat brain were performed using a polyclonal antibody raised to the DNSP-11 sequence. Intense immunoreactive signal of neuronal cell bodies was apparent in the locus coeruleus (LC) (Figure 6.2 and Figure 6.3), with strong signal in the ventral tegmental area (VTA), substantia nigra pars compact (SNc) (Figure 6.4) and glomeruli of the olfactory bulb (OB). Moderate signal was observed in granule-like cells of the dentate gyrus (DG) and CA1-CA3 of the hippocampus (Figure 6.5), the nucleus of Purkinje-like cells of the cerebellum, and neurons of the facial motor nucleus and mesencephalic nucleus of the trigeminal nerve. Weaker signal appeared in granule-like cells throughout the cortices of the brain, including but not limited to the olfactory cortex, accessory olfactory bulb, cerebral cortex (Figure 6.1). Both the antibodies to the rat and human DNSP-11 sequences showed comparable immunoreactivity, but the antibody raised against the human sequence resulted in more intense visualization and detection by ELISA to a lower concentration of the peptide. Pre-absorbing both antibodies with either the rat or human DNSP-11 sequence produced no non-specific signal.

It has been previously shown that DNSP-11 signal colocalizes to tyrosine hydroxylase positive neurons at post-natal day 10 in the Sprague Dawley rat (Figure 6.6) (Richardson, 2009; Bradley et al., 2010) and differential expression patterns of GDNF and DNSP-11 in embryonic and post-natal animals described (Richardson, 2009). Here, dual-fluorescence immunoreactive signals for DNSP-11 and GDNF in post-natal day 10 Fischer 344 rats revealed midbrain (Figure 6.7) and dorsal raphe (Figure 6.8) neurons containing DNSP-11 signal without GDNF. Furthermore, dual-fluorescence studies indicated some colocalization of DNSP-11 and GDNF signals within the neuronal cell bodies of the SN, which may represent the uncleaved prosequence; however, DNSP-11 signal was also found to be differentially localized from GDNF signal in the, SN and VTA (Figure
6.9) and the LC (Figure 6.10). Of note, DNSP-11 signal distinct from GDNF signal was observed in the nucleus, but not cytoplasm, of Purkinje-like cells of the cerebellum (Figure 6.11). DNSP-11 signal was present in the DG of the hippocampus without discernible GDNF signal, and DNSP-11 and GDNF signals were highly colocalized in the adult facial motor nucleus, the adult striatum, cerebellum, and medial lemniscus (not shown).

**Immunoprecipitation**

Peptide identification from the immunoprecipitated fractions were inconclusive. While compounds were pulled down by the antibody, none had the same LC retention time as the synthetic amidated rDNSP-11 peptide, and the mass spectroscopy identified breakdown pattern of synthetic rDNSP-11 was not identified in the immunoprecipitate. A total of three animals were used in this study, and one was excluded due to no detectable proteins pulled down by the immunoprecipitation column.

**Discussion**

**Limitations of the technique**

The antibody used in these studies was a custom, polyclonal antibody raised in rabbit against the synthetic 11-amino acid amidated DNSP-11 sequence. While its sensitivity in ELISA is high, and signal is blocked with excess of the synthetic DNSP-11 peptide, the specificity of the antibody is not known and the peptide cannot be used to quantitatively assay DNSP-11 concentrations in the brain. Previous studies have shown that the antibody is effective at identifying the distribution of DNSP-11 *in situ* after intracranial injection (Bradley *et al.*, 2010); however, the possibility of the polyclonal antibody binding to other compounds within the CNS is considerable. Indeed, the polyclonal antibody used in these studies strongly binds NPY as determined by ELISA. Taken at face value, this data only indicates that the antibody raised to synthetic DNSP-11 encounters binding partners in the observed regions of the brain, which may include at least the known preproGDNF and NPY proteins but may also include the DNSP-11 sequence. An exhaustive identification of the
binding partners of this antibody has not been performed, but based on immunoprecipitation results it is unlikely that the 11-amino acid DNSP-11 sequence exists as a free, isolated, amidated peptide. It should also be noted that the consensus cleavage site for the DNSP-11 sequence is conservatively mutated between rats (H40) and humans (R40), and that this introduces the possibility of minor differences in processing across species, based on in silico analysis (Duckert et al., 2004). Future studies would benefit from a sensitive, specific monoclonal antibody to the DNSP-11 sequence.

**Sequence homologs in the rat CNS**

The immunoreactivity presented in this study may be complicated by the presence of both neuropeptide Y (NPY) and TRK-Fused Gene product (Tfg) which contain partially homologous sequences to DNSP-11 (see Table 6.1). However, it has been reported in the literature that NPY does not localize to catecholaminergic neurons such as the LC, SN or VTA based on radiographic binding assays and immunohistochemical analysis (de Quidt and Emson, 1986a). Furthermore, Tfg immunoreactivity is restricted to the mesencephalic nucleus of the trigeminal nerve, the dorsal raphe nucleus, Purkinje cells of the cerebellum, and the vestibular and cochlear nuclei. As such, NPY and Tfg do not, individually or in combination, recapitulate the immunoreactive patterns observed in this study utilizing the custom polyclonal DNSP-11 antibody (see Table 6.1). In addition, the extensive differential localization of GDNF and DNSP-11 signals, both temporally and spatially, suggest the immunoreactivity is not explained by the presence of the unprocessed preproGDNF protein. Future studies may focus on the dual-fluorescence signals between DNSP-11, NPY and Tfg, or other potentially homologous proteins.

**Differential immunoreactive signals for DNSP-11 and GDNF in postnatal and young adult rats**

The DNSP-11 peptide sequence is theoretically processed from the pro-region of GDNF. In the absence of specific transport mechanisms GDNF and DNSP-11 should highly colocalize both spatially and temporally in the rat CNS. It
has been shown that the DNSP-11 peptide does not bind to GFRα1 or RET (Bradley et al., 2010), the canonical receptors of GDNF, and due to the differences in effects observed in the literature between GDNF and DNSP-11 (Bradley et al., 2010; Turchan-Cholewo et al., 2010; Kelps et al., 2011), it is hypothesized that GDNF and DNSP-11 are distinct biologically active compounds. The data obtained through dual-fluorescence immunohistochemical analysis showing differential localization of GDNF and DNSP-11 signals supports the hypothesis that DNSP-11 is present and is subjected to different transport or expression mechanisms than that of GDNF, and that there are cellular populations differentially sensitive to DNSP-11.

DNSP-11 and GDNF were found to colocalize at post-natal day 10, the time of maximal GDNF expression in the rat, but the signals did not exclusively colocalize. A subset of neurons of the developing mesencephalon were intensely immunoreactive for DNSP-11 while not exhibiting any detectable immunoreactivity to GDNF (Figure 6.7). While beyond the scope of this study, some possibilities present themselves: first, DNSP-11 may be processed from the pro-region of GDNF and undergoes transport by a mechanism specific to DNSP-11; and second, that splice variants of the GDNF gene may allow differential expression of DNSP-11 and GDNF. There are currently seven known splice variants of the GDNF gene in the human, and two of them (variant 005 and 006) produce a variant from the pro-region that omits a 27 amino acid section containing the DNSP-11 sequence (omitted section of human splice variants: GKRPEAPA EDRSLGRRRA PEALSSDS) (Flicek et al., 2013). While speculative, it should not be ruled out that an as yet unidentified splice variant of the GDNF gene may allow differential expression of DNSP-11 and GDNF.

Expression of GDNF is known to decrease markedly in the adult rat, but it was observed that the DNSP-11 signal remains intense in the mesencephalon of adult rats (Figure 6.9). This observation supports the hypothesis of differential expression levels or a prolonged half-life of endogenous DNSP-11 in the rat CNS. It may be speculated that endogenous DNSP-11 has a biological function
in the monoaminergic neuron populations identified here as containing DNSP-11 signal, such as the locus ceruleus, substantia nigra, and raphe nuclei.

The DNSP-11 sequence as a processed biological peptide

The highest intensity signal for DNSP-11 was observed in the locus ceruleus, the norepinephrinergic center of the CNS (Figure 6.10). It has been shown in the literature that the GDNF gene is necessary for the development and maintenance of the neurons of the locus ceruleus. In a conditional GDNF-null mouse model, it was shown that the approximate 60% reduction of GDNF mRNA and protein levels in these mice during adulthood results in the complete loss of the catecholaminergic neurons of the locus ceruleus with only moderate losses in the substantia nigra and VTA (Pascual et al., 2008), indicating that the GDNF gene is necessary for normal maintenance and survival of adult catecholaminergic neurons, especially the locus ceruleus. This is an interesting finding in light of the observation by others that the locus ceruleus contains no detectable mRNA for GDNF and only trace levels of mRNA for its receptors GFRα and RET in the rat (Trupp et al., 1997), contrasted with the substantia nigra which is characterized by extensive levels of GDNF receptor mRNA. The SN and VTA, with extensive GDNF receptor expression, only underwent moderate loss in these conditional knock-out animals, while the locus ceruleus, with very low levels of GDNF receptor expression, underwent a near total loss. This indicates that the locus ceruleus may be highly sensitive to GDNF gene product expression levels or the effects of GDNF gene products on receptors other than the canonical GDNF receptors.

The observed localization of the DNSP-11 signal in the rat CNS may provide evidence of the sensitivity of cell populations to DNSP-11 and may allow for increased therapeutic effect through targeting specific brain regions. Indeed, loss of the locus ceruleus in human Parkinson’s disease patients precedes and is greater than the loss of the substantia nigra neurons (German et al., 1992; Patt and Gerhard, 1993; Braak et al., 2003; Zarow et al., 2003). This loss contributes to many of the secondary symptoms and may exacerbate the primary motor symptoms. Shown within this work is data suggesting DNSP-11 affects
monoamine systems (Chapter Four), and while inconclusive the immunohistochemical studies of this chapter support the hypothesis that these nuclei interact with DNSP-11.

Immunoprecipitation studies indicate that the binding partners of the antibody raised to DNSP-11 do not match the LC-MS identity for the synthetic, amidated 11-amino acid DNSP-11 sequence. The results do not rule out the endogenous presence of DNSP-11, but suggest that it may be in a form other than that of the synthetic peptide. The endogenous DNSP-11 sequence may be covalently modified or it may be contained within a larger signal peptide. Additionally, endogenous DNSP-11 may degrade quickly under the conditions of the immunoprecipitation, or be present at concentrations too low to quantify. While the antibody used in these studies binds NPY and DNSP-11, immunoreactive signal was identified in brain regions not typically associated with NPY expression. Also, in other regions where NPY expression is found the antibody did not result in signal (Table 6.1). The detection and isolation of endogenous DNSP-11 would benefit from the production and use of a highly specific monoclonal antibody.

Although the studies in this chapter produced many questions, the insights gleaned may guide future studies on possible cell populations responsive to DNSP-11 and the possible existence of the DNSP-11 sequence as an active element of endogenous biological processes.
Table 6.1 – An overview of expression patterns for DNSP-11 and sequence homologues, GDNF, NPY, and Tfg in the adult.

<table>
<thead>
<tr>
<th>Homologous sequence</th>
<th>DNSP-11</th>
<th>GDNF</th>
<th>NPY</th>
<th>Tfg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homologous sequence</td>
<td>PPEAPAEDRSL-NH₂</td>
<td>-PPEAPAEDRSL-</td>
<td>-GEDAPAEDMAR-</td>
<td>-PPSAPTEDRSG-</td>
</tr>
<tr>
<td>Cortex</td>
<td></td>
<td>±</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Cortex</td>
<td>±</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Amygdala</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Substantia nigra</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ventral tegmental area</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Raphe nuclei</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Locus ceruleus</td>
<td>+++</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Mesencephalic nucleus of</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Trigeminal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellar Purkinje cell</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nucleus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellar Purkinje cell</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

This table summarizes the localization of various proteins related and/or homologous to DNSP-11 in the adult rat. Note that information for NPY, Tfg, and GDNF is based on a survey of the literature.
Figure 6.1 – Whole sagittal section of the rat brain immunoreacted for DNSP-11. An overview of immunoreactive hotspots of DNSP-11 signal in a sagittal view of the rat CNS. Especially intense intracellular signal was observed in the locus ceruleus, VTA and SN, glomeruli layer of the olfactory bulb (OB), and dentate gyrus of the hippocampus (DG). Diffuse signal was also present in the striatum (CPu). Representative section (n=6 sagittal, n=3 coronal).
**Figure 6.2** – DNSP-11 signal in the locus ceruleus and cerebellum in sagittal view.

A sagittal view of the locus ceruleus and cerebellum shows intense DNSP-11 signal in the neuronal cell bodies and projections of the locus ceruleus and light signal in the Purkinje cell layer of the cerebellum. (LC, locus ceruleus; PC, Purkinje cell layer of cerebellum; ic, inferior colliculus; 4V, fourth ventricle.)
Figure 6.3 – A detailed view of DNSP-11 signal in the locus ceruleus. Observed in this sagittal view of the locus ceruleus of naïve adult male Fischer 344 rats is intense immunoreactivity for DNSP-11, including cytoplasmic localization and vesicle-like concentrations in neuronal projections.
Figure 6.4 – DNSP-11 signal in the VTA and SN of adult rats. DNSP-11 signal was intense in neurons of both the VTA and substantia nigra pars compacta in the adult rat.
Figure 6.5 – DNSP-11 signal in the dentate gyrus and cornu ammonis of the hippocampus.

Moderate DNSP-11 immunoreactivity was observed in granule-like cells of the dentate gyrus (DG) and cornu ammonis (CA) formations of the hippocampus. (LV, lateral ventricle; 3V, third ventricle.)
Figure 6.6 – DNSP-11 signal highly colocalizes to TH-positive neurons in the midbrain of post-natal day 10 Sprague Dawley rats. In immunohistochemical experiments performed by April Dawn Richardson-Hatcher, Ph.D., contained within her dissertation work (Richardson, 2009), it was observed that DNSP-11 signal highly colocalizes with TH+ neurons at post-natal day 10.
Figure 6.7 – DNSP-11 signal, but not GDNF signal, is present in a developing post-natal day 10 midbrain cell.  

Shown here is a representative neuron in the midbrain of a post-natal day 10 Fischer 344 rat with only immunoreactive signal for DNSP-11.  DNSP-11 is thought to be processed from the pro-region of GDNF, suggesting a high degree of colocalization.  However, DNSP-11 signal does not exclusively colocalize with GDNF signal, suggesting DNSP-11 may have unique and efficient active transport mechanisms and specific reactive cell populations.
Figure 6.8 – DNSP-11 signal in the raphe of post-natal day 10 animals.

In the serotonergic raphe nucleus of post-natal day 10 animals, DNSP-11 signal is present in the cytoplasm of neurons, while GDNF signal is only weakly present and differentially localized. DNSP-11 signal in the raphe was also observed in the adult rat CNS.
Figure 6.9 – DNSP-11 signal remains intense in adulthood, while GDNF signal dissipates.

The time of maximal GDNF expression in the rodent is post-natal day 10, which would theoretically be the time of maximal DNSP-11 expression was well. While GDNF and DNSP-11 signals do colocalize extensively, they do not exclusively colocalize. In the adult, 3 month old Fischer 344 male rat, GDNF expression is markedly reduced, while the DNSP-11 signal remains intense. This suggests that GDNF and DNSP-11 may be differentially expressed or be subject to different post-signal processing or half-lives in vivo. (A-C) Post-natal day 10 animals. (A) Merged DNSP-11 and GDNF signals. DNSP-11: green, GDNF: red. (B) GDNF signal. (C) DNSP-11 signal. (D-F) Young adult animals. (D) Merged DNSP-11 and GDNF signals. DNSP-11: green, GDNF: red. (E) GDNF signal. (F) DNSP-11 signal.
Figure 6.10 – DNSP-11 signal is intense in the adult locus ceruleus where GDNF is not expressed.

In the adult male 3 month old Fischer 344 rat, DNSP-11 does not exclusively colocalize with GDNF. DNSP-11 signal is highly concentrated in the locus ceruleus (LC), a region with no mRNA for GDNF and only low levels of mRNA for the GDNF receptor GFRα and its co-receptor RET (Trupp et al., 1997). This suggests differential transport methods for DNSP-11 and GDNF. In addition, DNSP-11 is present in the cells of the mesencephalic nucleus of the trigeminal nerve (MeV), involved in proprioception of the muscles of mastication.
Figure 6.11 – DNSP-11 signal presence in the Purkinje cell layer of the adult cerebellum is differentially localized from GDNF signal. Here we see DNSP-11 signal localized to the nucleus of Purkinje-like cells of the cerebellum in the adult rat CNS. While GDNF signal is also present within these cells, it is restricted to small vesicle-like cytoplasmic concentrations.
Chapter Seven: Synopsis, Conclusions, and Future Directions

In this body of work, intranasally administered DNSP-11 has been shown to protect tyrosine hydroxylase positive cell bodies of the substantia nigra and striatal fiber densities in 6-OHDA lesioned young adult male Fischer 344 rats (Chapter Three); to modulate the dopaminergic neurochemistry of normal Fischer 344 rats and 6-OHDA lesioned rats (Chapter Four); and to prevent apomorphine-induced rotation behavior in 6-OHDA lesioned rats (Chapter Five). While future studies are needed, the data strongly supports the hypothesis that intranasally administered DNSP-11 has therapeutic potential for Parkinson’s disease. Furthermore, dual-fluorescence immunohistochemical visualization of post-natal day 10 and young adult Fischer 344 rat brain tissue provides evidence that DNSP-11 is endogenously present in the rat brain (Chapter Six).

The 6-OHDA lesion parameters used in the present study follow the parameters used in studies on the effects of GDNF authored by Kearns et al. in 1995 and 1997 also in the laboratory of Don M. Gash, Ph.D. In those studies, the lesion produced approximately 91% loss of TH+ neurons of the substantia nigra two weeks after lesioning, compared with 92.1% loss three weeks after lesioning in the present study. In those previous studies, when GDNF was administered as a single injection intranigrally 24 hours prior to 6-OHDA lesion induction, nigral TH+ cell loss was significantly reduced to 53% two weeks after lesioning. In the present study in which DNSP-11 was repeatedly administered for three weeks with the first dose 30 minutes prior to 6-OHDA lesion induction, TH+ cell loss was reduced to 75.2%. In the previous study published in 1997 that looked at the 3 week post-lesion time point, it was identified that pre-treatment with injected GDNF was most effective 6 hours prior to lesion induction, and both DA and DOPAC were significantly higher in the lesioned hemisphere of GDNF treated animals compared with citrate buffer control treated animals. In the present study, DA was significantly higher only in a subgroup of partially lesioned animals. In the animals simultaneously treated with GDNF and 6-OHDA, a 75% TH+ cell loss was observed, similar to the present study.
The similarity of the lesion and time points of analysis between this and the previous studies of GDNF allow more direct comparison of the effects of intranigral GDNF and intranasal DNP-11. While the effects observed in this intranasal DNP-11 study did not reach the same magnitude as the intranigral GDNF studies, the positive impact of intranasal DNP-11 is clear. It may be possible to reach effect parity with simple changes to the dose of DNP-11, the time point of initial treatment, or frequency of administration. Of note, no significant protective effects of GDNF were observed with pretreatment of less than 6 hours before 6-OHDA lesion induction in the previous study (Kearns et al., 1997), while the present study administered intranasal DNP-11 30 minutes prior to lesioning and repeatedly afterward. This could suggest that DNP-11 enters the CNS quickly and rapidly protects cell populations, or that DNP-11 treatment produces strong restorative effects after lesioning, as observed in previously published work on rats with a stable 6-OHDA lesion of the medial forebrain bundle four weeks prior to DNP-11 injection (Bradley et al., 2010).

The similarities of effects from intranasal DNP-11 and intranigral GDNF, along with the strong prevention of rotation behavior with intranasal DNP-11, provide exciting, positive data on the potential for an easy-to-administer, non-invasive preventive and restorative therapy for parkinsonism. Future studies should seek to rapidly advance this potential therapy toward clinical trials by focusing on faster assays and shorter time points of analysis to identify the sources of variability in the treatment.

**Answers and Questions**

This work began with the goal of determining whether the intranasal administration of DNP-11 could result in the transport of efficacious quantities of the peptide into the brain to produce a protective effect. This goal was accomplished, but many new questions were raised in the course of this study.

Data reported in Chapter Three indicates that DNP-11, when administered intranasally, is transported throughout the CNS in biologically relevant quantities. Repeated intranasal administrations of DNP-11 over a three week time period resulted in significantly higher numbers of tyrosine
hydroxylase positive substantia nigra neurons in a unilateral 6-hydroxydopamine lesion rat model. The projections of these neurons to the striatum were likewise higher in DNSP-11 treated rats. This supports the hypothesis that DNSP-11 has cellular protective effects in the rat. Furthermore, DNSP-11 treatment modulated monoamine neurotransmitter content and prevented apomorphine-induced rotation behavior, a hallmark of the unilateral 6-OHDA lesion model of Parkinson’s disease.

These data replicate previously published data on DNSP-11 injected intracranially, directly to the substantia nigra (Bradley et al., 2010; Fuqua, 2010; Littrell, 2011). In these studies it was shown that a single intra-nigral injection of DNSP-11 in stably lesioned animals reduced rotation behavior and modulated the dopaminergic system of the rat. DNSP-11 also protects cultured cells from a variety of cellular and mitochondrial toxins (Bradley et al., 2010; Turchan-Cholewo et al., 2010; Kelps et al., 2011).

**Effects of DNSP-11**

While this study resulted in many answers, many more questions were raised. The most important of these questions surrounds the modulation of dopamine release observed in both normal and lesioned animals. While it was found that dopamine turnover was increased in normal animals after three weeks of intranasal treatment this was due to increases in the metabolites of dopamine and not due to an increase in dopamine. A subgroup of partially lesioned animals in this study exhibited an increase in dopamine content of the striatum with DNSP-11 treatment. However, a small group of naïve animals infused with DNSP-11 during striatal microdialysis revealed a significant decrease in potassium-evoked, calcium-dependent release of dopamine in the striatum, suggesting acute effects. For an overview of the effects observed in this study, refer to Table 7.1.

What is the mechanism behind this modulation of the dopamine system? Based on the animals’ physiological parameters, observed health and behavior, histological, neurochemical and apomorphine-induced rotation metrics, it collectively and firmly supports that DNSP-11 produces protective or restorative
effects on the motor system. The loss of striatal dopamine content is the major factor in the motor impairments of Parkinson’s disease, and increases in striatal dopamine are believed to be the mechanism behind many of the symptomatic treatments currently available at the clinical level for Parkinson’s disease, such as L-DOPA and MAO-inhibitors.

While increases in striatal dopamine were observed in a subgroup of partially lesioned DNSP-11 treated animals in the present study, focusing solely on the dopamine system may be insufficient to explain the observed effects. Some treatments for Parkinson’s disease, such as deep-brain stimulation, produce their symptomatic relief through the targeting of downstream nuclei in the basal ganglia circuitry such as the subthalamic nucleus or substantia nigra pars reticulata. Other striatal inputs may play a role in Parkinson’s disease, such as serotonin inputs from the raphe nuclei, norepinephrinergic inputs from the locus ceruleus or glutamatergic input from the thalamus and cortex. Furthermore, data presented in this study and in the literature (Immonen et al., 2008) indicate that DNSP-11 may have profound effects on other nuclei in the body such as the raphe nuclei (Chapter Four, Chapter Six), the locus ceruleus (Chapter Six), and the hippocampus (Chapter Six). Many of these effects were beyond the scope of this study, but are deserving of future efforts.

Converging data points to the role of DNSP-11 on mitochondria. Protective effects of DNSP-11 against mitochondrial toxins in cell culture has been published in the literature (Bradley et al., 2010; Kelps et al., 2011) and strong changes in mitochondrial respiration and electrical potential are observed with DNSP-11 treatment (Turchan-Cholewo et al., 2010; Bradley et al., 2012). This may explain some of the cellular protective effects as well as neurochemical effects of DNSP-11. Synaptic mitochondria show a strong capacity to buffer calcium. While merely speculation, it is possible that DNSP-11 transiently increases mitochondrial respiratory reserve capacity through the increased buffering of calcium, leading to a decrease in cytosolic calcium and the calcium-dependent, potassium-evoked release of dopamine observed in this and other studies (Fuqua, 2010).
If DNSP-11 is a potent anti-apoptotic compound and can induce increases in mitochondrial respiration, it may also be useful as a treatment for a variety of other diseases of degeneration or metabolic imbalance, including metabolic syndrome (Mitchell et al., 2013), Alzheimer’s disease (Silva D. F. et al., 2012), and even cancer. It has been reported in the literature that cancerous cells upregulate aerobic glycolysis, shutting down their mitochondria and the mitochondrial apoptotic pathways. Stimulation of mitochondria and increases in their respiration reactivate these apoptotic pathways inducing cell death in cancerous cells (Scatena, 2012). Future work on the effects of DNSP-11 and its mechanisms may warrant the evaluation of its effects in other models and disorders.

The identification and characterization of the GDNF pro-region

While immunoprecipitation studies resulted in the elution of bound compounds, these compounds did not match the LC-MS/MS profile of the synthetic, amidated rat sequence of DNSP-11 (Chapter Six). The polyclonal antibody used in these studies was confirmed to bind both the rat and human DNSP-11 sequences to a high titer by ELISA, but the specificity of the antibody was not established. Indeed, it was noted that there exists homologous amino acid sequences in the central nervous system in the form of Neuropeptide Y (NPY) and TRK-Fused Gene product (Tfg). While data in the literature indicates the immunoreactive patterns observed in the CNS are inconsistent with both NPY and Tfg expression, the possibility remains that the observed signal in the CNS is from compounds other than the DNSP-11 sequence. The immunoprecipitation data suggests that, if the DNSP-11 sequence is endogenously expressed, it is covalently modified and/or processed to produce a peptide dissimilar to the synthetic amidated peptide, that it may degrade quickly under the conditions of the immunoprecipitation, or that it is present in quantities too low to detect.

The observed immunoreactive cell populations in the CNS (Chapter Six) and the observed neurochemical changes (Chapter Four), may provide insight into the effects of DNSP-11 in order to optimize its therapeutic use. While it is
hypothesized that the endogenous DNSP-11 sequence from the pro-region of GDNF produces biological effects, the acquisition of a sensitive monoclonal antibody to DNSP-11 would have benefits including the isolation and characterization of the endogenous DNSP-11 sequence. Furthermore, genetic knock-out studies focusing on the pro-region of GDNF could provide information on the developmental and maintenance roles of endogenous DNSP-11.

Mechanisms of action of DNSP-11 and the search for faster assays

The mechanisms of action of DNSP-11 are yet to be elucidated, but some clues do exist. DNSP-11 produces the strongest protective actions against mitochondrial toxins in cell culture, especially toxins against complex I, by inhibiting caspase 3 activation (Kelps et al., 2011; Bradley et al., 2012). DNSP-11 has also been shown to bind to GAPDH, an anti-apoptotic nuclear factor (Bradley et al., 2012), and to phosphorylate ERK1/2, part of an anti-apoptotic pathway (Turchan-Cholewo et al., 2010; Bradley et al., 2012) which suggests a possible membrane-bound trophic factor receptor. These many different proposed signaling pathways may be part of a multifaceted cascade, but the determination of the cellular target and downstream signaling pathways would provide a tool for optimizing the DNSP-11 peptide. It is also possible that DNSP-11, after rapid internalization, acts directly on downstream signaling cascades, bypassing a membrane-bound receptor. Having the ability to determine the activity of a cellular signaling pathway would allow the rapid evaluation of changes to the peptide, vehicle and delivery techniques.

The studies contained within this work required daily administration over three weeks, and although they show the efficacy of intranasal DNSP-11, the time and resources required to perform them are not ideal for quickly advancing DNSP-11 as a therapy. These studies were impaired by lengthy time periods, an administration routine prone to variability, and endpoints with low fidelity for the identification of the sources of variability. Any small change to the dosing regimen, peptide, or formulation would require an excessive length of time to test by this study design. Faster assays of the effects of intranasal DNSP-11 are required. One such possibility is in the radiolabeled $^{125}$I-DNSP-11 studies by
Mallory J. Stenslik. These studies can provide detailed information about the results of different time points and formulations on transport to the central nervous system, allowing for the identification of sources of variability from the dosing of the compound. However, an ideal assay would provide data on the biological effectiveness of the administration at such short time periods. Such an assay may be found in the cellular signaling cascades involved in the biological effects of DNSP-11, perhaps including ERK1/2 phosphorylation or mitochondrial effects previously shown in cell culture studies (Turchan-Cholewo et al., 2010; Bradley et al., 2012).

Furthermore, DNSP-11 may activate transcription factors producing long-term as well as short-term effects. The effects observed in the present study can be interpreted as long-term effects, but may be the result of short-term changes, for instance on mitochondrial potential, resulting in cellular protection as opposed to long-term changes in cellular transcription. It may be possible to positively identify the short-term effects as the dominant mechanism by blocking protein synthesis thus removing transcriptional activation as the possible protective effect. Strong and acute mitochondria effects in cell culture observed in published studies (Turchan-Cholewo et al., 2010; Bradley et al., 2012) may indicate the potential of an assay of ex vivo mitochondrial respiration and/or mitochondrial potential after intranasal administration. This assay would require euthanasia and is prone to variability in mitochondrial extraction. While these assays are interesting possibilities they are still not ideal for in vivo analysis.

Rapid changes to potassium-evoked release of dopamine was identified by microdialysis in a small group of naïve animals (Chapter Four) within two hours of the perfusion of a 50 µM DNSP-11 aCSF solution through a microdialysis probe placed in the striatum, but using microdialysis to assay changes to intranasal administration of the peptide may be unwieldy. Such acute changes may indicate the possibility of other assays, such as changes to blood-oxygen level dependent (BOLD) contrast by MRI after DNSP-11 administration. It may also be possible to implant electrodes to measure striatal dopamine, or other basal ganglia nuclei and neurotransmitters, with high precision both before
and after intranasal administration. The benefits of BOLD-MRI and electrode implantation are the rapid acquisition of the data and repeatability within the same animal. Furthermore, BOLD-MRI in particular is an assay with the potential to be easily translated to trials in non-human primates and even humans.

Regardless of the specific mechanism used, faster assays of the biological effects of DNSP-11 are necessary for the rapid advancement of DNSP-11 toward therapeutic use. While long-term studies provide the benefits of behavioral assays and safety studies in an animal model, they are cumbersome for the purposes of optimizing DNSP-11 as a therapeutic. Whether the assay evaluates mitochondrial respiration, cellular signaling cascades, or neuronal activity, the development of a rapid assay that positively identifies the effects of DNSP-11 soon after administration would provide the biggest advance in development of DNSP-11 by allowing high-throughput analysis of optimizations. As the treatment progresses, long term efficacy and toxicology studies can be performed across species and sexes.

**Conclusion**

Within this body of work, data has been shown that supports the hypothesis that intranasal administration of DNSP-11 does produce protective effects in a 6-hydroxydopamine lesion model of Parkinson’s disease. Tyrosine hydroxylase positive neurons were higher in DNSP-11 treated animals (Chapter Three), striatal dopamine content was higher by DNSP-11 treatment in a subgroup of partially lesioned animals (Chapter Four), and apomorphine-induced rotation behavior was prevented in the DNSP-11 treated animals (Chapter Five). This is strong evidence that intranasal DNSP-11 protected the basal ganglia dopamine system from the 6-OHDA lesion. It was also shown that DNSP-11 modulates the dopaminergic system in both normal and lesioned animals (Chapter Four). The acute effects of infused DNSP-11 on potassium-evoked dopamine release raise questions about the mechanism of action of DNSP-11 (Chapter Four). Animals treated with DNSP-11 did not lose excessive weight, exhibited no detrimental changes in body temperature or activity levels, and did not show signs of distress or discomfort from the treatment (Chapter Five)
providing evidence of the safety of the treatment. The endogenous DNSP-11 sequence was not positively identified, and the specificity of the polyclonal antibody to DNSP-11 was not validated, but endogenous immunoreactive patterns with the antibody do not match patterns of homologous sequences found in the brain, including that of NPY, Tfg, or the preproGDNF unprocessed parent protein (Chapter Six).

The data obtained in this study supports much of the data previously published on DNSP-11. DNSP-11 administered either intranasally or intracranially shares some effects with GDNF while having the potential to provide greater benefits than current therapies such as L-DOPA (Table 7.2). Intracranially injected DNSP-11 has been shown to rapidly enter the cytoplasm of tyrosine hydroxylase positive neurons, to increase dopamine and metabolite concentrations in the normal and stably lesioned rat striatum, and to reduce rotation behavior. Furthermore, DNSP-11 increases TH+ neuron count, neurite length and branching in primary cultured embryonic neurons. DNSP-11 also protects against known mitochondrial toxins in cell culture, reducing caspase-3 activation. (Bradley et al., 2010; Fuqua, 2010; Turchan-Cholewo et al., 2010; Kelps et al., 2011; Littrell, 2011; Bradley et al., 2012)

With the increasing average age of the population and increasing individual longevity, the impact of Parkinson’s disease is growing (Dorsey et al., 2007; Collier et al., 2011). The disease progresses insidiously and it profoundly impacts not just the individual but families as well as society. A protective therapeutic can potentially delay the manifestation of serious symptoms until much later in life, diminishing the personal, societal, and economic impact of this neurodegenerative disease. For the reasons provided in this dissertation, DNSP-11 is a good candidate for further development as a prevention and treatment for Parkinson’s disease.
Table 7.1 – Overview of the effects observed in this study

<table>
<thead>
<tr>
<th>Intranasal DNSP-11</th>
<th>Normal Rats</th>
<th>Lesioned Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Partial</td>
<td>Severe</td>
</tr>
<tr>
<td>Dopamine Content</td>
<td>inconclusive</td>
<td>↑↑</td>
</tr>
<tr>
<td>Dopamine Metabolite Content</td>
<td>↑↑</td>
<td>inconclusive</td>
</tr>
<tr>
<td>TH+ substantia nigra cell bodies</td>
<td>not tested</td>
<td>↑↑</td>
</tr>
<tr>
<td>TH+ striatal fiber density</td>
<td>not tested</td>
<td>↑↑</td>
</tr>
<tr>
<td>Baseline Dopamine Release</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>Baseline Dopamine Metabolites</td>
<td>no change</td>
<td>no change</td>
</tr>
<tr>
<td>K+-Evoked Dopamine Release</td>
<td>↓↓</td>
<td>no change</td>
</tr>
<tr>
<td>Apomorphine-induced rotations</td>
<td>not tested</td>
<td>↓↓</td>
</tr>
</tbody>
</table>

Overview of the effects observed with various endpoints in the studies composing this dissertation. Up arrows indicate positive changes with respect to controls, down arrows indicate negative changes.
Table 7.2 – Overview of the effects of DNSP-11 and GDNF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DNSP-11</th>
<th>GDNF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present study</td>
<td>(Bradley et al., 2010)</td>
</tr>
<tr>
<td>Administration</td>
<td>Intranasal</td>
<td>Intracranial</td>
</tr>
<tr>
<td>Organism</td>
<td>Rodent</td>
<td>Rodent</td>
</tr>
<tr>
<td>Lesion toxin</td>
<td>6-OHDA</td>
<td>6-OHDA</td>
</tr>
<tr>
<td>Lesion location</td>
<td>Acute SN</td>
<td>Stable MFB</td>
</tr>
<tr>
<td>Treatment timepoint</td>
<td>Repeated</td>
<td>Post-lesion</td>
</tr>
<tr>
<td>Post-lesion timepoint</td>
<td>3 weeks</td>
<td>5 weeks</td>
</tr>
<tr>
<td>TH+ nigral cell count</td>
<td>+</td>
<td>inconclusive</td>
</tr>
<tr>
<td>TH+ Striatal fiber density</td>
<td>+</td>
<td>inconclusive</td>
</tr>
<tr>
<td>Dopamine content</td>
<td>inconclusive</td>
<td>+</td>
</tr>
<tr>
<td>Dopamine metabolites</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Striatal dopamine release</td>
<td>inconclusive</td>
<td>++</td>
</tr>
<tr>
<td>Behavioral protection</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

This table represents data from the present work on intranasal DNSP-11, published reports on intracranial DNSP-11 (Bradley et al., 2010) and from work in the same laboratory evaluating the effects of GDNF using the same lesioning parameters (Kearns et al., 1995, 1997). Most significant from the Kearns articles, significant effects of GDNF were observed with pretreatment 6+ hours prior to lesion induction; simultaneous application of GDNF and the 6-OHDA toxin did not produce significant effects. While the most effective dose or timing of intranasal DNSP-11 administration may not yet be identified, intranasal treatment with DNSP-11 produced strong protective or restorative effects similar to GDNF treatment. This study has provided important evidence for the efficacy of a non-invasive administration route for the DNSP-11 peptide to protect or restore dopaminergic neurons from a 6-hydroxydopamine rodent model of Parkinson’s disease.
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