Intranasal Delivery of pGDNF Nanoparticles for Parkinson’s Disease

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by

Brendan Trevor Harmon

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Other committee members:

________________________________________ Date___________

________________________________________ Date___________

________________________________________ Date___________

________________________________________ Date___________

________________________________________ Date___________

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ABBREVIATIONS

6-OHDA: 6-hydroxydopamine
AAV: adeno-associated virus
BBB: blood-brain barrier
BDNF: brain-derived neurotrophic factor
CNS: central nervous system
CMV: cytomegalovirus (promoter)
CREB: cAMP-regulatory element binding protein
CSF: cerebrospinal fluid
DAB: 3,3-Diaminobenzidine
DAT: dopamine transporter
DDC: DOPA decarboxylase
DIC: differential interference contrast
eGFP: enhanced green fluorescent protein
ELISA: enzyme-linked immunosorbant assay
GDNF: glial cell line-derived neurotrophic factor
GFP: green fluorescent protein
GFR: GDNF family receptor
GP: globus pallidus
GPCR: g-protein coupled receptor
ICC: immunocytochemistry
IOD: integrated optical density
ICV: intracerebroventricular
IHC: immunohistochemistry
IN: intranasal
IP: intraperitoneal
ISH: in situ hybridization
MAO: monoamine oxidase
MFB: medial forebrain bundle
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NCAM: neural cell adhesion molecule
NGF: nerve growth factor
NP: nanoparticle (PEG-CK30)
OEC: olfactory ensheathing cells
ORN: olfactory receptor neurons
PD: Parkinson’s disease
pDNA: plasmid DNA
PEG: polyethylene glycol
PEG-CK30: polyethylene glycol coated cysteine-substituted lysine 30-mer nanoparticle
PI3K: phosphatidylinositol 3-kinase
RMS: rostral migratory stream
RNS: reactive nitrogen species
ROS: reactive oxygen species
SC: subcutaneous
SN: substantia nigra
SNC: substantia nigra pars compacta
SNr: substantia nigra pars reticulate
STN: subthalamic nucleus
SPECT: single-photon emission computed tomography
TH: tyrosine hydroxylase
UbC: polyubiquitin C (promoter)
VM: ventral midbrain (cultures)
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ABSTRACT

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that primarily affects the dopaminergic A9 nigrostriatal tract. For dopamine neurons specifically, glial cell-derived neurotrophic factor (GDNF) has been shown to promote their survival and proliferation both in culture and \textit{in vivo}. GDNF has also proven to be neuroprotective and restorative in various animal models of PD and some human clinical trials. However, its delivery to the brain has required invasive surgical routes which are not clinically practical for many patients. The main objective of this project was to test intranasal delivery to the brain of a nanoparticle vector incorporating an expression plasmid for GDNF (pGDNF). The intranasal route circumvents the blood-brain barrier, allowing larger sized vectors into the central nervous system while avoiding peripheral distribution. This approach would provide a renewable source of GDNF within the target areas of the brain, the striatum and the substantia nigra (SN) without the need for surgical injections or frequent re-dosing. A PEGylated polylysine compacted plasmid nanoparticle vector (PEG-CK30), developed by Copernicus Therapeutics, Inc., has been shown to transfet neurons and glial cells \textit{in vivo} while lacking the safety issues present with other vectors.

The first goal of this work was to determine if these PEG-CK30 compacted plasmid nanoparticles can successfully transfet cells and express the reporter protein, enhanced green fluorescent protein (eGFP) in the rat brain after intranasal administration. Initial \textit{in vivo} experiments utilized the expression plasmid pCG, expressing eGFP under the fast-acting cytomegalovirus (CMV) promoter. Intranasal administration of pCG nanoparticles resulted in evidence of transfection of brain cells, as shown both
qualitatively, by GFP-immunohistochemistry, and quantitatively, by GFP-ELISA. Expression was detected throughout the rat brain two days post-administration.

Following the proof-of-principle study with pCG, a new plasmid was created by Copernicus Therapeutics, Inc. to better mimic their long-lasting pGDNF plasmid while providing both GDNF as well as the reporter function of eGFP. This eGFP-GDNF plasmid was used to monitor expression and cell-types transfected. This expression plasmid, called pUGG, was first characterized in vitro to verify protein expression. Transfection experiments in SHEP-1 neuroblastoma cells, ventral midbrain cultures, and N27 dopaminergic cells all demonstrated that pUGG expressed bioactive eGFP and GDNF. However, cleavage of the two proteins did not occur and the expressed protein emerged as a fusion construct which was not detectable by GDNF-ELISA, although it was detected by GFP-ELISA.

The next goal was to determine if pUGG was able to transfect cells in vivo in rat brain. Direct striatal injection of pUGG nanoparticles showed significant eGFP expression at the site of injection both 7 and 14 days post-administration with no difference in eGFP expression between the two time-points. GFP-immunohistochemistry at the striatal injection site revealed expression of eGFP-positive cells as well as evidence of GDNF’s bioactivity as indicated by neurite outgrowth. Moving forward, we administered pUGG nanoparticles intranasally to rats and found significant expression seven days later throughout the brain, with highest levels in the forebrain areas (olfactory bulb and frontal cortex). Significant expression was also seen along the rostral-caudal axis of the brain compared with naked pUGG plasmid.
The final goal of this work was to examine whether intranasal pGDNF pre-treatment could generate sufficient GDNF to protect SN dopamine neurons after a unilateral 6-hydroxydopamine (6-OHDA) lesion, a common animal model for PD. Copernicus’ pGDNF plasmid was utilized for the neuroprotection experiments to avoid possible confounds due to the GFP fusion produced by pUGG. Tyrosine hydroxylase-immunostaining density was used as a marker for dopamine neurons in the SN and their nerve terminals in the striatum. Dopamine cell counts were also performed in the SN.

Intranasal delivery of pGDNF significantly protected dopamine neurons in the rat 6-OHDA model of PD. This was revealed in three ways. First, pGDNF treatments reduced amphetamine-induced circling behavior, suggesting a prevention of dopamine loss on the 6-OHDA-lesioned side. Second, pGDNF increased TH staining density and dopamine cell counts in the SN on the 6-OHDA-lesioned side. This result was direct evidence of neuroprotection of dopamine cell bodies. Third, pGDNF increased TH staining density in the striatum on the 6-OHDA-lesioned side. This result was direct evidence of protection of dopaminergic nerve terminals. Intranasal pGDNF nanoparticles provided greater neuroprotection than naked pGDNF for all measures. This result was consistent with our previous findings that pGDNF nanoparticles produce more GDNF in brain than the naked plasmid.

Collectively, these results demonstrate that intranasal delivery of Copernicus’ pGDNF nanoparticles has great clinical potential as a new, non-invasive and non-viral gene therapy approach for early stage Parkinson's disease. By promoting recovery of damaged neurons and preventing further cell loss, symptoms may be reversed and disease progression may be stopped.
I. INTRODUCTION

A. Statement of the Problem:

Gene therapy for CNS disorders is a rapidly growing research area primarily dominated by the use of viral vectors encoded to transduce and overexpress proteins known to provide a neuroprotective function. Although substantial, knowledge of viral behavior and genetics is not complete, interfering with our ability to prevent fatal immune responses or oncogenicity due to incorrect genetic insertion. The risks of invasive brain surgery for delivery of CNS gene therapy vectors are also of great concern, and largely limit the clinical applicability of this approach. Therefore, two major areas of interest for CNS gene therapy are developing effective non-viral vectors and applying them in a non-invasive manner.

There is a growing body of research into nanoparticle DNA vectors, heavily dominated by cationic liposomal-DNA formulations (termed “lipoplexes”). Their popularity is due to their customizability, allowing for a variety of lipid components and surface modifications to give them an array of targeting options and other unique characteristics. However, lipoplexes tend to have low transfection efficiencies, stemming from their inability to overcome barriers in order to deliver their DNA cargo to the nucleus. Viruses can inherently overcome these barriers, due to mechanisms not completely understood and hard to replicate in manufactured vectors (Thomas, 2003). As such, liposomes may be better suited for targeted delivery of siRNA or proteins, which do not require nuclear entry for their effectiveness.

The purpose of this thesis project was to advance non-viral gene delivery systems to the CNS using a novel route of administration, the intranasal pathway. Intranasal
administration circumvents the blood-brain barrier (BBB), providing a non-invasive means of targeting large molecular weight substances to the brain. Proteins have been shown to pass through spaces in the olfactory epithelium directly into the brain as no BBB exists at this interface (Thorne, 2001; Migliore, 2010). Focusing on a treatment for Parkinson’s disease, we combined the therapeutic potential of GDNF gene transfection with the non-invasive approach of intranasal delivery to the brain. Copernicus Therapeutics Inc. provided their optimized expression plasmids for GDNF and reporter proteins as well as their nanoparticles, which are polyethylene-glycol (PEG)-substituted poly-lysine (30-mer) constructs with an average diameter of 8-11 nm and a rod-like shape, known as PEG-CK30 (Liu, 2003; Yurek, 2009b). The PEG-CK30 vector has been shown to be non-immunogenic, non-inflammatory, small enough to allow entry into the cell’s nucleus, and able to successfully deliver expression plasmids and to transfect post-mitotic cells in the brain and retina for greater than one year (Ziady, 2003b; Yurek, 2009b; Cai, 2010).

If nasal delivery of pGDNF nanoparticles can successfully transfect cells in the striatum and SN, this approach could offer significant neuroprotection in the 6-OHDA rat model of PD, translating into a potentially long-lasting and non-invasive GDNF gene therapy approach.

B. Parkinson’s Disease: Symptoms and Treatment

Parkinson’s disease (PD) is a progressive neurodegenerative disorder that was first described by Sir James Parkinson as a “shaking palsy” in 1817 (Fahn, 2003). PD is now known to be caused primarily by destruction of the A9 tract of dopamine neurons
that project from the substantia nigra pars compacta (SNc) to the corpus striatum (made up of the caudate nucleus and putamen in primates). These areas of the brain are a major part of the basal ganglia circuitry that is crucial to the extrapyramidal control of movement, and as such, motor abnormalities (bradykinesia, resting tremor, postural instability, rigidity) are the major symptoms of PD. Generally, it is when greater than half of these SN dopamine neurons are lost that symptoms of the disease start to manifest in an individual, as 50-60% nigral cell loss results in 70-80% dopamine depletion (Hornykiewicz, 1973). This in and of itself is the greatest problem in curing PD, as the patient is already in an advanced stage of neuronal death when symptoms of the disease present themselves. At this point, it is too late to undo the loss of dopamine neurons that has already occurred, but not too late to prevent further loss and worsening of symptoms.

PD is highly correlated with age, with 1-2% prevalence in the U.S. population between the ages of 65 to 70 and increasing to 4-5% in those over 85 years of age (Tansey, 2007). PD patients have a 1.6 higher mortality rate than the age-matched members of the normal population, as many patients succumb to secondary health problems associated with ataxia and akinesia (Fahn, 2003). Almost 95% of cases are idiopathic, and the cause is hypothesized to be multifactorial due to a variety of extrinsic factors and intrinsic pre-determined genetic vulnerabilities.

Aside from A9 dopaminergic cell loss, PD causes degeneration in the amygdala, ventral tegmental area (VTA), locus coerules, raphe nuclei, and the vagal dorsal motor nucleus (Lang & Lozano, 1998). This leads to dysfunction in other neurotransmitter systems (cholinergic, adrenergic, serotonergic), resulting in the “non-motor” symptoms of PD which include autonomic dysfunction and cognitive decline (Schapira, 2009). In
addition, formation of Lewy bodies (cytoplasmic eosinophilic inclusions) made up primarily of a protein called α-synuclein are found throughout many neuronal populations and in post-mortem analysis of PD patients. However, their normal function and the role of α-synuclein itself in PD etiology and/or pathology are only recently being understood. Lewy bodies have been shown to first form in the olfactory bulb and brain stem, with later spread to the nigra and cortex. This may help to explain why anosmia is one of the first early non-motor indicators of PD (Schapira, 2009).

As loss of dopamine input to the striatum is the cornerstone of PD, the most widely used pharmacotherapies help to counteract that loss. These include dopamine precursors (L-DOPA), dopamine receptor agonists (pramipexole, bromocriptine, ropinirole), monoamine oxidase inhibitors (selegiline, rasagiline) and catechol-o-methyltransferase inhibitors (entacapone, tolcapone). However, as these drugs act only to salvage the remaining dopamine or mimic the dopamine lost due to cell death, they are not getting to the underlying root of the disorder and are thus only temporarily effective as further loss of dopamine neurons is imminent.

C. Parkinson’s Disease: Etiology

It is becoming clear that a single predisposing event or genetic flaw cannot accurately describe the vast pathology that underlies Parkinson’s disease. Current research points at a multifactorial interaction of many differing pathways and a variety of factors that eventually destroy the dopamine neurons. In fact, many argue that PD is actually a syndrome of various disorders that share the common outcome of dopamine neuron degeneration (Le, 2009). In particular, the nigral cell bodies are thought to be
primarily vulnerable due to the metabolism of dopamine, which can auto-oxidize if not kept in the low pH environment of the storage vesicles (Obeso, 2010). Dopamine metabolism generates several free radical species that can lead to oxidative stress and even cell death if normal antioxidant processes are overwhelmed or deficient (Jenner and Olanow, 1996).

The CNS environment and its adaptions to stress and damage differ greatly from those present peripherally, and these differences may contribute to PD pathology. The brain is unusually sensitive to oxidative damage as its reactions constitute 20% of the total oxygen used throughout the body, yet its enzymatic antioxidant defenses are quite lacking. In particular, the SN is thought to be in a more pro-oxidative state when compared to the rest of the brain, due a relatively greater lack of glutathione and increased dopamine metabolism (Tansey, 2007).

Several glial cell populations appear to play a role in PD etiology. The role of astrocytes and microglia is currently an area of intense research. Astrocytes are the most abundant glia in the brain and provide protection, support and eliminate toxins generated by neurons from their microenvironment (Mena, 2008). Astrocytes are also neuroprotective insofar as they are the predominant source of many neurotrophic proteins as well as the main sites where free radicals are scavenged. Observations of brains of post-mortem PD patients as well as comparable animal models show that astrocytes are found to be proportionally lower in the SN, which may explain either a cause of or an effect from the downstream mechanisms involved in the disease (Orr, 2002).

Microglia are the resident immune cells of the CNS, going from an inactive (quiescent) state to an active form in response to a variety of insults (Orr, 2002). Neurons
cannot be repaired and replaced like cells of peripheral tissues, so microglial-triggered local inflammation provides a much more controlled and limited process to prevent excessive damage (Orr, 2002). Microglia prevent the spread of infection and rid the microenvironment of any foreign invaders, dead cells or debris, but they contribute substantial amounts of damaging free radicals (Hirsch, 2009). Upon neuronal injury, microglia are activated and work in concert with T-cells to produce a variety of cytokines which promote pro-inflammatory as well as cytotoxic pathways (Block, 2007). To further stimulate the process, T-cells secrete additional cytokines to keep the microglia active, which could lead to large-scale damage to their surrounding neuronal environment if not carefully regulated (Orr, 2002). Thus, neuronal damage can initiate a long-lasting and self-propelling state of damage and inflammation, known as reactive microgliosis. This chronic cycle is thought to make neurons highly vulnerable to any additional insults, causing pro-inflammatory chemicals that generally serve a protective role in the short term to cause unchecked, progressive neuronal destruction (Block, 2007).

Other research ties the susceptibility of the SN dopamine neurons with their production of neuromelanin, the pigment which gives the region its characteristic black coloration. The exact role of neuromelanin is still unclear, but it has been shown to be derived from catecholamines and is suggested to be produced as a “sink” in those neurons producing large amounts of these neurotransmitters (Zecca, 2006). In addition, iron has a crucial role in neuromelanin production, as it is bound in a non-reactive ferric state (with the aid of ferritin, an iron binding protein), and this association serves as a means to prevent excess free radical production. It is thought that ferritin levels decrease over time, decreasing the scavenging properties of neuromelanin. This can lead to lipid
peroxidation and other means of free radical damage, which are plentiful from the metabolism of catecholamines (Zecca, 2006; Jenner and Olanow, 1996).

The natural vulnerability of dopamine neurons to oxidative stress and the unique environment of the CNS make intrinsic variations (genetic mutations) and extrinsic factors (environmental insults) play even larger roles in the disease. Analysis of the genomes of patients with familial PD brings new insights into PD pathology and highlights the large role of mutations and copy number variants in disrupting the dynamic equilibrium governing protein aggregation and mitochondrial dysfunction (Allain, 2008). Point mutations or multiplications in a variety of genes, including those for α-synuclein (SNCA), parkin (PARK2, a U3 ubiquitin ligase), PTEN induced putative kinase-1 (PINK1, a mitochondrial kinase), leucine-rich repeat kinase 2 (LRRK2, a mitochondrial membrane protein), and ubiquitin carboxyl-terminal esterase L1 (UCHL1, an enzyme involved in recycling ubiquitin) have all been implicated in familial PD.

These PD-related mutations may also provide insight into what can go wrong to cause idiopathic PD (Schapira, 2009). α-Synuclein is by far the most heavily researched of these, as a mere two-fold increase in the protein from gene duplication leads to profound PD-like phenotypes (Scott, 2010). It is thought that α-synuclein plays a crucial role in neurotransmitter release from the pre-synaptic terminal, although its exact function remains unknown (Lashuel, 2013). Elevated levels of α-synuclein prevent normal pre-synaptic proteins from properly releasing neurotransmitter and interfere with retrograde signaling to the SNc cell bodies, and these disruptions are presumed to be crucial toxic events that lead to neuronal degeneration (Scott, 2010).
There is much debate regarding the steps leading to cytotoxicity from α-synuclein. In the past, its toxicity was strongly linked to its propensity to natively aggregate. This aggregation was thought to overwhelm the ubiquitin-proteasome system in cells, forming huge amyloid plaques called Lewy bodies, a hallmark of PD found in post-mortem brain tissue. However, more recent research concludes that the native form of the protein is a stable tetramer that largely resists aggregation (Bartels, 2011). This means that a major part in the pathogenesis of α-synuclein relies on the breakdown of this stable tetramer to form the more toxic species. The three point mutations in the SNCA gene that cause autosomal dominant Parkinson’s disease all convey changes in the protein that greatly accelerate its propensity to aggregate (Irvine, 2008). The soluble oligomer (or pre-fibril form) of α-synuclein seems to be the most toxic variant and many now also believe that the Lewy bodies are actually a failsafe to contain these toxic species of the protein in an insoluble, nondestructive form (Irvine, 2008).

A recent finding by Luk et al. (2012) has found that a single intrastriatal injection of α-synuclein oligomers led to cell-to-cell transmission between interconnected brain areas in wild-type nontransgenic mice. This presence of α-synuclein oligomers caused a pronounced decrease in A9 dopamine neurons marked by the presence of Lewy bodies after 30 days, and these spread throughout the brain after 180 days (Luk, 2012). This gives support to Braak’s “dual-hit” theory, which posits that α-synuclein starts aggregating in the GI tract and olfactory region (due to a toxin, pathogen or inflammatory insult), with oligomers spreading into the brain via the vagus and olfactory nerves. This dual attack is hypothesized to initiate the degeneration found in PD (Hawkes, 2009).
This progression may explain the anosmia, constipation and sleep problems commonly found as early symptoms in PD patients (Lee, 2011).

The involvement of α-synuclein, combined with that of other proteins involved in familial PD, underscore a critical need for proper functioning of mitochondrial energy homeostasis as well as the ubiquitin-proteasome system for maintaining proper targeting and degradation of misfolded, defective or excessive proteins, such as α-synuclein (Schapira, 2009). Studies using transgenic and knockout animals manipulating α-synuclein and other PD-linked genes are providing a better understanding of the dynamics involved in the pathogenesis of PD and what is needed to develop animal models that more accurately mimic disease progression.

Environmental factors have also been implicated in the etiology of PD. For example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a synthetic analog of meperidine (a drug of abuse), caused parkinsonism in those exposed (Langston and Ballard, 1984; Helmuth, 2000). This was later found to be due to MPTP’s conversion to toxic MPP+ by monoamine oxidase-B, where MPP+ is readily taken up into dopamine neurons, specifically by the dopamine transporter (Irvine, 2008). In the neuron, it binds to complex 1 of the mitochondria and causes widespread oxidative stress through mitochondrial dysfunction. Many other environmental toxins linked to PD are derived from herbicides and insecticides (such as rotenone) and behave in a similar manner. These mitochondrial toxins contributed to our hypothesis about the role of mitochondrial dynamics in PD (Irvine, 2009). More recent studies have shown that there is a critical link between genetic polymorphisms and exposure to pesticides. Those people that were exposed to pesticides (such as DDT) and also share a polymorphism in the gene that
expresses P-glycoprotein (which normally doesn’t show any correlations to Parkinson’s disease), had a 3.5-fold increased risk of developing PD (Dutheil, 2010). In addition, viral pathogens have been shown to cause PD (Takahashi and Yamada, 1999). For instance, patients that had contracted encephalitis lethargica during a 1920’s outbreak later developed PD (Calne and Lees, 1988).

In summary, the dopamine neuron damage underlying PD can be thought to fit a “multihit model” wherein any combination of genetic susceptibilities (causing early cell loss and/or high vulnerability) coupled with environmental factors, aging, inflammation or insult can lead to degradation (Le, 2009).

D. Dopamine: Synthesis, Metabolism and Receptors

Much of the pioneering work with dopamine was done in the 1950’s by Arvid Carlsson. He showed that dopamine acts as a neurotransmitter and mapped the location of dopamine neurons in the brain using a formaldehyde gas fluorescence histochemical method (Carlsson and Waldeck, 1958; Ungerstedt, 1971; Fahn, 2003). Dopamine is a catecholamine, synthesized locally in the cell bodies and at the nerve terminals of dopamine neurons starting from the amino acid tyrosine. The first reaction involves the enzyme tyrosine hydroxylase (TH), which converts tyrosine to L-DOPA. As such, TH is commonly used as a marker of dopamine neurons. L-aromatic amino acid decarboxylase, or DOPA decarboxylase, catalyzes the reaction of L-DOPA to dopamine. However, the enzyme is found throughout the body as a general aromatic amino acid decarboxylase, so it is not catecholamine neuron-specific. Carlsson also demonstrated that administration of L-DOPA could reverse the motor symptoms of PD in patients as it is able to cross the
blood-brain barrier (BBB), making it the first and still widely used treatment for PD. Dopamine itself is less effective as a treatment because it cannot cross the BBB.

After release from nerve terminals, unbound dopamine is taken back up into the presynaptic neuron by the dopamine transporter (DAT), or broken down by either monoamine oxidase (MAO) or catechol-O-methyltransferase (COMT). MAO degrades dopamine to 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide, whereas COMT metabolizes dopamine to 3-methoxytyramine (3-MT) (Youdim, 2006). **Figure 1** shows a detailed schematic illustration of the synthesis and release of dopamine in a presynaptic dopamine neuron.

![Figure 1: Synthesis, metabolism and release of dopamine](image_url)

**Figure 1**: Synthesis, metabolism and release of dopamine. In a nigrostriatal dopamine neuron, the amino acid tyrosine is transformed by tyrosine hydroxylase (TH) to L-DOPA, which is then converted by DOPA decarboxylase (DDC) to dopamine. The neurotransmitter is placed into synaptic vesicles where it is released to act on the postsynaptic neuron in the striatum (such as binding to D_1 and D_2 receptors). The dopamine transporter (DAT) terminates the signal in the synapse, and excess dopamine is broken down by monoamine oxidase-A (MAO-A) in the neuron. Figure adapted from (Youdim, 2006).

Dopamine has its effect by binding to dopamine receptors, which are G-protein coupled receptors (GPCRs) first discovered as existing in two subtypes: D_1 and D_2 (Kebabian and Calne, 1979). There are now known to be five dopamine receptors that fall into either the D_1-like family or the D_2-like family. The D_1-like family includes the D_1 and D_5 receptors, which are coupled to G_s and activate adenylyl cyclase (increasing
cAMP and activating downstream signaling cascades). The D$_2$-like family includes the D$_2$, D$_3$, and D$_4$ receptors, and are coupled with G$_{i/o}$ and inhibit cAMP formation or couple to other effector systems (Fahn, 2003).

E. Neuronal Circuitry of Parkinson’s Disease

Voluntary movements result from the activation of the pyramidal (corticospinal) system, which is modulated by input from the extrapyramidal motor system. The extrapyramidal motor system is composed of the basal ganglia and associated nuclei, including the caudate nucleus and putamen (together comprising the corpus striatum), globus pallidus (GP), substantia nigra pars compacta (SNc – the A9 dopamine cell group), the SN pars reticulata (SNr) and subthalamic nucleus (STN). Dopamine is a key regulator of this system, acting on striatal efferent neurons expressing D$_1$ and D$_2$ receptors. These GABAergic neurons are the medium spiny neurons that make up >95% of the corpus striatum. These striatal efferents have dual projections. They project to the SNr and GP internal segment (GPi), which is termed the “direct pathway”, and they also project to the GP external segment (GPe), the first stage of the “indirect pathway”. Activation of the striatonigral “direct pathway” neurons inhibits those of the GPi/SNr. This directly removes the inhibition placed on the thalamus to facilitate movement (Lewis, 2003). Dopamine achieves a similar function mediated through inhibition of striatopallidal neurons of the indirect pathway, which in turn stimulates GPe inputs to the STN and inhibits the STN's glutamatergic projections on the GPi/SNr. This inhibition of the indirect pathway causes the same end result of GPi/SNr inhibition (Lewis, 2003).
In PD, the lack of striatal dopamine leads to excessive inhibitory output from the basal ganglia system (SNr/GPi) which in turn results in a decrease in pyramidal motor output. **Figure 2** shows a summary of the distinct brain regions involved in the basal ganglia circuitry showing the origination of motion control via SNC dopamine signals and ending in glutamatergic activation of the cortex. Lowered dopamine levels dramatically reduce cortical drive by this circuitry, making movements slow and difficult to control.

**Figure 2**: Basal ganglia model of movement. Dopamine released from the SNC into the putamen can act to activate the “direct pathway” or inhibit the “indirect pathway”. Activation of the “direct pathway” inhibits GPi/SNr GABAergic neurons, allowing the thalamus’ glutamatergic neurons to activate the cortex and produce movement. Inhibition of the “indirect pathway” blocks striatopallidal inhibition on the GPe, which allows its GABAergic neurons to inhibit the STN. The STN cannot excite the GPi/SNr GABAergic neurons. These combined actions of dopamine on the direct and indirect pathways reduce the inhibition on the thalamus, allowing activation of the cortex (Lewis, 2003).

**F. The 6-OHDA Model of Parkinson’s Disease**

To better understand the pathogenesis of PD, neurotoxic agents that can selectively ablate nigrostriatal dopamine neurons are used to provide animal models that mimic the damage done in the disease. 6-Hydroxydopamine (6-OHDA) is one of the...
most popular neurotoxin-induced methods to model PD (Dauer and Przedborski, 2003). It is used to generate a rat model of PD. However, no animal model can mimic the range of behaviors observed in humans with PD. Species as well as strain differences also impart great effects on the resulting phenotype (Deumens, 2002; Bove, 2005).

6-OHDA is a hydroxylated analog of dopamine, and as such, does not cross the BBB and must be stereotaxically injected directly into the brain. It is taken up by both the dopamine and norepinephrine transporters, and can induce a lesion of both neuronal populations. Proper care must be taken to block the norepinephrine transporter to prevent noradrenergic damage (Bove, 2005).

Neurodegeneration results from oxidative damage caused by reactive oxygen species, formation of reactive quinones (Figure 3), and inhibition of mitochondrial complexes I and IV (Asanuma, 2004). Usually, researchers refrain from performing bilateral 6-OHDA injections due to the generation of severe lesions, resulting in aphagic and adipsic animals that often die shortly after the procedure. As a result, 6-OHDA is normally injected unilaterally, and the contralateral side is used as an unlesioned control. Previous research in our lab has shown, however, that the unlesioned side is not “normal” and is not truly a control relative to the lesioned side (Waszczak, 2006; White-Cipriano & Waszczak, 2006). Nevertheless, the unilateral 6-OHDA model is still used widely as a rat model for PD. The site of 6-OHDA administration is critical at determining the extent of the lesion generated and the time course of lesion development. Typically, 6-OHDA is injected into the SN, striatum, or the medial forebrain bundle (MFB) (Deumens, 2002;
Bove, 2005). Striatal lesions are generated via retrograde transport of the neurotoxin to the SN cell bodies and tend to form a more progressive partial lesion. Injections into either the SN or the MFB, on the other hand, can result in a rapid decrease in both the number of A9 nigrostriatal TH+ neurons, as well as the SN and striatal TH fiber density (Yuan, 2005). However, 6-OHDA injections via the MFB may cause additional destruction of the A10 dopaminergic neurons in the VTA, which can complicate experimental analysis (Deumens, 2002). In our previous work, Migliore used a less concentrated dose of 6-OHDA into the MFB to limit the severity of the lesion (Migliore, 2009), as this allows for a partial lesion of dopamine neurons, making it possible to reverse the damage and better gauge the neuroprotection and neurorestorative properties of the treatment (Truong, 2006).

G. Neurotrophic Factors: Glial Cell-Line Derived Neurotrophic Factor

One of the most profound breakthroughs in the field of neuroscience has been the concept of adult neurogenesis; the ability of the brain to grow new neurons. In studying the mechanisms that allow brain development and neurogenesis to occur, scientists have discovered proteins known as neurotrophic factors that play a variety of roles in both the developing and postnatal brain. Generally, these proteins are secreted by the neuron’s target (tissue or neuron) during development, where vast numbers of neurons are competing to make a synaptic connection. Without neurotrophic factor activation, the competing axon terminals die back and those that were successfully activated will strengthen their neuronal connection with their target (Peterson, 2008). Postnatally, neurons serve a much more permanent role than most peripheral tissue cells and as such
need constant nourishment and protection to remain healthy. As mentioned previously, the continuing processes of neurotransmitter synthesis and metabolism generate large amounts of damaging oxidative stress, and neurotrophic factors have evolved to bring the cell a multitude of neuroprotective and neurorestorative effects (Peterson, 2008). Since neuronal death is at the heart of neurodegenerative disorders like PD, neurotrophic factors have been heavily researched for their roles in its pathology and as potential therapeutics.

The three major families of neurotrophic factors are: 1) the neurotrophin family, 2) the glial cell-line derived neurotrophic family of ligands (GFL), and 3) the mesencephalic astrocyte-derived neurotrophic factor (MANF) family (Peterson, 2008). Each family possesses its own distinct signaling by which activation of pro-survival pathways are accomplished. Members of the neurotrophin family bind to Trk (tropomyosin-related kinase) receptors and consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3), and neurotrophin 4/5 (NT-4/5). Proteins in the GFL interact with GFRα (GDNF family receptor-α) co-receptors, which are normally linked to a tyrosine kinase known as Ret. The GFL is made up of glial cell-line derived neurotrophic factor (GDNF), neurturin, artemin and persephin. Finally, the MANF family consists of MANF and conserved dopamine neurotrophic factor (CDNF), although little is known about their receptors (Peterson, 2008).

GDNF has received the most attention related to PD as in 1993, the Lin group isolated the protein from rat B49 glial cell line culture medium and found it to be relatively specific for promoting the neurite outgrowth, increasing cell size and the amount of TH in cultured ventral midbrain dopamine neurons (Lin, 1993). Protein
characterization of GDNF yielded a monomeric weight of around 15 kDa, however, GDNF is normally found as a glycosylated disulfide-bonded homodimer with a weight around 30-40 kDa (Lin, 1994).

GDNF is first synthesized in a precursor form known as pre-pro-GDNF (Lin, 1993). The GDNF gene actually encodes two different mRNAs produced by alternative splicing: pre(α)- and a shorter pre(β)-proGDNF, which are cleaved to (α)long proGDNF and (β)short proGDNF by proprotein convertase, respectively (Glerup, 2013). In vitro studies have indicated that both forms are secreted from neurons, but secretion of the (β)short-pro-GDNF and its mature GDNF is activity-dependent, whereas (α)long pro-GDNF and its mature GDNF are secreted constitutively (Lonka-Nevalaita, 2010). Other researchers have found that a significant amount of proGDNF cleavage (to mature GDNF) occurs by the enzyme furin (and other proprotein convertases) which exist outside the cell in the extracellular matrix (Lonka-Nevalaita, 2010). However, other GDNF and proGDNF splice variants may exist and have an as of yet undiscovered signaling role.

H. GDNF Receptors and Signal Transduction

The GDNF family receptor is a unique multi-subunit receptor with both a ligand binding and a signal transduction domain (Treanor, 1996). The ligand binding domain consists of an extracellular receptor, GFRα anchored to the outer plasma membrane by glycosylphosphatidylinositol (GPI), putting the receptors in a special lipid raft microenvironment (Treanor, 1996; Paratcha, 2008). Four GFRα receptors have been identified, GFRα1-4. GDNF binds preferentially to GFRα1, neurturin to GFRα2, artemin
to GFRα₃, and persephin to GFRα₄ (Figure 4). However, there appears to be some cross activation of the GFRα receptors between GDNF, neurturin, and artemin (Treanor, 1996).

Wang et al. (2000) examined the biological significance of GFRα receptors cross activation by creating GFRα₁ knockout mice, and they found that GFRα₁ is an essential mediator of GDNF’s ability to promote dopamine neuron survival. GDNF binding to GFRα₁ occurs at the cysteine rich central region of the receptor, on domains 2 and 3 (residues 145-348), and it initiates signal transduction (Leppänen, 2004). Alanine scanning mutagenesis studies have identified the following critical amino acid residues

The tyrosine kinase, Ret (Rearranged during Transfection), acts as the signal transduction domain that is activated upon GFRα stimulation. Trupp et al. (1996) showed that c-ret mRNA is highly expressed in SN dopamine neurons, and that GDNF can protect Ret-positive neurons in the SN from destruction by 6-OHDA. This suggests that GDNF’s neuroprotective effects on SN dopamine neurons are mediated by Ret receptor activation.

Further research into GDNF signaling revealed that there is great complexity in the mechanisms involved with signal transduction. One of the major issues found early on was that there were several regions of the brain where GFRα was expressed and Ret was not. This led to the proposed models of “Ret-dependent” and “Ret-independent” signaling of GDNF binding GFRα₁ (Saarma, 2003). Studies of normal Ret-dependent signal transduction suggest that GDNF stimulates the formation of GFRα₁ homodimers, which then bind and dimerize two Ret receptors (Sariola & Saarma, 2003). In a more recent view on GDNF pharmacodynamics (Figure 5), Ret and GFRα₁ are shown to exist in a dynamic equilibrium, switching between monomers and homodimers with themselves as well as forming both active and inactive heterodimers and heterotetramers with each other. GDNF binding shifts the equilibrium towards the active heterotetramer state, where an active Ret homodimer is bound to an active GFRα₁ homodimer, causing the tyrosine kinase domain to auto-phosphorylate and initiate its downstream signaling cascade (Bespalov, 2007).
The RAS/MAP kinase pathway and phosphatidylinositol 3-kinase (PI3K) pathways are the two main pathways that become activated following GDNF binding. Ultimately, the transcription factor cAMP response element binding protein (CREB) becomes phosphorylated leading to increased expression of another transcription factor, c-fos, which ultimately increases expression and synthesis of cell survival and growth proteins (Sariola & Saarma, 2003). In particular, this signaling cascade is found to increase both TH and GFRα1 expression levels in SN neurons, leading to GDNF-elicited neurotrophic benefits (Pruett, 2010). Additional evidence supporting the roles of MAPK and PI3K as mediators of GDNF neurotrophic activity comes from in vitro studies of dopamine neurons, where GDNF increases cell survival in dopamine neurons treated with

*Figure #5: Dynamic equilibrium models for GDNF, GFRα1 and Ret. GFRα1 and Ret exist in a dynamic equilibrium with each other, where multiple conformations of homodimers and heterodimers are possible. It is thought that GDNF shifts this equilibrium towards the active heterotrimer state, where two homodimers of Ret and two homodimers of GFRα1 combine to interact with GDNF (which itself is normally found as a homodimer) (Bespalov, 2007).*
6-OHDA. However, this neuroprotective effect is blocked by adding either MAPK or PI3K inhibitors (Ugarte, 2003).

Newer research into GDNF’s function has focused on the transcription factor Pitx3, which has been shown to be crucial to GDNF’s effects, especially in its selectivity towards promoting A9 dopamine neuron survival (Peng, 2011). GDNF was found to induce transcription of Pitx3, which in turn activated the expression of BDNF. In midbrain dopamine cell cultures derived from Pitx3 knockout mice, only treatment with BDNF (and not GDNF) was able to protect dopamine neurons after a 6-OHDA insult. Intrastriatal delivery of GDNF in adult rats also increased Pitx3 and BDNF expression in the SNc. Together, these results show the region-specific feed-forward effects of GDNF on BDNF production through the necessary expression of Pitx3, and may point to a more targeted treatment option for PD using BDNF or Pitx3 activators (Peng, 2011).

Pitx3 and CREB have also been known for their crucial role in dopamine neuron development, specifically through activation of another transcription factor, the nuclear receptor Nurr1 (Jacobs, 2009). Nurr1 expression and activation has been shown to increase levels of Ret, TH, DAT and other dopaminergic proteins, leading to examination of its role in PD (Jacobs, 2009). Work by Decressac et al. (2012) revealed that exogenously applied GDNF in rats increased nigral Nurr1 expression as expected, however these effects were blocked by overexpression of α-synuclein which lead to downregulation of Nurr1. This downregulation of Nurr1 made dopamine neurons heavily susceptible to α-synuclein's damaging effects, primarily by decreasing expression of the Ret receptor, which mediates GDNF's neuroprotective actions (Decressac, 2012). An interesting observation was also made in that retrograde transport of GDNF from the
striatum to the SN was also dramatically lowered by α-synuclein overexpression. Nurr1 overexpression was able to attenuate this effect of α-synuclein and was also able to protect nigral neurons without additional GDNF, implying Nurr1 may be another viable target for PD therapeutics by restoring the ability of neurons to be affected by endogenous GDNF (Decressac, 2012).

In addition to the canonical models of GDNF signal transduction via Ret, GDNF has also been shown to signal in a “Ret-independent” manner. Using a neuronal cell line expressing only GFRα receptors, Trupp et al. (1999) showed that GDNF could activate Src kinases, phosphorylate CREB, and upregulate the expression of c-fos mRNA in the absence of Ret. However, high affinity binding of GDNF to GFRα₁ has only been demonstrated when Ret and GFRα₁ are co-expressed (Vieira, 2003).

Studies on additional GFL receptors lead to an alternative signaling method for those neurons that lack a combined GFRα and Ret phenotype. These studies uncovered neural cell adhesion molecule (NCAM) as an alternative to Ret for GFRα co-receptor activation. NCAM signaling normally occurs by short-range interaction with other NCAMs on neighboring cells, and it is thought that GFRα acts as an inhibitor of this signaling. Paratcha et al. (2003) have determined that GDNF can mediate long-range NCAM signaling, with GFRα₁ allowing for NCAM’s downstream activation of two different cytoplasmic tyrosine kinases, Fyn and FAK (which activate the MAPK pathway). Later research found that GDNF-GFRα-NCAM signaling is crucial to migration of neuronal precursors in the rostral migratory stream (RMS) (Paratcha, 2008). For a summary of GDNF binding and the receptors and cell types involved, see Figure 6.
While it is clear is that GDNF’s pleiotropic effects are not limited to Ret
activation, its neurotrophic effects are completely dependent upon GDNF’s ligand-
receptor interaction with GFRα1 and requires TGF-β as a cofactor (except in motor
neurons) (Krieglstein, 1998). Application of neutralizing antibodies that inactivate TGF-
β completely inhibits GDNF’s neurotrophic effects. In addition, TGF-β has been shown
to increase neuronal responsiveness to GDNF by initiating GFRα1 translocation to the cell
membrane, and not by upregulation of GDNF or GFRα1 receptor mRNA expression as
one may expect (Sariola & Saarma, 2003).

<table>
<thead>
<tr>
<th>Biological functions</th>
<th>Cell type</th>
<th>Receptor system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroblast Proliferation</td>
<td>Enteric progenitors</td>
<td>Ret/GFRαx</td>
</tr>
<tr>
<td>Migration/chemoattraction</td>
<td>Enteric progenitors, MGE-derived progenitors, RMS-derived progenitors</td>
<td>Ret/GFRαx, 7/GFRαx, NCAM/GFRαx</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Enteric progenitors, MGE-derived progenitors</td>
<td>Ret/GFRαx</td>
</tr>
<tr>
<td>Neuron Axonal growth</td>
<td>Sensory and sympathetic neurons, Motor neurons, Hippocampal neurons, Cortical GABAergic neurons</td>
<td>Ret/GFRαx*, Ret/GFRαx, NCAM/GFRαx</td>
</tr>
<tr>
<td>Neuron Axon guidance</td>
<td>Spinal chord motor neurons</td>
<td>Ret/GFRαx*, Ret/GFRαx</td>
</tr>
<tr>
<td>Survival</td>
<td>VM dopaminergic neurons, Spinal chord motor neurons, Sympathetic, parasympathetic and sensory neurons, Enteric neurons</td>
<td>Ret/GFRαx</td>
</tr>
<tr>
<td>Synapse formation</td>
<td>Midbrain dopaminergic neurons, Motor neurons, Hippocampal and cortical neurons</td>
<td>Ret/GFRαx, NCAM/GFRαx</td>
</tr>
</tbody>
</table>

*Figure #6: GDNF’s actions on particular cell types and the receptors involved. (Paratcha, 2008)*

I. Anatomical Distribution of GDNF and its Receptors

*In situ* hybridization (ISH) has been used to determine the anatomical cellular
location of GFRα1 receptor mRNA, Ret mRNA, and GDNF mRNA. As shown in
Table 1, Trupp et al. (1997) have conducted extensive ISH studies showing a wide
expression range of GDNF and its receptors throughout the adult rat CNS. Concerning the A9 nigrostriatal tract, GDNF mRNA was found in low levels in the striatum (caudate-putamen) but not in the SN, whereas GFRα1 and Ret mRNA were heavily expressed in the SNc but not in the striatum. A second ISH study in adult mice (Golden, 1998) found low GDNF mRNA expression in both striatum and SN, along with heavy expression of GFRα1 and Ret mRNA. Thus, although GDNF was initially discovered as a glial cell-derived factor from rat embryonic midbrain cultures (Lin, 1993), its expression is downregulated in adulthood. Moreover, postnatal production of GDNF in the striatum is generally confined to neurons (Pascual, 2008). Recent work by Hidalgo-Figueroa et al. (2012) used GDNF-LacZ mice that allowed for sensitive X-gal immunohistochemistry (IHC) to detect locations of GDNF expression. They discovered that GDNF is mainly expressed, both normally and after injury, by a distinct set of parvalbumin (PV)-positive GABAergic interneurons. These neurons represent only a small fraction of the striatal neurons (0.7%), but more than 95% of GDNF-expressing cells (Hidalgo-Figueroa, 2012).

<table>
<thead>
<tr>
<th>Brain structure</th>
<th>GDNF</th>
<th>GDNFR-α</th>
<th>RET</th>
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<tbody>
<tr>
<td>Olfactory system</td>
<td>++</td>
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<td>+</td>
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<tr>
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<tr>
<td>External plexiform layer of olfactory bulb</td>
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<tr>
<td>Olfactory nerve layer</td>
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<td>Ventroposterolateral/medial nucleus</td>
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<td>Anteromedial and dorsal thal nuclei</td>
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<td>Anteroventral thalamic nuclei</td>
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<tr>
<td></td>
<td>Lateral habenular nucleus</td>
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<td>Medial habenular nucleus</td>
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<tr>
<td></td>
<td>Mesencephalon</td>
<td>Substantia nigra compacta and scattered cells of SN reticulata</td>
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<td></td>
<td>Ventral tegmental area</td>
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<td></td>
<td>Interpeduncular nucleus</td>
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<td>Supramammillary nucleus</td>
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<td>Red nucleus</td>
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<td>Dorsal raphe nucleus</td>
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<td>Cerebellum</td>
<td>Granular layer (not granule cells)</td>
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<td>Purkinje layer (not Purkinje cells)</td>
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<td></td>
<td>Molecular layer</td>
<td>−</td>
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<td>Deep cerebellar nuclei</td>
<td>+</td>
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Table #1: GDNF, GFRα1, and Ret mRNA expression locations in the rat brain. (Trupp, 1997)
IHC has been notoriously difficult in revealing the physical location of mature GDNF and its receptors, due to the low-level expression in the adult brain and the various isoforms of GDNF that can be present (Lonka-Nevalaita, 2010). In the adult brain, it is generally believed that GDNF is primarily produced and secreted in the striatum and retrogradely transported to the SN via interaction with GFRα1 and Ret (Tomac, 1995b; Coulpier, 2004). In addition to striatum, weak GDNF immunoreactivity was found in the SN, olfactory neurons, olfactory bulb, spinal trigeminal, cuneate, solitary, vestibular and cochlear nuclei (Buckland & Cunningham, 1999; Del, 2002). GDNF was also found in the vagus nerve, ventral grey column, hypoglossal nucleus, medullary reticular formation, pontine grey, pontine tegmentum, locus coeruleus, tectal plate, trochlear nucleus, raphe nuclei, linear nuclei, and cuneiform nucleus (Del, 2002).

Other studies show faint to heavy expression of the GFRα1 receptor mRNA and protein throughout the brain in the cerebellum, olfactory bulb, diagonal band, oculomotor nucleus, trochlear nuclei, substantia innominata, zona incerta, thalamus, cerebellar cortex, cranial nerves, spinal motor neurons, medial forebrain bundle, and SN (Trupp, 1997; Golden, 1998; Buckland & Cunningham, 1999; Kawamoto, 2000; Matsuo, 2000). GFRα1 exists as a soluble intracellular form as well, so GFRα1-IHC may reveal a diverse spread of receptor locations, especially in long neuronal tracts (Pruett, 2013). IHC also reveals wide expression of GFRα1 in various peripheral tissues and glands. For instance, the kidneys, testes, developing teeth, tongue papillae, and the gastrointestinal tract ganglia are all found to have GFRα1, and these receptors are responsible for GDNF’s peripheral effects on these tissues (Nosrat, 1997). They may also present issues of side-effects from GDNF delivered systemically.
Recent studies show that excessive GDNF signaling activates the sorting receptor SorLA to initiate endocytosis of the GFRα1-GDNF complex, allowing for recycling of GFRα1 and degradation of GDNF via the lysosome (Glerup, 2013) (Figure 7). Along those lines, a SorLA knock-out mouse had elevated levels of GDNF in the striatum, making the protein a key regulator of GDNF signaling and potential target for PD.

*Figure 7: The sorting receptor SorLA is involved with the recycling of GDNF’s receptors and termination of the GDNF signal.* The SorLA/GFRα1 complex targets GDNF for lysosomal degradation, while GFRα1 is recycled via the trans-Golgi network (TGN). SorLA/GFRα1 targets RET for endocytosis and influences GDNF-induced signaling (Glerup, 2013).

**J. Experimental Evidence of GDNF’s Neurotrophic Effects**

As previously stated, GDNF has great therapeutic potential in the treatment of PD due to its selective neurotrophic and neuroregenerative functions on dopaminergic neurons. However, the mechanism by which GDNF exerts its neuroprotective effects on dopamine neurons is not yet fully understood. Microdialysis studies performed on the rat hippocampus demonstrated that GDNF significantly reduces free radical production, and
that it increases the activities of glutathione peroxidase and superoxide dismutase (free radical scavengers) following kainate-induced excitotoxicity (Cheng, 2004). In addition, studies have suggested that the already low levels of GDNF in the adult SN are decreased by as much as 19.4% per neuron in patients with PD (Chauhan, 2001; Hurelbrink, 2004). This suggests that supplementation of GDNF may restore neurotrophic antioxidant properties to the remaining dopamine neurons, as well as decrease free radical production and ultimately help to arrest the neurodegeneration associated with PD.

*In vitro* studies using midbrain dopamine neurons have demonstrated that GDNF is a potent neurotrophic factor with an EC$_{50}$ of only 1 pM (or 40 pg/mL) (Lin, 1994). This suggests that only small quantities of GDNF may be required to reach target areas in the basal ganglia (striatum and SN) in order to produce a therapeutic effect for PD. Other *in vitro* studies using recombinant human GDNF (rhGDNF), have demonstrated that GDNF selectively increases the survival of dopamine neurons in culture by 2.7 ± 0.5 times when compared to control, and it causes a 2.5-3 fold increase in dopamine uptake per TH-positive neuron (Lin, 1993). In addition, rhGDNF has been shown to increase dopamine cell body size and synaptic terminals, as well as cause sprouting of their dendritic processes *in vitro* (Lin, 1993; Bourque and Trudeau, 2000). Akerud et al. (1999) confirmed that GDNF improves neuron survival, increases soma size, and induces neuronal sprouting in cultures of midbrain dopamine neurons. These authors were also the first to show that GDNF was significantly effective at protecting dopamine neurons from 6-OHDA’s oxidative damage in an *in vivo* rat model of PD. By implanting GDNF secreting fibroblasts into the SNc, GDNF not only protected SN dopamine neurons from the neurotoxic effects of 6-OHDA, but it also induced an increase in their cell size and
generated dendritic sprouting. In contrast, dopamine neuronal cultures lacking GDNF have been demonstrated to die via a non-mitochondrial, death receptor-dependent pathway, suggesting the need for constant GDNF stimulation (Yu, 2008).

It is important to know whether GDNF can stimulate adult dopamine neurogenesis, or if A9 dopaminergic neurogenesis is even possible. The generation of new neurons from neuronal precursor cells could theoretically replace the lost dopamine neurons in those suffering with PD if neurogenesis were sufficient to offset cell death. Experimental evidence suggests that GDNF does increase cell proliferation in the adult rat SN by 52%, but the new cells formed were glial cells, not neurons (Chen, 2005). Although it seems no new dopamine neurons can be generated, an increase of glial support may help to save those dopamine neurons still present from further damage. With the promise of stem cells replacing the dopamine neurons lost to PD, neurotrophic factors may play an important role in stimulating their maturation and differentiation into functional dopaminergic neurons (Trzaska, 2009). In fact, it is known that GDNF is crucial to the survival of adult catecholaminergic neurons. The Pascual group (2008) confirmed GDNF’s “absolute” requirement using conditional GDNF knockout mice that showed near complete ablation of the locus coeruleus, SN and VTA when GDNF production was abolished.

Since GDNF does not stimulate dopamine cell neurogenesis, future therapies using GDNF must act before the dopamine cell death has reached levels that are symptomatic (>50-60%). Advances in imaging technology now allow for pre-symptomatic detection of PD, making neuroprotective treatment of early PD possible. The same principles also apply when studying PD animal models. GDNF treatments
should ideally show significant efficacy both if administered prior to the generation of a 6-OHDA lesion (neuroprotection) as well as rescue damaged neurons after the lesion has stabilized (neuroregeneration). Ding et al. (2004) exposed cultured SN dopamine neurons to varying concentrations of 6-OHDA in the absence or presence of GDNF and found that GDNF was only neuroprotective when applied at the earlier time points following a 6-OHDA exposure. In addition, Kearns et al. (1997) conducted an in vivo rat time course study where they found that maximal protection of SN TH-positive neurons from an intranigral 6-OHDA lesion occurred when GDNF was administered 6 hours prior to the lesion, and little benefit was shown when given 1 hour prior or concurrently when compared to lesioned control animals. Furthermore, it appeared that GDNF’s neuroprotective effect was dependent upon protein synthesis, because pretreatment with cycloheximide (an inhibitor of protein synthesis) resulted in decreased numbers of SN TH-positive neurons (Kearns, 1997).

The above-mentioned studies regarding time-sensitivity aren’t definitive, however, as Hoffer et al. (1994) showed that intranigral administration of GDNF four weeks after a medial forebrain bundle 6-OHDA lesion in rats still decreased apomorphine-induced rotations and increased dopamine levels in the ipsilateral SN. These results indicate a neuroprotective or regenerative effect of GDNF when given even weeks after the lesion. Aoi et al. (2000) also demonstrated that intrastriatal GDNF injections increased dopamine fiber density and TH-positive cell numbers even when administered four weeks after a 6-OHDA partial lesion in rats, although most of the benefit was found to be due to its retrograde transport to the SN dopamine cell bodies. Furthermore, Tomac et al. (1995a) showed that intranigral GDNF significantly increased
dopamine levels when administered 1 week after systemic MPTP in a mouse model of PD. Taken together, these studies indicate that GDNF may in fact protect dopamine neurons from neurotoxin-induced damage when administered hours before, concurrently, or even weeks after administration of the toxin. But they also show how the location of the lesion, the site of GDNF administration, and the concentration of both the neurotoxin and GDNF play important roles beyond the time course of GDNF dosing. The evidence to date suggests that GDNF exerts both a neuroprotective effect when given before the lesion, as well as a neuroregenerative effect to save damaged neurons if given after the lesion.

Research into long-term effects of GDNF overexpression has also been done, particularly by the Kirik group. Using healthy rats, they have shown that at 13 months post-injection of a lentiviral GDNF expression vector (that allows constant production of GDNF) into the striatum, GDNF levels in the SN and striatum increase by 25 and 100 fold, respectively. This effect substantially reduced TH mRNA by 72% in the SN (39% in the VTA) and caused TH-positive striatal innervation to decrease from 25-52% (Rosenblad, 2003). No change was found in dopamine and dopamine receptor levels at 13 months, leading the authors to conclude that GDNF might increase the capabilities of neurons to store, release, and turnover dopamine, allowing the cell to downregulate TH levels as a compensatory mechanism. Later work by Georgievsk et al. (2004) confirmed that this compensatory mechanism leading to a decrease in TH starts after 6 weeks of continuous lentiviral GDNF expression (reaching levels of 2-4 ng/mg tissue), and it was needed to maintain dopamine levels in the normal range. On the other hand, similar experiments have shown the opposite when studied in non-human primates, i.e.
that TH levels actually increase as an effect of prolonged overexpression of GDNF (Eslamboli, 2005). Why the differences in primate and rat models exist remains in question, but it does support the possibility of using GDNF as a successful treatment in humans. The Kirik group also sought to find whether there was an optimal level of GDNF expression that could protect neurons from an intrastriatal 6-OHDA lesion without affecting normal dopamine neuron function. They showed that a unilateral intrastriatal injection of an adeno-associated viral (AAV) vector overexpressing GDNF in marmosets at a level of 0.04 ng/mg tissue (only three-fold over baseline GDNF levels) did not affect TH or dopamine levels but did provide ~85% protection of the nigrostriatal neurons following a 6-OHDA lesion (Eslamboli, 2005). Taken together, these results suggest that modest increases in GDNF in the SN may be neuroprotective and restorative of dopamine cells, but extreme increases may be counterproductive.

The extensive pre-clinical data summarized above prompted investigators to begin considering GDNF use in human subjects. The results of the first multicenter, double-blind, placebo-controlled study involving GDNF administration were published in 2003 by Nutt et al. The treatment group received intracerebroventricular (ICV) GDNF doses ranging from 25-4000 µg at monthly intervals for 8 months. Disappointingly, all GDNF doses failed to improve PD symptoms as rated by the Unified Parkinson’s disease rating scale (UPDRS). However, it was later found that the ICV delivery method did not allow GDNF to penetrate brain tissue to reach the nigrostriatal dopamine neurons. As a result, doctors in England devised a catheter for continuous intrastriatal GDNF delivery in humans, and a second clinical study was initiated. Patients received an average of 14.4 µg of GDNF/striatum/day for the first 18 months of the trial, and then the GDNF dose
was doubled in 4 patients for the remaining 6 months of the study (Patel, 2004). In contrast to the previous ICV infusions, intrastriatal administration of GDNF decreased UPDRS motor scores by 39% during drug free periods (lower scores correspond to better PD symptom control), improved “activities of daily living” scores by 61%, and decreased dyskinesias by 64% (Gill, 2003). Furthermore, $^{18}$F-dopamine uptake in the striatum, a quantitative measure of dopamine terminal density, was shown to increase by as much as 28% after 18 months of treatment. Patients enrolled in this clinical study continued to receive GDNF for an additional 2 years, and were reported to have a decrease in UPDRS motor scores of 57%, and an improvement in their “activities of daily living” of 63% (Patel, 2005). In addition, no severe adverse effects were reported.

The limited numbers of patients enrolled, and the fact that this clinical trial had been an open-label study (i.e. patients knew that they were receiving GDNF) undermined the significance of this study. The objectivity of PD clinical trials has always been affected by the “placebo effect”. The mere knowledge that a treatment is being administered is enough to induce symptomatic relief in PD patients. In fact, de la Fuente-Fernandez et al. (2001) demonstrated that placebo can elicit a significant increase in striatal dopamine release in PD patients. As a result, Amgen conducted the first multicenter, double-blind, placebo-controlled trial involving intrastriatal administration of recombinant human GDNF (Liatemr®). Thirty-four patients were randomized to receive either GDNF (15 µg/striatum/day), or placebo (Lang, 2006). Unexpectedly, GDNF treatment was found not to be significantly different from placebo in measuring UPDRS scores. Furthermore, nine patients experienced “device related serious adverse events,” two patients required catheter repositioning, one patient required catheter
removal, and one patient suffered a hemorrhagic stroke. These findings, together with the results of a toxicology study showing the presence of anti-GDNF antibodies in plasma, and that 4 out of 15 monkeys (administered 100 µg of GDNF/day) experienced Purkinje cell loss, prompted Amgen to abruptly discontinue its GDNF clinical trial in February 2005 (Lang, 2006).

It is now thought that the failure of the Amgen trial was due to the participants’ greater severity of PD than in the British study, perhaps to a level that is beyond therapeutic intervention (Lang, 2006). Additionally, the double-blind Amgen study used a different catheter to deliver GDNF to the striatum than the open-label trial (Lang, 2006). Recent analysis has also found that the most likely explanation for the pathology and the development of antibodies was due to GDNF leakage into the cerebrospinal fluid (CSF) or blood from the cannula implanted in the striatum and/or the infusion pump implanted under the skin (Bartus, 2012). These factors aside, it is clear that removing the risk of dangerous surgical interventions for GDNF therapy would be of great clinical value, and this is a large impetus for the presented research.

K. Blood-Brain Barrier: Obstacle to CNS Drug Delivery

One of the biggest obstacles to delivering a protein therapeutic such as GDNF to the CNS for neurodegenerative conditions is the presence of the BBB, a dynamic barrier that separates the systemic circulation from the brain and CSF. It acts to impede brain penetration of pathogens and toxic substances, as well as actively transports necessary nutrients and electrolytes from the systemic circulation to the brain. The BBB is compromised of endothelial cells, pericytes, the basal lamina, and astrocyte projections
(Hawkins & Davis, 2005). In particular, brain endothelial cells possess tight junctions as well as numerous efflux pumps (i.e. P-glycoprotein) and degradative enzymes (i.e. cytochrome P450s and peptidases) which combine to make the BBB a highly-resistant and challenging obstacle for many CNS drug candidates (Cecchelli, 2007). In terms of conventional drug delivery, only small, lipophilic compounds are able to penetrate the BBB. Small, polar molecules that are essential for brain functioning such as amino acids, glucose and water are shuttled in by their respective transporters found in the endothelial cells. Larger molecules, such as proteins, are limited to receptor-mediated endocytosis, which allows permeation of certain proteins (such as insulin) that have a particular receptor on the BBB (Hawkins & Davis, 2005).

Bypassing the BBB is a unique challenge. Methods such as BBB disruption with hyperosmotic agents as well as using chimeras that allow for BBB receptor-mediated endocytosis comprise some of the pioneering work in this area. The focus in our lab, however, is to deliver drugs to the brain using a route that begins in the nasal mucosa. Here, the absence of a BBB has led to successful attempts to transport drugs deemed normally undeliverable to the brain via the intranasal route of administration.

L. Mechanisms Involved with the Intranasal Route of Administration

It has been known for quite some time that the nasal mucosa is rich in blood vessels where direct absorption into the bloodstream can provide a rapid and minimally invasive route for drugs that have limited oral bioavailability. Currently, numerous small molecule drugs and vaccines are formulated for intranasal use (for a list see Pires, 2009). Recent advances in intranasal delivery include drug atomizers (ViaNase™) to saturate the
nasal cavity, gels that aid binding to the nasal epithelium, and nano-sized vectors for targeting and added protection of the therapeutic agent (Mistry, 2009). However, the novelty of this approach for delivery of CNS therapeutics comes from ground-breaking work done by Thorne et al. (1995) that the nasal mucosa is rich with sensory neurons that provide conduits for transport of xenobiotics into the brain, essentially bypassing the BBB. The nasal route clearly provides a much safer route than direct surgical administration.

The mechanisms by which intranasally delivered substances enter the CNS have not been fully elucidated, but it is thought to involve a combination of neuronal, vascular and lymphatic transport methods, largely dependent on the region where the delivered agent is placed within the nasal cavity and the physicochemical properties of the therapeutic being administered (Dhuria, 2010). The neuronal elements that exist in the nasal epithelial layer are essential to intranasal uptake to the brain. The olfactory nerve pathway contains olfactory receptor neurons (ORNs) which extend from the olfactory bulb into the olfactory epithelium. Here, ORN dendritic processes are exposed to the external environment, creating intracellular and extracellular pathways that lead into the olfactory bulbs of the brain and cerebrospinal fluid (CSF). In addition, the trigeminal nerves extend from the respiratory mucosa into caudal regions of the brain, allowing for a second main route by which therapeutics can travel into the CNS (Figures 8 and 9).
Drugs and biomolecules administered intranasally have two main mechanisms that utilize these neuronal connections to the brain: transcellular transport and extracellular transport (Dhuria, 2010). Passive transcellular diffusion occurs “through cells” and primarily applies to small, lipophilic molecules that are taken up by the nerve terminals of olfactory neurons. Molecules that have receptors on ORNs may also be taken up transcellularly through receptor-mediated endocytosis. Both passive diffusion and endocytosis can lead to retrograde transport of molecules along their axons, a process that can take hours or days. However, since numerous experiments show consistently rapid uptake (within 30 minutes to an hour post-administration) of intranasally-administered drugs to the CNS, it is unlikely that slow retrograde transport accounts for more than a small percentage of intranasal delivery to the brain (Dhuria, 2010).

The second route of nose-to-brain transport involves extracellular mechanisms that arise from the unique environment of the nasal mucosa. ORNs are constantly...
recycled every 3-4 weeks from basal cells due to their exposure to the outside environment. This constant regeneration is dependent on special olfactory ensheathing cells (OECs) that act similar to Schwann cells and wrap around the ORNs, providing trophic support. The OECs, along with olfactory nerve fibroblasts, form a structure known as the *fila olfactoria* which wraps around as many as 100 axons, subdivided into fascicles forming stable, fluid-filled perineuronal channels that normally allow for the passage of cells such as erythrocytes and macrophages. Since these channels surround the ORNs, they allow drugs that entered the *lamina propria* to bypass the BBB and go through the pores in the cribiform plate directly to the olfactory bulb region and the surrounding CSF (Li, 2005). Through these mechanisms, the successful delivery of drugs, proteins, nanoparticles, and even cells to the brain has been demonstrated after intranasal administration (Liu, 2001; Danielyan, 2009; Migliore, 2010; Jiang, 2012; Renner, 2012; Reitz, 2012).

As stated above, vascular and lymphatic systems are thought to also play a large role in nose-to-brain transport. Since the olfactory and respiratory mucosa are highly vascularized, small molecular weight drugs are able to be absorbed into the systemic circulation (Dhuria, 2010; Lochhead, 2012). Although this may present a large concern for side effects, newer research shows a large proportion of intranasally delivered molecules avoid systemic absorption. However, they are delivered to the CNS via perivascular spaces associated with blood vessels (Dhuria, 2010). Within the brain, these perivascular spaces act as a lymphatic system, allowing neuron-derived substances, CSF, and substances within CSF to be drained from interstitial fluid. Acting in reverse, arterial pulsations and bulk flow mechanisms provide the driving force by which substances can
be transported throughout the parenchyma, giving this distribution system the name “perivascular pump” (Hadaczek, 2006). In other words, perivascular spaces are able to act as a sort of delivery path, allowing the spread of intranasally delivered therapeutics throughout the brain via the “perivascular pump”. This mechanism works along with the perineuronal channels that surround the ORNs to allow molecules to move from the nasal mucosal surface into deep regions of the brain in a relatively short time (<1 hour).

Recent work done by Scranton et al. (2011) also suggested the importance of the rostral migratory stream (RMS) in the retrograde flow of intranasally applied therapeutics from the olfactory bulb to the rest of the brain. When the RMS was surgically ablated, intranasal administration of the fluorescent tracker, CellTracker™ Green (Invitrogen) was reduced in its delivery to selected brain areas by 80%. In addition, peripheral administration was increased as a consequence of the decreased CNS penetrability (Scranton, 2011).

Thorne and Nicholson (2006) estimated that the extracellular spaces within the brain have a maximum width of approximately 64 nm, limiting larger particles from traversing through these extracellular spaces within tissue. How this impacts transport of biomolecules to sites deep within the CNS after intranasal delivery remains unclear. Other factors to be taken into consideration are the vast differences between nasal passages between animals and humans, the extent of systemic exposure, the variable environment of the nasal passages, the risk of nasal damage or irritation, correct administration techniques, dosing, formulations, and the use of adjuvants. All of these considerations will require further research to make this route a viable one for therapeutic development (Dhuria, 2010; Pires, 2009).
In addition, there are factors which operate to oppose the delivery of molecules to the brain from the nasal cavity. Efflux transporters and degradative proteins found in the BBB are also present in the nasal epithelium, as are many metabolic Phase I and Phase II metabolic enzymes. These may present a major barrier to intranasal drugs, but they are still necessary to provide protection from inhaled toxicants (Wong, 2010). Another obstacle to nasal delivery is the nasal mucus layer. Drugs must first permeate through this constantly recycling mucus (produced by goblet cells) before reaching the nasal epithelium (Talegaonkar & Mishra, 2004).

M. **Intranasal Delivery: Proteins, Nanoparticles and Other Therapeutics**

The intranasal route of administration can potentially open many new doors for drugs that cannot normally cross the BBB. In particular, the delivery of proteins and nanoparticle formulations may provide a means of therapy for many CNS disorders,
including PD, the primary focus of our lab and this thesis project. A number of previous studies have confirmed the feasibility of the approach, reviewed briefly below.

Thorne et al. (2004) demonstrated that $^{125}$I-insulin-like growth factor I (IGF-I) can be effectively delivered to rat brains via the olfactory route of administration. Intranasal administration resulted in >100 fold higher $[^{125}]$IGF-1 counts in brain than intravenous administration, as well as widespread CNS distribution. A similar brain distribution pattern was observed in adult non-human primates one hour after intranasal administration of $^{125}$I-interferon-β (Thorne, 2008). More relevant to the current research, $^{125}$I-nerve growth factor (NGF), a well-characterized neurotrophic factor, has also been administered intranasally in a rodent model of Alzheimer’s disease (Thorne and Frey, 2001). Although transport to the brain was rapid in each case, the delivery efficiency was low with an estimate of 0.006 to 0.023% of the administered dose reaching brain (Thorne & Frey, 2001; Thorne, 2004 & 2008).

Intranasally administered NGF has also been shown to reverse the neurodegeneration that is characteristic of the AD11 mouse model of Alzheimer’s disease (Capsoni, 2002; De Rosa, 2005). Similarly, nasally administered fibroblast growth factor (FGF) was demonstrated to increase motor activity and decrease rigidity in a mouse MPTP model of PD (Kucherianu, 1999; Thorne & Frey, 2001). More recent studies showed that the neurotrophic proteins brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), EPO (erythropoietin) and neurotrophin-4 (NT-4) are all delivered reliably to the brain within minutes of intranasal delivery and exert their receptor-specific effects (Alcala-Barraza, 2010). Finally, intranasal administration of peptides and other substances has also been attempted in humans. Peptides such as
vasopressin, corticotropin releasing hormone, growth hormone releasing hormone, insulin, and oxytocin have all been demonstrated to exert CNS effects in humans when given intranasally (Thorne & Frey, 2001; Born, 2002; Guastella, 2010).

It is important to understand that intranasal administration delivers less than 1% of the total dose to the brain, with larger accumulation in the olfactory bulbs and along the trigeminal nerve path (Alcala-Barraza, 2010). There is little evidence for any correlation of molecular weight to the distribution pattern, although presence of a corresponding receptor may cause sequestration of the administered protein in particular areas (Alcala-Barraza, 2010). Thus, determining the correct dose for a neurotrophic effect is a challenge that must be overcome in order for this approach to succeed as a treatment. Moreover, since very small quantities enter brain, a prerequisite for intranasal administration is an extremely high potency and efficacy of the administered agent.

Within the past decade, there has been a surge of research into nano-sized vectors to aid drug delivery into the CNS. As mentioned before, the extracellular perineuronal channels (that are the most likely path of brain entry following intranasal delivery) have a diameter around 10-15 nm, giving nanoparticles a size limit for rapid delivery to the brain (although larger sized particles have been shown to travel via retrograde neuronal transport) (Mistry, 2009). Liposomes are one example of nanoparticles for drug delivery that have attracted significant attention because of their ability to increase delivery to the target site of drug action. Liposomes are simply vesicles made from phospholipids, which contain an inner hydrophilic core and an outer lipophilic membrane readily amenable to surface modifications. The basic concept behind liposomal drug delivery is that liposomes can be loaded with the drug of interest, either in their inner core (if
hydrophilic) or in their outer membrane (if lipophilic). The encapsulation will offer protection from plasma and tissue hydrolytic enzymes and allow the drug to reach its cellular destination. Uptake into cells is by endocytosis. One of the main benefits to liposomes is their ease of adding surface modifications for cell targeting (i.e. monoclonal antibodies), evasion from the immune system (i.e. polyethylene-glycol), and/or detection (i.e. fluorescent tags). A range of lipids can be used to generate cationic, neutral or anionic vectors. For example, a liposome having an overall cationic charge will greatly increase cell-binding as well as aid in condensation of DNA (forming lipoplexes). This may be of particular interest to intranasal delivery, as cationic liposomes are thought to bind sialic acid residues in the nasal cavity and increase residence time at the nasal mucosa. In addition, various “helper lipids” aid with destabilization of the endosome after liposomal endocytosis, ensuring that the content of the liposome successfully escapes the liposome and avoids degradation in the lysosome (Szoka & Papahadjopolous; 1980; Hafez, 2001).

Previous research in our lab showed successful intranasal delivery of the labeled protein Alexa 488-ovalbumin (Migliore, 2010) as well as GDNF to the brain (Migliore, 2009; Bender, 2009) using a liposomal preparation. The liposomes rapidly delivered larger amounts of protein to the brain than an equivalent dose in PBS solution. As these liposomal formulations had a size range around 150 nm, their transport throughout the brain challenges the view that there is a strict size cut-off for perivascular flow. Since distribution throughout the brain was evident in less than 1 hour in these intranasal studies, it can be rationalized that the protein was protected from degradation in the nasal
mucosa, but possibly released from liposomes prior to transport through the perivascular channels and extracellular spaces.

Numerous other nanoparticle formulations are currently being investigated in addition to liposomes. Of interest to the current project are polymeric nanoparticles, which are composed of biologically compatible materials (such as amino acids) to form a shell in which therapeutic agents can be loaded. Like liposomes, these nanoparticles can be utilized not only to deliver protein cargo, but can be complexed to expression plasmids and employed for non-viral gene therapy, to be discussed in the next section.

N. Gene Therapy: GDNF

As described previously, a major pitfall in GDNF protein therapy was the need for surgical implantation of mechanical hardware into brain to provide continuous delivery and dispersion for long periods of time. This approach had a poor safety profile as malfunctions could cause protein leakage with spread to off-target sites, causing major side-effects and greatly reducing the effectiveness of the treatment (Bartus, 2012). By comparison, gene therapy approaches ideally require a single directed treatment, greatly improving clinically applicability.

An effective vector for PD gene therapy should ideally be able to: 1) carry its genetic cargo to cells of interest (those cells in the striatum and SN) without degradation, 2) reach the nucleus of cells at the target sites, including non-dividing cells such as neurons (transfection), 3) produce a significant concentration of a protein of interest, and 4) have an appropriate safety profile (non-immunogenic, non-toxic, non-inflammatory, non-oncogenic). Viral vectors for gene delivery hold great promise and are the most
widely used for gene therapy, with adenoviruses, adeno-associated viruses (AAVs) and retroviruses making up the clear majority used in clinical trials (Edelstein, 2007). However, our knowledge of viral behavior and genetics is not complete, limiting our ability to fully understand and prevent fatal immune responses or oncogenic problems due to incorrect genetic insertion. As such, equally efficacious non-viral means for gene delivery are a much sought after and heavily researched goal.

As described previously, the combination of oligonucleotides or plasmid DNA with a liposome is termed a lipoplex, and lipofection currently makes up 7.6% of gene therapy clinical trials and is the most popular non-viral vector (Edelstein, 2007). The popularity of lipoplexes is due to their safety, ease of manufacturing, ability to carry a wide range of therapeutics, and adaptability to surface modifications to give them a variety of potential targeting options and other unique characteristics. However, a common problem using non-viral vectors is their low transfection capability when compared with their viral counterparts. The low transfection efficiency of non-viral vectors stems from the aforementioned barriers that they must overcome to deliver their DNA cargo to the nucleus, a feat which a virus inherently can perform by mechanisms not completely understood and hard to replicate in manufactured vectors (Thomas, 2003). In addition, the engineering of expression plasmids that can avoid the silencing and degradative mechanisms that exist once inside the cell is still an area of research that has much room for growth.

In the case of GDNF gene therapy, there have been numerous successes in animal models of PD using liposomal (Lu, 2002; Lu, 2004; Zhang, 2009) and adeno- (or adeno-associated) viral vectors (Bilang-Bleuel, 1997; Choi-Lundberg, 1997; Björklund, 2000;
Kordower, 2000; Kozlowski, 2001; Dowd, 2005; Eslamboli, 2005). The GDNF gene has been delivered to either the SN or the striatum in PD animal models and successfully reduced dopamine cell loss and motor symptoms. This research has heralded several clinical trials using direct injection of AAV viral vectors, including Ceregene’s clinical trial with CERE-120 (encoding neurturin, which acts via a mechanism similar to GDNF), into the striatum. In other examples, Oxford BioMedica administered ProSavin (encoding TH, DDC, and GTP-cyclohydrolase 1, which together produce dopamine), and Neurologix administered NLX-P101 (encoding glutamic acid decarboxylase) to produce the inhibitory neurotransmitter GABA in the STN.

The CERE-120 trial is the most relevant to the current study since it’s a neurotrophic gene therapy, and it generated many useful discoveries and benefits. Preliminary Phase 2 studies seemed to show significant lowering of UPDRS scores (Muramatsu, 2010). However, postmortem analyses of patients given the CERE-120 treatment found a surprising lack of neurturin in the SNc (Bartus, 2012). It is now thought that retrograde axonal transport from the striatum to dopamine cells in the SN is severely reduced in those with even moderate cases of PD, requiring the need for the additional targeting to the SN. As such, the CERE-120 trial was retooled for dual injection sites with optimized dosing strategies, and it is currently undergoing new Phase 1/2b studies (Bartus, 2012). The clinical trial for NLX-P101 showed a three year dramatic improvement in one patient and a 37% average improvement in the others (Edelstein, 2007). Finally, the ProSavin treatment showed a 53% maximum improvement in motor function and a 34% average improvement when compared to patients’ pre-treatment motor function (Muramatsu, 2010). Although these gene therapy
options are an incredible step forward for treating PD, they still required intracerebral surgery to deliver the gene construct. The therapeutic practicality of intracerebral injections is very questionable and may be too risky for most PD patients when compared with normal pharmacotherapies.

O. Copernicus Therapeutics’ Expression Plasmids and Nanoparticles for Gene Therapy of PD

The tremendous potential of non-viral CNS gene therapy using intranasal delivery led our group to collaborate with Copernicus Therapeutics, a company that has successfully developed compacted plasmid nanoparticles which are able to transfect brain cells both in vivo and in vitro. These particles are primarily composed of poly-lysine (30-mer), with polyethylene-glycol (PEG) substituted at the N-terminal cysteine (PEG-CK30). Cationic polymers such as poly-lysine compact negatively-charged DNA, allowing for great reductions in size when compared to naked pDNA. PEG instills the nanoparticles with increased solubility, decreased protease binding and immune recognition, and it prevents their aggregation. PEG-CK30 can be shaped into rods using acetate counterions during mixing, giving them a diameter as little as 8-11 nm, small enough to contain and deliver a singular plasmid DNA molecule (Liu, 2003; Yurek, 2009b).

Probably the most unique feature of these nanoparticles is one of the mechanisms by which they enter cells. It was found by Chen et al. (2008) that PEG-CK30 can act as a ligand to the cell-surface protein nucleolin, which shuttles the nanoparticle through the nuclear pore complex (NPC) and directly to the nucleus. Nucleolin has been shown to be
associated with glucocorticoid receptors, which can assemble with heat-shock proteins and adaptor proteins into a nuclear import complex (Chen, 2011). Nuclear transport via nucleolin seems to be dependent on microtubules, where the receptor-nanoparticle complex is rapidly transported through the action of the dynein motor complex (Chen, 2011). Together, it was found that glucocorticoid agonists such as cortisone actually aid transfer of PEG-CK30 nanoparticles to the nucleus by up to 100%, but only when administered after the nanoparticles (Figure 10). Once in the nucleus, the nanoparticles dissociate from the pDNA which allows for protein expression (the entire process occurring within 18 hours post-administration) (Liu, 2003; Chen, 2008). This mechanism of cellular entry greatly reduces the hurdles that most other nanoparticles encounter such as classical endocytosis and the eventual need to escape the endosome before reaching the degradative lysosome. Blocking nucleolin expression via siRNA reduced PEG-CK30’s nuclear entry dramatically, again reinforcing nucleolin’s role as a receptor for these nanoparticles (Chen, 2008). The normal physiological role of nucleolin on the cell surface is currently unknown, but it has been found to interact with viral particles, apoB/E lipoproteins and growth factors (Chen, 2008; Hovanessian, 2010). Most of the discoveries by Chen et al. (2008 & 2010) surrounding nucleolin’s function focused on lung epithelial cells. A newer study by Hovanessian et al. (2010) showed that surface nucleolin is induced in tumor cells both in vitro and in vivo and may actually have a major role in cell proliferation and cancer cell growth. However, the role and distribution of nucleolin in brain has not been studied. In brain, cell-surface nucleolin may or may not be involved in the mechanism of PEG-CK30 nanoparticle uptake into cells.
Copernicus has engineered numerous expression plasmids, including those for GDNF and various reporter proteins such as enhanced green fluorescent protein (eGFP) and luciferase. They have further shown that their compacted plasmid nanoparticles transfect mammalian non-dividing cells in brain, lung and retina in vivo as episomes, a great technical achievement as generally only viral vectors have been able to accomplish this feat (Liu, 2003; Ziady, 2003a; Fink, 2006; Farjo, 2006; Yurek 2009a,b; Fletcher, 2011). Table 2 summarizes the plasmids used in this thesis.

Figure #10: Mechanism for nuclear entry of PEG-CK30 nanoparticles. Copernicus’ nanoparticles (CK30-NP) interact with nucleolin at the cell surface, which is normally associated with lipid rafts. This interaction causes the complex to further associate with a dynein motor complex and glucocorticoid receptor (GCR), which shuttles the NP via microtubules through the nuclear pore complex (NPC) and into the nucleus. (Adapted from Han, 2011)
<table>
<thead>
<tr>
<th>Name</th>
<th>Promoter</th>
<th>Protein product(s)</th>
<th>Plasmid map</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCG</td>
<td>Cytomegalovirus (CMV)</td>
<td>Enhanced green fluorescent protein (eGFP)</td>
<td></td>
</tr>
<tr>
<td>pGDNF</td>
<td>Polyubiquitin C (UbC)</td>
<td>Glial cell line-derived neurotrophic factor, splice variant 1b (hGDNF1b)</td>
<td></td>
</tr>
<tr>
<td>pUGG</td>
<td>Polyubiquitin C (UbC)</td>
<td>eGFP + hGDNF1b</td>
<td></td>
</tr>
</tbody>
</table>

**Table #2: Copernicus’ engineered expression plasmids used in this project.** The pCG plasmid contains a cytomegalovirus enhancer sequence (CMV enh) and promoter region (CMV prom) to drive expression of eGFP. The pGDNF plasmid contains an E1 enhancer with a polyubiquitin C promoter region (UbC prom) to drive expression of the human GDNF splice variant 1b (hGDNF). The pUGG plasmid contains the same enhancer and promoter region as pGDNF while coding for eGFP in addition to hGDNF. A simian virus 40 late polyadenylation signal sequence (SV40 pA) allows for mRNA stability and export from nucleus. Scaffold/matrix attachment regions (S/MAR) provide proper nucleosome formation and overall stability (Jenke, 2004).

This project was dependent on the abilities of the plasmids and nanoparticles designed by Copernicus to achieve transfection in rat brain. Although proprietary technical detail was not shared by Copernicus, we were given the basic plasmid maps for each one used, detailing their major constituents. Each plasmid contains a simian virus 40 late polyadenylation signal sequence (SV40 pA), which maintains proper polyadenylation upon transcription. Also, scaffold/matrix attachment regions (S/MAR) provide the plasmid with the ability to attach to the nuclear chromatin and maintain itself through replication as an episome (Jenke, 2004).

The expression plasmid pCG expresses the fluorescent protein eGFP coupled to the cytomegalovirus (CMV) promoter. The CMV promoter is well known for high-level expression in many mammalian cell lines and species. However, it is one of the most variable in terms of the specific cell-types transfected (Qin, 2010). pGDNF expresses the full-length human GDNF splice variant-1b (hGDNF1b) that was shown to yield
maximum expression of GDNF after transfection (Wang, 2008; Fletcher, 2011). GDNF is produced under the mammalian polyubiquitin C promoter (UbC). Expression with UbC is more ubiquitous throughout many species and cell types, but is characteristically much weaker compared with CMV (Qin, 2010). However, UbC is known for its ability to resist attenuation and can maintain protein expression in vivo for greater than 6 months, giving it a clear advantage for long-term studies or in treating chronic conditions (Gill, 2001). The pUGG plasmid is a fusion construct utilizing the same plasmid backbone as pGDNF but with the gene for eGFP placed upstream of the hGDNF1b. This fusion plasmid was created by Copernicus specifically for use in this project and was thus characterized extensively.

Our first introduction to Copernicus’ plasmids came from Dr. David Yurek’s lab’s work with pGDNF for PD. His studies in rats analyzed the effects of intrastriatally injected pGDNF NPs, which provided prolonged overexpression of GDNF (around fivefold over control levels) 1-3 weeks after injection, with minimal evidence of any inflammatory or toxic effects (Yurek, 2009b). Later studies have shown that intrastriatal injection of pGDNF can protect nigrostriatal cells in the 6-OHDA rat model of PD as well as increase survival of fetal dopamine neuron grafts implanted at the injection sites (Yurek, 2009a). Showcasing the long-lasting expression of the UbC promoter, striatal injection of pGDNF NPs resulted in prolonged GDNF expression (>300% of baseline) 6 months after a single injection (Fletcher, 2011). Dr. Yurek and his lab worked with us through characterization of the pUGG plasmid and his experiments helped to guide the design of many studies throughout my work.
In summary, Copernicus’ compacted pGDNF plasmid nanoparticles are an effective gene therapy vector with a proven ability to transfec brain cells and express protein for long periods of time. They have already been shown to be therapeutic in animal models of PD, making them an ideal gene therapy vector for use in these intranasal studies.
II. SPECIFIC AIMS

SPECIFIC AIM 1: To test the feasibility of the intranasal route as a means of delivering plasmid DNA nanoparticles to the rat brain.

The goal of Specific Aim 1 was to compare transfection of cells in brain after intranasal delivery of a reporter plasmid encoding enhanced green fluorescent protein (eGFP). Originally, the plasmid, pCG, was to be supplied in Copernicus’ PEG-substituted polylysine vectors or formulated at Northeastern in lipoplexes. Lipoplex formulations of Copernicus’ reporter plasmids were to be designed in consultation with Dr. Amiji. The two formulations were to be tested *in vitro* using a SHEP-1 neuroblastoma cell line for transfection efficiency. The superior liposomal vector *in vitro* was to be carried forward to *in vivo* intranasal delivery experiments.

The next studies of this Aim were to examine the time course of protein expression in rat brain following intranasal administration and to assess the distribution of cells in brain that express the reporter protein. For the distribution study, rats were to be sacrificed at a single time point, i.e. the time of peak expression. The location of reporter protein-positive cells was to be mapped using Bioquant® image analysis software in sections taken along the rostral-caudal axis of the brain. This study would reveal whether transfection and expression reach the striatum and SN, the target brain regions for PD gene therapy. The results would establish proof of principle that intranasal delivery could lead to transfection of cells in brain, and they would guide studies of Specific Aim 2, which was to utilize the GDNF-expressing plasmid, pGDNF.

However, in consultation with the thesis committee, a decision was made to delay moving forward to work with pGDNF. Instead, it was decided to examine the expression...
profile of pGDNF using a GFP reporter. This required engineering a new plasmid, pUGG, which expresses the reporter protein eGFP and GDNF as a fusion construct. This plasmid had the advantage of being identical to the pGDNF construct, with the same promoter as the one to be used in efficacy studies of Specific Aim 3. The fusion plasmid was to be characterized in similar in vitro and in vivo experiments to determine its protein expression and suitability in future Aims.

SPECIFIC AIM 2: To evaluate the transfection of rat brain cells using intranasal pGDNF-encoding nanoparticles.

Specific Aim 2 was to follow a sequence similar to that of the first Aim, with the goal of demonstrating transfection of cells in rat brain after intranasal delivery of nanoparticles containing the GDNF plasmid supplied by Copernicus. Using the newly available pUGG, the studies of this Aim examined transfection both in vitro and in vivo. The in vitro studies were performed in three cell culture systems. The in vivo studies involved direct injection of pUGG in rat brain, as well as intranasal administration. Maximal expression time-points were to be determined by evaluation of reporter protein expression. Transfection and expression were assessed by GFP-ELISA, immunohistochemistry and fluorescence microscopy.

SPECIFIC AIM 3: To test the efficacy of pGDNF-encoding nanoparticles in the 6-OHDA model of PD.

This Aim was focused on testing the neuroprotective efficacy of nasal administration of the pGDNF construct in the 6-OHDA rat model of PD. pGDNF, rather
than pUGG, was used to avoid any confounding effects on bioactivity due to the GFP fusion. 6-OHDA was surgically injected into the rat brain on one side to cause a partial unilateral lesion of the A9 dopamine neurons. Rats were to be pretreated intranasally with naked pGDNF or the pGDNF-nanoparticle formulation at the optimal time determined by the previous study. The 6-OHDA lesion would be made on the day of peak expression of GDNF to insure that GDNF levels were maximal when the neurotoxin was present in brain and actively damaging dopamine neurons. An increased survival of dopamine neurons in rats given intranasal pGDNF nanoparticles would be evidence that the gene therapy approach was successful.

**SPECIFIC AIM 4: To evaluate the toxicity of pGDNF nanoparticles on the nasal mucosa.**

This Aim was originally put forward as an optional study intended to test the potential nasal toxicity of the nanoparticle formulation before taking the approach forward to clinical application. The goal was to measure a range of inflammatory cytokines and other recognized apoptotic markers in nasal lavage fluid. However, this Aim was abandoned after Copernicus indicated they had already performed many of these studies with their compacted plasmid nanoparticles.
III. MATERIALS AND METHODS

Materials used:

- 6-hydroxydopamine hydrobromide: Sigma Aldrich Chemical Company, St. Louis, MO.
- ABC Vectastain elite kit (PK-6100): Vector Laboratories, Burlingame, CA.
- Antifoam Y-30 Emulsion (#A6457): Sigma Aldrich Chemical Company, St. Louis, MO.
- Ascorbic Acid: Sigma Aldrich Chemical Company, St. Louis, MO.
- BCA™ protein assay kit (23225): Pierce (Thermo Scientific), Rockford, IL.
- Biotinylated anti-rabbit secondary IgG antibody (BA-1000): Vector Laboratories, Burlingame, CA.
- Biotek ELx800 microplate reader: Biotek, Wisnooski, VT.
- Bioquant® Nova image analysis software version 6.90.1: Nashville, TN.
- Bovine serum albumin (BSA) Fraction V (A3059-10G): Sigma Aldrich Chemical Company, St. Louis, MO.
- Buprenex®: Reckitt Benckiser Pharmaceuticals, Richmond, VA.
- Chloroform: Sigma Aldrich Chemical Company, St. Louis, MO.
- Cholesterol: Avanti Polar Lipids, Alabaster, AL.
- Cyclone Phosphorimaging System: Perkin-Elmer, Boston, MA.
- Desipramine hydrochloride: Sigma Aldrich Chemical Company, St. Louis, MO.
- Dioleoylphosphatidylcholine (DOPC): Avanti Polar Lipids, Alabaster, AL.
- Dithiothreitol (DTT): Sigma Aldrich Chemical Company, St. Louis, MO.
• Donkey Serum (#D9663): Sigma Aldrich Chemical Company, St. Louis, MO.
• Dulbecco’s modified Eagle’s medium (DMEM): Invitrogen, Carlsbad, CA.
• ECL™ Western Blotting Analysis System (#RPN2108) containing anti-rabbit (#NIF824) and anti-mouse (#NIF825) secondary antibodies conjugated to HRP: GE Healthcare UK, Buckinghamshire, UK.
• Ethylene glycol: Sigma Aldrich Chemical Company, St. Louis, MO.
• Fetal bovine serum (FBS): Invitrogen, Carlsbad, CA.
• Fluoromount-G: Electron Microscopy Sciences, Hatfield, PA.
• Fugene-HD: Promega, Madison, WI.
• GDNF ELISA DuoSet® (#DY212): R&D Systems, Minneapolis, MN.
• Gen5® software: Biotek, Winooski, VT.
• Glycerol: Sigma Aldrich Chemical Company, St. Louis, MO.
• Graph Pad Prism® statistical analysis software version 4.03: San Diego, CA.
• Heater VL-20F: Fintronics, Orange, CT.
• Heparin: Baxter Health Care Corporation, Deerfield, IL.
• HEPES: Sigma Aldrich Chemical Company, St. Louis, MO.
• Hydrogen peroxide (30%): Sigma Aldrich Chemical Company, St. Louis, MO.
• Igepal CA-630: Sigma Aldrich Chemical Company, St. Louis, MO.
• Isoflurane (IsoFlo®): Abbott Animal Health, Abbott Park, IL.
• Ketamine: Fort Dodge Animal Health (Wyeth), Madison, NJ.
• Lane Marker Sample Buffer (Loading Buffer, #39000): Pierce (Thermo Scientific), Rockford, IL.
• Lipofectamine-LTX with PLUS reagent: Invitrogen, Carlsbad, CA.

• Mouse anti-GFP capture antibody (#G-6539): Sigma Aldrich Chemical Company, St. Louis, MO.

• Nano SPECT/CT with InVivoScope software suite: Bioscan, Washington, DC.

• Neg-50 frozen section medium: Richard Allen Scientific, Kalamazoo, MI.

• Normal goat serum (S-1000): Vector Laboratories, Burlingame, CA.

• Non-fat dry milk (generic)

• OPTI-MEM reduced serum media: Invitrogen, Carlsbad, CA.

• Paraformaldehyde: Sigma Aldrich Chemical Company, St. Louis, MO.

• PE20 tubing: Braintree Scientific, Braintree, MA.

• Peroxidase substrate kit DAB (#SK-4100): Vector Laboratories, Burlingame, CA.

• Phosphate buffered saline (PBS): Fisher Scientific, Fair Lawn, NJ.

• Polyvinylpyrrolidone (PVP): Sigma Aldrich Chemical Company, St. Louis, MO.


• Precise Tris-HEPES 4-20% gradient polyacrylamide gel (#25224): Pierce (Thermo Scientific), Rockford, IL.

• Protease Inhibitor Tablets (#88660SPCL): Pierce (Thermo Scientific), Rockford, IL.

• Rabbit anti-green fluorescent protein (#Ab290): Abcam, Cambridge, MA.

• Rabbit anti-tyrosine hydroxylase polyclonal antibody (#AB152): Chemicon, Temecula, CA.

• Sodium chloride 0.9% preservative free: Hospira Inc., Lake Forest, IL.
- Sodium dodecyl sulfate (SDS): Sigma Aldrich Chemical Company, St. Louis, MO.
- Sodium hydroxide: Sigma Aldrich Chemical Company, St. Louis, MO.
- Sonicator, water bath type: Laboratory Supplies Co. Inc., Hicksville, NY.
- Sprague-Dawley male rats: Taconic, Germantown, NY.
- Stainless steel surgical blades: Miltex, York, PA.
- Stearylamine: Avanti Polar Lipids, Alabaster, AL.
- Streptavidin-HRP (#890803): R&D Systems, Minneapolis, MN.
- SureBlue™ TMB microwell peroxidase substrate (#52-00-01): KPL, Gaithersburg, MD.
- Sucrose: Sigma Aldrich Chemical Company, St. Louis, MO.
- Syringe filters, 0.2 μm: Nalgene Nunc International, Rochester, NY.
- TMB Stop Solution (#50-85-05): KPL, Gaithersburg, MD.
- Triton X-100: Sigma Aldrich Chemical Company, St. Louis, MO.
- Tween-20: Sigma Aldrich Chemical Company, St. Louis, MO.
- Vetbond tissue glue: 3M Animal Care Products, St. Paul, MN.
- XCell SureLock™ Electrophoresis Cell: Invitrogen, Carlsbad, CA.
- Xtremegene 9: Roche, Nutley, NJ.
- Xylazine: Vetus Animal Health, Owings Mills, MD.
- ZetaPALS Phase Analysis Light Scattering ultra-sensitive zeta potential analyzer (Ver. 3.28): Brookhaven Instruments Corp., Holtsville, NY.
- ZetaPALS 90 Plus Light Scattering particle size analyzer (Ver. 3.57): Brookhaven Instruments Corp., Holtsville, NY.
Methods:

A. Preparation of liposomal formulations for transfection

Liposomes were created from dioleoylphosphatidylcholine (DOPC), cholesterol and stearylamine (Avanti), where stearylamine was the cationic lipid. Briefly, lipid components were dissolved in chloroform and mixed in a round-bottom flask, using nitrogen to eventually evaporate the organic solvent. Next, the resultant lipid film was freeze-dried for 2 hours to eliminate any remaining chloroform. Using the same initial volume as the DOPC constituent, PBS was added to reconstitute the lipids. Liposome packing took place by repeated (five to seven iterations) vortexing for 5 min, then placing the lipid solution on ice for 2 min alternating with a heat bath at 37°C. These hot and cold packing steps densely pack lipids into a lipid layer. Next, the solution was sonicated in a bath sonicator until the solution was clear, indicating the uniform production of liposomes.

To form lipoplexes, the correct amount of stock cationic liposome needed for a certain amount of pDNA was determined via the N/P ratio, which is the nmol nitrogen (in the cationic lipid) divided by the nmol phosphate (in the pDNA; 1 μg = 3 nmol phosphate). The formula used to determine the volume of cationic liposome stock is:

\[
\frac{(\text{μg of pDNA} \times \text{nmol of phosphate per μg} \times \text{desired N:P ratio})}{(\text{nmol per μL cationic lipid stock})}
\]

The final volume of pDNA and cationic liposome solutions was determined by the size of the well containing the cells to be transfected, with OPTI-MEM reduced serum media (Invitrogen) used as the diluent for both liposome and pDNA solutions. The solutions were mixed thoroughly, with a 30 min incubation allotted for pDNA-
liposome complexation. Samples from each preparation were removed for particle size and zeta potential analysis using a Particle Size and Zeta Potential Analyzer (Brookhaven Instruments).

B. Cell culture and transfections

SHEP-1 cells were grown at 37°C in 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% heat inactivated Fetal Bovine Serum (FBS) and 10% Donor Horse Serum (DHS) supplemented with 1% penicillin-streptomycin. N27 cells were grown under the same conditions using RPMI-1640 media (Sigma) containing 10% FBS and 1% penicillin-streptomycin. Cells were grown in 75 cm² or 25 cm² cell culture flasks and were then passaged onto coverslips inside 24-well plates (BD Falcon) at seeding densities of 50,000-125,000 cells/well at least 3 hours before transfection.

Transfection experiments were performed using combinations of PEG-CK30 vectors, liposomal constructs, Fugene-HD (Promega), Xtremegene 9 (Roche) and/or Lipofectamine-LTX, in each case using 1 µg pDNA per well (24-well plate) or 4 µg pDNA per well (6-well plate). OPTI-MEM reduced serum media (Invitrogen) was used as the diluent. A total volume of 50 µL/well pDNA solution was combined with 50 µL/well lipid transfection agent in a microcentrifuge tube. The solution was mixed and incubated at room temperature for 30 min for pDNA-lipid complexation. This volume was increased four-fold when using a 6-well plate. The solution was then dropped onto cells and allowed to incubate for four hours. Media was then replaced and transfection was observed two days later.
To assess transfection efficiency or to examine eGFP-immunohistochemistry, cells were fixed to coverslips by immersion in 4% paraformaldehyde solution for 15 min. Cells that were harvested for protein analysis had their media decanted, were lysed using 0.5 mL of a PBS-Triton buffer (containing 0.1% Triton-X 100 with 1% protease inhibitor cocktail solution) and then this solution was collected into microcentrifuge tubes and placed on ice on a shaker for 20 min. Lysates were then centrifuged at 14,000 g for 10 min at 4°C and the supernatant was collected. For most experiments, the decanted media was also analyzed to detect the secreted protein.

C. Immunocytochemistry to detect eGFP

Coverslips with fixed cells in wells were washed with PBS (x3, 5 min each) and placed in a 0.3% H₂O₂ solution for 15 min to inactivate endogenous peroxidases. Cells were washed again with PBS (x3, 5 min each) and incubated in the primary antibody (rabbit anti-GFP, Abcam Ab290) in a 1:1,000 dilution for 1 hour. Cells were washed with PBS and incubated in the secondary antibody (donkey anti-rabbit, conjugated to horseradish peroxidase (HRP), GE #NIF824) in a 1:1,000 dilution for an additional hour. The peroxidase substrate, 3,3′-diamino-benzidine (DAB; Vector Labs), which reacts with HRP to form a brown stain upon reaction, was added and incubated with the coverslips for 5 min (or until accurate color development occurred). The presence of eGFP in cells was visualized by light microscopy using DIC optics.
D. **Animal treatments**

All procedures used male Sprague-Dawley (SD) rats weighing 225-250 g (Taconic) that were anesthetized with ketamine and xylazine (90/20 mg/kg, i.p.) in accordance with an approved Northeastern University IACUC protocol. For intranasal delivery, rats were placed in the supine position with their noses upright and their heads flat on the surface. Rats were given solutions of either naked plasmid (4.3 mg/mL), compacted nanoparticles (4.4 mg/mL) or saline via a Hamilton syringe fitted with a short piece of polyethylene tubing. Intranasal doses were given in 2.5 µL increments alternating nares for a total of either 10.0 µL or 12.5 µL per side (20 or 25 µL total, respectively). Rats remained supine for 30 minutes post-treatment.

For intracerebral injections, 4 µL of the same solutions were surgically injected into the left striatum at the following stereotaxic coordinates: +2.5 mm lateral to lambda, +0.7 mm anterior to bregma, -5.8 mm ventral to the surface of the skull. The solution was injected at a rate of 1 µL/min. The needle was left at the injection site for 10 min to ensure solution fully dispersed and was slowly removed. The entire stereotactic surgery was done under aseptic technique and skin was closed using Vetbond (3M). Rats were given an injection of buprenorphine (0.05 mg/kg, s.c.) before recovery from anesthesia to minimize post-surgical pain.

E. **Preparation of brain tissue**

Brains being processed for immunohistochemistry or other direct microscopic analysis required perfusion prior to sectioning. Rats were anesthetized as described above and a midline incision was made to expose the heart for transcardial perfusion.
Heparin (1 mL; 1000 U/mL) was injected directly into the left ventricle to prevent blood coagulation. At the same injection site, a needle attached to the perfusion line was placed, and PBS was allowed to flow to flush the vasculature. Next, approximately 200 mL of 4% paraformaldehyde (PFA) was allowed to flow through the vasculature to fix the brain. Brains were removed, post-fixed in 4% PFA for an additional 18 hours, and then transferred to 30% sucrose for 2-4 days until they sunk to the bottom of the vial. The brains were sectioned at 30 μm using a cryostat. The sections were stored at -20°C in a cryoprotectant solution, until further analysis or assay.

Brain tissue used for the enzyme-linked immunosorbant assay (ELISA) was not subjected to fixation. Instead, rats were decapitated under isoflurane anesthesia and their brains were rapidly removed. Sections were made by razor cuts using a Plexiglas brain matrix. The fresh brain sections were flash frozen in either liquid nitrogen (N₂) or on dry ice (CO₂) before being stored at -80°C. Prior to the ELISA, each section was homogenized in 1 mL of lysis buffer (1% Igepal, 10% glycerol, and 1:100 protease inhibitor in PBS) and centrifuged at 4°C for 30 minutes at 14,000 RPM. The resulting supernatant was used as the medium in the ELISA to detect the protein of interest.

**F. Immunohistochemistry for detection of eGFP and TH**

Brain sections stored in cryoprotectant were first washed with PBS (x3, 10 min each) and then treated with 0.9% H₂O₂ for 15 min to inhibit endogenous peroxidases. For eGFP-IHC, wash steps took place using a PBS-Triton Buffer containing 0.1% Triton-X 100 and occurred between each incubation step (x3, 10 min each). Tissue was blocked with 2.5% normal donkey serum (NDS) in PBS for 30 min at room temperature to
decrease non-specific binding. Following the blocking step, brain sections were incubated for 2 hours at room temperature on a rotating wheel with a 1:5,000 dilution of a rabbit anti-GFP antibody (Abcam #Ab290) in 2.5% NDS. Next, a donkey secondary anti-rabbit antibody conjugated to HRP was added to the sections in a 1:3,000 dilution in PBS for 2 hours (GE #NIF824). DAB (Vector Labs) was prepared according to kit instructions and incubated with the sections for 12 min.

For tyrosine hydroxylase (TH)-IHC, wash steps took place using PBS and similarly occurred between each incubation step (x3, 10 min each). Tissue was blocked with 5% normal goat serum (NGS) in PBS for 30 min at room temperature to decrease non-specific binding. A rabbit anti-TH antibody (Chemicon #Ab152) was diluted 1:4,000 in 5% NGS and was used as the primary antibody. Brain sections were incubated overnight at 4°C on a rotating wheel. The next day, sections were incubated for 1 hour with a 1:250 dilution (in PBS) of biotinylated anti-rabbit secondary IgG antibody raised in goat (Vector Labs #BA-1000). Following this, the sections were incubated for 1 hour with ABC reagent (Vectastain Elite ABC kit, Vector Labs #PK-6100) made-up according to kit instructions. DAB solution, prepared as above, was added for colorimetric detection. Sections were incubated for exactly seven minutes with DAB to standardize color development.

G. Detection of eGFP and GDNF expression by ELISA

Enzyme-linked immunosorbant assay (ELISA) was used as a quantitative method to measure eGFP and GDNF expression in cell lysates, media, and in brain tissue. For each ELISA, a 96-well plate was used and volumes of all solutions used were maintained
at 100 µL/well. Plates were incubated at room temperature with continuous shaking. The wells were first coated with capture antibody diluted in PBS (for eGFP-ELISA, 1:4,000 mouse anti-GFP, Sigma #G-6539; for GDNF-ELISA, 2.0 µg/mL mouse anti-human GDNF, R&D Systems #840189), and then incubated overnight at 4°C. After this and each subsequent step, the plate was washed with wash buffer (0.05% Tween-20 in PBS, 3x) and blotted dry using paper towels. Blocking buffer (5% non-fat dry milk in PBS) was then added at room temperature for 1 hour. Next eGFP or GDNF standards made-up in reagent diluent (1% BSA in PBS) were added to rows of wells along one side of the plate. The standards were serially diluted from 1,000 pg/mL to 7.81 pg/mL.

Tissue samples for the GDNF-ELISA required an acid-shock step before adding to plate. This involved addition of 1 N HCl (10 µL/sample) for 20 min, followed by neutralization with 1 N NaOH (10 µL/sample). Reagent diluent was used for blanks. Samples and blanks were then added, and the plate was incubated at room temperature for 2 hours. Next, detection antibody diluted in reagent diluent (for eGFP-ELISA, 1:4,000 rabbit anti-GFP, Abcam #Ab290; for GDNF-ELISA, 100 ng/mL biotinylated goat anti-human GDNF, R&D Systems #840190) was added, and the plates were incubated for another 2 hours. For detection, an additional 45 min incubation step with secondary antibody was required. For the GFP-ELISA, the secondary antibody was an anti-rabbit, conjugated to HRP (GE #NIF824) and was diluted in reagent diluent 1:4,000. For the GDNF-ELISA, the plates were incubated with a streptavidin-HRP solution (R&D Systems #890803) in a 1:200 dilution in reagent diluent. Color development took place using SureBlue TMB substrate (KPL #52-00-01). The plate was incubated for 10-20 min until full color development took place. The reaction was stopped with 1N HCl. An older variant of the
eGFP-ELISA protocol was used for pCG-related experiments. This involved the use of a
different detection antibody (1:2,000 rabbit anti-GFP conjugated to HRP, Novus
Biologicals #NB100-1184) that did not require an additional HRP conjugation step. All
ELISAs had optical density read at 450 and 570 nm using a microplate reader (Biotek
ELx800) and analyzed using Gen5® software. To express protein in terms of mg protein
present in the samples, a BCA protein assay (Pierce BCA Protein Assay Kit, Thermo
Scientific #23227) was run concurrent with each ELISA in accordance with the
manufacturer’s protocol.

H. Detection of eGFP by Western blot

Cell lysates (30 µL) were incubated at 95°C for 5 min with the reducing agent
dithiothreitol (DTT; 0.5 M, 4 µL) and loading buffer (Pierce #39000; 16.7 µL). A 30 µL
aliquot of each sample was loaded into wells on a Precise Tris-HEPES 4-20% gradient
polyacrylamide gel (Pierce #25224) along with standards and ladder (Invitrogen). Gel
electrophoresis was performed using a Tris-HEPES running buffer (composed of 12.1 g
Tris-HCl, 23.8 g HEPES and 1 g SDS in 1 L dH2O) at 100 mV for 45 min using an XCell
SureLock™ Electrophoresis Cell (Invitrogen). Semi-wet protein transfer to a
nitrocellulose membrane occurred using a Tris-glycine transfer buffer (composed of 3.03
g Tris-HCl, 14.4 g glycine and 0.1 g SDS in 1 L dH2O) at 20 mV for 30 min using the
same apparatus. The membrane was transferred to a shallow plastic vessel, washed with
0.1% Triton-X in PBS (x3, 10 min each) and blocked with blocking buffer (5% non-fat
dry milk in PBS) overnight. The next day, rabbit anti-GFP primary antibody diluted in
PBS 1:4,000 (Abcam #Ab290) was added at a volume ensuring complete coverage, and
the membrane was incubated for 1 hour at room temperature on a shaker. Membranes were washed as before and the membrane was then incubated with the secondary antibody, anti-rabbit, conjugated to HRP (GE #NIF824) for an additional hour. Membranes were washed a final time and an ECL detection reagent (GE #RPN2108) was used to generate a chemiluminescent signal, which was visualized using Kodak Image station hardware and software.

I. 6-hydroxydopamine (6-OHDA) lesioning

Rats received a unilateral 6-OHDA lesion on the left side of the brain. Sterile surgical equipment and aseptic techniques were used for all procedures in compliance with Northeastern University IACUC policy and the approved protocol. Rats were anesthetized as previously described, and just before surgery were given desipramine (15 mg/kg; i.p.), a norepinephrine reuptake inhibitor, in order to prevent uptake of 6-OHDA into noradrenergic neurons. The animals were then placed in the stereotaxic instrument, and their body temperature was maintained at 38°C by using a heating pad (Fintronics). The rat’s head was shaved, and the skin over the skull was wiped three times in alternating fashion with 70% isopropyl alcohol and betadine. A sterile scalpel was used to create a rostral to caudal incision on the scalp, exposing the lambdoid suture. A drill (Dremel) was used to form a 3.0 mm hole at the following stereotaxic coordinates: +1.2 mm lateral to lambda, +4.4 mm anterior to the lambdoid suture, and -8.3 mm ventral to the surface of the skull. A needle, attached by a length of PE tubing to a 10 µL Hamilton syringe, was lowered through the hole. A total of 4 µL of 6-OHDA solution (2 µg/µL in 0.1% ascorbic acid) was injected into the left medial forebrain bundle. The solution was
injected at a rate of 1 µL/min using an infusion pump (Harvard Apparatus) that displaced water to push 6-OHDA out of the needle. At the end of the 6-OHDA infusion, the needle was left in place for 15 minutes and then removed slowly in order to minimize tracking of the neurotoxin up the needle track. The incision was then closed using veterinary tissue adhesive (Vetbond®) and rats were administered a dose of buprenorphine (0.05 mg/kg). Rats were allowed to recover from the 6-OHDA lesion for 3 weeks, at which time further behavioral assessment and animal sacrifice occurred.

**J. Behavioral assessment of unilateral dopamine cell loss**

To assess the extent of the 6-OHDA lesion, all lesioned rats were evaluated for rotational behavior following an amphetamine challenge. Just prior to sacrifice 3 weeks after the 6-OHDA lesion, rats were given a dose of *d*-amphetamine sulfate (5 mg/kg, i.p.) and then placed in a large circular bucket. A video camera mounted above the pail was used to observe and record rotations. The number of rotations was counted for a total of 30 min, beginning 15 min after amphetamine injection. Net rotations were expressed as the number of ipsilateral rotation (toward the lesion; counterclockwise) minus the number of contralateral rotations (away from the lesion; clockwise).

**K. Microscopy**

All microscopic analyses took place using an Olympus BX51 microscope equipped with X-cite fluorescence and DIC optics for contrast enhancement. Bright field microscopy was used to detect chromogenic signal from DAB after GFP-IHC and TH-
IHC. Fluorescence microscopy to observe eGFP was done with a FITC excitation/emission filter set. Sections were mounted on slides using Fluoromount-G (Southern Biotech).

L. Bioquant® and ImageJ analysis of TH-immunohistochemistry

Bioquant® Nova version 6.90.1 was used to analyze TH immunostaining density in each treatment group following the 6-OHDA lesion. For SN sections, the TH density measurements were analyzed under a 4X zoom and the RGB threshold was set to R: 162/9, G: 134/6, B: 130/9 to selectively capture the brown staining from DAB. Light settings and white balance were kept constant for all sections examined. For striatal sections, threshold was set to R: 184/41, G: 165/54, B: 170/47. TH density measurements in the SN were done by tracing the perimeter of the unlesioned SN and using that same shape flipped and copied exactly over the contralateral structure on the opposite side of the brain using the Bioquant® software. Striatal density measurements were done similarly, except five circular samples (measuring 350 microns in diameter) were performed on each side using Bioquant®. Six evenly-spaced coronal sections corresponding to different anterior-posterior levels of the substantia nigra and striatum were analyzed for each brain. The integrated optical density of staining on the lesioned and intact sides were divided and subtracted from 100 to obtain the percentage lesion for each section. Sections from the SN and striatum of each rat were averaged to give the percentage lesion in each region for each animal.

Dopamine cell counts were determined manually from captured SN images on the lesioned and unlesioned side using ImageJ version 1.46 and the CellCounter plug-in.
This plug-in allows accurate quantification of cells using mouse clicks to track cells and prevent double counting. All cells in an SN-outlined perimeter were counted and compared as before with the unlesioned side to obtain the percentage lesion (or % cell loss) when subtracted from 100 for each section.

M. SPECT Imaging

SPECT imaging of the dopamine transporter was used to assess 6-OHDA lesion severity in a subset of the lesioned rats. β-CIT [2β-carbomethoxy-3β-(4-iodophenyl)tropane] was labeled with $^{125}$I on the day of use. Rats were anesthetized with isoflurane, and 0.4 mCi of the radioligand (specific activity ~2,000 Ci/mmol) was administered via tail vein injection 2 hours prior to imaging. SPECT acquisitions were conducted by Ben Gershman in the Center for Translational Neuroimaging at Northeastern University using a Nano SPECT/CT® (Bioscan, Inc). A total of 48 projections were taken at a rate of 100 sec/projection over 20 min. Gamma detection for SPECT was achieved using four NaI detectors, measuring 215 x 230 mm2, coupled with photo-multiplier tubes for signal amplification. Each detector employed a 9-pin-hole aperture, $\varnothing = 2.5$ mm, as the image-forming element, and a photodiode-array detector with 1024 x 2048 pixels measuring 48 $\mu$m. Post-processing and data analyses were performed using the InVivoScope suite (Bioscan, Inc). MRI, CT, and SPECT reconstructed volumes were co-registered using manual and automated techniques. The CT-SPECT co-registration transformation is stable, well-calibrated and automatically applied by InVivoScope. CT and MRI datasets were manually aligned using the skull as
a fiducial. Left-right differences were calculated from cylindrical volumes of interest (VOI) defined using anatomical landmarks in the MR image.

N. Autoradiography

Two hours after completion of SPECT imaging, rats were sacrificed, their brains were collected, and coronal sections (~1.5 mm thick) were cut. Brain sections were dried overnight on slides and then placed on phosphorimaging plates. The plates were exposed for 1 hour using a Cyclone Phosphorimaging System (Perkin-Elmer). Left-right differences in striatal DAT radioligand binding were quantified from autoradiograms.

O. Statistical Analysis

Statistical analyses were performed using Graph Pad Prism® software version 4.03. The α-level for significance was set at p<0.05. Values are presented as mean ± standard error of the mean (SEM). Comparisons between multiple groups of data were conducted by analysis of variance (ANOVA) with the appropriate post-hoc test to determine the differences between groups. Comparisons between two groups of data were performed using the unpaired Student’s t-test. Correlations were analyzed using linear regression analysis.
IV. RESULTS

SPECIFIC AIM 1

A. Testing transfection efficiency: *in vitro* pCG transfection

The goal of the first Aim was to demonstrate proof of principle that the intranasal route could be used to deliver gene therapy vectors to the brain that could produce reporter proteins where successful transfection occurred. This would provide a means to track where intranasal administration delivered the therapeutic vector and allow for testing of the cell-types transfected. Our collaboration with Copernicus Therapeutics, Inc. came from our desire to utilize a non-viral vector that had already been shown to be effective for transfection *in vitro* and in the brain. The first series of experiments thus employed the enhanced green fluorescent protein (eGFP) expression plasmid pCG, which expresses eGFP under the cytomegalovirus (CMV) promoter. Copernicus informed us that this plasmid has a maximal expression time-point of 48 hours post-administration.

We first examined the transfection efficiency of pCG nanoparticles, and the naked plasmid, in transfecting SHEP-1 cells (a neuroblastoma-derived line) in culture. Since SHEP-1 cells lack nucleolin, Fugene-HD (a proprietary blend of lipids) was used with both nanoparticles and naked plasmids as it forms a lipid shell that aids in endocytosis of the nanoparticles and plasmids into cells. In the studies described below, cultured SHEP-1 cells were treated with pCG NPs or naked pCG plasmid with Fugene-HD (1 μg pDNA per well, 3.8 mg/mL). Cells were fixed onto coverslips and analyzed for fluorescence two days after transfection (Figure 11). Successful transfection could be observed by direct fluorescence microscopy and eGFP expression readily was detected in cultures treated with both naked pCG (Figure 11a, b) and the pCG nanoparticle formulations.
mixed with Fugene-HD (Figure 11c, d). NP and naked pCG lacking Fugene-HD showed no transfection, as expected (Figure 11e). There was no difference in apparent transfection efficiency between naked pCG and pCG NP (with Fugene-HD) at the observed time-point, although this was not calculated. The results of this study showed that SHEP-1 cells were an ideal system to test transfection in vitro and proved the pCG plasmid has the capacity to transfect brain-derived cells and express eGFP. Although the original plan was to compare pCG transfection using NPs vs. liposomes, the experiments with lipoplex formulations were put off until work was done with a plasmid more resembling the pGDNF used in the final Aim of this thesis.

Figure 11: Transfection of SHEP-1 cells using pCG: a, b. Transfection by naked pCG (with Fugene-HD); c, d. Transfection by pCG nanoparticle (with Fugene-HD); e. Transfection by pCG nanoparticle (without Fugene-HD). All views at 20X magnification with fluorescent overlay. Scale bars = 100 microns.
B. Direct striatal injection of PEG-CK30 pCG nanoparticles successfully transfects brain cells

The next studies were introduced to examine transfection ability by pCG in vivo, and ultimately after intranasal administration. Since intranasal delivery was expected to be an effective, but inefficient means of delivery of nanoparticle vectors to the brain, initial experiments were conducted to qualitatively demonstrate eGFP expression in rat brain after a direct injection of pCG NPs. A dose of 4 μL (3.8 mg/mL) pCG NPs was injected stereotaxically into the left striatum, and rats were sacrificed two days post-injection (n=4). GFP expression was visualized using GFP-IHC and by fluorescence microscopy (Figure 12 a-c).

Results revealed heavy, local expression of eGFP confined to the area directly surrounding the needle track. GFP-IHC was a more sensitive means of detection, as shown in the figures below. A larger number of GFP-positive cells and a wider area of transfection adjacent to the needle track were apparent by IHC compared with direct fluorescence. Cells successfully expressing eGFP were densely clustered and overlapping, making it difficult to assess whether they were neurons, glia or both. GFP-ELISA on striatal sections, including the injection site, did not reveal the presence of eGFP. This was likely due to the low sensitivity of the ELISA assay at the time this study was done.
This study confirmed that PEG-CK30 NPs were capable of transfection of cells in rat brain when directly injected into the striatum.

**C. Intranasal pCG nanoparticles transfect and cause eGFP expression throughout rat brain**

The next studies examined whether intranasal administration of pCG NPs could transfect cells in brain and lead to protein expression. For this study, an improved GFP-ELISA was needed to detect much lower protein concentrations. Sensitivity was improved by using different capture and detection antibodies than in the previous ELISA. Rats received an intranasal dose of 25 μL of either pCG NP (3.9 mg/mL, n=7) or naked pCG plasmid (3.9 mg/mL, n=5). Control rats received intranasal saline (n=5), and each group was sacrificed 2 days post-administration. Levels of eGFP expression were analyzed in ~2 mm thick coronal sections spanning the rostral-caudal axis of the brain.

*Figure #12: GFP-IHC in striatal tissue after pCG injection.* eGFP expression in rat striatal tissue sacrificed 2 days post-injection of PEG-CK30 pCG nanoparticles. (a) 20X and (b) 40X view of injection site track after GFP-IHC. (c) Native eGFP fluorescence along needle track (20X). Scale bar = 50 microns.
Each coronal section was further divided into dorsal and ventral areas, and the 13 regions were assayed by GFP-ELISA.

Expression of eGFP from pCG was detectable in sections throughout the entire rat brain, with levels in the pCG NP treatment group greatly exceeding all other groups (Figure 13). No specific section showed a significantly higher eGFP expression as a result of treatment, but one-way ANOVA revealed significance of the pCG NP treatment group relative to the naked pCG (p<0.05) and saline control groups (p<0.01). Figure 14 represents the same ELISA data as a percent above control, more clearly showing the eGFP expression achieved above background in each section. There was a clear trend toward higher eGFP expression in the more rostral and caudal sections, which was expected due to the key roles of the olfactory and trigeminal nerve pathways in intranasal delivery. Results indicated that pCG nanoparticles, (and to a lesser extent, naked pCG) can reach and transfect cells throughout the rat brain after intranasal administration.
Figure #13: GFP-ELISA of rat brain sections two days after intranasal administration of pCG. GFP levels were normalized to protein found in each section. Markedly higher expression was found in each of thirteen sections in those rats given pCG NPs or naked pCG. The effects of treatment and section were found to be significant using 2-way ANOVA (p<0.0005), although no particular section showed significance. A one-way ANOVA revealed a significant eGFP expression effect by treatment (p=0.0062). Bonferroni’s post-test showed pCG NP treatment was significant vs. saline (p<0.01) and naked pCG (p<0.05) (D: dorsal, V: ventral).
The second part of this study was to determine whether transfection and eGFP expression could be detected in brain by fluorescence microscopy and GFP-IHC. Another set of rats were administered the same intranasal pCG treatment, and control rats were given intranasal saline. The rats were sacrificed two days after intranasal treatments by transcardial perfusion, and brains were sectioned as above to observe qualitative evidence of pCG transfection. Brain sections spanning the entire rostral-caudal axis showed sparse but obvious eGFP expression using direct fluorescent microscopy (Figure 15). Although eGFP fluorescence was detectable, it appeared that only those cells with large amounts of expression were able to be seen above background autofluorescence. The sparse distribution of these green fluorescent cells may therefore under-estimate the actual ability of pCG nanoparticles to elicit eGFP expression. In addition, as was shown with GFP-ELISA, naked pCG plasmid can also cause sparse transfection of brain cells after intranasal administration (Figure 15b). In both cases, cells producing eGFP were often found along the edges of capillaries, suggesting they might be capillary endothelial cells, but other larger cells with neuronal morphology were also observed (Figure 15d).
Some sections from each of the treated rats were also subjected to GFP-IHC, since our previous study showed GFP-IHC may allow for greater detection of faint eGFP expression. Immunohistochemical expression patterns were similar to direct fluorescence, with those rats given intranasal pCG nanoparticles having much higher eGFP-immunostaining in brain sections than rats given intranasal naked pCG or saline (Figure 16). Cells expressing eGFP often appeared clustered together, and like those seen by fluorescence microscopy, seemed to align along capillaries and resemble capillary endothelial cells. Double-label IHC would be useful for determining the types of cells expressing eGFP. These studies were not performed as part of this thesis.
An effort was made to quantify relative eGFP expression levels in the treated rats by counting GFP-immunostained cells from each treatment group (cell counts performed by Amanda Nadeau). A representative section from each of six brain regions (A through E) was examined for immunostaining and the total number of GFP-positive cells was quantified for an equivalent section of each brain, and results were normalized to total section area (Figure 17). Rats from the pCG NP treatment group had numbers of eGFP-positive cells far above naked pCG and saline control rats. Section D, comprising the midbrain, appeared to show the greatest number of GFP-positive cells.

Figure #16: GFP-IHC in rat brain after intranasal administration of pCG. Sections from a saline control (a) and naked pCG treatment (b) show no cellular immunostaining for eGFP. (c, d) Sections from rats given pCG NP treatment show heaviest eGFP expression. (e) Optical zoom reveals immunostained GFP-positive cells resemble capillary endothelial cells in a rat given intranasal pCG NPs. Scale bars = 50 μm.
D. The fusion plasmid pUGG causes expression of eGFP and produces the neurotrophic effects of GDNF expression in vitro

Specific Aim 2 had a design similar to that of the previous Aim, but focused on characterizing transfection and expression from the pUGG plasmid. Initially, we proposed that this Aim would move directly to intranasal delivery of pGDNF nanoparticle vectors with detection of GDNF instead of reporter proteins. However, GDNF produced from pGDNF can only be detected above background GDNF levels in brain, making its production difficult to measure or visualize compared with a reporter protein like eGFP. In addition, the two plasmids studied for this Aim and the last vary greatly, making pCG a poor surrogate for pGDNF. The pCG plasmid produces eGFP
under the fast-acting CMV promoter whereas the pGDNF plasmid produces GDNF under the long-lasting UbC promoter. In consultation with the thesis committee, a decision was made to make (or ask Copernicus to make) a new fusion plasmid that produces both proteins while maintaining the same plasmid backbone as pGDNF. This would allow for detection of eGFP as a reporter of successful transfection while being produced in parallel with GDNF. This plasmid would more closely mimic the properties of pGDNF, which was to be the construct used in neuroprotection studies of Aim 3.

To this end, Copernicus developed the pUGG fusion plasmid by removing the signal peptide sequence from the GDNF gene construct and inserting the eGFP sequence. According to Linas Padegimas at Copernicus, removal of the signal sequence should reduce the secretion of the protein once expressed (Figure 18). The GDNF sequence was the hGDNF_1b variant, which has a 26 amino acid splicing sequence removed. This variant was characterized by Copernicus and found to optimally express GDNF (Figure 19). The eGFP and GDNF proteins are theoretically separated by a sequence that normally allows for cleavage of the pro-GDNF sequence. This cleavage should allow for two separate proteins to be expressed with pUGG transfection.

![GFP-GDNF fusion protein precursor](image)

**Figure #18:** Design of the GFP-GDNF fusion plasmid, pUGG. pUGG utilizes the UbC promoter to produce a fusion protein consisting of eGFP linked to hGDNF_1b upon successful transfection. GDNF’s signal sequence was replaced with the eGFP sequence. The hGDNF_1b variant has a 26 amino acid intron added in the pro-GDNF precursor sequence.
One of the main reasons for the creation of pUGG was to use it in liposomal formulation experiments. The optimal lipoplex formulation would be taken forward to compare with Copernicus’ pUGG NPs in *in vitro* transfection studies. However, since the pUGG plasmid had not been evaluated before, it was first necessary to determine its transfection and expression characteristics before proceeding with liposomal and *in vivo* intranasal experiments. Transfection experiments were first done using a variety of proprietary lipid formulations to find conditions for optimal eGFP expression in SHEP-1 cells. Lipofectamine-LTX with PLUS reagent was compared to Xtremegene-9 in preparations that were optimized according to the manufacturer’s protocols. Optimization experiments determined that maximum transfection occurred using a Lipofectamine:pDNA ratio of 4:1 (µL:µg) and Xtremegene 9:pDNA ratio of 3:1. Cells were transfected at 60-80% confluency and harvested after two days. Results showed that transfection and expression of eGFP was dramatically greater using Lipofectamine-LTX with PLUS reagent than with Xtremegene 9 (*Figure 20*). Lipofectamine-LTX with PLUS reagent was therefore used for all further *in vitro* experiments with pUGG.
Transfection with the previous plasmid, pCG, caused maximum protein expression after two days due to the fast-acting CMV promoter. The use of the UbC promoter with pGDNF, on the other hand, was shown to cause less expression than pCG, but that expression persisted for a much longer time (>6 months) following direct injection into rat striatum (Fletcher, 2011). This difference was seen in vitro with pCG transfection producing far greater levels of eGFP than pUGG in SHEP-1 cells (Figure 21). Determining the maximum time-point for transfection and expression for pUGG was not possible in vitro due to the constant cell division that would cause over-confluent cultures leading to the death of the cells that were initially transfected. Transfection efficiency experiments, however, revealed an average of 39.7% cells transfected with pCG-Lipofectamine and 15.7% of cells transfected with pUGG-Lipofectamine at two days after transfection (n=6-8 culture wells/treatment) (Figure 22). This result may be skewed in favor of pCG since it expresses higher levels of eGFP at this time-point.
GFP-ELISA further showed the difference in expression between pCG and pUGG (Figure 23). Transfection using pCG yielded exponentially greater levels of detectable eGFP after two days than transfection with pUGG. eGFP was found both intracellularly, i.e. in cell lysates (I), and secreted into media (S), and levels were similar in each. This was surprising, as the pUGG construct was designed to limit secretion of the expressed protein.
pUGG transfection was also evaluated in ventral midbrain cultures by Dr. David Yurek at the University of Kentucky. These cells represent the closest in vitro counterpart of the brain area to be targeted by intranasal pGDNF, and they can provide a bioassay of the neurotrophic properties of GDNF expression by increasing dopaminergic phenotypes. Dr. Yurek sent the cell lysates and media from these cultures for us to assay by GFP-ELISA. Initial evaluation of ventral midbrain cultures revealed that pUGG transfection using Lipofectamine-LTX with PLUS reagent caused successful transfection and production of eGFP both secreted into media and intracellularly (n=4 culture samples/treatment) (Figure 24).
Dr. Yurek also examined GDNF expression in ventral midbrain cultures transfected with pUGG or pGDNF in Lipofectamine-LTX. He assayed by ELISA both secreted and intracellular levels of GDNF and found, unexpectedly, that GDNF was only minimally detectable in cells treated with pUGG (Figure 25a), although cultures treated with pGDNF showed typically high levels of both secreted and intracellular GDNF. However, despite the failure to detect GDNF by ELISA, the VM cultures treated with pUGG showed increased survival of dopamine neurons comparable to cultures transfected with pGDNF or treated with 10 ng/mL GDNF protein. Dopamine cell survival was assessed by immunocytochemistry for tyrosine hydroxylase (TH; a marker of dopamine cells) (Figure 25b). We thus hypothesized that either pUGG produces a form of GDNF that is unable to be recognized by the GDNF-ELISA or that the fusion protein is not being cleaved, and eGFP occludes GDNF’s immuno-detection by ELISA.

*Figure 24: GFP-ELISA of ventral midbrain cultures transfected with pUGG.* Ventral midbrain cells were transfected using pUGG and were harvested two days later. Surrounding media were collected to determine secreted (S) protein levels. Cell lysates were collected for intracellular protein (I) levels. pUGG transfection yielded expression of eGFP in media as well as in lysates with no significant difference (n=4 samples/treatment group).
To test whether the protein expressed by pUGG remains uncleaved, a denaturing GFP-Western blot was developed. The pUGG construct consists of the eGFP sequence (27 kDa) coupled to the pro-GDNF domain (precursor peptide, 8 kDa), which normally gets cleaved to mature GDNF (16-18 kDa per monomer). Uncleaved, the expected molecular weight of the fusion protein is around 51-53 kDa, combining the eGFP, linker segment and hGDNF1b. Figure 26 shows the results of a GFP-Western blot on SHEP-1 cell lysates two days after pUGG transfection. Almost all detectable eGFP was found at a band located at the predicted molecular weight of the fusion protein, confirming the hypothesis that eGFP and GDNF are not cleaved after expression.

Figure #25: Comparison of transfection using pUGG and pGDNF in rat embryonic ventral midbrain cultures. a) GDNF was undetectable by GDNF-ELISA in pUGG-transfected VM cultures yet was readily found both intracellularly (I) and secreted into media (S) when transfected with pGDNF (which produces GDNF only). One-way ANOVA indicated a significant difference between groups (*p<0.001) and Tukey's post-test revealed a significant difference between both pGDNF treatment groups vs. both pUGG treatment groups (*p<0.001). b) Tyrosine hydroxylase (TH) immunocytochemistry in VM cultures showed that bioactive GDNF was produced by pUGG transfection as the number of TH-positive cells is similar to that of cultures transfected with pGDNF or treated with GDNF protein (10 ng/mL). One-way ANOVA revealed significant difference between groups (p<0.001) and Tukey's post-test showed a significant difference between untreated control TH cell counts and all other treatment groups (*p<0.05). Data provided by Dr. David Yurek, University of Kentucky.
Although GDNF was not detectable by ELISA or western blot on lysates from SHEP-1 cells transfected with pUGG, the increased survival of dopamine neurons in ventral midbrain cultures suggested that bioactive GDNF protein was nevertheless being expressed by the plasmid. To further examine the neurotrophic properties of the fusion protein, an N27 dopaminergic cell line was transfected with pUGG as before using Lipofectamine-LTX with PLUS reagent or using pUGG NPs (Figure 27). Untreated cells received no plasmid. Even a modest level of transfection by pUGG in Lipofectamine, indicated by the few GFP-positive cells that were found, induced marked morphological changes in the N27 cells in culture, such as neurite outgrowth. Cells treated with pUGG NP did not get transfected, most likely due to the lack of surface nucleolin, as is true in most cell culture lines. These morphological changes suggest that GDNF produced from pUGG can be bioactive and that N27 cells are sensitive to the

**Figure #26**: GFP-Western blot of SHEP-1 cell lysates transfected by pUGG. SHEP-1 cell lysates were collected two days after transfection using pUGG-Lipofectamine. Molecular weights (MW) are listed for the expressed components of the pUGG fusion plasmid. Results indicated a heavy band at the 51-53 kDa MW, confirming that cleavage failed to occur and a single eGFP-GDNF fusion protein was produced.

- MW eGFP: 27 kDa
- MW hGDNF1b monomer: 16-18 kDa
- MW of linker: 8 kDa
- 51-53 kDa fusion protein
- MW of eGFP standard (with 6X-His tag): 33 kDa
neurotrophic effects of GDNF, consistent with their dopaminergic lineage and the previous findings in VM cultures.

**Figure #27:** *Transfection of pUGG in dopaminergic N27 cells.* Untreated N27 cells (a) had a flat, circular morphology. Cells treated with pUGG NPs did not exhibit morphological changes (b). Those cells successfully transfected with pUGG using Lipofectamine-LTX (c, d, e) had a drastically altered morphology, with formation of neurite outgrowths and a neuronal phenotype. Assuming that eGFP and GDNF are produced and secreted in equal amounts, only a small amount of the fusion protein is needed to induce a neuronal phenotypes in many cells in the culture (e). DIC = differential interference contrast, FL = fluorescent overlay. Scale bars = 100 microns.
E. Lipoplex formulations fail to achieve pUGG transfection in SHEP-1 cells

As part of my IGERT fellowship, a main goal of Aim 1 was to formulate lipoplexes and compare their transfection efficiency to Copernicus’ PEG-CK30 nanoparticles. A major reason for the creation of the fusion plasmid pUGG was to utilize it \textit{in vitro} for these studies. The best formulation would be the one taken forward to the \textit{in vivo} studies in rats.

Liposomes were composed of dioleoylphosphatidylcholine (DOPC), cholesterol and cationic stearylamine in a 50:30:5 molar ratio, respectively. These particular lipids were chosen as they had been shown to successfully deliver proteins to the brain after intranasal administration in our previous work (Migliore, 2006; Migliore, 2007; Bender, 2009; Migliore, 2009; Migliore, 2010). However, initial zeta potential results showed that these liposomes were only weakly cationic (~5.5 mV), which would not be suitable for complexing pDNA while maintaining a positive charge to interact with cells (data not shown). Therefore, liposomes were prepared by adding a 10-fold greater concentration of stearylamine, producing a 50:30:50 molar ratio. Various lipoplex formulations were then generated by changing the amount of cationic liposome to pDNA (known as the N/P ratio). A range of N/P ratios were tested: 1, 3, 5, 10 and 20, with each well of SHEP-1 cells receiving 1 μg pUGG. Analysis of particle size and zeta potential was done for each lipoplex formulation. \textbf{Figure 28} shows that higher N/P ratios, containing proportionally more cationic stearylamine, reduced the size of the lipoplexes from approximately 800 nm to 350 nm. As expected, increasing cationic charge had a direct relationship with increasing the zeta potential (\textbf{Figure 29}). Plain liposomes, lacking pUGG, had the highest zeta potential due to the lack of negative charge from the pDNA.
High zeta potentials, i.e. above 30 mV, are usually found to be undesirable for transfection studies due to potential toxicity and an inability for pDNA to escape from the lipid. For example, Lipofectamine-LTX was found to have a zeta potential of 23.3 mV with pUUG (data not shown). All lipoplex formulations of pUUG, when tested in
SHEP-1 cell cultures, failed to show transfection, as indicated by expression of eGFP under fluorescence microscopy (Figure 30). In addition, lipoplexes with an N/P ratio of 20 had significant toxicity, as indicated by a dramatic loss of viable cells in the cultures 2 days after transfection.

A final lipoplex formulation was tested in which the molar ratio of stearylamine was lowered to 30 (making it 50:30:30 of DOPC:cholesterol:stearylamine). Initial testing showed an inability of these nanoparticle formulations to transfect SHEP-1 cells using the same N/P ratios as above (data not shown). It was therefore decided that no further work with lipoplex formulations would be done, and in vivo transfection studies would focus on Copernicus’ PEG-CK30 nanoparticles.

**Figure #30:** Transfection of SHEP-1 cells using pUGG-cationic lipoplex formulations. Lipoplexes with N/P ratios ranging from 1 through 20 (increasing levels of cationic liposome) all failed to transfect SHEP-1 cells (a–e). Lipoplexes with an N/P ratio of 20, containing the highest level of cationic stearylamine, were actually shown to be toxic to cells (e). Naked pUGG plasmid did not readily transfect cells in culture (f). FL=fluorescence filter. Scale bars = 100 microns.
F. Direct striatal injection of pUGG nanoparticles successfully transfects brain cells

Before taking the transfection studies into rats, it was decided that more sensitive assays would be needed for detection of expressed eGFP. Both the GFP-ELISA and GFP-IHC protocols were modified to improve sensitivity. The new methods use the same antibodies as in the Western blot (Abcam #Ab290 rabbit anti-GFP and GE #NIF824 anti-rabbit-HRP). We first tested the GFP immunocytochemistry (ICC) assay to verify specificity of this antibody combination on fixed SHEP-1 cells transfected with pUGG and Lipofectamine-LTX (Figure 31). The brown DAB reaction product from GFP-ICC co-localizes well with the brightest green fluorescent cells, although not all DAB immuno-positive cells appeared to be fluorescent. The GFP-ICC assay therefore allows better detection of faint eGFP production than fluorescence microscopy.

Moving forward, it was necessary to determine the time at which protein expression was maximal in rat brain after administration of Copernicus’s pGDNF
PEG-CK30 NPs. This time course could not be tested *in vitro*. Previous work by Dr. David Yurek using the pGDNF construct found that a time-point of 7 days provided maximum expression of GDNF in rat brain after intracerebral injection into striatum, with levels gradually decreasing thereafter (Fletcher, 2011). We therefore examined this time-point along with 14 days post-administration to determine the time of maximum transfection and expression by pUGG in rat brain. GFP-IHC and GFP-ELISA were used to monitor expression.

Rats were stereotaxically injected with of 4 μL (17.6 μg) pUGG NP into their left striatum and were sacrificed 7 or 14 days later. Their forebrains were cut into 3 coronal sections and analyzed by GFP-ELISA (n=4). Control animals received saline injections (n=4). The 3 sections of rostral striatum were further blocked to isolate the side of the injection (A: frontal striatum, B: mid-striatum, C: caudal striatum). The ELISA data *(Figure 32)* showed modest GFP expression above background control levels in the striatum of rats injected with pUGG NPs, with a 2-way ANOVA showing a significant effect by treatment (p<0.0019). The frontal striatal sections (A) showed significance above control for both 7 days (p<0.01) and 14 days (p<0.01). There were no differences in eGFP levels between 7 and 14 days post-injection (Bonferroni post-test) *(Figure 32).*
eGFP expression was also assessed by IHC and fluorescence microscopy. GFP-IHC analysis at the needle track was done on sections from perfused rats given the same pUGG NP treatment and sacrificed either 7 or 14 days post-injection (Figure 33). GFP expression was found surrounding the needle track and sparsely in sites distal to the injection site. At higher power, it was clear that the labeling was cellular in nature. In addition, direct fluorescence microscopy on tissue from the same treatment group showed native eGFP fluorescence in a fine line at the needle track (Figure 34). The cell-types transfected by pUGG NPs could not be ascertained.
Figure #33: GFP-IHC in striatal tissue along needle track after injection of pUGG nanoparticles. Rats were injected with pUGG NPs into the left striatum and sacrificed 7 days (a, b) and 14 days (c-e) later. Cells expressing eGFP were found along the needle track and in some distal sites. eGFP expression was found in fiber-like projections which may be neurite extensions (a, e), although many transfected cells appear to be small, non-neuronal cells (b-d). BF = bright field microscopy; DIC = differential interference contrast microscopy. (Scale bars = 50 µm)
Although it was clear that eGFP was expressed in brain adjacent to the needle track after pUGG injection in vivo, it was not possible to use IHC to visualize the GDNF component due to the inability of GDNF antibodies to recognize the fusion protein. However, in previous studies GDNF produced from pUGG seemed to cause localized neurotrophic effects, i.e. increased survival of midbrain dopamine neurons in cultures, marked neurite outgrowth of N27 cells in culture, and fiber outgrowth in the rat striatum near the injection site. To assess whether pUGG caused a dopamine cell-specific neurotrophic effect in the injected striatum, tyrosine hydroxylase (TH)-IHC was performed on sections from the injected rats. TH-IHC revealed a dense band of staining along the injection track, indicating up-regulation of the enzyme in dopaminergic nerve terminals (Figure 35).
G. Intranasal delivery of pUGG nanoparticles successfully transfects cells and expresses eGFP throughout the entire brain

Having demonstrated that pUGG was capable of in vivo transfection and expression in rat brain after direct injection, we next attempted intranasal administration of pUGG nanoparticles or the naked plasmid to anesthetized rats (88 μg DNA; 20 μL in 2.5 μL increments alternating sides). Since the previous study did not find a detectable difference between the 7 or 14 day post-administration time-points for maximal eGFP expression, we selected a 7 day time-point for sacrifice of rats after intranasal pUGG.

Rat brains were divided into seven coronal slices (Figure 36). Expression in each section was determined by GFP-ELISA and compared with animals given naked pUGG. Greatest eGFP expression was found in Section A, which included the frontal cortex and olfactory bulbs (Figure 37). All other sections along the rostral-caudal axis (except...
Section E, the hindbrain) displayed a significantly higher expression of eGFP in rats treated with pUGG NPs versus those given the naked plasmid (Figure 38). The comparatively high level of eGFP found in Section A after intranasal treatment with the naked plasmid suggests that transfection can occur in rat brain even with the plasmid alone, albeit to a much lower extent than with NPs. These results demonstrated that intranasal delivery of Copernicus’ PEG-CK30 NPs can achieve transfection and significant expression of GDNF in the target areas for PD, i.e. the striatum and substantia nigra (SN), and provided the impetus to move forward to testing the approach in the 6-OHDA animal model of PD.

Figure #36:  **Rat brain regions assayed by GFP-ELISA after intranasal pUGG.**  Section A = olfactory bulb / frontal cortex; B = early striatum; C = mid- and late-striatum; D = midbrain; E = hindbrain; F = cerebellum; G = brainstem.
Figure #37: GFP-ELISA of olfactory bulbs (OB) / frontal cortex section of rat brain following intranasal administration of pUGG NPs vs. naked pUGG. GFP-ELISA results are shown for section A as described in the previous figure; graph compares pUGG NP-treated rats vs. naked pUGG-treated rats seven days post-administration. Greatest eGFP expression was detected in this most rostral section, although the variability between subjects limited the significance.

Figure #38: GFP-ELISA of sections spanning the rostral-caudal axis of rat brain following intranasal administration of pUGG NPs vs. naked pUGG. GFP-ELISA results are shown for sections B through G, as described in Figure 36. 2-way ANOVA indicated significant effect by treatment (p<0.0001) and by section (p<0.0056). Bonferroni post-tests showed significant differences between pUGG nanoparticles vs. naked pUGG 7 days after intranasal administration (*p<0.01, #p<0.05).
SPECIFIC AIM 3

H. Correlation of 6-OHDA lesion severity with SPECT imaging and autoradiography assessed by DAT radioligand binding

This thesis had the goal of developing a GDNF neuroprotective treatment for PD, and Aim 3 tested the approach in the rat 6-hydroxydopamine (6-OHDA) animal model of the disease. The 6-OHDA model is one of the gold standards for testing neuroprotective therapeutics for PD as the neurotoxin selectively destroys a percentage of SN dopamine neurons in a dose-dependent manner. A unilateral lesion causes a left-right difference in the brain that allows for the assessment of protection on the lesioned side as compared to the unlesioned (control) side. If we could monitor the lesion severity in the same rat over time, we would gain considerable insight into how a treatment affects lesion development, neuroprotection, and the time course of recovery, including the effect of multiple doses and the timing of doses. To assess whether single photon emission computed tomography (SPECT) imaging of the dopamine transporter (DAT) can be used to provide an accurate and sensitive in vivo measurement of lesion severity, we generated unilateral 6-OHDA lesions of varying severity and then compared left-right differences in striatal DAT radioligand binding using SPECT imaging and autoradiography. SPECT imaging analyzing DAT density after a 6-OHDA lesion was previously shown to correlate with TH-IHC dopamine cell counts in the SN (Scherfler, 2002) and dopamine content in the striatum (Gleave, 2011). Rats were lesioned with a series of 6-OHDA doses (0.5-2 µg/µL in 0.1% ascorbic acid) given in a 4 µL solution into the medial forebrain bundle. Left-right differences in striatal densities were determined three weeks later using the radioligand $^{125}$I-$\beta$-CIT [2$\beta$-carbomethoxy-3$\beta$-(4-iodophenyl)tropane],
which is selective for the DAT localized on the striatal nerve terminals. SPECT imaging was used to view and quantify the left-right differences in dopamine nerve terminals in live rats, and then within 2 hours of imaging, the animals were sacrificed and their brains were cut into ~1.5 mm coronal sections. The sections were mounted onto glass slides and placed on phosphorimaging plates. The plates were exposed for 1 hour on a Cyclone Phosphorimaging system (Perkin-Elmer). Left-right differences from SPECT analysis were then compared with autoradiography of brain slices to determine the degree of correlation between the two approaches.

Left-right differences in binding of $^{125}$I-β-CIT to DAT ranged from 4.3% to 78% when determined by SPECT imaging. Left-right differences in striatal $^{125}$I-β-CIT binding in the same brains analyzed by autoradiography ranged from 8.7% to 76% (Figure 39). A near perfect correlation ($r = 0.988$) was achieved between left-right differences in striatal $^{123}$I-CIT binding by SPECT imaging and autoradiography, confirming the ability of SPECT to accurately measure graded differences in lesion severity in the 6-OHDA lesion model of Parkinson’s disease (Figure 40).

Our results show that SPECT imaging of the DAT holds potential as a tool for longitudinal in vivo assessment of neuroprotective and regenerative treatments in small animal models of Parkinson’s disease. I originally proposed using this technology to evaluate the neuroprotective efficacy of intranasal pGDNF nanoparticles in 6-OHDA lesioned rats. However, the imaging center at Northeastern has since disbanded their NanoSPECT imaging component, and this approach could not be used to assess lesion severity in the 6-OHDA-lesioned rats of my thesis.
Figure #39: Left-right differences in striatal $^{125}$I-β-CIT binding determined by SPECT imaging and their corresponding autoradiograms. SPECT images (left) and autoradiograms (right) show reduced radioligand signal on the left side of coronal sections due to the 6-OHDA lesion. The top images were taken from a sham-lesioned rat. The remaining images were taken from rats given 6-OHDA lesions of varying severity, with increasing lesion severity displayed from top to bottom.

Figure #40: Left-right differences in striatal $^{125}$I-β-CIT binding determined by SPECT imaging and their correlation with those determined by autoradiography in the same brains. Left: The similarities between values from each method by animal number. Right: Left-right differences detected by the two methods showed a near-perfect correlation ($r=0.988$).
I. **Intranasal pGDNF NPs and naked pGDNF have efficacy in protecting dopamine neurons from a 6-OHDA lesion**

Before progressing to testing intranasal pGDNF in the rat 6-OHDA model, a study was performed in collaboration with Ph.D. candidate Amirah Aly to confirm that intranasal pGDNF treatments would increase GDNF expression above background levels in brain. In our previous experiments with intranasal pUGG, it was not possible to measure GDNF production directly by ELISA, so it was of interest to measure GDNF production by pGDNF. Rats were given intranasal saline (control), naked pGDNF or pGDNF NPs as before (88 μg DNA; 20 μL in 2.5 μL increments, alternating sides; n=6/group). Seven days later, coronal sections were collected as before (Figure 36) and analyzed by GDNF-ELISA (Figure 41). Results showed that both naked pGDNF and pGDNF NPs increased GDNF levels in brain compared to saline-treated controls. A 2-way ANOVA showed significant differences in GDNF levels by treatment (p=0.0005) and a 1-way ANOVA confirmed this difference (p<0.0005). Tukey’s post-test showed significant differences in GDNF expression between intranasal naked pGDNF and saline (p<0.001) and between pGDNF NP and saline (p<0.001). Unlike results with pUGG, pGDNF did not display greater protein expression in the olfactory bulb/frontal cortex region. However, the data did reveal that both intranasal pGDNF treatments significantly increased GDNF production across rostral to caudal brain regions, although the increases were not significant for individual sections. It is important to note that the levels of GDNF measured in this assay reflect endogenous GDNF as well as GDNF derived pGDNF. Taking into consideration the low basal levels in brainstem, the most caudal area showed the highest percentage increase above baseline of all regions.
Having confirmed that intranasal pGDNF increases GDNF levels throughout brain, the next studies tested the neuroprotective efficacy of intranasal pGDNF in 6-OHDA-lesioned rats. The pGDNF treatment groups were the same as in the previous study (n=7-8/group). Seven days after intranasal treatments, 6-OHDA (4 μL of 2 mg/mL at 1 μL/min; 8 μg) was injected into the left medial forebrain bundle. Desipramine (15 mg/kg, i.p.) was given 30 min prior to surgery to spare norepinephrine neurons from the insult. Rats recovered for three weeks, and they were then behaviorally assessed using an amphetamine rotational challenge. Amphetamine (5 mg/kg, i.p.) causes rotational behavior due to the imbalance of dopamine being released from striatal nerve terminals on the lesioned versus unlesioned side. Rats rotate ipsilateral to the lesion (counter-clockwise), with severe lesions generally correlating with a high degree
of rotation frequency. Immediately after the amphetamine challenge, rats were sacrificed via transcardial perfusion and their brains were processed for TH-IHC. See Figure 42 for a summary of the experimental design.

In previous studies, overexpression of GDNF has been found to cause significant weight loss in subjects, and this has been thought to indicate an action mediated outside the nigrostriatal pathway or from excessive doses (Zhang, 1997; Manfredsson, 2009). As such, it was important to examine whether pGDNF treatments caused weight loss. Rats were weighed before their intranasal treatment and four weeks later before sacrifice. Results show that no significant weight loss occurred from pGDNF treatment, with the pGDNF NP treated rats actually gaining significantly more weight compared to saline controls (p<0.05) and those given intranasal naked pGDNF (p<0.01; Figure 43).

**Figure 42: Timeline of the intranasal pGDNF efficacy study.** Rats (n=6-8/group) were pretreated with intranasal doses of either saline, naked pGDNF or pGDNF NPs. One week later, a 6-OHDA lesion was made in the left medial forebrain bundle. Lesion stabilization occurred over three weeks. Rotational assessment after amphetamine took place just prior to sacrifice. Brains were collected and the SN and striatum were assayed for TH-IHC.
To monitor the rotational response to amphetamine challenge, rats were placed in a large bucket 15 min after an injection of d-amphetamine (Figure 44a). Net ipsilateral rotations were calculated from video recordings during the following 30 min interval after amphetamine was given. Results showed that rats given intranasal saline generally had the greatest ipsilateral rotational response to amphetamine, although there was considerable variability in this measure with several of the rats exhibiting little net rotation (Figure 44b). One-way ANOVA did, however, show a significant effect by treatment (p=0.011) and Tukey’s post-test found significance between intranasal saline vs. naked pGDNF (p<0.05) and saline vs. pGDNF NP (p<0.05). This result suggests that both pGDNF NPs and naked pGDNF had a marked ability to attenuate the dopaminergic...
imbalance caused by a unilateral 6-OHDA lesion. This is consistent with a neuroprotective effect of intranasal pGDNF in this model.

**Figure #44:** Amphetamine-induced rotations following 6-OHDA unilateral lesions. (a) Rats were placed in a bucket with a video camera mounted above to record rotation following the amphetamine challenge. (b) Net rotations were counted during a 30 min interval following *d*-amphetamine (5 mg/kg, i.p.) challenge. Saline control rats had the greatest rotational response to amphetamine. One-way ANOVA showed a significant effect by treatment (*p*=0.011), and Tukey’s post-test revealed significance between saline vs. naked pGDNF (*p*<0.05) and saline vs. pGDNF NP treatment groups (*p*<0.05).

After completion of the behavioral assessment, rats were sacrificed by transcardial perfusion and sections from both the striatum and SN were collected for TH-IHC. Bioquant® image analysis software was used to calculate TH staining density from both the lesioned (L) and unlesioned (R) SN from six representative sections per rat. The integrated optical density on the lesioned side versus the unlesioned side was subtracted from 100 to determine the % lesion. Results showed a significant reduction in % lesion in the pGDNF NP group compared to saline controls, indicating a large
reduction in lesion severity in the SN of rats given pGDNF NPs (Figure 45). The neuroprotective effect was significant by one-way ANOVA (p=0.0081), and Tukey’s post-test showed significance between pGDNF NP vs. saline-treated rats (p<0.01).

Figure 46 shows representative images of the SN of rats from each treatment group, illustrating the average 77.7% ± 7.4% lesion in saline-treated rats (top), the 45.0% ± 12.1% lesion in rats given naked pGDNF (middle), and 28.9% ± 10.5% lesion in rats given pGDNF NPs (bottom).

Figure #45: Percent lesion comparing the lesioned vs. unlesioned SN in rats treated with intranasal pGDNF or saline. Six representative sections from the SN of each rat were analyzed for TH staining density using Bioquant® integrated optical density (IOD) methods. The IOD of the lesioned (L) side was divided by that of the unlesioned side (R) and then subtracted from 100 to calculate the % lesion. One-way ANOVA indicated a significant effect from treatment (p=0.0081) and Tukey’s post-test showed significance for the pGDNF NP group vs. saline control (*p<0.01). The right panel shows the individual values for each rat.
**Figure 46**: Representative TH-IHC images of the ventral midbrain of 6-OHDA-lesioned rats used for SN density and dopamine cell count analysis. The 6-OHDA lesioned (L) side was compared to the unlesioned (R) side. Saline controls showed the overall greatest lesion severity. Naked pGDNF provided a substantial, but non-significant attenuation of lesion severity using TH staining density as the measure. Intranasal pGDNF NP provided the greatest neuroprotection, with a significant reduction in lesion severity relative to saline-treated rats.
In addition to the TH immunostaining density measures, dopamine cell counts were also done in the SN of the lesioned rats to further assess the neuroprotective effect of the pGDNF treatments. **Figure 47** shows that both the intranasal naked pGDNF and pGDNF NP provided significant protection of dopamine cells from a 6-OHDA lesion. Saline control rats had an average loss of 72.3% ± 9.3% of dopamine cells. However, this was reduced by intranasal naked pGDNF to 42.6% ± 7.6%, and by pGDNF NPs to only 20.0% ± 6.8%.

**Figure 47**: Percent dopamine cell loss comparing lesioned vs. unlesioned SN dopamine neurons in rats treated with intranasal pGDNF or saline. Four representative sections spanning the SN for each rat were examined. TH+ cell bodies were counted using ImageJ cell counting software. The cell count from the lesioned (L) side was divided by the unlesioned side (R) and then subtracted from 100 to calculate the % cell loss. One-way ANOVA indicated a significant effect from treatment (p= 0.0012) and Tukey’s post-test showed significance for the pGDNF NP group vs. saline control (*p<0.001) and for naked pGDNF vs. saline control (#p<0.05). The right panel shows the % cell loss results for each rat.

TH-immunostaining densities were also quantified from striatal sections in each treatment group. Alexandra Christodoulou assisted in this measure. Densities were determined in Bioquant® using five non-overlapping circular areas placed randomly throughout each striatum, and the values from four sections spanning the entire rostral-
caudal striatum were averaged for each rat. Both intranasal naked pGDNF and pGDNF NPs significantly protected striatal dopamine nerve terminals from 6-OHDA (Figure 48). The reduction in lesion severity was significant by one-way ANOVA (p=0.0008), and Tukey’s post-test showed significance between saline vs. naked pGDNF (p<0.05) and between saline vs. pGDNF NP (p<0.001). Figure 49 shows representative striatal images illustrating the neuroprotection effect. There was an average 83.1% ± 6.3% lesion in saline-treated rats (top), a 50.7% ± 10.0% lesion in naked pGDNF-treated rats (middle), and a 32.8% ± 4.8% lesion in pGDNF NP-treated rats (bottom). In summary, pretreatment with intranasal pGDNF 1 week before a 6-OHDA lesion resulted in a significant neuroprotective effect on dopamine cells in every measure considered. These results confirm the potential of intranasal pGDNF as a strategy for treatment of PD.

**Figure #48:** Percent lesion comparing lesioned vs. unlesioned striatum in rats treated with intranasal pGDNF or saline. Four sections throughout the striatum of each rat were analyzed for TH staining density using Bioquant® IOD. The lesioned (L) side was divided by the unlesioned side (R) and then subtracted from 100 to calculate the % lesion. One-way ANOVA indicated a significant effect from treatment (p=0.0008) and Tukey’s post-test showed significance for the pGDNF NP group vs. saline control (*p<0.001) and for naked pGDNF vs. saline control (#p<0.05). The right panel shows the % lesion for each rat.

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Figure #49: Representative images of the striatum of 6-OHDA-lesioned rats stained for TH. The 6-OHDA lesioned-side (L) was compared to the unlesioned side (R). Saline controls showed the overall greatest lesion severity. Naked pGDNF provided a significant protection of staining density. pGDNF NP treated-rats showed the greatest protection of TH staining density, with a large and significant reduction in lesion severity relative to saline-treated rats.
V. DISCUSSION

In Parkinson’s disease (PD), the dopamine neurons of the nigrostriatal pathway degenerate due to a combination of genetic susceptibility, aging, environmental insults and lack of trophic support (Le, 2009). Current pharmacotherapies do not address the underlying death of these neurons, only providing temporary symptomatic relief. Glial cell-line derived neurotrophic factor (GDNF) was discovered to be a protein critically involved in the maintenance of dopamine neurons, providing essential neurotrophic support from early postnatal development into adulthood (Lin, 1993; Paratcha, 2008; Pascual, 2008). Since its discovery, GDNF has consistently shown the ability to protect dopamine neurons from a variety of insults and to have efficacy in various animal models of PD (Schapira, 2009) and some human clinical trials (Gill, 2003). Almost all of these approaches required invasive surgical delivery of GDNF directly to the brain, as it cannot cross the BBB. The first successful clinical studies found efficacy in continuous delivery of GDNF through a catheter system (Gill, 2003). However, this method was found in subsequent studies to cause many device-related complications (Lang, 2006).

Gene therapy has the potential to provide the benefits of continuous delivery of GDNF in a single administration. Neuroprotective and neurorestorative effects toward dopamine neurons have been demonstrated using many types of viral vectors encoding GDNF: adeno-associated virus (Björklund, 2000), lentivirus (Georgievskka, 2004), herpes simplex virus (Natsume, 2001), and adenovirus (Björklund, 2000). Although viral vectors are effective, our incomplete knowledge of viral behavior and genetics interferes with the ability to use them without concern about fatal immune responses or oncogenic potential due to incorrect genetic insertion (Thomas, 2003). Therefore, two areas of
research that require extensive focus for CNS gene therapy to be successful are in the
development of effective non-viral vectors and the application of them in a non-invasive
manner. The blood-brain barrier is the main obstacle for non-invasive gene therapy to
the CNS, and finding ways to bypass it would allow an unprecedented opportunity for
treatment options (Frey, 2002).

Intranasal delivery offers a largely unexplored and unexploited means by which
larger molecular weight therapeutics can gain direct access to the brain. Numerous
groups have shown success utilizing the intranasal route to deliver proteins, siRNA, viral
vectors and even stem cells to the brain (Liu, 2001; Danielyan, 2009; Migliore, 2010;
Jiang, 2012; Renner, 2012; Reitz, 2012). Our lab's previous work showed that intranasal
delivery of GDNF is neuroprotective in the 6-OHDA model of PD and that a multiple
dosing strategy provided the most protection against the lesion (Bender, 2009; Migliore,
2009). However, successful intranasal gene therapy for brain disorders, such as PD,
using non-viral vectors has not been previously reported. Demonstrating the feasibility
of this approach was the goal of this thesis.

Using Copernicus’ PEG-CK30 vectors and expression plasmids, we showed that
brain-wide expression was possible through intranasal delivery of pCG, a reporter
plasmid with no therapeutic value but useful for establishing proof of principle.
However, the plasmid contains a different promoter than the pGDNF plasmid to be used
in later studies. In addition, one goal of the thesis was to design lipoplex formulations
and to test them in vitro, with the aim of selecting the best vector for in vivo transfection
experiments. For these studies, it was also desirable to use a plasmid with properties
similar to the pGDNF plasmid. The fusion construct, pUGG, which produces both eGFP
and GDNF, was created by Linas Padegimas at Copernicus in order to provide a construct with the benefits of having reporter protein expression along with GDNF expression. Experiments were done with pUGG lipoplexes and PEG-CK30 NPs to determine which formulation to take forward for intranasal delivery to the CNS in rats. Copernicus’ pGDNF NPs were ultimately chosen as the vector for in vivo efficacy studies in the rat 6-OHDA model, a common animal model of PD. The major findings in each component of the project are discussed below.

A. Intranasal pCG nanoparticles transfect cells throughout rat brain

For a non-viral gene therapy approach to ultimately succeed clinically in treating disorders of the brain, the nanoparticle construct would have to be capable of transfecting cells that allow expression of the protein of interest in the target regions affected by the disease process. To provide proof-of-principle, we used a plasmid (pCG) encoding the enhanced green fluorescent protein (eGFP) gene under the cytomegalovirus (CMV) promoter. Direct injection of pCG NPs into the rat striatum resulted in a clear and obvious expression of eGFP in cells near the needle track one week after injection. eGFP was visible both by direct fluorescence and by GFP-IHC, although the latter means of detection was more sensitive in revealing transfected cells. Nasal delivery of pCG caused widespread expression of eGFP throughout brain, with the nanoparticle treatment group greatly exceeding that of naked pCG plasmid. The largest increases in eGFP expression from pCG NP treatments were in the far rostral sections of brain (encompassing the olfactory bulbs and frontal cortex) and the far caudal sections (encompassing the hindbrain, cerebellum and brainstem). This pattern is consistent with the findings of
Thorne et al. (1995 & 2004) who showed that molecules administered by the nasal route travel along the olfactory and trigeminal nerves to achieve their highest levels in the forebrain and brainstem, respectively.

An unexpected result was that intranasal delivery of the naked pCG plasmid also resulted in protein expression in brain, as detected by GFP-ELISA. This was unusual for several reasons. First, the nasal environment is host to numerous metabolic and degradative enzymes that would be expected to reduce the amount of intact DNA reaching the brain from the nasal cavity (Wong, 2010). Presumably, the large concentration of pDNA administered intranasally was sufficient to offset this effect to some extent. However, eGFP expression by naked pCG plasmid seemed to exceed background levels only in the olfactory bulb and prefrontal cortex, the areas closest to the nasal cavity. This may be due to a combination of factors, i.e. a lower transfection efficiency of the naked plasmid as well as its inability to reach more distal sites in the brain, perhaps due to inactivation by DNAses along the transport pathway. Second, the fact that naked pCG was capable of transfection, albeit to a much more limited extent than NPs, calls into question the role of nucleolin, and highlights the lack of comparability of in vitro and in vivo transfection studies. PEG-CK30 NPs are thought to achieve transfection primarily through nucleolin receptor-mediated endocytosis. This novel mechanism has been examined in recent years, although it is still incompletely understood. Chen et al. (2008 & 2011) discovered that the nucleolin-NP interaction in HeLa cells initiates formation of a dynein motor complex with glucocorticoid receptor to directly shuttle the NP into the nucleus. However, whether nucleolin is absolutely required, its exact role, and its distribution in brain are still undetermined. Nucleolin may
play an important role in determining which cell-types are transfected by PEG-CK30 nanoparticles, but it may not be obligatory for transfection of others. Indeed, transfection with naked pCG may not be reliant on nucleolin at all but may be mediated by a different transfection mechanism. Future studies will be needed to determine the requirements and limitations of both PEG-CK30 nanoparticles and naked plasmid DNA for transfection in brain, and to delineate the factors which influence transfection of cells in brain after nasal delivery.

Although GFP-ELISA revealed significant expression along the rostral to caudal axis of brain after intranasal pCG NPs, no specific section was found to have significant eGFP expression above the naked pCG or saline treatment group. GFP-IHC and direct fluorescence microscopy both revealed that eGFP expression was diffuse and sporadic, but labeled cells were found primarily around capillaries and in clusters. Moreover, these labeled cells were much more common in rats given pCG NPs than the naked plasmid, suggesting higher transfection rates with NPs. Considering the hypothetical means of distribution of intranasally administered substances by perivascular flow (Hadaczek, 2006), the cells that are likely to first interact with the NPs as they travel in the brain might be those lining perivascular spaces. These cells include the major constituents of the blood-brain barrier, including endothelial cells, pericytes and astrocytic endfeet (Figure 50).
When GFP-immunostained cells were quantified per section, the number of GFP-positive cells was highest in the midbrain but similar increases were seen in other areas. It is not clear to what extent non-specific staining in these sections may have impacted the results from the cell counting study. Many cells in brain, including those in the vasculature, express endogenous peroxidases that can convert the DAB substrate into the insoluble brown stain. In the IHC protocol, hydrogen peroxide is used to inactivate peroxidases in tissue, thereby reducing non-specific background staining. However, it is possible that a certain percentage of stained cells may have been false positives due to incomplete inactivation of vascular peroxidases. Regardless, the total number of stained cells was significantly higher in the pCG NP group compared with the naked pCG- and saline-treated groups.

The pattern of cellular labeling suggested that transfection and expression of eGFP seemed to occur primarily in non-neuronal cells. Previous work by Yurek et al. (2009b) found that direct intracerebral injection of PEG-CK30 nanoparticles that utilized the CMV promoter resulted in transfection of both neurons and astrocytes at the earliest time-points examined. However, expression by neurons decreased at later time points,
suggesting that neurons may have a mechanism that eventually inactivates or reduces the efficiency of the CMV promoter. Neither the Yurek et al. (2009a) study nor our own determined whether non-neuronal, non-glial brain cells could be transfected by PEG-CK30, such as the aforementioned cells comprising the BBB. Studies are currently underway to examine this issue.

Transfection by PEG-CK30 nanoparticles may be occurring at any point throughout the intranasal pathway. Although GFP-IHC provided substantial qualitative evidence that cells in brain are expressing eGFP and are thus being transfected by pCG NPs, the transfection patterns do not accurately match that of GFP-ELISA and the numbers of eGFP-positive cells are sparse compared to what might be expected given the amount of eGFP measured. This presents the possibility that cells lining the olfactory or trigeminal nerves may be preferentially transfected even before the entire dose reaches the brain. These cells might serve as a depot for expression of protein and could result in a similar distribution throughout brain. It is known that eGFP can be secreted through non-classical pathways in mammalian cells (Tanudji, 2002), so this mechanism could contribute to the detection of eGFP even in areas where cellular transfection and protein expression were limited.

Although many CNS disorders would benefit specifically from neuronal expression of a therapeutic protein, the lack of long-term expression by neurons would not inherently be a limitation of this approach for our application. For example, any cell that is able to be transfected by pGDNF should also be able to secrete the protein and thus provide trophic support to nearby neurons. In Parkinson’s disease, where there is a deficit in key neuronal populations, reliance on specialized expression in the neurons
affected by the disease would not be the ideal approach. Expression by non-neuronal cells in the areas affected by the disease would be a preferred strategy.

In summary, these studies represent the first demonstration that intranasal delivery of Copernicus’ PEG-CK30 nanoparticles can lead to the expression of a protein of interest in rat brain. This non-invasive approach was proof-of-principle for continued use of these vectors throughout the experiments of the thesis work, with the ultimate goal of intranasal delivery of pGDNF NPs for expression of GDNF in the 6-OHDA animal model of PD.

B. pUGG expresses eGFP and GDNF as a single fusion protein

The pUGG plasmid was designed to use the UbC promoter that the pGDNF plasmid has while producing both eGFP and GDNF as separate proteins. SHEP-1 cells transfected with pUGG using Lipofectamine-LTX had greatly reduced expression of eGFP relative to the pCG plasmid. This was expected from the different nature of the promoters. UbC is a weaker expressing promoter that has been shown to allow for protein expression for longer than 6 months after intrastriatal injection using pGDNF NPs (Fletcher, 2011). The longer expression profile of the UbC promoter makes pGDNF and presumably pUGG capable of long-term expression by transfected cells, a particular advantage for our use.

Further characterization of pUGG in ventral midbrain cultures and N27 dopaminergic cells revealed an inability of GDNF-ELISA to detect GDNF after transfection, although there was a clear neurotrophic effect in both cell culture systems. For example, the number of TH-positive neurons was increased in midbrain cultures
transfected with pUGG, and N27 cells showed evidence of neurite outgrowth, both indicative of GDNF-like bioactivity. However, GFP-Western blot analysis on cell lysates after pUGG transfection lead us to the conclusion that the expressed protein was not being cleaved but produced as a single fusion construct. We thus concluded that the fusion protein retains the fluorescent properties of eGFP and the neurotrophic capabilities of GDNF, but is only able to be immuno-assayed by GFP-ELISA. The most likely explanations are either: a) eGFP expressed by pUGG directly blocks a GDNF antibody recognition site, or b) the extended GFP-GDNF construct abolishes the tertiary conformation necessary for recognition by the GDNF antibodies. Since GDNF normally exists as a homodimer, a non-denaturing GFP-Western blot may reveal whether the expressed fusion protein also dimerizes. This would present another potential reason for interference in the GDNF-ELISA.

C. Lipoplex formulations of pUGG failed to transfect SHEP-1 cells

One goal of this thesis was to compare Copernicus’ PEG-CK30 NPs with our own cationic lipoplex formulations to assess the differences in transfection and expression by each. Lipoplexes suffer from a lack of transfection efficiency, stemming from their inability to overcome the numerous barriers which prevent delivery of their DNA cargo to the nucleus (Thomas, 2003). For this reason, liposomes may be better suited for targeted delivery of siRNA or proteins, which do not require nuclear entry. Having characterized the pUGG plasmid, the next stage of work was to generate a lipoplex formulation that was effective for transfection and gene delivery. The formulation with
the greatest transfection efficiency would be taken forward to the intranasal delivery experiments.

For this study, dioleoylphosphatidylcholine (DOPC) and cholesterol were used as the “helper lipids” while stearylamine was used as the cationic lipid. The ratios used in previous studies in our lab for intranasal delivery of proteins were a 50:30:5 molar ratio of DOPC:cholesterol:stearylamine (Migliore, 2006; Migliore, 2007; Bender, 2009; Migliore, 2009; Migliore, 2010). In contrast to these past studies, which involved liposomal delivery of proteins, the current goal was to generate lipoplexes that were moderately cationic even after complexation with negatively charged pDNA. As such, this initial formulation, with a zeta potential of ~5.5 mV without pDNA, was deemed to be not cationic enough for use in compacting pDNA. The molar ratios were therefore changed to 50:30:50 (DOPC:cholesterol:stearylamine) thus increasing the cationic stearylamine component 10-fold. In addition to the molar ratios of lipids, the ratio of the amount of pDNA to the cationic lipid (known as the N/P ratio) is a critical factor in lipoplex formulation. N/P ratios ranging from N/P=1 (highest relative amount of pDNA) to N/P=20 (highest relative amount of cationic lipid) were tested using pUGG. Due to the higher cationic density of the lipids, increasing the N/P caused a consistent decrease in particle size while increasing the zeta potential. However the zeta potential values, which ranged from +34 mV to +45 mV, were highly cationic and outside the “ideal” range for transfection. By comparison, Lipofectamine-LTX complexed with pUGG had a zeta potential of +23.3 mV (data not shown).

SHEP-1 transfection using all lipoplex formulations of pUGG at the 50:30:50 molar ratio (DOPC:cholesterol:stearylamine) resulted in a complete lack of transfection.
This overall failure to achieve transfection may have been due to the relatively high concentration of stearylamine in the formulations, which either prevented the pUGG from escaping the endosome before being degraded in the lysosome, or the cationic lipid formed too strong of a complex with the pDNA to allow pUGG to enter the nucleus.

One additional lipoplex formulation was created that lowered the relative cationic strength of stearylamine to 50:30:30 (DOPC:cholesterol:stearylamine). However, this formulation tested at the same N/P ratios in SHEP-1 cells also failed to induce transfection. Although these studies were not successful in designing a candidate lipoplex formulation for in vivo testing, research into a range of non-viral vectors for transfection of brain should continue in order to improve upon the efficiency of transfection using nanoparticle delivery systems.

D. pUGG nanoparticles transfect cells throughout rat brain after intranasal administration

Before testing intranasal pUGG NPs in the rat 6-OHDA model, it was necessary to know if the naked plasmid and NPs could transfect cells in brain, and if so, the optimal time-point for maximal protein expression. The pUGG plasmid utilizes the long-acting UbC promoter. As mentioned previously, work in Dr. Yurek’s lab showed that the UbC plasmid encoding GDNF had the ability to generate protein in rat brain for an extended period of time, i.e. greater than 6 months. Peak GDNF expression occurred at his initial time-point, 7 days after direct injection of pGDNF NPs into rat striatum (Fletcher, 2011). Our own striatal injection studies of pUGG NPs showed similar eGFP expression along the injection track at both 7 and 14 day time-points. In addition, GDNF appeared to exert
a neurotrophic effect on surrounding neurons in striatum, as indicated by neurite extensions adjacent to the immunostained cells and a marked increase in TH-immunostaining density in dopaminergic nerve terminals along the needle track compared with the un-injected side. The small number of labeled cells and the modest increases in striatal eGFP detected by ELISA at both time-points may have been due to modest spread of injected volume in tissue and the comparatively large amount of tissue assayed.

Since expression was similar and presumably maximal at both 7 and 14 days, 7 days was chosen as the time-point for intranasal delivery studies. GFP-ELISA was used to assess the distribution of eGFP in seven coronal sections spanning the entire brain after intranasal administration of pUGG NPs or naked pUGG plasmid. In rats treated with intranasal pUGG NPs, eGFP expression was ten-fold higher in the most rostral section, which included the olfactory bulbs and frontal cortex (section A), than in rats given naked pUGG. However, there was considerable variability among rats given pUGG NP, so eGFP levels measured in section A were not significantly different from those in rats given naked pUGG. Indeed, treatment with the naked plasmid showed greater eGFP expression in this section than that observed for pUGG NPs or naked pUGG in most of the remaining more caudal brain areas. This evidence of transfection by the naked plasmid was similar to the results of the intranasal pCG study. This again suggests that, in high enough doses, plasmid DNA is able to transfect cells without the aid of vectors. It also brings to light again our limited understanding of the mechanisms of transfection by naked pDNA in vivo. Relevant to this point, other researchers have reported successful transfection after direct injection of naked pDNA directly into muscle tissue,
however, liposomal and other nanoparticle vectors greatly increased transfection efficiency (Wolff, 2005; Yurek, 2009a).

All other brain sections caudal to section A (except for one, hindbrain) showed significant eGFP expression after intranasal pUGG NP treatment vs. naked pUGG. The significant levels of protein expression found throughout the brain after a single intranasal dose of pUGG NPs was a monumental feat that was not observed in the intranasal pCG study. Notably, the amount of eGFP expression detected by ELISA after intranasal pUGG delivery was far above that observed after striatal injection. The higher level of expression may be due to the larger dose of pDNA used with intranasal delivery, or it may reveal that intraparenchymal transfection after direct injection is limited compared with the widespread transfection of cells in the nasal epithelium and/or vascular endothelium of the brain after nasal administration. In addition, the cells which are transfected after nasal delivery may be more adapted for secretion of the expressed protein than neurons or glial cells, making them better targets for gene therapy vectors. Whatever the reasons, the stark differences in expression after direct injection and intranasal delivery of pUGG have important implications for gene therapy of brain disorders. A higher level of transgene expression in brain may be achievable with intranasal administration than with direct injection. Moreover, the fact that transfection occurs throughout the brain offers advantages in cases where protein expression would be desirable in multiple brain areas affected by a neurodegenerative disease, such as the striatum and SN in Parkinson’s disease. On the other hand, the widespread nature of the expression may present disadvantages such as off-target effects. Balancing these pros and cons will be a necessary step in translating these results into a clinical application.
In summary, these studies with pUGG provided proof-of-principle that intranasal delivery of PEG-CK30 NPs (and to a lesser extend, naked plasmid), can produce widespread transfection and protein expression in brain. With that, we were confident in moving forward with the efficacy studies in the 6-OHDA model. Copernicus’ pGDNF construct was chosen for these studies to avoid any possible loss of efficacy or other confounds that might occur from expression of the GFP-GDNF fusion protein.

**E. SPECT imaging correlates with autoradiography for in vivo quantification of 6-OHDA lesion severity**

At the outset of this research, it was anticipated that efficacy of intranasal pGDNF treatments in the 6-OHDA-lesioned rats would be assessed by two means: *in vivo* SPECT imaging of the dopamine transporter on striatal nerve terminals and *ex vivo* analysis of TH immunohistochemical staining in the SN and striatum. SPECT imaging offers a means of assessing the efficacy of treatments *in vivo* as they develop, whereas TH-IHC data must be collected post-mortem at a single time-point. Observing the progress of a treatment’s ability to ameliorate a disease state would provide an opportunity to gauge the impact of a treatment over time, and guide decisions regarding re-dosing. PET and SPECT technology (with MRI) have progressed to a point of being able to precisely localize a radioligand’s binding and measure the intensity of its signal. We compared this to classical autoradiography to gauge the sensitivity of SPECT in measuring the left-right differences in rats with unilateral 6-OHDA-lesions. Using the radioligand $^{125}$I-β-CIT to label the dopamine transporters in the striatum, rats were assessed after being given unilateral 6-OHDA lesions of varying severity using our standard protocol
(Migliore, 2009). We found a near perfect correlation of the left-right differences in SPECT compared to autoradiography, confirming the potential for SPECT imaging to be used in this project for longitudinal in vivo assessment of neuroprotection following intranasal pGDNF treatments. However, the Center for Neuroimaging at Northeastern subsequently closed their SPECT imaging facility, making this component of the project impossible. TH-IHC remained the sole means by which neuroprotection from pGDNF was assessed.

F. **Intranasal pGDNF provides neuroprotection in the rat 6-OHDA model of PD**

The final studies of this thesis focused on evaluation of the neuroprotective efficacy of intranasal pGDNF in the unilateral 6-OHDA model. As before, a preliminary study was performed to verify that GDNF protein expression was increased in brain 7 days after intranasal delivery of pGDNF. GDNF-ELISA revealed significantly higher GDNF expression throughout the brain in pGDNF NP-treated animals relative to that observed in rats given intranasal naked pGDNF or saline. The elevated levels of GDNF were a general effect of treatment, although no particular section showed significance. The seemingly more modest increase in protein expression compared with that observed with intranasal pUGG may be due to the fact that GDNF is endogenously expressed in brain and the additional amounts derived from pGDNF transfection are a small percentage of the total present in each sample. Endogenous GDNF brain levels could also be highly variable due to factors such as age (Georgievskia, 2004), further complicating detection of that which was derived from pGDNF. Finally, some proportion of GDNF could be bound to receptors, preventing its detection by ELISA.
Despite these mitigating factors, both intranasal pGDNF treatments significantly increased whole-brain GDNF expression. Since levels of GDNF needed for neuroprotection are known to be in the picomolar range (Lin, 1994), even modest increases in GDNF, especially if sustained over weeks to months, could be sufficient to protect dopamine neurons from damage and cell death.

When intranasal pGDNF NPs and naked plasmids were assessed for efficacy in the unilateral 6-OHDA lesion model, results confirmed the ability of both pGDNF treatments to provide significant protection against the effects of the lesion. This protection was revealed both behaviorally and by TH-IHC of dopamine cells within the SN and their nerve terminals in the striatum. Considering first the behavioral evidence of neuroprotection, intranasal pGDNF (naked or NPs) significantly reduced amphetamine-induced ipsilateral rotations compared to saline controls. Although there was considerable variability in this measure, the data do demonstrate that intranasal pGDNF abolishes behavioral evidence of the 6-OHDA lesion.

Next, considering the IHC evidence of neuroprotection, Table 3 shows the average percent lesion in the SN and striatum for rats pretreated with intranasal saline, naked pGDNF, or pGDNF NPs 7 days prior to 6-OHDA. Several observations can be made from the data in the Table. First, comparing the integrated optical density of TH staining in the SN with dopamine cell counts, it is clear that these two measures were consistently very similar within each treatment group. For instance, in the intranasal saline group, the percent lesion was 77.7% using TH staining density and 72.3% using cell counts. This pattern was also observed in the naked pGDNF and pGDNF NP groups, although the treatments attenuated lesion severity to different degrees. This suggests that
a close correlation exists between these two measures of dopamine cell integrity and lesion severity. Second, the percent lesion in the SN was generally consistent with that in the striatum within each treatment group, with only slightly higher values for the percent lesion in striatum versus SN. This confirms the internal consistency of the 6-OHDA lesioning protocol, the intranasal treatments, and the immunohistochemical methods used to detect the effects of the intranasal treatments. The similarities seen with the three TH-IHC measures also indicate that the lesion causes parallel changes in dopaminergic functional status at both cell bodies and nerve terminals. Finally, the most important outcomes of the study are that intranasal pGDNF significantly reduces lesion severity by every measure considered. Copernicus’ pGDNF NPs provided roughly twice as much neuroprotection as the naked pGDNF. These results confirm the tremendous potential of intranasal pGDNF, and the nanoparticle vector, as means of protecting and rescuing dopamine neurons from a severe neurotoxic insult.

<table>
<thead>
<tr>
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<th>% lesion ± S.E.M.</th>
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<tr>
<td>Saline</td>
<td>Naked pGDNF</td>
</tr>
<tr>
<td>SN TH density (IOD)</td>
<td>77.7% ± 7.4%</td>
</tr>
<tr>
<td>SN dopamine cell count</td>
<td>72.3% ± 9.3%</td>
</tr>
<tr>
<td>Striatum TH density (IOD)</td>
<td>83.1% ± 6.3%</td>
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*Table #3: Average percent lesion assessed by TH-IHC in each treatment group.* The % lesion represents the value on the 6-OHDA-lesioned side divided by that on the unlesioned side, subtracted from 100. TH density in the SN and striatum are the integrated optical density (IOD) of staining in each region. Dopamine cell counts were determined in the SN of the same sections used for TH density analysis.

Unlike current symptomatic treatments for PD, a gene therapy approach aimed at increasing expression of a neurotrophic factor in the nigrostriatal system provides a direct means of counteracting the underlying processes responsible for damage and the loss of
dopamine neurons. This approach may also be applied to numerous other CNS disorders where GDNF has been shown to be of benefit, including treatment of addiction (Carnicella, 2009), ischemic stroke (Kilic, 2003), and age-related obesity (Manfredsson, 2009). Taking this even further, intranasal delivery may provide a means of delivery of other gene therapy vectors to the CNS, enabling production of numerous therapeutic proteins with applications well beyond Parkinson’s disease.

G. Future considerations regarding intranasal gene therapy

Although many factors intertwine to influence transport of molecules or nanoparticles to the brain by the intranasal route, size of the vector seems to not be very limiting. As mentioned previously, even mesenchymal stem cells were shown to reach the brain after intranasal administration, and cells are far larger than any nanoparticle formulation (Danielyan, 2009). Most sources concur, however, that transport by the nasal route is extremely inefficient. Intranasal administration results in delivery to brain of less than 1% of the total dose (Illum, 2003; Thorne & Frey, 2001; Thorne, 2008). In fact, Thorne estimated that only 0.006% to 0.02% of intranasally administered β-interferon and nerve growth factor reached the brain, respectively (Thorne, 2008; Thorne & Frey, 2001). Although low transport efficiency may be considered a limitation for nasal delivery of proteins, successful gene transfection has the potential to generate long-lasting production of the desired protein, offsetting this limitation. Even if relatively few cells in brain are transfected, they may generate enough protein to provide trophic support to neighboring neurons, especially if the potency of the protein is very high. In addition, the low transport efficiency brings to light another unique advantage of the
nasal route, avoidance of systemic absorption. The remainder of the nasally administered dose is mostly swallowed (Chien, 1989; Migliore, 2010) and will typically be degraded and eliminated through the GI track. Taken together, it is clear that the method of nasal delivery, dose and formulation are all important variables determining successful delivery of a gene therapy vector to the brain, and each of these should be further studied to optimize the overall concentration of pDNA that is able to reach and transfect cells in the brain.

The ability of the intranasal route to deliver a gene therapy vector to the brain is entirely dependent on the overall transfection efficiency of the expression plasmid and the nanoparticle vector. The success of this project is largely due to the continued progress of Copernicus Therapeutics to improve their PEG-CK30 vectors and engineer plasmids that ensure maximum protein expression. Also important is the ability of the vector to penetrate barriers. This is critical in tissues where the target sites lie below a mucus layer. The mucus barrier at the nasal epithelium presents an obstacle for intranasally administered substances to gain access to the brain. Mucus penetrating particles (MPPs), which have a coating of low molecular weight polyethylene glycol (PEG), have been shown to rapidly penetrate mucus (Lai, 2009; Ensign, 2012) and even facilitate penetration of nanoparticles within brain (Nance, 2012). Copernicus’ PEG-CK30 NPs should therefore also benefit from the trans-mucosal properties of PEG, thereby promoting intranasal delivery by facilitating penetration through the mucus layer surrounding the nasal epithelium (Vila, 2005).

One potential limitation to the intranasal gene therapy approach used in these studies is the lack of targeting to the nigrostriatal dopamine neurons. Although the cell
type(s) transfected may provide trophic support to these neurons by virtue of their ability
to secrete the protein of interest, expression of GDNF in off-target sites has been shown
to cause side-effects in humans such as loss of appetite (Kordower, 1999; Manfredsson,
2009), hallucinations (Kordower, 1999) and depression (Kordower, 1999). It is thus of
clinical importance to investigate means of targeting of pGDNF transfection after
intranasal administration to brain regions affected by Parkinson’s disease, and perhaps to
dopamine neurons themselves via a tyrosine hydroxylase promoter, or a similar cell-type
specific promoter.

H. Summary and conclusions

This thesis provides the first evidence of successful gene transfection and
expression in brain after intranasal administration of a gene vector. The results represent
important advances in several key areas of neuroscience and nanomedicine. First, they
demonstrate the utility and tremendous potential of the intranasal route for delivery to the
brain of a non-viral vector encoding a therapeutic protein. The use of Copernicus’
plasmid DNA nanoparticles allows for long-lasting but non-permanent expression of the
encoded protein. Moreover, the nasal route results in widespread transfection and protein
expression throughout the brain, exceeding that produced locally by direct injection at a
single site in brain. Expression was greatest in cells lining the capillary endothelium,
suggesting distribution by the perivascular pump system, which is postulated to transport
nasally-administered substances throughout the brain. Importantly, protein expression
was significantly increased in the brain regions targeted in our study, the substantia nigra
and the striatum.
Since the goal of this project was to develop a neuroprotective treatment for Parkinson’s disease, the most important result of this thesis was demonstration of the ability of intranasally administered pGDNF to protect nigrostriatal dopamine neurons in the rat 6-hydroxydopamine model of Parkinson’s disease. Both the naked pGDNF plasmid and pGDNF nanoparticle pretreatments showed neuroprotection, but the later provided significantly greater efficacy, confirming the ability of Copernicus’ PEG-CK30 nanoparticles to promote transfection and GDNF expression. If similar neuroprotective efficacy can be achieved against the factor(s) underlying dopamine cell loss in Parkinson’s disease, intranasal pGDNF would become the first non-invasive gene therapy capable of stopping progression of early stage Parkinson’s disease and promoting recovery.
VI. FUTURE DIRECTIONS

Intranasal delivery as a whole is limited by the highly complex and variable nature of the nasal environment combined with the lack of nasal-targeted devices that can effectively deliver therapeutics in a controlled manner for animal studies. Much of this work is thus subject to inherent variability among experimental animals and the variability in dosing, which can mask the significance of the intranasal delivery method. In addition, classic pharmacological dose-response curves (i.e. the relationship between dose of pDNA and expression of protein) are very difficult to obtain due to these limitations. Therefore, further research into effective delivery devices, adjuvants for mucosal penetration and methods for increasing consistency between dosing are all areas that should be focused on in future work. In addition, a number of follow-up studies are necessary to complement and add value to the results of this thesis.

First, it would be interest to determine which cell types in brain are susceptible to transfection after intranasal delivery of expression plasmids (either naked or compacted into nanoparticles). This study was one of the initial reasons for requesting the reporter protein expression plasmids. The sparse but widespread expression in brain following intranasal administration will make this study a major undertaking. However, using double-label IHC for eGFP and multiple cell-specific markers, it should be possible to examine in a systematic way the proportion of eGFP-positive cells that are vascular endothelium-associated cells, glial cells, or neurons, and the relative proportions of each cell type in different brain regions.

Second, it would be of value to gain a better idea of the localization and time-course of protein expression after intranasal pDNA delivery. For these studies, it may be
beneficial to utilize the pUL plasmid, which expresses luciferase under the same plasmid backbone as the pGDNF and pUGG plasmids. Luciferase activity in brain could be visualized after luciferin injection using several methods, including live, whole-body imaging using the IVIS 200 bioluminescent imager or similar device. This could provide the means for tracking regional expression of protein in brain, as well as a way to monitor the time-course of expression after intranasal delivery in the same animal.

Along the same lines, it would be worthwhile to measure regional mRNA transgene levels in brain after intranasal administration. This complementary assay would establish to what extend the protein detected in the brain is a product of local transfection of brain cells versus transport of the protein from rostral sites of transfection, i.e. the nasal cavity or olfactory bulb and frontal cortex.

Additional studies should also be performed in the rat 6-OHDA model to determine if intranasal treatments with pGDNF NPs (or naked pGDNF) can reverse damage and promote the recovery of dopamine neurons after a toxic insult has occurred. The studies of this thesis only examined GDNF’s neuroprotective effects, since intranasal delivery occurred 7 days before a 6-OHDA lesion. Giving intranasal pGDNF after lesioning, or both before and after the lesion, would test the whether it is possible to achieve a neurorestorative effect toward surviving dopamine neurons. Delivery after the lesion would more closely model the intended use of pGDNF gene therapy in Parkinson’s patients who already have ongoing neurodegeneration at the outset of treatment.

Finally, although intranasal pGDNF provided evidence of neuroprotection in the 6-OHDA lesion study, its effects in intact animals have not been tested. A parallel study in unlesioned rats is important because it will show whether there is a neurotrophic effect
of pGDNF treatment on the dopaminergic system independent of the 6-OHDA lesion. Direct assessments of dopaminergic staining intensity would have to be compared between treatment groups, rather than side-to-side evaluations in the same brain, since there will be no left-right difference in unlesioned animals. This baseline data will also provide a basis for determining whether there was a neurotrophic effect of pGDNF treatments on the unlesioned side in the 6-OHDA-lesioned rats.
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