Intranasal Delivery of GDNF for the Treatment of Parkinson’s Disease

Thesis Presented

By

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Program:  Department of Pharmaceutical Sciences

This project satisfies all research requirements for the Doctoral Degree

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ABSTRACT

Glial cell-line derived neurotrophic factor (GDNF) has been shown to exert neuroprotective and neuroregenerative effects on midbrain dopamine neurons and has great potential in the treatment of Parkinson’s disease (PD). However, GDNF does not cross the blood-brain barrier, limiting its administration to invasive intracerebral infusions and rendering GDNF too risky for widespread clinical use. In this thesis, the intranasal route of administration was selected in order to target GDNF to the brain while limiting peripheral distribution. This approach exploits transport by the olfactory pathway that bypasses the blood-brain barrier while avoiding peripheral distribution and associated potential adverse effects.

The goals of this thesis were to: 1) to evaluate the feasibility of the intranasal route of administration for delivery of GDNF to brain, and 2) to develop a cationic liposomal formulation suitable for intranasal administration of proteins to the brain. In initial studies, cationic liposomes were loaded with a model protein, ovalbumin (OVAL; MW= 45 KDa), and were intranasally administered to rats. Ovalbumin was chosen because it has a molecular weight similar to GDNF (GDNF MW= 30 KDa), and because it is not endogenously expressed in the rat brain making it easier to detect the delivered protein. Two preparations of Alexa 488-OVAL and 111In-OVAL were administered intranasally to rats. Preparation # 1 consisted of adding the protein to preformed cationic liposomes and vortexing. Preparation # 2 consisted of adding the protein to the aqueous phase during liposomal formation, followed by sonication of the liposomal preparation to
obtain the desired particle size. The ability of the two preparations to deliver OVAL to the brain was assessed using both qualitative and quantitative methods.

In qualitative studies, the liposomes were loaded with Alexa 488-OVAL (50 µg dose per rat) and delivery of the labeled protein to the brain was assessed by fluorescence microscopy in coronal sections taken along the rostral-caudal axis of the brain. By 6 and 24 hours after administration, Alexa 488-OVAL deposits were widely distributed throughout the brain, with apparent cellular uptake in the ventral midbrain by 6 hours after administration. In quantitative studies, liposomes were loaded with $^{111}$In-OVAL, and distribution to brain and peripheral tissues was monitored by gamma counting at 1 hour, 4 hours, 6 hours, and 24 hours after administration. At all time points, the liposomal preparation yielded higher $^{111}$In-OVAL concentrations in brain than intranasal administration of $^{111}$In-OVAL in PBS. For both preparations, the highest brain concentrations were achieved at the shortest time point, 1 hour. A 25 µl dose of a more concentrated form of the liposomal OVAL preparation (2 µg/µl) yielded a higher percentage of dose in the brain, and a lower percentage of dose in the stomach and intestines, than 50 µl of a 1 µg/µl preparation. As a result, the higher concentration (2 µg/µl) was used in all subsequent OVAL studies, and later with GDNF. These studies demonstrated that intranasal administration of cationic liposomes may provide a novel, non-invasive strategy for delivering therapeutic proteins and neurotrophic factors such as GDNF to the brain for the treatment of central nervous system disorders.

The second goal of this thesis was to generate a cationic liposomal preparation of recombinant human GDNF (rhGDNF) for intranasal administration to rats. Cationic liposomes incorporating GDNF had an average particle size of 158 ± 26 nm, a zeta
potential (surface charge) of $32 \pm 8$ mV, and a protein loading efficiency of $95 \pm 3\%$.

When liposomal GDNF (2 µg/µl, 50 µg total GDNF) was administered intranasally to rats, the protein was detected by immunohistochemistry in both the substantia nigra (SN) and striatum 6 and 24 hours later. However, abundant endogenous expression of GDNF in the SN, and the GDNF antibody’s inherent inability to discriminate between recombinant human GDNF (rhGDNF) and rat GDNF, limited the immunohistochemical identification of the administered protein. In the striatum and cortex, where the endogenous expression of GDNF is modest, there was qualitatively more punctate GDNF immunoreactivity (IR) than in saline treated rats.

In addition, two therapeutic studies were conducted where intranasal administration of the formulation was evaluated in a 6-hydroxydopamine (6-OHDA) rat model of PD. The first study consisted of intranasally administering the formulation to rats 1 hour prior to the generation of a medial forebrain bundle partial lesion. The second study was a multiple administration study, and it consisted of intranasally administering the formulation three times: 24 hours prior to the lesion, 1 hour prior to the lesion, and then 24 hours after the lesion for a total dose of GDNF of 150 µg. In both the single and the multiple administration studies, intranasally administered GDNF, in liposomes or in PBS, was shown to protect against a 6-OHDA lesion. Significant neuroprotection was demonstrated by a higher overall density of tyrosine hydroxylase in the lesioned SN. In addition, the intranasal GDNF groups showed a higher number of dopamine cell bodies in the lesioned SN. These results indicate that intranasal administration of GDNF is an effective, non-invasive means of delivery of the protein to brain and may provide a useful approach to the treatment of PD.
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I. INTRODUCTION

Statement of the Problem:

Parkinson’s disease (PD) is a progressive neurodegenerative disease resulting from the destruction of dopaminergic neurons of the A9 nigrostriatal pathway originating in the midbrain. PD is a debilitating condition, which causes motor dysfunction, akinesia, and eventually death. The currently available treatment strategies for PD do not arrest disease progression and only provide patients with temporary symptomatic relief. Neurotrophic factors are proteins that promote the growth, regeneration, and survival of neurons (Gash et al., 1998; Ikeda et al., 2000; Markus et al., 2002). Glial cell line-derived neurotrophic factor (GDNF) has been demonstrated to exert neuroprotective and neuroregenerative effects on midbrain dopamine neurons, and has potential as a treatment for PD. However, GDNF has poor blood-brain barrier penetration, and its administration is limited to invasive intracerebral infusions (Kastin et al., 2003). This route of administration poses significant surgical risks including life-threatening infections, intracerebral hemorrhages, embolic strokes, and even death. These risks are more pronounced in the elderly, which make up about 60% of all PD patients.

The goal of this thesis project was to develop a non-invasive means of delivering GDNF to the brain using the intranasal route of administration. Intranasal administration was selected because it exploits transport by the olfactory and trigeminal pathways present in the nasal cavity. These pathways bypass the blood-brain barrier (BBB), while avoiding peripheral distribution and associated potential side effects. In addition, this research evaluated a means for incorporating the protein into a cationic liposomal carrier, thereby increasing tissue residence time, stability, and uptake of GDNF at the nasal...
mucosal surface so that clinically relevant doses can reach the brain. If successful, such a strategy could move GDNF from an experimental treatment to a clinical reality, and conceivably provide the first “curative” therapy for Parkinson’s disease.

**Review of the Literature:**

**A. Parkinson’s Disease**

Parkinson’s disease (PD) is a progressive neurodegenerative disease resulting from the destruction of dopaminergic neurons of the A9 nigrostriatal pathway (Figure 1). PD interferes with normal motor function and eventually results in akinesia and death. In fact, PD patients have a 1.6 higher mortality rate than age-matched members of the normal population (Fahn, 2003). In the US, the incidence of PD in people over 65 yrs old is approximately 1% (www.cds.gov/nchs). This translates to 1.5 million people in the US alone (www.cds.gov/nchs). In addition, as our population ages, the number of people afflicted by PD will dramatically increase. Currently, there is no cure for PD, and all available treatment strategies only provide temporary symptomatic relief, and don’t stop its progression.
Figure 1: Mapping of the distribution of dopamine neurons in the developing brain (a) and in the adult (b) showing the A9 nigrostriatal pathway affected by Parkinson’s disease (Bjorklund and Dunnett, 2007a).

Parkinson’s disease was so named in honor of James Parkinson who first described the disease as a “shaking palsy” in 1817 (Fahn, 2003). James Parkinson described the symptoms as: “involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from walking to a running pace: the senses and intellects being uninjured” (Fahn, 2003).

PD is characterized by four cardinal symptoms: rigidity, bradykinesia or slowness of movement, resting tremor, and postural abnormalities resulting in a shuffling gait and a tendency to fall. The most significant pathological features of PD include progressive destruction of dopamine neurons of the substantia nigra (SN) pars compacta which innervate the corpus striatum, and the formation of Lewy bodies in the SN, which are cytoplasmic inclusions composed of 10-14 nm fibrils, ubiquitin, and α-synuclein (Figure 2) (Gibb, 1992; Dawson and Dawson, 2003).
Figure 2: Lewy bodies in substantia nigra dopamine neurons from Parkinson’s disease patients (http://www.sfn.org/index.cfm?pagename=brainBriefings_alphaSynucleinAndParkinsonsDisease#fullsize).

Other brain structures affected by PD are the amygdala, ventral tegmental area, locus ceruleus, raphe nuclei, the vagal dorsal motor nucleus, and the olfactory system (Lang and Lozano, 1998; Hawkes et al., 1999). As a matter of fact anosmia, or the loss of the sense of smell, may be one of the first symptoms of idiopathic PD (Wenning et al., 1995; Hawkes et al., 1999; Haehner et al., 2007; McKinnon et al., 2007; Kranick and Duda, 2008). The pathology of anosmia in PD appears to be related to the presence of Lewy bodies, and subsequent neuronal loss in the olfactory bulb and tract, and not to a decrease in olfactory bulb volume (Pearce et al., 1995; Mueller et al., 2005). However, the cardinal symptoms of PD result from decreased dopamine neurotransmission due to degeneration of nigrostriatal dopamine neurons. In fact, post-mortem examination of PD patients revealed a >80% decrease in dopamine levels in the corpus striatum, with lower levels in the putamen than in the caudate nucleus, the two components of the human striatum (Hornykiewicz, 1973; 1998). The typical onset of PD symptoms is around 60
years of age. The cause of PD cannot be ascertained in most cases, with only a few cases being familial and resulting from a genetic mutation in the \(\alpha\)-synuclein gene (Barzilai and Melamed, 2003). Other genes and loci have been implicated in the development of familial PD and are described in Table 1.

Mutations in the \(\alpha\)-synuclein gene are thought to result in misfolding of the synuclein protein and alterations of its homeostasis, which leads to protein aggregation and possibly cell death (Gates, 2006). Nevertheless, inheritance of a dominant mutation of one of these genes does not necessarily correlate with the development of clinical PD (Sulzer, 2007). For instance, it has been shown that only 24% of patients with dominant \(LRRK2\) mutation develop PD (Sulzer, 2007).

<table>
<thead>
<tr>
<th>Locus (gene)</th>
<th>Inheritance</th>
<th>Clinical presentation</th>
<th>Pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1 (alpha-synuclein)</td>
<td>AD</td>
<td>PD, DBL</td>
<td>Typical PD, DBL for A53T/E46K mutations</td>
</tr>
<tr>
<td>PARK2 (Parkin)</td>
<td>AR, pseudodominant?</td>
<td>Early onset PD, slow progression</td>
<td>Nigral loss, no LBs</td>
</tr>
<tr>
<td>PARK3</td>
<td>AD</td>
<td>Typical PD</td>
<td>Typical PD, NFTs</td>
</tr>
<tr>
<td>PARK5 (UCLH1)</td>
<td>AD?</td>
<td>Typical PD</td>
<td>No reports</td>
</tr>
<tr>
<td>PARK6 (PINK1)</td>
<td>AR</td>
<td>Early onset PD, slow progression</td>
<td>No reports</td>
</tr>
<tr>
<td>PARK7 (DJ-1)</td>
<td>AR</td>
<td>Early onset PD, slow progression</td>
<td>No reports</td>
</tr>
<tr>
<td>PARK8 (LRRK2)</td>
<td>AD</td>
<td>Typical PD</td>
<td>Variable, typical PD with or without LB</td>
</tr>
<tr>
<td>PARK9</td>
<td>AR</td>
<td>Atypical PD with dementia, spasticity, supranuclear gaze palsy</td>
<td>No reports</td>
</tr>
<tr>
<td>PARK10</td>
<td>Unclear</td>
<td>Typical PD</td>
<td>No reports</td>
</tr>
<tr>
<td>PARK11</td>
<td>AD</td>
<td>Typical PD</td>
<td>No reports</td>
</tr>
</tbody>
</table>

\(AD = \text{autosomal dominant}; AR = \text{autosomal recessive}; DBL = \text{diffuse LB disease}; LB = \text{Lewy body}; NFT = \text{neurofibrillary tangle}; PD = \text{Parkinson's disease.}\) From Douglas 2007.[8]

**Table 1:** Loci and genes associated with an increased risk of developing Parkinson’s disease (Siderowf, 2007).
Idiopathic PD, on the other hand, has been attributed to many different intrinsic and extrinsic factors (Calne and Langston, 1983). Normal aging, for instance, results in loss of dopaminergic neurons and the prevalence of PD increases more than 40 fold between 55-85 years of age (Sulzer, 2007). However, PD symptoms do not appear until approximately 50-70% of SN dopamine neurons have been destroyed and striatal dopamine levels are decreased by 60-80% (Hornykiewicz, 1998; Barzilai and Melamed, 2003). This suggests that PD development may be multi-factorial, and/or that PD may be influenced by genetics, with some people being predisposed to developing the disease. Other intrinsic factors involved in the development of PD, are defects in energy metabolism, an excessive generation of free radicals, and/or insufficient antioxidant synthesis (Cohen et al., 1997). These intrinsic factors may also be under genetic control accounting for the predisposition of individuals who do not have one of the more obviously-linked mutations.

Dopamine neurons are particularly susceptible to oxidative damage because dopamine metabolism to DOPAC results in the production of hydrogen peroxide. Hydrogen peroxide is then converted to hydroxyl radicals by the Fenton reaction (Figure 3). In addition, dopamine can be oxidized to 6-hydroxydopamine (6-OHDA), which can then generate more reactive oxygen species (ROS), form reactive quinones, and inhibit mitochondrial complexes I and IV (Asanuma et al., 2004; Sulzer, 2007). Excessive production of free radicals and ROS may damage cell membranes through lipid peroxidation, ultimately resulting in cell death.
Another factor which may increase dopamine neuronal susceptibility to oxidative stress is the presence of neuromelanin. Neuromelanin is the dark substance that gives the substantia nigra is characteristic dark color (“substantia nigra” means black substance) in humans, and which contains abundant quantities of iron and the oxidative byproducts of dopamine (Fasano et al., 2006; Sulzer, 2007). Neuromelanin accumulates throughout life, suggesting that cells are unable to efficiently break it down (Sulzer, 2007). A critical threshold may be reached where neuromelanin stores exceed cell capacity, and may result in excessive ROS production and cell death (Figure 4).

Figure 3: Formation of hydroxyl radicals from hydrogen peroxide by the Fenton reaction (http://www.gpo.or.th/rdi/images/iron.gif).

Figure 4: Neuromelanin and reactive oxygen species (Fasano et al., 2006).
An additional intrinsic factor which may be involved in the development of idiopathic PD is mitochondrial complex I impairment. Decreased mitochondrial complex I activity increases free radical production, and is believed to play a role in the development of PD (Dawson and Dawson, 2003).

Environmental factors have also been implicated in the development of PD. For example, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an analog of the synthetic opioid meperidine, and pesticides such as rotenone, have been shown to cause PD by inhibiting mitochondrial complex I activity (Langston and Ballard, 1984; Helmuth, 2000). In addition, viral pathogens have also been shown to cause PD (Takahashi and Yamada, 1999). For instance, patients that had contracted encephalitis lethargica during the outbreak of the 1920’s later developed PD (Calne and Lees, 1988).

Current pharmacologic treatment strategies for PD are aimed at ameliorating the symptoms of the disease by administering a dopamine precursor (levodopa), dopamine receptor agonists (ex: bromocriptine, pramipexole, and ropinorole), inhibiting dopamine metabolism (ex: seleginine and entacapone), and/or by blocking muscarinic acetylcholine receptors. Unfortunately, these treatment strategies do not delay the progression of the disease, and often lose their effectiveness over time.
B. Dopamine: Synthesis, Metabolism, and Receptors

In the 1950’s, Carlsson identified dopamine as a neurotransmitter (not just a precursor to norepinephrine synthesis) and noted that reserpine administration, which depletes catecholamines from nerve terminals, resulted in parkinsonian symptoms (Carlsson and Waldeck, 1958; Carlsson, 2001). Furthermore, Carlsson was the first to demonstrate that L-DOPA, the immediate precursor in the synthesis of the neurotransmitter dopamine, could reverse reserpine-induced parkinsonian symptoms in experimental animals (Figure 5). This finding ultimately resulted in the development of oral levodopa in 1967 by George Cotzias, a drug which is still the mainstay treatment of PD today (Bjorklund and Dunnett, 2007a).

Carlsson and colleagues also demonstrated that dopamine was present in the brain, and they mapped the location of dopamine neurons in the brain by using spectrophotofluorimetry, an adaptation of the formaldehyde gas fluorescence histochemical method developed by Falck and Hillarp in 1961 (Carlsson and Waldeck, 1958; Ungerstedt, 1971; Fahn, 2003; Bjorklund and Dunnett, 2007a, b). The Falck and
Hillarp method consisted of exposing freezed-dried tissue to formaldehyde gas, which would then convert dopamine and norepinephrine molecules to isoquinolines. These isoquinolines would then emit a green-yellow fluorescence visible under fluorescence microscopy (Bjorklund and Dunnett, 2007a).

![Image](http://nobelprize.org/nobel_prizes/medicine/laureates/2000/carlsson-lecture.pdf)

**Figure 6:** Fluorescence histochemical identification of dopamine neurons in the substantia nigra by the Falck and Hillarp method (http://nobelprize.org/nobel_prizes/medicine/laureates/2000/carlsson-lecture.pdf).

Dopamine is a catecholamine, which is unable to cross the blood-brain barrier (BBB). Therefore, it must be synthesized locally in the cell bodies and at the nerve terminals of dopamine neurons from the amino acid tyrosine by the enzyme tyrosine hydroxylase (TH). TH is often used as a histochemical marker for dopamine neurons. Catecholamines are synthesized as follows in Figure 7:
Figure 7: Catecholamine synthesis. The rate limiting step in dopamine synthesis is the conversion of tyrosine to DOPA by tyrosine hydroxylase (http://web.indstate.edu/thcme/mwking/catecholaminesynthesis.jpg).

Following its synthesis, dopamine is stored within the presynaptic terminal in storage vesicles. Depolarization results in the release of dopamine from these vesicles into the synapse. Dopamine can then bind to its receptors, either on the presynaptic nerve terminal that released it or on postsynaptic neurons. Unbound dopamine can either undergo presynaptic reuptake by the dopamine transporter (DAT), or it can be metabolized enzymatically within the synapse or presynaptic terminal. In presynaptic neurons, dopamine may be repackaged in vesicles for future release or be broken down in the cytoplasm. Dopamine metabolism is carried out by the enzymes monoamine oxidase (MAO) and/or catechol-O-methyl transferase (COMT) into DOPAC and HVA as shown in Figure 8.
Dopamine receptor activation and cell signaling mechanisms were elucidated by Greengard, and his research won him the Nobel Prize in Physiology and Medicine in 2000, along with Dr. Arvid Carlsson (Iversen and Iversen, 2007). Signaling begins with the binding of dopamine to its receptor(s). There are five dopamine receptors, divided into two different groups: the D1-like group and the D2-like group (Kebabian and Calne, 1979). The D1-like group includes D1 and D5 receptors, and they activate adenylyl cyclase via Gs and Golf coupling, and increase cAMP. In addition, D1-like receptors have been reported to activate phospholipase C (PLC), hydrolyze phosphatidylinositol,
and increase calcium signaling in brain tissue (Lee et al., 2004). However, recent data indicates that this effect on PLC is actually a result of D1 and D2 receptor co-activation on the same neuron, and subsequent coupling to Gq (Lee et al., 2004). The D2-like group includes the D2, D3, and D4 receptors, and they couple with Gi or Go and inhibit cAMP formation (Fahn, 2003). D2-like receptors may also couple to other effectors besides cAMP, such as PLC resulting in a blockade of calcium release from intracellular stores (Vallar and Meldolesi, 1989). This effect is mediated through receptor coupling with Gq. In addition, D2-like receptors can potentiate potassium channels resulting in hyperpolarization and inhibition of neuronal firing (Lacey et al., 1987).

C. Neuronal Circuitry of Parkinson’s Disease

Ungerstedt and colleagues used stereotaxic lesioning techniques to map the monoamine pathways in the brain, and much of our understanding of the nigrostriatal circuitry stems from this work (Ungerstedt, 1971). Voluntary skeletal muscle movements result from a delicate interaction between the pyramidal and the extrapyramidal systems. The pyramidal motor system is composed of neurons that extend from the cerebral cortex to the brainstem and spinal cord. On the other hand, the extrapyramidal motor system is composed of the basal ganglia and associated nuclei, including the caudate nucleus, putamen, globus pallidus, substantia nigra and subthalamic nucleus (STN). The caudate nucleus and putamen make up the corpus striatum and are often referred to as simply the striatum. The basal ganglia’s function is to modify and regulate the pyramidal motor system. PD symptoms result from the loss of striatal dopamine, which leads to a defect in basal ganglia output, which in turn results in unbalanced pyramidal motor output.
According to the traditional model of the basal ganglia proposed in the early 1990’s, SN pars compacta dopamine neurons project from the midbrain into the striatum and release dopamine, which then binds to either D1 receptors on striatal efferent neurons of “the direct pathway”, or it binds to D2 receptors on striatal efferent neurons of “the indirect pathway” (Alexander and Crutcher, 1990). In the direct pathway, D1 receptor binding is postulated to couple to Gs, and result in activation of the striatonigral neurons, and in the release of GABA in the globus pallidus internal segment (GPi) and the SN pars reticulata (SNr). GABA is an inhibitory neurotransmitter, and striatal GABA released onto the GPi/SNr inhibits the release of GABA from GPi/SNr projections to the thalamus. In a manner of speaking, it removes the inhibition that the GPi/SNr normally has on the thalamus, and as a result, the excitatory neurons of the thalamus become activated, glutamate is released in the cerebral cortex, and movement occurs.

Dopamine released from nigrostriatal neurons also binds to D2 receptors (which couple to Gi and Go), and results in the inhibition of striatopallidal neurons, the first cell group of the indirect pathway (Alexander and Crutcher, 1990). These neurons project from the striatum to the globus pallidus external segment (GPe), and dopamine receptor activation results in the inhibition of GABA release in the GPe. The GPe projects into the subthalamic nucleus (STN), and the decreased GABA input into the GPe results in an increase in GABA output transmission, which then inhibits glutamatergic release from the STN to the GPi/SNr. The lack of excitatory glutamate input into the GPi/SNr results in a diminished inhibitory effect on the thalamus, and an increase in glutamatergic transmission from the thalamus to the cerebral cortex. The net result of dopamine’s
action on both the direct and indirect pathways is that movement is facilitated by removing the GABAergic inhibition on the thalamus (Figure 9).

Figure 9: Basal ganglia model of movement in the normal patient (a), and in the Parkinson’s disease patient (b). Red indicated the glutamatergic pathways, green the GABAergic pathways, and yellow the dopaminergic pathways. Thickening of the lines indicates increased activity, while thinning indicates decreased activity when compared to the normal control. Winding lines indicate changes in firing patterns (Cenci, 2007).

It should be noted that subsequent research in our laboratory and many others has shown that this model is an oversimplification, and in some respects incorrect. For instance, D1 and D2 receptors are not segregated in their expression to separate populations of striatal neurons as shown in Figure 9. Rather they are extensively co-expressed on striatal efferent neurons (Dickerson and Waszczak, 2005; Waszczak et al., 2006; Dickerson and Waszczak, 2007). There is also evidence that when the two receptors are co-expressed, they interact as dimers with a different G protein than either
does alone (Gq/11) and presumably activate a unique signaling cascade (Lee et al., 2004). These new findings are not in agreement with the traditional basal ganglia model, and the functional ramifications have not been fully worked out.

Nevertheless in PD, the neurons innervating the striatum from SN pars compacta (SNC) die, and as a consequence, inhibitory transmission (in the form of GABA) from the GPi/SNr increases, and glutamatergic excitatory transmission from the thalamus to the cerebral cortex decreases. There are also important changes in the pattern of firing in the basal ganglia output nuclei to a more bursting and oscillatory mode. These alterations collectively give rise to the decreased movement and tremor of parkinsonism (Albin et al., 1989). In addition, abnormal basal ganglia output from the SNr to the pedunculopontine nucleus (PPN) results in some of the other phenotypic signs of PD, for example the shuffling gait (Lewis et al., 2003).

D. Animal models of Parkinson’s Disease

Several neurotoxin animal models have been developed to better understand the pathogenesis of PD, and to study potential therapeutic agents. The most commonly used neurotoxins for the development of PD animal models are 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and 6-hydroxydopamine (6-OHDA) (Dauer and Przedborski, 2003). All three agents are believed to induce the death of dopamine neurons by generating reactive oxygen species, and by inhibiting mitochondrial respiration (Asanuma et al., 2004).

In the 1980’s, MPTP emerged as the cause of a PD outbreak amongst young drug addicts. MPTP was accidentally synthesized from the illicit manufacture of 1-methyl-4-
phenyl-4-propionoxypiperidine (MPPP), an analog of meperidine (Dauer and Przedborski, 2003). MPTP was found to cause severe and irreversible symptoms of PD in humans, primates, and in some strains of mice. However, rats were unaffected by this neurotoxin (Bove et al., 2005). MPTP readily crosses the BBB, is oxidized to MPP⁺ (its active form) by monoamine oxidase B (MAO-B), and then undergoes uptake into presynaptic dopamine neurons by the dopamine transporter (DAT; Figure 10) (Dauer and Przedborski, 2003). Once inside the dopamine neuron, MPP⁺ inhibits mitochondrial complex I of the electron transport chain, depletes the neuron of ATP, and stimulates the production of reactive oxygen species.

Figure 10: MPTP uptake into dopamine neurons (Dauer and Przedborski, 2003).

The rotenone model of PD is much less frequently used. Rotenone is a herbicide/pesticide that readily crosses the BBB, and it causes neurotoxicity by inhibiting mitochondrial complex I, microtubule formation, and by activating microglia (Gao et al., 2002; Dauer and Przedborski, 2003; Bove et al., 2005). Rotenone is not used more
widely because of the unpredictability and inconsistency of rotenone lesions, which makes it difficult to standardize from one animal to another.

The 6-OHDA lesion model of PD, on the other hand, was developed in the late 1960’s, and was the first neurotoxin model that selectively targeted dopamine neurons of the SN (Ungerstedt, 1968). 6-OHDA is a hydroxylated analog of dopamine, and as such, does not cross the BBB. It is presynaptically taken up by both the dopamine and norepinephrine transporters (Bove et al., 2005). Therefore, 6-OHDA lesions must be performed by stereotaxic injections directly into the brain, and care must be exercised to inhibit its uptake into noradrenergic neurons. 6-OHDA neurodegeneration results from oxidative damage caused by reactive oxygen species, formation of reactive quinones, and inhibition of mitochondrial complexes I and IV (Figure 11) (Asanuma et al., 2004).

![Figure 11: Oxidation reaction of 6-OHDA (Bove et al., 2005).](image)

More often than not, 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, most scientists have opted to inject 6-OHDA unilaterally, and use the contralateral side as a control. However, the assumption that the unlesioned side is truly a control has been challenged by other work in our laboratory, which has indicated that changes do occur on the side contralateral to
the lesion (White-Cipriano and Waszczak, 2005; Waszczak et al., 2006; White-Cipriano and Waszczak, 2006, 2007). Furthermore, the site of 6-OHDA administration is critical at determining the extent of the lesion generated. Typically, 6-OHDA is injected into the SN, striatum, or the medial forebrain bundle (MFB) (Bove et al., 2005). Striatal lesions using 6-OHDA are generated via retrograde transport of the neurotoxin to the SN (Dauer and Przedborski, 2003; Yuan et al., 2005). However, intrastriatal administration of 6-OHDA results in a graded lesion, with an approximately 50% reduction in the number of TH positive neurons in the SN, and a 45-50% reduction in striatal TH optical density (Yuan et al., 2005). Furthermore, since the striatum is a large structure, it is difficult to produce a uniform partial lesion with a single injection of the toxin into this structure.

A MFB lesion, on the other hand, can result in a >90% decrease in the number of TH positive neurons in the SN, and in an 80% decrease in striatal TH optical density (Yuan et al., 2005). Unlike MPTP, 6-OHDA can be effectively used in all species to generate consistent nigrostriatal lesions. This thesis employed the MFB 6-OHDA rat model of PD to test the efficacy of intranasal GDNF at protecting dopamine neurons from neurotoxin-induced cell death. This was accomplished by first determining conditions for generating a consistent partial lesion, using the same stereotaxic coordinates for a MFB lesion, but administering a smaller dose and less concentrated solution of 6-OHDA (Truong et al., 2006). The reason for this strategy was that GDNF is known to protect dopamine neurons against death from a toxic stimulus, but there is some controversy as to whether or not GDNF is capable of stimulating neurogenesis or just protects damaged or dying neurons from destruction. Therefore, a lesion model that results in more than a 90% death of SN dopamine neurons may not be the best method to determine if sufficient
GDNF reaches the brain to afford neuroprotection. In other words, a nearly complete lesion may be too severe for it to be reversed by GDNF treatments.

E. Blood-Brain Barrier

As previously stated, PD has no cure, and currently available treatments only alleviate its symptoms but do not arrest its progression by preserving dopamine neurons. The biggest obstacle to the development of new protein therapeutics for PD, and other neurodegenerative conditions, is the presence of the BBB. It has been estimated that more than 98% of all small drug molecules, and approximately 100% of large drug molecules are excluded from the brain by the BBB (Figure 12) (Pardridge, 2005). In order for even small drug molecules to cross the BBB in therapeutically sufficient quantities, they must have a molecular mass of less than 500-Da, and be highly lipophilic such that they form less than 8-10 hydrogen bonds with water in solution (Pardridge, 2005).

**Figure 12:** Autoradiogram of a mouse following IV administration of radiolabelled histamine (a molecule of only 100 Da) showing whole body distribution of the radiolabel, except for the brain and spinal cord (Pardridge, 2005).
The blood-brain barrier is a dynamic barrier that separates the systemic circulation from the brain. The BBB not only impedes brain penetration of some substances, but it also actively transports necessary nutrients and electrolytes from the systemic circulation to the brain. The BBB is comprised of capillaries with a single layer of endothelial cells, pericytes, the basal lamina, and astrocyte projections (Hawkins and Davis, 2005). It has been estimated that there are >100 billion capillaries in the human brain, separated by a distance of approximately 50 µm (Figure 13) (Pardridge, 2005). As a result of this small distance between brain capillaries, it has been hypothesized that each neuron is perfused by its own blood capillary (Pardridge, 2005). Furthermore, this translates to a molecular diffusion distance of 25 µm within brain parenchyma following intravascular administration of drugs that either penetrate the BBB, or bypass the BBB as is the case for intranasal administration (Pardridge, 2005).

![Figure 13: Demonstration of high vascular density within the adult rat brain following India ink administration (Pardridge, 2005).](image)

Brain endothelial cells are different from other endothelial cells in that they have no fenestrations, they possess tight junctions, and they express a large number of mitochondria. As a result of the presence of tight junctions, brain capillaries restrict
paracellular transport, and are 50-100 times tighter than the peripheral microvasculature (Abbott, 2002). Pericytes, which attach to the basolateral side of endothelial cells, are relatively undifferentiated vascular smooth muscle cells believed to play an important role in the formation of BBB tight junctions by increasing the expression of occludin (a major component of tight junctions; Figure 14) (Hawkins and Davis, 2005). However, the exact origin and function(s) of pericytes are still under debate. Pericytes are believed to originate from microglia, and they may play a role in endothelial cell growth and differentiation (Hawkins and Davis, 2005).

![Anatomical features of the blood-brain barrier](https://www.expertreviews.org)

**Figure 14:** Anatomical features of the blood-brain barrier (www.expertreviews.org, 2003).

The basal lamina, or the basement membrane, encloses both the endothelial cells and pericytes, and is also believed to be part of the BBB (Hawkins and Davis, 2005). The basal lamina has a thickness of approximately 30-40 nm, and it is composed of extracellular matrix proteins including collagen IV (Hawkins and Davis, 2005).
Astrocytic processes termed “glial feet,” which encircle over 99% of cerebral endothelial cells, provide trophic support for neurons as well as inducing the formation of the BBB (Neuwelt, 2004). In addition, astrocytes partake in the transport of some substances across the BBB.

Tight junctions, or zonula occludens, are intercellular connections that restrict the passage of molecules from one cell to another. They form at the level of the apical membrane, and are composed of the “outer leaflets” of adjoining cells’ plasma membranes (Tsukita and Furuse, 1998). Tight junctions are not limited to the BBB, as they can also be found in olfactory epithelia, testis, and gastrointestinal tracts.

To date, occludin, claudin, and the ZO proteins are believed to be the key players in tight junction formation. Occludin is a four transmembrane protein with a molecular weight of 65 KDa (Stevenson, 1999). The amino acid sequence of occludin is approximately 90% conserved in mammals, exemplifying its importance across the different species (Tsukita and Furuse, 1998). However experimentally, it has been observed that occludin knockout mice are able to survive, and form tight junctions without any observable deficits (Tsukita and Furuse, 1998).

Claudins are a group of 16 different proteins with varying distributions and expressions in different parts of the body (Stevenson, 1999). Claudins, much like occludin, are also four transmembrane proteins (MW= 22 KDa) (Tsukita and Furuse, 1998). Claudins, together with occludin, are believed to influence the intercellular permeability of tight junctions (Stevenson, 1999).

Lastly, the ZO proteins are guanylate kinases that associate with plasma membrane components (Brown and Davis, 2002). Serine/threonine phosphorylation of
occludin is believed to trigger tight junction formation, and ZO proteins may be involved in this process (Tsukita and Furuse, 1998). After occludin becomes phosphorylated, claudin and occludin associate, and ZO-1 then binds to occludin’s intracellular loop and interacts with actin to form tight junctions (Brown and Davis, 2002).

F. Transport Across the Blood-Brain Barrier

Several different techniques have been attempted to deliver drugs and therapeutic proteins across the BBB and into the brain. In order to understand these techniques and how to best employ them, we must first understand the main transport mechanisms that naturally exist in the blood-brain barrier. There are six main transport mechanisms: paracellular, transcellular, facilitated transport, receptor mediated endocytosis, adsorptive endocytosis, and carrier mediated efflux transport (Neuwelt, 2004).

Paracellular transport involves the movement of very small water soluble molecules, down their concentration gradient, through the tight junctions of cerebral endothelial cells (Neuwelt, 2004). Transcellular transport, on the other hand, involves the movement of low molecular weight, highly lipophilic substances across the plasma membranes of endothelial cells via passive diffusion (Neuwelt, 2004). Facilitated transport, or carrier mediated transport, is an energy independent mechanism that employs membrane bound proteins that undergo conformational changes, and transport substances down their concentration gradient (Neuwelt, 2004). Conversely, receptor mediated endocytosis involves binding of ligands to cell surface receptors on endothelial cells, which then results in a conformational change that terminates in endocytosis of the receptor-ligand complex. Some examples of substances that undergo receptor-mediated
endocytosis are: insulin, transferrin, leptin, insulin-like growth factor (IGF) I and II (Neuwelt, 2004).

Adsorptive mediated endocytosis, on the other hand, is not specific and induces endocytosis through the binding of positively charged molecules to the surface of the negatively charged plasma membrane of cerebral endothelial cells (Drin et al., 2003; Neuwelt, 2004). Cationic liposomes may undergo adsorptive endocytosis at the level of the nasal epithelium (Friend et al., 1996; Miller et al., 1998; Briane et al., 2002).

Lastly, BBB efflux transport is carried out by the p-glycoprotein efflux transporter on the apical membrane of brain endothelial cells, the multidrug resistance-associated protein (MRP), the peptide transport system (PTS-1), and the organic anion transporter (OAT) (Neuwelt, 2004). These processes act in reverse of those permitting passage of substances from blood into brain. They carry out the reverse, i.e. elimination of substances from brain endothelium into blood (Figure 15).

**Figure 15:** Transport mechanisms across the blood-brain barrier (Neuwelt, 2004).
G. Drug Delivery Across the Blood-Brain Barrier

As previously stated, several techniques have been utilized to deliver therapeutic moieties across the blood-brain barrier. The four main approaches are: BBB disruption, bypassing the BBB, using chimeric translocating proteins, and using microparticles to deliver drugs to the brain.

BBB disruption takes advantage of the anatomical characteristics of tight junctions, and attempts to increase paracellular (or in between cells) transport by interfering with tight junction formation and/or integrity. However, long-term disruptions of the BBB could potentially result in the passage of toxic substances and/or pathogens into the CNS. Broman and Olsson, in 1940, were the first to attempt to deliver a substance to the brain by disrupting the BBB by using contrast dyes (Kroll and Neuwelt, 1998). However, it was Rapoport and colleagues who first proposed using hyperosmolar solutions to increase the delivery of substances to the brain (Kroll and Neuwelt, 1998). Hyperosmolar solutions are believed to cause a disruption of the BBB by causing endothelial cell dehydration and shrinking, thereby loosening tight junctions and increasing intercellular space. The most commonly used hyperosmotic agent for BBB disruption is intravenous mannitol because it is FDA approved, safe, effective, inexpensive, and its effects are temporary. However, agents that disrupt the integrity of the BBB are non-specific, and can also increase BBB permeability to toxins and pathogens making this approach a less attractive alternative.

Bypassing the BBB is another method that has been employed in order to deliver drugs to the brain. The presence of an incomplete BBB in the nasal mucosa has led to
attempts to deliver drugs via the intranasal route of administration. An extensive
discussion on this subject will follow.

Chimeric peptide technology has also been utilized to improve BBB permeability. This approach takes advantage of the blood-brain barrier’s receptor-mediated endocytosis mechanism. Currently, this approach is being attempted by using drugs (or drug carriers) conjugated with an anti-transferrin antibody, in order to take advantage of the relative abundance of the transferrin receptor in the BBB (Bradbury et al., 2000). In addition, insulin has also been utilized to deliver proteins across the BBB because it undergoes receptor-mediated endocytosis in cerebral endothelial cells (Bradbury et al., 2000). TAT-peptide conjugates have also been used to deliver drugs, proteins, peptides, plasmids, and other molecules to the brain. TAT is an HIV virus protein that contains an 11 amino acid peptide transduction domain (PTD) (AA= 47-57). TATp is composed of positively charged residues like arginine and lysine, which allows this peptide (and its cargo) to translocate across plasma membranes and into cells through adsorptive-mediated endocytosis (Torchilin et al., 2001; Drin et al., 2003). For example, β-galactosidase was fused with TATp, and was successfully delivered to brain following an intraperitoneal injection in mice (Schwarze et al., 1999).

Finally, another approach to delivering drugs to the CNS is by using nanoparticles as drug delivery vehicles. Liposomes are an example of such nanoparticles, and have attracted significant attention because of their ability to increase drug delivery to the target site of drug action. Liposomes are nothing more than vesicles made from phospholipids, which contain an inner hydrophilic core and an outer lipophilic membrane. 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, and the drug will be protected from degradation by plasma and tissue hydrolytic enzymes. My project utilized two of the above strategies: cationic liposomes and intranasal administration to help deliver GDNF from the nose to the brain.

H. Brain Transport and the Intranasal Route of Administration

In recent years, the intranasal route of administration has emerged as an attractive method for delivering brain impermeable drugs and proteins to the CNS. This is because intranasal drug administration is generally well tolerated, non-invasive, and because the olfactory route of administration completely bypasses the blood brain barrier. As a result, drugs and/or proteins can be transported directly from the nasal epithelium into the brain (Graff and Pollack, 2005).

Due to their functions of smell and sensory perception, the olfactory and trigeminal neurons’ nerve terminals are exposed to the nasal cavity, and serve as the interface between the outside world and the brain. In addition, there is an anatomical connection between the olfactory system and the midbrain (Figure 16). Brain regions involved in olfactory perception send axonal projections directly to the limbic areas of the brain. This has been demonstrated by distribution studies using intranasally administered horseradish peroxidase (HRP; Balin et al., 1986). Unilateral intranasal administration of HRP resulted in ipsilateral labeling of the ventral tegmental area (VTA), SN pars reticulata, and ventral pallidum (Hawkes et al., 1999). Furthermore, since most infectious agents enter the body through the mucous membranes of the nose and mouth, the connection between the olfactory neurons and the midbrain may help
explain the theory that PD is a result of a CNS infection caused by either viral or bacterial pathogens (Hawkes et al., 2007). This anatomical connection between the midbrain and the olfactory system may become a major advantage in the treatment of PD by providing a direct access for drugs (and/or therapeutic proteins) to the areas of the brain most devastated by PD.

Figure 16: Anatomical connection between the olfactory neurons and the limbic system (www.sfn.org/content/Publications/BrainBriefings/smell.htm).

I. Mechanisms of Intranasal Drug Absorption

In order for drug uptake to occur in the nasal cavity, drugs must first come into contact with the nasal epithelium, which is constantly covered by a protective mucus layer. This layer of mucus is recycled every 10-15 minutes, removing all particulate matter (including drugs) to the back of the throat for elimination (Talegaonkar and Mishra, 2004). Therefore, it is crucial to maximize residence time (how long the drug or protein is present) in the nasal cavity. For that reason, cationic liposomes were used in
this research to encapsulate GDNF. It was postulated that electrostatic attraction between the positive charges on the liposomes and the negative charges of sialic acid residues in the nasal epithelium would prolong GDNF’s presence in the nasal cavity and facilitate its uptake. Once through the mucus layer, there are 3 main mechanisms by which drugs undergo nose-to-brain transport: transcellular, paracellular, and axonal transport (Illum, 2003). Transcellular (or across cells) drug transport involves endocytosis through the olfactory epithelial cells, which surround the olfactory neurons (see Figure 16). Transcellular transport can be either receptor mediated, or through passive diffusion, and results in both brain and systemic transport (Illum, 2003). Lipophilic, low molecular weight (MW) molecules typically undergo transcellular transport. For example, fentanyl, which is very lipophilic, is 80% bioavailable when administered intranasally (Illum, 2003). On the other hand, small polar molecules are only 10% bioavailable, and peptides are less than 1% bioavailable when administered intranasally (Illum, 2003). Transport of small lipophilic drugs via this route is very fast; it occurs within minutes (Frey 2002).

Alternatively, the paracellular (around cells) route involves the passage of drug molecules between the intercellular tight junctions of olfactory epithelial cells (Figure 17) (Graff and Pollack, 2005). The average size of these intercellular channels is approximately 10 Å, and the molecular weight cut off was last reported to be approximately 1000 Da (Illum, 2003). However, insulin-like growth factor 1 (IGF-1; MW= 7.6 KDa), nerve growth factor (NGF; MW= 26.5 KDa), and HRP (MW= 40 KDa) have been demonstrated to undergo brain uptake via the paracellular route following intranasal administration (Balin et al., 1986; Chen et al., 1998; Liu et al., 2001b). This route can also mediate very rapid transport of molecules to the brain and is presumably
the route by which intranasal GDNF could enter the brain. Lastly therapeutic molecules can also be taken up by olfactory neurons with subsequent axonal transport to the olfactory bulb, and then distributed to the rest of the brain (Graff and Pollack, 2005). However, this method of drug transport can be very slow, usually taking anywhere from hours to days (Frey II, 2002). Wheat germ agglutinin-horseradish peroxidase (WGA-HRP, MW 62 KDa) is an example of a protein that undergoes axonal transport following endocytosis by olfactory neurons (Thorne et al., 1995). WGA-HRP binds to glycoproteins on the surface of olfactory neurons and undergoes adsorptive endocytosis, and subsequent brain transport (Thorne et al., 1995).

![Figure 17: Paracellular transport of xenobiotics through the tight junctions of the olfactory epithelium.](http://www.nastech.com/img/img_junction_structure.jpg)

There are several intrinsic factors of solutes that can affect both the rate and the extent of intranasal drug transport, and they include molecular weight, molecular size or diameter, pKa of the molecule, pH of the solution, and volume and concentration administered. In very general terms, brain transport following intranasal administration is greater when molecules have molecular weights of less than 1000 Da, and when peptides have less than 20 amino acids (Talegaonkar and Mishra, 2004). Diffusion of
substances once in brain is also limited by particle diameter, which determines their movement through the extracellular space of brain. Thorne and Nicholson (2006) have estimated that substances with a width of < 38-64 nm may be transported through the fluid-filled pores of extracellular space, but larger sized solutes would likely not be transported.

For drugs, the pKa should be such that the drug will be present in its unionized form, and the pH should be between 4.5-6.5 to minimize irritation to the nasal mucosa (Talegaonkar and Mishra, 2004). In addition, the most concentrated form should be used, and the volume administered should be limited in order to prevent the drug from leaking out of the nasal cavity and/or from being swallowed, as we found experimentally in studies with $^{111}$In-OVAL (Wolfe and Bernstone, 2004; Migliore et al., 2006). Other factors that can affect intranasal absorption are the rate of mucociliary clearance, metabolism and hydrolysis, the presence of the p-glycoprotein efflux pump (Graff and Pollack, 2003), nasal cycling (periodic cycles of congestion and decongestion between one nostril and the other), and hypersecretory conditions like allergic rhinitis.

Administration of drugs by the intranasal route does have some disadvantages. Frequent administration of drugs intranasally could result in nasal irritation, mucosal damage and/or ulceration, and anosmia (loss of sense of smell) (Talegaonkar and Mishra, 2004). Another disadvantage to intranasal drug administration is that brain transport in the rat may not fully correlate to drug delivery in humans due to basic anatomical differences between the species (Graff and Pollack, 2005). For instance, the nasal mucosa of humans only makes up about 8% of the total nasal epithelium. However in rats, the nasal mucosa makes up as much as 50% of the total nasal epithelium (Figure 18).
As such, caution must be exercised when attempting to extrapolate animal intranasal data to humans.

![Image](https://www.cce.umn.edu/pdfs/cpe/conferences/nano/Gunter_Oberdorster.pdf)

**Figure 18:** Comparison between rat and human nasal cavities. Olfactory bulbs are represented in red and olfactory mucosa in blue. Note the vast difference in size of the olfactory mucosa and olfactory bulbs between the species (http://www.cce.umn.edu/pdfs/cpe/conferences/nano/Gunter_Oberdorster.pdf).

### J. Intranasal Brain Delivery of Drugs, Proteins, and Neurotrophic Factors

Several attempts have been made to deliver substances, which do not normally cross the BBB, from the nose to the brain. For instance, dopamine was administered intranasally to mice in the form of $^3$H-dopamine to one nare, and it was found that after four hours, 0.12% of the administered dose was still present in the ipsilateral side of the brain (Thorne and Frey, 2001). In addition, Wang and colleagues demonstrated that intranasally administered methotrexate was able to bypass the BBB in rats (Wang et al., 2003a). When compared to intravenous administration, intranasal methotrexate achieved levels that were significantly higher in the olfactory bulb, olfactory tract, cerebrum, and
cerebellum. Also, Gozes et al. (1996) demonstrated that the 28 amino-acid protein, $^{125}$I-VIP (Vasoactive Intestinal Peptide; MW= 3.3 KDa) can successfully be delivered to the brain following intranasal administration in rats. Furthermore, the protein interferon-β 1b (Betaseron®; MW=20 KDa), which is used for the treatment of multiple sclerosis, has been successfully delivered to the brain via intranasal administration in rats, and to non-human primates (Ross et al., 2004; Thorne et al., 2008).

Thorne et al. (2004) also demonstrated that $^{125}$I-Insulin-like growth factor I (IGF-I; MW= 7.65 KDa) can be effectively delivered to rat brains via the olfactory route of administration. $^{[125]}$IGF-1 was intranasally administered via a pipette to instill 6-8 µl drops into the rats’ nostrils over a period of 18.5 min in an alternating fashion. $^{[125]}$IGF-1 was also administered intravenously into the femoral vein over a period of 1-2 minutes. Blood and cerebral spinal fluid (CSF) samples were collected from both groups and $^{[125]}$IGF-1 concentrations were determined by gamma counting. The intravenous $^{[125]}$IGF-1 dose chosen was that dose which resulted in similar area under the curve (AUC) levels as a fixed dose of intranasal $^{[125]}$IGF-1. This was done to standardize the intranasal and intravenous doses administered, in order to compare the two methods of administration. Intranasal administration resulted in >100 fold higher $^{[125]}$IGF-1 counts in brain than intravenous administration. In a separate experiment, rats were intranasally administered a high specific activity solution of $^{[125]}$IGF-1, were sacrificed 30 min after the start of the administration, and brain autoradiography imaging was conducted (Figure 19). Autoradiography demonstrated widespread CNS distribution of $^{[125]}$IGF-1 following intranasal administration, but not after intravenous administration.
Figure 19: $^{125}$I-IGF-1 autoradiography following intranasal administration showing widespread brain distribution (Thorne et al., 2004).

$^{125}$I-nerve growth factor (NGF; MW= 26.5 KDa), which is a powerful neurotrophic factor, has also been administered intranasally in a rodent model of Alzheimer’s disease (Thorne and Frey, 2001). The resulting CNS concentrations were between 0.1 and 4.0 nM, which constitute a 2 to 3 fold increase when compared with a standardized dose of NGF given intravenously. These brain concentrations were more than the concentration required in order for NGF to be therapeutically effective, as NGF’s EC$_{50}$ has been reported to be 6 pmol/L (Thorne and Frey, 2001).

Transforming growth factor beta-1 (TGF-$\beta$1), a 25 KDa homodimer, has also been administered intranasally to rats. Brain levels of TGF-$\beta$1 following intranasal
administration were determined by ELISA, and were found to be highest 1 hour after administration in most brain areas (Ma et al., 2007). In general, TGF-β1 levels were increased five-fold in the olfactory bulb, and 2–3 fold in the striatum, thalamus, cortex, and hippocampus relative to control levels in normal animals (Figure 20) (Ma et al., 2007).

Figure 20: Brain distribution and quantitative analysis of TGF-β1 following intranasal administration. Striatum levels are shown in bar # 3, and midbrain levels are shown in bar # 9 at all time points (Ma et al., 2007).

In non-human primates, intranasal administration of $^{125}$I-interferon-β 1b (IFN-β1b) resulted in rapid, widespread brain distribution with the highest levels observed in
the olfactory bulbs and trigeminal nerve, but with the next highest levels in the striatum and SN, the brain areas affected by PD (Figure 21) (Thorne et al., 2008). Measurable brain levels were detected by 60 min after intranasal administration, indicating a rapid nose-to-brain transport presumably along the olfactory and trigeminal nerve pathways.

Figure 21: $^{125}$I-IFN-β1b autoradiography following intranasal administration in monkeys. Striatal sections shown in A and B, and midbrain sections shown in the right panel (Thorne et al., 2008).

In addition to the above quantitative experiments, other researchers have demonstrated the nose-to-brain delivery of substances by providing functional evidence. For instance, intranasally administered NGF reverses the neurodegeneration that is characteristic of the AD11 mouse model of Alzheimer’s disease (Capsoni et al., 2002; De Rosa et al., 2005). Nasally administered fibroblast growth factor (FGF) was also demonstrated to increase motor activity and decrease rigidity in a mouse MPTP model of PD (Kucherianu et al., 1999; Thorne and Frey, 2001). Intranasally administered insulin-like growth factor I has also been shown to reduce the infarct size, and improve functional recovery in a middle cerebral artery occlusion model of stroke (Liu et al.,
Similarly, activity-dependent neurotrophic factor (ADNF; MW= 14 KDa), a powerful neurotrophic factor, was shown to improve rat performance in a water maze test following intranasal administration (Gozes et al., 2000). Finally, intranasally administered fibroblast growth factor-2 (FGF-2) and heparin-binding epidermal growth factor (HB-EGF) were shown to increase neurogenesis in the mouse subventricular zones (SVZ) and subgranular zones (SGZ) of the hippocampus (Jin et al., 2003). Neurogenesis was determined by measuring the cellular incorporation of bromodeoxyuridine (Jin et al., 2003).

Intranasal administration of peptides and other substances has also been attempted in human subjects. Peptides such as vasopressin (MW= 1100 Da), corticotropin releasing hormone (CRH; MW= 21,423 Da), growth hormone releasing hormone (MW= 3358 Da), insulin (MW= 6000 Da), and an analog of cholecystokinin (CCK-8) have all been reported to exert CNS effects in humans when administered intranasally (Thorne and Frey, 2001).

Finally, liposomal preparations have also been attempted for brain delivery of centrally-active molecules. Law et al. (2001), compared intranasally administered cationic and anionic liposomal preparations of desmopressin, as well as desmopressin in solution, in rabbits. They found that the cationic liposomal preparation of desmopressin resulted in higher nasal mucosal permeability, and greater anti-diuretic effect than the anionic liposomal preparation or desmopressin in solution (Law et al., 2001). In their cationic liposomal preparation, Law et al. used the same combination of lipids (DOPC, cholesterol, and stearylamine) that were used in this project. The conclusion for this
study was that the positively charged liposomal preparation interacted with the negatively charged mucosal membrane to increase residence time and mucosal permeability.

K. Liposomes

Liposomes are spherical vesicles that resemble cells in that they contain an inner hydrophilic core, and a relatively impermeable outer lipophilic phospholipid bilayer (Szoka and Papahadjopoulos, 1980). Bangham and colleagues first demonstrated more than 40 years ago that phospholipids spontaneously formed bilayered, enclosed structures when exposed to an aqueous medium (Bangham, 1963, 1972; Torchilin, 2005). However, it wasn’t until the early 1970’s that Gregoriadis and Ryman suggested that liposomes had the potential to be used as drug delivery vehicles (Gregoriadis and Ryman, 1971). Over the last couple of decades, liposomes have gained considerable attention as drug delivery carriers because they are biocompatible, non-toxic, can deliver both hydrophilic and lipophilic drug molecules, protect their cargo from degradation by plasma enzymes, and transport their load across biological membranes (Torchilin, 2005).

Liposomes can be made from several different types of lipids, however phospholipids are most commonly used to generate liposomes as drug carriers (Szoka and Papahadjopoulos, 1980). Although liposome formation is spontaneous when a lipid film is mixed with an aqueous solution, it can also be expedited by applying force in the form of shaking by using either a homogenizer or a sonicator, or by using an extrusion apparatus (Rawat et al., 2008). Generally, extrusion devices are preferred over homogenizers, not only because homogenizers apply excessive pressure and heat to the
mixture, but also because extrusion devices provide a means of controlling the size and the structure of the liposome (Rawat et al., 2008).

Several other additives may be added to liposomes in order to modify their structure and properties. For instance, either cholesterol or sphingomyelin may be added to the liposomal mixture in order to help stabilize the liposomal structure, and to prevent the leakage of the liposomal inner cargo (Szoka and Papahadjopoulos, 1980). Polyethylene glycol has also been added to the surface of liposomes in order to prevent liposomal aggregation in solution, to decrease liposomal uptake by the reticulo-endothelial system, and to increase the half-life of the liposomal formulation (Gabizon et al., 1994; Torchilin, 2005). These types of sterically stabilized liposomes are called stealth liposomes.

Depending on the method of preparation, there can be several different types of liposomes vesicles. Spontaneous formation of liposomes, and gentle shaking produce big multilamellar vesicles (MLV). MLV are liposomes with multiple concentric lipid layers, with up to fourteen layers, each separated by an aqueous solution (Kozubek et al., 2000; Torchilin, 2005). MLV tend to be present as a heterogenous mixture, with vesicle sizes ranging from 500-5000 nm (Torchilin, 2005). Extrusion, homogenization, or sonication of MLV can then result in either small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV) (Kozubek et al., 2000). SUV’s are liposomes whose structure contains only one lipid layer, and whose average diameter ranges from 25-100 nm (Kozubek et al., 2000; Torchilin, 2005). LUV’s, on the other hand, are liposomes that contain a single lipid layer, and whose diameter can range from 200-800 nm (Torchilin, 2005). Both the
SUV’s and the LUV’s tend to form more homogenous mixtures in terms of both size and shape of the liposomes (Kozubek et al., 2000).

Although liposomes contain an outer lipophilic membrane that increases their permeability across membranes, some biological barriers such as the blood-brain barrier remain impenetrable. Consequently, scientists have attempted to modify the liposomal structure in order to improve liposomal penetration across biological membranes, and into their target organs. One approach has been to create immunoliposomes, to take advantage of receptor-mediated endocytosis, in order to deliver drugs across membranes (Torchilin, 2005). Immunoliposomes are liposomes whose outer structure contains embedded antibodies against a particular target (Figure 22) (Gregoriadis et al., 1981). The antibodies can either be attached directly onto the surface of the liposomes, or they can be attached to the polyethylene glycol groups (PEG) on the liposomes. The latter is a more effective formulation of immunoliposomes because the PEG groups provide the liposomal structure with greater stability and a longer half-life (Torchilin, 2005). An example of immunoliposomes, are anti-HER2 immunoliposomes. Anti-HER2 antibodies recognize the HER2 receptor which is over expressed in tumor cells. Liposomes encapsulating cytotoxic agents and with anti-HER2 antibodies on their surface provide a more effective means of targeting such tumor cells (Torchilin, 2005). Immunoliposomes have also been used to facilitate liposomal transport across the blood-brain barrier. Liposomes coated with anti-transferrin antibodies have been used to improve liposomal penetration to the brain. For example, Shi and Pardridge demonstrated that they could successfully deliver plasmids encoding luciferase and β-galactosidase to the brain by using anti-transferrin immunoliposomes (2000).
Another approach that has been used to target liposomes across membranes involves using cationic liposomes to take advantage of adsorptive endocytosis (Miller et al., 1998; Briane et al., 2002). Adsorptive-mediated endocytosis occurs when the positively charged liposomes bind to the negatively charged plasma membrane. The binding of the liposomes to the outer membrane initiates a sequence of events that results in the endocytosis of the liposomes and their contents.

Lastly, an additional approach to targeting liposomes across biological barriers utilizes the trans-activating transcriptional activator (TAT) peptide to coat the surface of the liposomes. TAT is a peptide that allows the HIV-1 virus to easily get across plasma membranes (Torchilin, 2006). The mechanism by which TAT is able to translocate across cell membranes has been controversial. However, we do know that it is an energy
independent process, and recent research suggests that TAT may undergo endocytosis, bypassing cellular lysosomal degradative mechanisms (Torchilin, 2006).

Liposomes have been used clinically as delivery systems for therapeutic drug delivery of chemotherapeutic agents, antibiotics, and antifungals. This is because liposomal preparations have been shown to increase the margin of safety of many drugs, and also their efficacy (Kozubek et al., 2000). For instance, several chemotherapeutic agents (ex. Doxil®), antifungals (ex. Ambisome®), and antibiotics (ex. liposome-entrapped Rifampin) have been shown to be safer and more efficacious than their non-liposomal counterparts. However, to date no published work has used liposomes to deliver a growth factor, such as GDNF, to the brain by the intranasal route of administration.

### L. Glial Cell-Line Derived Neurotrophic Factor

As previously stated, the goal of this thesis project was to develop a non-invasive means of delivering GDNF to the brain using the intranasal route of administration. Glial cell-line derived neurotrophic factor (GDNF) is a heparin-binding basic protein originally isolated from rat B49 glial cell line culture medium. Its identification was prompted by its ability to promote dopamine uptake in midbrain cultures (Lin et al., 1993; Lin et al., 1994). Lin et al purified GDNF 34,000 fold by using heparin-sepharose chromatography, molecular sieving chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and reverse-phase high performance liquid chromatography (RP-HPLC). Protein characterization using HPLC and SDS-PAGE yielded a molecular weight of 33-45 KDa, and showed that GDNF was a glycosylated
protein indicated by a broad smear on SDS-PAGE. The glycosylation state of GDNF was further confirmed by using N-glycanase, which reduced its molecular weight from 20 KDa to 15 KDa. Further characterization of GDNF revealed it to be a disulfide-bonded homodimer because exposure to both reducing and non-reducing SDS gels resulted in bands of different molecular weights (i.e. 32-42 KDa for non-reducing gels and 18-22 KDa for reducing gels). GDNF’s isoelectric point (pI), as determined by its amino acid sequence, is 9.55 (http://www.embl-heidelberg.de/cgi/pi-wrapper.pl).

After purifying and characterizing the GDNF protein, Lin et al. were able to clone GDNF genes in both rats and humans using probes targeted against the amino terminal sequence of GDNF. It was determined that the rat and human GDNF protein have a sequence homology of 93% (Figure 23). Cloning further revealed that GDNF is synthesized as a precursor protein and that the mature protein contains 134 amino acid residues per monomer, with two potential N-glycosylation sites (Lin et al., 1993). However, glycosylation does not appear to be an essential component of GDNF’s structure-activity relationship since recombinant human GDNF, which lacks glycosylation, has been shown to be just as potent as the endogenous protein in vitro (Lin et al., 1994). On the other hand, GDNF’s interchain disulfide bond on Cys 101 residues appears to be essential for GDNF activity. Although GDNF’s secondary structure is only minimally affected by eliminating this disulfide bond, GDNF’s neurotrophic activity is destroyed by the reduction of this bond (Lin et al., 1994; Li et al., 2002).
Figure 23: Sequence of rat and human GDNF, and the relationship of GDNF with the TGF-β family (Lin et al., 1993).

GDNF was later found to be a member of the transforming growth factor-β (TGF-β) superfamily because it contains the characteristic seven conserved cysteine residues of the TGF-β family. However, GDNF only has a 20% sequence homology with other members of the TGF-β subfamilies, and it was determined to be the first member of a new subfamily (Lin et al., 2003). The GDNF family of neurotrophic factors now also includes neurturin, persephin, and artemin, which all share some conformational similarities with TGF-β2 and bone morphogenetic protein-7.
(Saarma, 2000). However GDNF, unlike the TGF-β superfamily, does not employ serine/threonine receptor kinases for signal transduction. Instead, signal transduction for the GDNF family employs tyrosine kinase receptors (Saarma, 2000).

GDNF protein tertiary structure was elucidated by x-ray crystallography, and revealed that GDNF has a cysteine knot motif, a single interdimer disulfide bond which is not part of the cysteine knot motif, two β-sheets called “fingers,” and two α-helices (Figure 24) (Chen et al., 2000). Deletion mutagenesis studies demonstrated that the α-helix containing amino acids 76-91 is essential for GDNF neurotrophic activity (Chen et al., 2000). GDNF’s actions were also abolished if the C-terminus 17 amino acids were deleted (Chen et al., 2000). However, it does not appear that amino acids 1-39 in the amine terminus are necessary for biological activity (Chen et al., 2000). Alternatively, insertion mutagenesis studies showed that inserting a GGGS amino acid mutation, which would disrupt the β-sheet on finger 2, would also destroy GDNF’s activity (Chen et al., 2000).
M. GDNF Receptors and Signal Transduction

The GDNF family receptor is a multi-subunit receptor with separate ligand binding and signal transduction domains (Treanor et al., 1996). The ligand binding domain consists of an extracellular receptor, GFRα (GDNF family receptor α), anchored to the outer plasma membrane by glycosylphosphatidylinositol (GPI) (Treanor et al., 1996). Four GFRα receptors have been identified, GFRα1-4. GDNF binds preferentially to GFRα1, neurturin to GFRα2, artemin to GFRα3, and persephin to GFRα4 (Figure 25). However, there appears to be some cross activation of the GFRα receptors between GDNF, neurturin, and artemin (Treanor et al., 1996). Wang et al. (2000) examined the biological significance of GFRα receptors cross activation by creating GFRα1 knock-out 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 et al., 2004). Alanine scanning mutagenesis studies have identified the following critical amino acid residues for GDNF binding to GFRα1: Asparagine 152 and 153, Arginine 259, Serine 316, Asparagine 317, and Serine 318 (Wang et al., 2004).

Figure 25: GDNF family of neurotrophic factors and receptors (Saarma, 2000).

In contrast, the signal transduction domain of the GDNF multicomponent receptor consists of the transmembrane tyrosine kinase receptor Ret. Trupp et al. (1996b) 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. GFRα1 receptor localization will be discussed in detail below.

Two models of GDNF receptor activation/signal transduction have been proposed, and will be referred to as the “Ret-dependent” mechanisms for the rest of the
The first model of Ret-dependent signal transduction suggests that binding of GDNF to GFRα1 stimulates the formation of homodimers, which then bind and dimerize two Ret receptors (Sariola and Saarma, 2003). Dimerization of the Ret receptors results in transphosphorylation of their tyrosine kinase domains, activation of the RAS/MAP kinase pathway, and phosphatidylinositol 3-kinase (PI3K) pathways. Ultimately, GDNF receptor activation results in cAMP response element binding protein (CREB) phosphorylation, increased expression of c-fos, increased protein synthesis, and cell survival (Sariola and Saarma, 2003). Additional evidence supporting the roles of MAPK and PI3K as mediators of GDNF neurotrophic activity comes from in vitro studies using dopamine neurons, the neurotoxin 6-OHDA and inhibitors of MAPK and PI3K (Ugarte et al., 2003). GDNF increases cell survival in dopamine neurons treated with 6-OHDA. However, this neuroprotective effect is blocked by adding either MAPK or PI3K inhibitors, suggesting that GDNF’s neurotrophic actions are mediated via these signaling cascades (Ugarte et al., 2003).

The second model of Ret-dependent signaling suggests that GFRα1 may associate with Ret prior to GDNF binding (Eketjäll et al., 1999; Saarma, 2000). Site-directed mutagenesis studies conducted by Eketjäll et al. (1999) demonstrated that a mutant form of the GDNF protein still retained its full Ret-activating ability, although it possessed poor GFRα1 receptor affinity (Figure 26). Further evidence suggesting that GDNF may interact more directly, and may be in direct contact with the Ret receptor is that GDNF can be chemically cross linked with Ret (Trupp et al., 1996a). In addition, experiments using Ret overexpressing cell lines demonstrated that GFRα and Ret can undergo low affinity binding in the absence of GDNF, and that this binding occurs in GFRα’s central
domain (Trupp et al., 1998; Scott and Ibanez, 2001). The site responsible for GFR α1’s interaction with Ret in the absence of GDNF was localized to its central domain (in close proximity to its ligand binding site; Figure 26) (Scott and Ibanez, 2001).

Figure 26: GDNF binding to the GFRα1 receptor showing key GDNF amino acid residues for receptor binding (Leppänen et al., 2004).
In addition to the previously mentioned models of GDNF signal transduction, GDNF has also been shown to signal in a “Ret-independent” manner. Recent data using Ret deficient cells has demonstrated that GDNF can activate intracellular signaling directly by just binding to GFRα1 (Saarma, 2000). 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. Further evidence that GDNF and GFRα1 can function independently from Ret activation was provided by Jain et al. (2006) who generated Ret conditional reporter knock-out mice, which lacked Ret specifically in dopaminergic neurons. A conditional reporter deletion of Ret was necessary because much like GDNF null mice, Ret null mice are not viable (Jain et al., 2006). Their results indicated that a Ret deficiency did not affect dopamine neuronal number and size in the SN and VTA of adult mice. Tyrosine hydroxylase positive fiber density was also unaffected. Functionally, these Ret conditionally deficient mice did not demonstrate any motor abnormalities (Jain et al., 2006). However, it is important to note that although Ret signaling is not essential, the high affinity binding of GDNF to GFRα1 has only been demonstrated when Ret and GFRα1 are co-expressed (Cik et al., 2000; Vieira et al., 2003). In addition, the rate of GFRα1 receptor internalization is slowed two fold in cells co-expressing Ret, making more receptors available for GDNF binding (Vieira et al., 2003). This indicates that the co-expression of Ret with GFRα1 facilitates GDNF binding to its receptor.

What is clear is that, although GDNF’s signaling may not be dependent on Ret, its neurotrophic effects are completely dependent upon GDNF’s ligand-receptor interaction with GFRα1. Moreover, it requires TGF-β as a cofactor (except in motor neurons).
Krieglstein et al. (1998) demonstrated by using neutralizing antibodies that inactivation of TGF-β completely inhibits GDNF’s neurotrophic effects. TGF-β and GDNF signaling involves the activation of phosphatidylinositol-3 (PI-3) kinase as an intermediate step. This was shown experimentally by using wortmannin, which is a PI-3 kinase inhibitor (Krieglstein et al., 1998). In addition, TGF-β has been shown to increase neuronal responsiveness to GDNF by initiating GFR α1 translocation to the cell membrane surface, and not by upregulation of GDNF or GFR α1 receptor mRNA expression as one might expect (Peterziel et al., 2002; Sariola and Saarma, 2003). These studies indicate that TGF-β is an essential co-factor for GDNF to exert its neurotrophic effects.

N. Anatomical Distribution of GDNF and its Receptors

In situ hybridization has been used to determine the anatomical location of GFRα1 receptor mRNA, Ret mRNA, and GDNF mRNA. Immunohistochemistry, on the other hand, has been used to determine the location of the respective expressed proteins. The presence of the mRNA in a region indicates the location of the cells, or neuronal cell bodies, that make the protein but does not necessarily mean that the protein exerts a local effect in that area. In fact, both the GDNF and GFRα1 receptor proteins may be transported down axons and be expressed by nerve terminals some distance from the cell bodies where the mRNA exists. The anatomical distribution of GFRα1 and Ret receptors mRNA will be reviewed first, followed by a discussion of immunohistochemical localization of their expressed proteins, and concluding with a review of what is known about GDNF mRNA and protein distribution.
Several in situ hybridization studies conducted in adult rodents, have shown that the GFRα1 receptor mRNA is widely expressed in the central nervous system (Golden et al., 1998). GFRα1 receptor mRNA expression was detected in: the forebrain (including the olfactory tubercle, the septum, diagonal band of Broca’s area, piriform cortex, neocortex, and ventral pallidum), olfactory bulb, locus coeruleus, midbrain including the substantia nigra (high levels in the pars compacta but limited labeling in the pars reticulata; Figure 27), the ventral tegmental area (VTA), periaqueductal gray, rostral raphe, interfascicular nucleus, and in Edinger Wesphal nucleus, and tectum (Nosrat et al., 1997; Golden et al., 1998). GFRα1 receptor mRNA is also present in the oculomotor nucleus, cerebral cortex, red nucleus, thalamus (high levels in the reticular nucleus), hypothalamus, hippocampus, brainstem, spinal cord (high expression in the motor neurons of the ventral horn), and cerebellum (Nosrat et al., 1997; Golden et al., 1998). In the striatum, GFRα1 receptor mRNA expression has only been shown during development, with maximum expression occurring between post-natal days 10 and 14 (Nosrat et al., 1997; Cho et al., 2004).

**Figure 27:** Adult mouse coronal sections showing GFRα1 receptor mRNA (A), and Ret mRNA (C) localization in the substantia nigra and VTA (Golden et al., 1998).
Conversely, peripheral nervous system GFRα₁ receptor mRNA expression is localized to: brachial and lumbar motor neurons (Oppenheim et al., 2000), Schwann cells, the superior cervical ganglion, trigeminal ganglion, nodose ganglion, and the dorsal root ganglion (Nosrat et al., 1997; Golden et al., 1998). These peripheral sites of GFRα₁ mRNA expression correlate with important sites of GDNF action. For instance, in the dorsal root ganglion, GDNF has been shown to attenuate hyperalgesia in a rat model of neuropathic pain (Wang et al., 2003b; Dong et al., 2006). Also, in the spinal cord, GDNF secreting primary fibroblasts were shown to induce axonal regeneration and remyelination in a rat model of spinal cord injury (Blesch and Tuszynski, 2003). The GFR α₁ receptor mRNA is also widely expressed in various peripheral tissues and glands. For instance, GFRα₁ receptor mRNA expression is abundant in the kidneys, in developing teeth, in tongue papillae, and in the gastrointestinal tract ganglia (Nosrat et al., 1997). In addition, GDNF protein, GFRα₁ receptor mRNA, and Ret mRNA expression have been detected in the anterior pituitary, and may play a role in the development of pituitary tumors (Urbano et al., 2000; Japón et al., 2002).

Ret mRNA expression, on the other hand, does not always correlate with GFRα₁ receptor mRNA expression. For instance, no Ret mRNA has been found in the striatum, hippocampus, Schwann cells, and in tongue papillae (Nosrat et al., 1997; Golden et al., 1998). Ret mRNA expression has been found in both developing and adult dopamine neurons of the SN and VTA (Figure 27), in α motor neurons, thalamus, ruber and oculomotor nuclei, habenular complex, septum, cerebellum, and brainstem (Nosrat et al., 1997). Ret mRNA expression is also present in the dorsal root ganglia, sympathetic
ganglia, trigeminal ganglia, developing teeth, inner ear, retina, olfactory epithelium, kidneys, and the gastrointestinal tract ganglia (Nosrat et al., 1997).

Turning next to the localization of GFRα1 and Ret protein expression, immunohistochemistry has revealed that GFRα1 receptor protein is present in the SN, cerebellum, olfactory bulb, diagonal band, oculomotor nucleus, trochlear nuclei, substantia innominata, zona incerta, thalamus, cerebellar cortex, cranial nerves, spinal motor neurons, and medial forebrain bundle (Buckland and Cunningham, 1999; Kawamoto et al., 2000; Matsuo et al., 2000). Ret protein immunoreactivity, on the other hand, has only been demonstrated in the SN, VTA, and weakly in α motor neurons (Nosrat et al., 1997). The location of both GFRα1 and Ret protein in the SN, but not the striatum, suggests that the most important target for GDNF in our studies would be the SN. However, the widespread localization of the receptor proteins at other central and peripheral sites suggests that systemic distribution of GDNF could lead to unwanted effects at many non-target sites.

The expression of GDNF mRNA has also been studied extensively and was found to be somewhat different than the localization of its receptor and the protein itself. GDNF mRNA expression in rodents has been found in the striatum, nucleus accumbens, thalamic nuclei, olfactory tubercle, hippocampus, cerebellum, cingulate cortex, and olfactory bulb, but interestingly not the SN (Nosrat et al., 1997; Trupp et al., 1997). The images below are sagittal sections of an adult mouse brain showing GDNF mRNA distribution and intensity. These images were taken from the on-line Allen brain atlas (http://www.brain-map.org.www.allenbrainatlas.org), which used in situ hybridization to map the location of thousands of different mRNA’s throughout the adult mouse brain.
The first image, Figure 28, shows the anatomical location of different brain regions in pastel colors, and GDNF gene expression is represented in the form of dots, with different colors to indicate relative mRNA abundance in that region. The second image, Figure 29, only shows GDNF mRNA expression, without showing the distinct brain regions. The third image is a brain atlas image showing the anatomical location of different brain regions for comparison (Figure 30). These images should be viewed together for better clarity. Note that the GDNF mRNA signal in the midbrain is modest, which may indicate a low level of expression in the area of the SN and VTA of the adult brain.

**Figure 28:** Three dimensional representation of a sagittal brain section showing the distribution and intensity of GDNF gene expression (http://www.brain-map.org.www.allenbrainatlas.org).
Figure 29: Sagittal brain section showing the distribution and intensity of GDNF gene expression (http://www.brain-map.org/www.allenbrainatlas.org).

Figure 30: Sagittal brain section identifying key brain regions. The substantia nigra and SN are identified by a red circle. Adapted from The Rat Brain Atlas (Paxinos and Watson, 1998).

For comparison, Figure 31 shows the distribution of GFRα mRNA. The Allen brain atlas did not differentiate between the different GFR alpha subtypes, but did show
that GFRα mRNA is present in the ventral midbrain where the SN and VTA are located. In addition, it is interesting to note that for unclear reasons it was not possible to map Ret mRNA expression in the adult mouse brain. Nevertheless, it is apparent that GFRα mRNA distribution throughout the brain is scarce in comparison with that of the GDNF mRNA. This may prove to be an advantage in our research, in that intranasally administered GDNF will only be able to act in areas where GFRα is present thereby minimizing effects at non-target sites.

![Sagittal brain section showing the distribution and intensity of the GFRα gene expression](http://www.brain-map.org.www.allenbrainatlas.org).

**Figure 31:** Sagittal brain section showing the distribution and intensity of the GFRα gene expression.

The apparent mismatches between GDNF and GFRα mRNA expression may be due to their differential down-regulation during post-natal maturation and aging. In humans, one in situ hybridization study conducted on post-mortem brain tissues (mesencephalon and striatum) from eight PD patients and six aged-matched control patients failed to detect any GDNF mRNA expression (Hunot et al., 1996). This effect was apparently not due to a faulty assay because Hunot et al. used the same assay on
brain tissue obtained from biopsies of astrogloma patients and on the brain of a newborn and found that GDNF mRNA was expressed in these two groups. These findings suggest that GDNF mRNA is down-regulated in adulthood, and that only small quantities are present in the adult human brain.

Despite the low levels of its mRNA, GDNF protein is expressed in some brain areas in adulthood. In fact, a Western blot analysis of adult human brains revealed that the GDNF protein was present in both the frontal cortex and cerebellum. In rodents, GDNF immunoreactivity was found to be present in the SN, olfactory neurons, olfactory bulb, spinal trigeminal, cuneate, solitary, vestibular, and cochlear nuclei (Buckland and Cunningham, 1999; Del et al., 2002). GDNF immunoreactivity was also found in the vagus nerve, ventral grey column, hypoglossal nucleus, medullary reticular formation, pontine grey and pontine tegmentum, locus coeruleus, tectal plate, trochlear nucleus, raphe nuclei, linear nuclei, and cuneiform nucleus (Del et al., 2002). These results suggest that some GDNF protein is expressed in the adult brain (Kawamoto et al., 2000), however its normal role may be during early neurogenesis and its expression may be down regulated during post-natal development and maturation.

GDNF is also expressed in a variety of tissues outside the brain. In the periphery, GDNF mRNA expression has been found in dental pulp cells (Nosrat et al., 2004), and in stromal keratocytes in the eye (where it stimulates the proliferation of corneal epithelial cells) (You et al., 2000). In the enteric nervous system, GDNF is essential due to its role in enteric neuron proliferation during development. A deficiency of GDNF can result in Hirschsprung’s disease (obstruction of the distal intestine due to aganglionosis) (Gianino et al., 2003). GDNF expression has also been confirmed in the kidneys where it is
responsible for nephrogenesis during development (Orth et al., 2000). GDNF’s role in nephrogenesis has been verified in GDNF knock-out mice, which display a short-lived phenotype and are born without kidneys (Pichel et al., 1996; Sánchez et al., 1996). Also, GDNF has been implicated in the development of Wilms tumor, a childhood solid tumor of the kidney (Séguier-Lipszyc et al., 2001). Furthermore, GDNF may play a role in the development of glomerulosclerosis and renal failure, since patients with chronic renal failure demonstrate higher plasma GDNF levels (Orth et al., 2000). These pathological changes in states of GDNF-overexpression underscore the point that peripheral distribution of GDNF must be kept to a minimum in order to avoid peripheral adverse effects. This project attempted to deliver GDNF to the brain, bypassing the peripheral circulation, in order to avoid GFRα1 receptor activation in peripheral organs and any accompanying unwanted systemic side effects.

O. Regulation of GDNF and GFRα1 expression

Little is known about how GDNF and its receptors are regulated. However, certain factors that modulate their expression have been identified. For instance, GFRα1 and GDNF expression have been shown to be upregulated in the brain and spinal cord after an ischemic event, thus resulting in neuroprotection (Sakurai et al., 1999; Miyazaki et al., 2001; Sarabi et al., 2003; Tokumine et al., 2003b; Tokumine et al., 2003a). In addition, GDNF exogenous administration has been demonstrated to have a profound neuroprotective effect on the cerebral cortex of rats undergoing middle cerebral artery (MCA) ligation (Wang et al., 1997). These data suggest that GDNF may play an important role in protecting the CNS from ischemic events and reperfusion injury.
GDNF expression has also been shown to be regulated by various neurotransmitters and neuroactive drugs. Serotonin application to a C6 glioblastoma cell culture, for instance, resulted in a dose-dependent increase in GDNF mRNA expression (Hisaoka et al., 2004). This finding suggests that GDNF may play a role in the pharmacological activity of antidepressant drugs. GDNF levels can also be altered by D1 and D2 receptor agonists (Ohta et al., 2003). Bromocriptine, a selective D2 receptor agonist, can significantly decrease GDNF expression in mouse astrocytes cultures. Conversely, pergolide and cabergoline, which are mixed D1 and D2 receptor agonists, significantly increase GDNF expression in vitro. Therefore, mixed D1 and D2 receptor agonists could play a role in delaying the progression of PD by increasing GDNF availability.

Another factor that can modulate the expression of GDNF and its receptors is phencyclidine (PCP) (Semba et al., 2004). Semba et al. (2004) conducted in situ hybridization studies and demonstrated that rats chronically administered PCP have higher expressions of GDNF and Ret mRNA in their SN pars compacta and ventral tegmental area (VTA). GDNF upregulation in response to chronic PCP administration may represent a neuroprotective mechanism against the neurotoxic effects of PCP. GDNF receptor expression may also be affected by other neurotoxic stimuli, such as administration of 6-OHDA. GFRα1 and Ret mRNA levels are briefly upregulated in the SN following striatal administration of 6-OHDA (Marco et al., 2002). However, after only 3 days post-lesioning, GFRα1 and Ret become downregulated (Marco et al., 2002). Smith et al. (2003) provide further evidence that 6-OHDA modulates GDNF receptor expression. By creating a medial forebrain bundle lesion in adult rats, Smith et al.
demonstrated that the GDNF protein level is unaffected in the striatum over a period of 60 days, but that mRNA expression of GFRα1 and Ret become downregulated in both the SN and VTA. This downregulation of GFRα1 and Ret mRNA expression in the SN and VTA may simply reflect the 6-OHDA-mediated loss of dopamine neurons since these cells express the receptor in the ventral midbrain. Additionally, the finding that GDNF protein levels are not upregulated in striatum after a 6-OHDA lesion (Smith et al., 2003) suggests that any increases in striatal GDNF provided by intranasal administration can be directly attributed to that which was exogenously administered, and not to an upregulation of endogenous GDNF production after the 6-OHDA lesion.

**P. Experimental Evidence of GDNF’s Neurotrophic and Neuroprotective Effects**

The therapeutic potential of GDNF in the treatment of PD is due to its selective neurotrophic and neuroregenerative effects on dopaminergic neurons. The mechanism by which GDNF exerts these effects on dopamine neurons is not yet fully understood. Microdialysis studies performed on rat hippocampus demonstrated that GDNF significantly reduces free radical production and increases the activities of glutathione peroxidase and superoxide dismutase (free radical scavengers) following kainate-induced excitotoxicity (Cheng et al., 2004). A similar mechanism may operate in the SN since dopamine neurons are particularly susceptible to oxidative damage. In fact, endogenous dopamine metabolism generates several free radical species (Jenner and Olanow, 1996). When dopamine is metabolized to DOPAC, hydrogen peroxide is also formed, which then gets converted to hydroxyl radicals by the Fenton reaction. The Fenton reaction is fully dependent upon the availability of ferrous ions, which are found in abundance in the
Increased oxidative stress, coupled with diminished antioxidant stores, can lead to lipid peroxidation and cell death.

In addition, studies have suggested that GDNF levels are decreased by as much as 19.4% per SN neuron in patients with PD (Chauhan et al., 2001; Hurelbrink and Barker, 2004). This suggests that supplementation of this growth factor may restore this imbalance, decrease free radical production, increase antioxidant levels, and ultimately arrest the progression of PD. In this review, the extensive evidence supporting the use of GDNF as both a neurotrophic and neuro-restorative factor for dopamine neurons, and its potential role in the treatment of PD, will be summarized.

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 et al., 1994). This suggests that only small quantities of GDNF may need to reach the basal ganglia in order to produce a therapeutic effect in 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 et al., 1993). In addition, rhGDNF has been shown to increase dopamine cell body size and to cause sprouting of their dendritic processes in vitro (Lin et al., 1993). Furthermore, in rat ventral tegmental area cultures, GDNF doubled the number of synaptic terminals in TH positive neurons as determined by synapsin-1 immunofluorescence (Bourque and Trudeau, 2000). GDNF has also been shown to inhibit apoptotic cell death in dopamine neuron cultures (Burke et al., 1998; Oo et al., 2003; Li et al., 2007). Burke et al. observed that GDNF-treated postnatal dopamine neurons were able to survive the natural
process of apoptosis, which normally occurs during the first two weeks after birth. In contrast, sympathetic neuronal cultures lacking GDNF have been demonstrated to die via a “nonmitochondrial caspase-dependent pathway,” suggesting that unassociated Ret may directly activate caspases (Yu et al., 2003). Akerud et al. (1999) confirmed that GDNF improves neuron survival, increases soma size, and induces neuronal sprouting in cultures of midbrain dopamine neurons. What’s more, these authors showed that GDNF was very effective at protecting dopamine neurons from 6-OHDA’s oxidative damage in the standard in vivo rat model of PD. By implanting GDNF secreting fibroblasts into the SN pars compacta, Akerud et al. found that 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.

The question has arisen as to whether GDNF can also stimulate neurogenesis, i.e. the generation of new neurons from neuronal precursor cells, thereby regenerating the lost dopamine neurons in the Parkinsonian brain. Recent evidence suggests that GDNF does increase cell proliferation in the adult rat SN by 52%, but none of the new bromodeoxyuridine (BrdU) positive cells were TH positive neurons (Chen et al., 2005). This indicates that although GDNF can stimulate proliferation of glial cells in the SN, it does not generate new dopamine neurons. In fact, it is controversial as to whether or not neurogenesis can even occur in the adult SN. Several lines of evidence suggest that neurogenesis does not occur in the adult SN, even after a neurotoxic stimulus like a 6-OHDA lesion (Frielingsdorf et al., 2004; Liu et al., 2006). On the other hand, other studies suggest that neurogenesis can occur in the adult SN (Zhao et al., 2003). Further research is needed to resolve this controversial subject.
Assuming GDNF does not stimulate dopamine cell neurogenesis, then GDNF must act before the dopamine cells have died in order to exert its neuroprotective effect. This suggests that administering GDNF either concurrently, or just prior to the generation of a 6-OHDA lesion, may yield a more significant neuroprotective response. Administering GDNF after the lesion has already formed might prove to be less useful because GDNF would only be able to rescue and promote the recovery of damaged neurons, but would not be able to regenerate dopamine neurons to replace the ones that died. Evidence that GDNF administration is time-sensitive was provided by Ding et al. (2004) by using dopamine neurons in culture, and by Kearns et al. (1997) in vivo. Ding et al. exposed dopamine neurons to varying concentrations of 6-OHDA in the absence or presence of GDNF. They 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 a time course study where GDNF was administered directly into the right SN pars compacta at 0 hours (i.e. concurrently), 1, 6, 12, and 24 hours prior to a 6-OHDA lesion. They found that maximal protection of SN TH positive neurons occurred at the 6-hour time point. GDNF administration at the 1-hour and 0-hour time points provided no statistically significant increase in SN dopamine neuronal survival when compared to control. Furthermore, it appears that GDNF’s neuroprotective effect is dependent upon protein synthesis, because pretreatment with cycloheximide (an inhibitor of protein synthesis) resulted in decreased numbers of SN TH positive neurons (Kearns et al., 1997).

On the contrary, Hoffer et al. (1994) showed that intranigral administration of GDNF, administered four weeks after a medial forebrain bundle 6-OHDA lesion in rats,
decreased apomorphine-induced rotations, and increased dopamine levels in the ipsilateral SN, both indices of a neuroprotective effect. Aoi et al. (2000b) 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. Furthermore, Tomac et al. (1995) demonstrated that intranigral GDNF significantly increased dopamine levels when administered 1 week after systemic administrations of MPTP in a mouse model of PD. A more recent study comparing GDNF with a newly identified neurotrophic factor, CDNF, demonstrated that intrastriatal GDNF (10 µg) given four weeks after a striatal 6-OHDA lesion in rats resulted in significant SN dopaminergic neuroprotection 12 weeks post-lesion (Figure 32) (Lindholm et al., 2007). The neuroprotective effects of GDNF have also been shown to be long lasting, even against a medial forebrain bundle 6-OHDA lesion. Sullivan et al. (1998) confirmed that intranigral GDNF, administered prior to a 6-OHDA lesion, increased the number of TH positive neurons in the SN and ventral tegmental area even after 13 weeks. Taken together, these studies indicate that GDNF may protect dopamine neurons from neurotoxin-induced damage when administered hours before, concurrently, or even weeks after administration of the toxin, and the neuroprotection is long-lasting.
Intrastriatal GDNF administration given 4 weeks following a striatal 6-OHDA lesion in rats results in significant (\(P< 0.05\)) SN dopaminergic neuroprotection (Lindholm et al., 2007).

GDNF’s neurotrophic effects are not only time-dependent, but they are also reliant upon the route and site of administration. Both intracerebroventricular and intraparenchymal administrations of GDNF protein have been attempted. Intraparenchymal administrations of GDNF protein have been limited to the corpus striatum and the SN. Intranigral GDNF injections, administered 6 hours prior to a unilateral intranigral 6-OHDA lesion in rats, prevent the loss of TH positive dopamine neurons in the SN (Kearns and Gash, 1995; Kearns et al., 1997). Furthermore, Cass and Manning (1999) showed that intranigral GDNF administration 6 hours prior to a unilateral intranigral 6-OHDA lesion could effectively increase dopamine levels in both the SN and the striatum. Bowenkamp et al. (1995) confirmed that intranigral GDNF administration can significantly increase the number of SN TH positive neurons. Behavioral and microdialysis studies conducted on 6-OHDA lesioned rats also showed that a single intranigral GDNF injection was capable of decreasing apomorphine-induced rotation, increasing locomotor activity, and increasing dopamine levels for up to four
weeks after administration, all evidence of a neuroprotective effect (Hoffman et al., 1997). In another study, intranigral GDNF has been reported to be effective in rescuing SN dopamine neurons from retrograde 6-OHDA oxidative damage, although it was ineffective at protecting against local tissue damage caused by the striatal 6-OHDA injection (Winkler et al., 1996; Kirik et al., 2004). In addition, intranigral GDNF administration has also been demonstrated to protect SN dopamine neurons from a medial forebrain bundle axotomy (Beck et al., 1995; Tseng et al., 1997).

Intrastriatal administration of GDNF exerts significant neurotrophic effects on the nigrostriatal dopamine system, although most of the benefits of intrastriatal GDNF are attributed to its retrograde transport to the cell bodies of SN dopamine neurons (Aoi et al., 2000b; Oo et al., 2003). For instance, intrastriatal GDNF injections can inhibit apoptosis of SN dopamine neurons by as much as 61% in rats (Oo et al., 2003). These authors also demonstrated, by administering intrastriatal injections of anti-GDNF antibodies, that endogenous GDNF can suppress apoptosis 2-3 fold in nigral dopamine neurons. Finally, unilateral intrastriatal administration of GDNF was shown to increase the firing rates of striatal neurons bilaterally in aged rats (Stanford et al., 2005). All of these studies provide evidence of the neuroprotective and neurotrophic effects of GDNF on the nigrostriatal dopamine system.

Thus far, discussion of GDNF’s neuroprotective effects has been centered on in vitro and in vivo rodent animal studies. GDNF has also been proven to be neuroprotective, increase dopamine release, improve motor function, and to decrease parkinsonian symptoms in non-human primates (Gash et al., 1995; Gash et al., 1996; Zhang et al., 1997; Gash et al., 1998; Grondin et al., 2003). For instance, in aged rhesus
monkeys continuous intrastriatal infusions of GDNF (at a dose of 22.5 µg/day) increased the number of ipsilateral SN TH positive neurons by 18%, and increased their cell body size by as much as 28% (Ai et al., 2003). In addition, Gerhardt et al. (1999) used a unilateral MPTP rhesus monkey model of PD to demonstrate that a single intracerebroventricular GDNF injection not only improved parkinsonian symptoms, but it also significantly increased dopamine levels in the ipsilateral SN. Grondin et al. (2002) also provided evidence that chronic GDNF infusions (5 or 15 µg/day) increase SN dopamine cell size by more than 30%, SN TH positive neurons by more than 20%, and striatal TH positive fiber density by 5 fold in MPTP-treated rhesus monkeys.

Q. GDNF Clinical Trials

The extensive pre-clinical data summarized above prompted investigators to begin considering GDNF use in human subjects. Kordower et al. (1999) published the first case report of GDNF administration to a PD patient almost 10 years ago. GDNF was administered monthly via an indwelling right intracerebroventricular (I.C.V.) catheter. The patient had been part of a placebo-controlled double-blind phase I study of GDNF and had received monthly injections of either 75 µg of GDNF or vehicle. Upon completion of this study, the patient continued to receive I.C.V. injections of GDNF at doses escalating up to 300 µg (for a total of 6 additional doses until his death) (Kordower et al., 1999). Unfortunately, GDNF proved to be ineffective at decreasing this patient’s parkinsonian symptoms, and he experienced several adverse effects as a result of GDNF administration. Some of the side effects included weight loss, nausea, anorexia, tingling sensations, hyponatremia, hallucinations, depression, and L’hermitte’s phenomenon (a
tingling sensation going from the neck to the arms and lower back when the patient bends
their neck).

The results of the first multicenter, double-blind, placebo-controlled clinical trial
involving I.C.V. GDNF administration were published in 2003 (Nutt et al.). Fifty PD
patients were randomized to either the placebo group or the GDNF treatment group. The
treatment group received 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 standardized Unified Parkinson’s disease rating scale (UPDRS), and similar adverse
effects were reported as those described by Kordower et al. in 1999. So the question
remains, why did this first clinical trial fail when the pre-clinical animal studies had been
so promising? The consensus view was that GDNF may never have reached its intended
sites of action, the SN and striatum.

Intracerebroventricular GDNF administration presupposes that GDNF would be
distributed from the cerebrospinal fluid (CSF) to brain parenchyma, which may not really
occur to a great extent. After I.C.V. administration, distribution is severely limited by the
rapid clearance of the CSF into both the lymphatic, and the venous systems (Thorne and
Frey, 2001). In addition, the ventricles of the brain are lined by ependymal cells bound
together by tight junctions, which form a barrier against the distribution of substances
from the CSF to the brain, the so-called CSF-brain barrier. Therefore, delivery of a
therapeutic protein or a drug into the CSF does not directly correlate with its delivery to
the brain.

As a result, neurologists in England devised a catheter for continuous intrastriatal
GDNF delivery in humans, and a second clinical study was initiated (Figure 33). Briefly,
five patients with idiopathic PD underwent stereotaxic surgery to permanently implant catheters (four patients bilaterally) into their posterior-dorsal caudate putamen (Gill et al., 2003). In addition, these patients also had continuous-infusion pumps implanted into their abdominal cavities. 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 I.C.V. infusions, intraparenchymal 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 et al., 2003). In addition, two patients experienced increased sensitivity to Levodopa treatment resulting in a decrease in their Levodopa dose, and three patients regained their sense of smell and taste after only six weeks of GDNF infusions.

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. An increase in striatal dopamine uptake indicates an increase in the presence of healthy and biologically active dopamine nerve terminal transporters. On the other hand, GDNF’s side effects were less pronounced than those observed in the I.C.V. trial and did not include weight loss and anorexia. 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 et al., 2005). In addition, no severe adverse effects were reported.
Figure 33: Catheter used for intrastralial administration of GDNF in humans, and illustration of GDNF’s retrograde transport to the SN (Patel, 2004).

Further evidence of GDNF’s neurotrophic and neuroprotective effect in humans, was obtained from post-mortem immunohistological examination of the brain of one of the British study participants (who died of unrelated natural causes). This patient suffered from hemiparkinsonian symptoms, and therefore received only contralateral GDNF infusions. Tyrosine hydroxylase immunohistochemistry demonstrated a five-fold increase in striatal staining in the GDNF treatment site when compared to the contralateral side, suggesting that GDNF stimulated sprouting of dopamine nerve terminals in this area (Love et al., 2005).

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” (de la Fuente-Fernandez et al., 2001). 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. demonstrated that placebo can elicit a significant increase in striatal dopamine release in PD patients.

As a result, Amgen sponsored the first multicenter, double-blind, placebo-controlled trial involving intrastriatal administration of recombinant methionyl human GDNF (r-metHuGDNF; Liatemin®). Thirty four patients were randomized to receive either GDNF (15 µg/stripatum/day), or placebo bilaterally (Lang et al., 2006). Unexpectedly, GDNF treatment was found not to be significantly different from placebo as assessed by the UPDRS motor scores, even though a significant difference of 32.5% in $[^{18}F]$-dopamine influx constant was observed in the GDNF treatment group (Lang et al., 2006). In addition, UPDRS motor scores off of PD medications were decreased by 10% in the GDNF group and by 4.5% in the control group, a difference that was not statistically significant with a $P= 0.53$ (Lang et al., 2006). 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. Additionally, 18 out of 34 patients tested positive for serum anti-rhGDNF binding antibodies (Tatarewicz et al., 2007). Four out of those eighteen patients actually demonstrated immunoglobulin class switching from IgM to IgG, and developed neutralizing activity against GDNF, although the significance of this finding is still unknown. However, one patient involved in the British trial also developed serum anti-GDNF antibodies, and has not experienced any adverse effects. On the contrary, this patient continued to experience GDNF’s neurotrophic effects even 3 yrs after discontinuing treatment, as assessed by UPDRS motor scores.
These findings, together with the results of a 6-month toxicology study in monkeys which showed Purkinje cell loss following intrastriatal administration of GDNF (Figure 34), prompted Amgen to abruptly discontinue its GDNF clinical trial in February 2005 (Lang et al., 2006; Hovland et al., 2007). In this toxicology study, rhesus monkeys were treated with a unilateral intraputametal infusion of GDNF at the following daily doses: 0 µg (citrate buffered saline vehicle), 15 µg, 30 µg, or 100 µg. A total of 59 monkeys were tested at the various GDNF daily doses. Cerebellar toxicity was only seen in 4 animals that received 100 µg of GDNF/day (out of a total of 15 animals in this group). The fact that cerebellar toxicity was seen in only a few monkeys at the highest dose tested suggests that the effect was likely due to a non-specific mechanism. Moreover, since GFRα distribution in the cerebellum is modest, it was probably not attributable to GDNF acting specifically on its receptors. Endogenous GDNF mRNA, on the other hand, is relatively abundant in the cerebellum, suggesting that GDNF may already be present at higher levels in the cerebellum. Nevertheless, in humans this toxicological effect did not appear to be present. Neurological examinations and MRI studies done on nine of the participants of the Amgen trial revealed that these patients experienced no significant changes in their cerebella when compared to imaging studies obtained just prior initiation of GDNF infusions, and they exhibited no clinical symptoms of cerebellar dysfunction (Chebrolu et al., 2006). Altogether, these clinical results suggest that the cerebellar cell loss observed in a small sub-group of monkeys given high doses of GDNF was likely to have been a non-specific rather than a pharmacological effect of the treatment.
What factors could account for the different findings between the open-label trial and the placebo-controlled trial? The strongest factor could be patient selection. The open-label trial employed PD patients suffering from less severe motor symptoms and whose PD was not as advanced, whereas the participants in the Amgen study had more advanced PD (Lang et al., 2006). Consistent with the previously mentioned pre-clinical data, this might suggest that GDNF was more effective at protecting surviving dopamine neurons.
neurons from degeneration rather than generating new neurons (Chen et al., 2005). Additionally, the double-blind study used a different catheter to deliver GDNF to the striatum than the open-label trial (Lang et al., 2006) raising the possibility that delivery was not as effective in the Amgen trial. However, what is clear from this clinical trial is that this route of administration resulted in several unacceptable adverse effects and an inconsistent clinical response.

R. Alternative GDNF Delivery Strategies

The development of an improved drug delivery system that would allow the safe and effective delivery of GDNF to the brain is integral to bringing GDNF into clinical practice. Accordingly, several attempts have been made to find alternative methods to deliver either the GDNF protein, or its gene to the brain. For instance, Jiao et al. (1996) successfully delivered therapeutically relevant quantities of GDNF to the brain by osmotically disrupting (i.e. opening) the BBB through mannitol administration. Other researchers developed sustained-release drug-delivery carriers to encapsulate the GDNF protein for surgical implantation into target brain areas (Aubert-Pouëssel et al., 2004). Jollivet et al. (2004a; b), for example, implanted rat striata with poly D,L-lactic-co-glycolic acid (PLGA) microspheres loaded with GDNF and demonstrated protection against a 6-OHDA lesion. Another strategy that has been used to deliver GDNF across the BBB is to construct a TAT-GDNF fusion protein (Kilic et al., 2003). As previously stated, TAT is an 11 amino acid peptide derived from the human immunodeficiency virus’s transduction domain, and it is effective at penetrating cell membranes and the
BBB. Kilic et al. demonstrated that sufficient quantities of TAT-GDNF cross the BBB after intravenous administration to confer neuroprotection in a mouse model of stroke.

In the case of gene delivery, adenoviral vectors, lentiviral vectors, and adeno-associated viral vectors have all been used to deliver the GDNF gene to either the SN or the striatum (Björklund et al., 2000). However, all gene transfer methods share the common disadvantage of being irreversible and involving use of potentially harmful viral vectors. In the event of an adverse effect, these treatments could not be discontinued. In addition, targeting the vector to a particular brain area requires surgical implantation and its associated risks. Despite these drawbacks, GDNF gene therapy remains an active area of research.

Adenoviral vectors have been used to transfect cells because of their capacity to carry large quantities of genetic information, and their ease of replication (Björklund et al., 2000). Furthermore, adenoviral vectors can transfect both dividing and non-dividing cells (such as neurons). Both intrastriatal and intranigral injections of adenoviral vectors containing the GDNF gene have been used successfully in rats to prevent dopaminergic neuron degeneration after a 6-OHDA lesion (Bilang-Bleuel et al., 1997; Choi-Lundberg et al., 1997). This was also true in aged rats who received an intrastriatal 6-OHDA lesion (Connor, 2001). In addition, spinal injections with an adenoviral vector encoding the GDNF gene have also been demonstrated to significantly transfect, and protect spinal motor neurons from degeneration after a lesion (Watabe et al., 2000). Significant GDNF expression has also been shown in non-human primates after an intrastriatal injection of adenoviral vectors containing the GDNF gene (Kozlowski et al., 2001). However, the disadvantage to adenoviral vector use is that transfection with these vectors may also
result in expression of adenoviral proteins, which can then lead to inflammation and activation of an immune response (Björklund et al., 2000).

Lentiviral vectors, on the other hand, are made from virulent retroviruses, such as the Human Immunodeficiency virus (HIV) (Björklund et al., 2000). From a safety standpoint, there is still a significant concern even though lentiviral vectors only have 10% of their own genome remaining (Kordower, 2003). Then again, lentiviral vectors do have the advantages of being able to efficiently infect non-diving cells, they have large loading capacities, and they are capable of long term gene expression and high yields (Björklund et al., 2000). Arvidsson et al. (2003) demonstrated in rats that intrastriatal lentiviral vector delivery of the GDNF gene resulted in high levels of GDNF expression. However, it did not confer protection against a middle cerebral artery (MCA) occlusion. Conversely, intrastriatal and intranigral lentiviral GDNF injections in non-human primates resulted in significant GDNF gene expression over long periods of time (up to 8 months), and it protected against MPTP induced nigrostriatal degeneration (Kordower et al., 2000). Furthermore, in rats lentiviral delivery of GDNF protected against a medial forebrain bundle 6-OHDA lesion (Dowd et al., 2005).

Adeno-associated viral vectors (AAV) have also been used to deliver the GDNF gene in both rat and primate models of PD. The advantages of using adeno-associated viral vectors are that they can effectively and stably transflect non-diving cells, and they are less immunogenic because 96% of the viral genome has been deleted (Björklund et al., 2000). The disadvantages are that AAV’s cargo capacity is limited to 5 kb, and gene expression in the host cell can be delayed over a few days (i.e. onset of action is delayed). Despite these disadvantages, various groups have shown that an AAV carrying the
GDNF gene provides protection of dopamine neurons against a 6-OHDA lesion in rats (Mandel et al., 1997; Kirik et al., 2000). Similar protection against MPTP has been demonstrated in non-human primates following AAV delivery of the GDNF gene (Eslamboli et al., 2003; Eslamboli et al., 2005).

Besides viral vectors, there are other effective methods to deliver genes to the CNS. One such approach is to surgically implant fibroblasts previously modified to overexpress the protein of interest. For instance, Pérez-Navarro et al. (1999) demonstrated that intrastriatal implantation of GDNF secreting fibroblasts in rats afforded neuroprotection against the excitotoxic effects of quinolinic acid. Another approach employs liposomes to deliver recombinant plasmids (Roessler and Davidson, 1994; Vitiello et al., 1996). In fact, liposomes have even been used to deliver GDNF plasmids within the CNS (Lu et al., 2002; Lu et al., 2004). Lu et al. (2002; 2004) injected liposomes, cationic and non-cationic, loaded with the GDNF gene directly into the spinal cords of rats with spinal cord injuries and demonstrated transfection of their spinal motor neurons after only 1 week. In these animals, GDNF was able to protect motor neurons from degeneration and restored hind limb locomotion.

However, all of these alternative methods to deliver either the GDNF protein or its gene to the brain are still very invasive and pose many significant risks for the patient. Improved methods are badly needed if GDNF is to achieve clinical use. In this thesis project, cationic liposomes were evaluated as a possible delivery vehicle for intranasal administration of the GDNF protein to brain. The underlying hypothesis is that non-viral vectors pose fewer risks and can easily be terminated in the event that adverse effects develop, while the intranasal route of administration will provide a noninvasive method
for delivering the protein to the brain by bypassing the BBB. Conversely, there may also be disadvantages of the intranasal delivery of GDNF to the brain, e.g. unintended neurotrophic effects at non-target CNS sites and the risk of antibody development. However, the occurrence and severity of such side-effects can only be assessed after first demonstrating the feasibility of such an approach.
II. SPECIFIC AIMS OF THESIS

AIM #1: Develop and characterize a cationic liposomal formulation of a model protein for intranasal administration, and demonstrate brain delivery both qualitatively and quantitatively.

The goal of this AIM was to develop a cationic liposomal formulation for the intranasal delivery of a model protein, ovalbumin, which would then serve as a starting point for preparing a cationic liposomal formulation for the intranasal delivery of GDNF. Ovalbumin (OVAL) was chosen as the model protein because it has a similar molecular weight to GDNF (OVAL MW = 45 KDa; GDNF MW = 30 KDa), is readily available, not native to rat brain, relatively inexpensive, and commercially available as a conjugate with the green fluorophore Alexa 488 (Molecular Probes). The intent was to develop a formulation that would have a protein loading capacity sufficient to permit administration of the desired dose of GDNF (50 µg) in a 25 µl volume of solution or less per nostril. The formulation should also protect the encapsulated protein from proteolytic degradation by enzymes present in the nasal mucosa. In addition, the ideal liposomal preparation should have a positive surface charge of about +20 mV in order to increase residence time at the nasal mucosa, and increase the paracellular transport of the protein from the nose to the brain. It should also provide sufficient physical and chemical stability to deliver the intact, biologically active protein to the target structures, substantia nigra (SN) and striatum.
AIM # 2: Develop and characterize a cationic liposomal formulation of GDNF for intranasal administration, and demonstrate that liposomal preparation does not compromise GDNF protein integrity.

The goal of this AIM was to develop a cationic liposomal formulation for the intranasal delivery of recombinant human GDNF (rhGDNF), and to characterize the formulation for particle size, zeta potential, protein loading efficiency, and protein integrity. To confirm protein integrity of the liposomal preparation, a GDNF functional ELISA assay was developed and optimized. The GDNF receptor, GFRα1, was used as the “capture antibody”, and the anti-GDNF antibody as the “detection antibody”. The idea behind this sandwich ELISA was that only intact GDNF would bind to its receptor, thereby permitting determination of whether the GDNF protein remained intact through the liposomal preparation process, which included sonication. To assess delivery and integrity of the administered protein in brain, GDNF immunohistochemistry was also proposed. The expectation was that the rhGDNF would be detectable above low background levels of the endogenous protein in brain.

AIM # 3: Determine the therapeutic efficacy of intranasal GDNF in a 6-hydroxydopamine rat model of Parkinson’s disease, and determine which method of administration is most efficacious: single dosing vs. multiple dosing.

The goal of this AIM was to assess whether intranasally administered GDNF is neuroprotective against a 6-OHDA lesion in a rat model of Parkinson’s disease. This AIM consisted of two studies. The first study was an acute dosing study to determine if a
single 50 µg dose of intranasally administered GDNF protected against a 6-OHDA lesion, and to assess whether there was an advantage to the liposomal formulation.

The second study was a multiple dosing study to assess whether 3 doses of GDNF given at one day intervals (1 day before, 1 hour before, and 1 day after the lesion) would provide greater neuroprotection than the single dose. The dose administered was 50 µg in 25 µl per administration, for a total dose of 150 µg of GDNF over 3 days. In both studies, the rats were allowed to recover for 3-4 weeks, and then they were sacrificed by perfusion with 4% paraformaldehyde. The brains were collected and processed for TH immunohistochemistry. Quantification of the extent of the lesion, and the degree of neuroprotection, was performed by comparing dopamine cell counts and the density of TH immunolabeling on the lesioned and intact sides of the brain.
III. MATERIALS AND METHODS

A. Materials

**ABC Vectastain elite kit (PK-6100):** Vector Laboratories, Burlingame, CA.

**AffiniPure donkey anti-goat IgG conjugated to Texas Red (705-075-147):** Jackson ImmunoResearch Laboratories, West Grove, PA.

**Alexa 488-ovalbumin:** Invitrogen, Carlsbad, CA.

**Apomorphine hydrochloride, hemihydrates:** Sigma Aldrich Chemical Company, St. Louis, MO.

**Ascorbic Acid:** Sigma Aldrich Chemical Company, St. Louis, MO.

**BCA™ protein assay kit (23225):** Pierce (now Thermo Scientific), Rockford, IL.

**Biotinylated anti-rabbit secondary IgG antibody (BA-1000):** Vector Laboratories, Burlingame, CA.

**Bioquant® Nova image analysis software version 6.90.1:** Nasville, 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.

**Desipramine hydrochloride:** Sigma Aldrich Chemical Company, St. Louis, MO.

**Dioleoylphosphatidylcholine (DOPC):** Avanti Polar Lipids, Alabaster, AL.

**Donkey anti-goat IgG polyclonal antibody conjugated to horseradish peroxidase (6420-05):** SouthernBiotech, Birmingham, AL.
Donkey Serum (D 9663): Sigma Aldrich Chemical Company, St. Louis, MO.

Dulbecco’s phosphate buffered saline (PBS): Sigma Aldrich Chemical Company, St. Louis, MO.

Ethylene glycol: Sigma Aldrich Chemical Company, St. Louis, MO.

Fluoromount-G: Electron Microscopy Sciences, Hatfield, PA.


GDNF E-max® Immunoassay ELISA kit (Cat # G7620): Promega, Madison, WI.

Goat polyclonal anti-GDNF antibody (AF-212-NA): R&D systems, Minneapolis, MN.

Goat polyclonal anti-rabbit secondary IgG antibody coupled to Texas Red: Jackson ImmunoResearch Laboratories, West Grove, PA.

Graph Pad Prism® statistical analysis software version 4.03: San Diego, CA.

Heparin: Baxter Health Care Corporation, Deerfield, IL.

Hydrogen peroxide (30%): Sigma Aldrich Chemical Company, St. Louis, MO.

Ketamine: Fort Dodge Animal Health (Wyeth), Madison, NJ.


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

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

Ovalbumin from chicken egg white (A7642-1VL): Sigma Aldrich Chemical Company, St. Louis, MO.

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): Roche Diagnostic Corporation, Indianapolis, IN.

Polyvinylpyrrolidone: Sigma Aldrich Chemical Company, St. Louis, MO.

Rabbit anti-ovalbumin polyclonal IgG antibody: Chemicon, Temecula, CA.

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

Recombinant human GFRα1/Fc Chimera (714-GR): R&D systems, Minneapolis, MN.

RIPA Lysis buffer: Sigma Aldrich Chemical Company, St. Louis, MO.

Sodium chloride 0.9% preservative free: Hospira Inc., Lake Forest, IL.

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.

SureBlue™ TMB microwell peroxidase substrate (52-00-01): KPL, Gaithersburg, MD.

Sucrose: Sigma Aldrich Chemical Company, St. Louis, MO.

Surgical suture, black monofilament nylon: Ethicon Inc., Piscataway, NJ.

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.

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.

6-hydroxydopamine hydrobromide: Sigma Aldrich Chemical Company, St. Louis, MO.
B. Methods

1. Preparation of protein loaded liposomal formulations

Cationic liposomes were prepared using a protocol developed by Dr. Robert Campbell, and modified by Dr. Tushar Vyas. Dioleoylphosphatidylcholine (DOPC), cholesterol, and stearylamine were each dissolved in chloroform at a concentration of 10 µmol/ml, and were then mixed together in a volume ratio of 50:30:5. The mixture was placed in a rotary evaporator at 42°C for 30 min to eliminate the chloroform, and the resulting lipid film was freeze-dried for 2 hours to remove any remaining trace of the organic solvent. Two versions of this cationic liposomal formulation were originally prepared for the OVAL experiments in AIM 1. Preparation # 1 was made by adding phosphate buffered saline (PBS) to the lipid film in a volume of 50 parts relative to the lipid volumes above. The mixture was then vortexed for 1 min to mix. The liposomal preparation was then placed on ice for 2 min, vortexed, and then placed in a 37 ºC water bath for another 2 min and then vortexed again. This cold/warm temperature cycle was repeated five times to help pack the liposomal bilayer. The resulting cationic liposomes were then sonicated for 5 min, and then the protein (either Alexa 488-OVAL, \(^{111}\)In-OVAL, or plain OVAL) was added to the preformed liposomes to achieve a final concentration of 1 µg of protein/µl of liposomal solution. The mixture was again vortexed.

Preparation # 2, on the other hand, was prepared by adding the protein (either Alexa 488-OVAL, \(^{111}\)In-OVAL, or plain OVAL) to the lipid film during the hydration step. In this case, the protein was dissolved in PBS in a volume of 50 parts relative to the
lipid components above. The mixture was then cycled five times between cold and warm
temperatures, and vortexed after each cycle as above. Two forms of preparation # 2 were
made: one at a final concentration of 1 µg of protein/µl, and one at 2 µg/µl. The
liposomal solution was vortexed, and then sonicated to achieve the smallest particle size
without causing precipitation of the protein. Depending on the strength of the sonicator,
these times varied from 2.5 to 10 min. Following sonication, the liposomes were filtered
through a 0.2 µm syringe filter to obtain the desired particle size (<500 nm). The filtered
liposomal preparation was then centrifuged for 5 min at 10,000 g to separate the
liposomes from the unassociated protein remaining in solution. The bottom layer,
containing the protein-loaded liposomes, was collected and analyzed for particle size and
surface charge. Particle size was determined by light scattering using the ZetaPALS
90Plus (Brookhaven Instruments Corp., Holtsville, NY; Ver. 3.57). The surface charge
was measured using the ZetaPALS Phase Analysis Light Scattering ultra-sensitive zeta
potential analyzer (Brookhaven Instruments Corp., Holtsville, NY; Ver. 3.28). The
liposomes were then administered to rats, or stored in a sealed vial, in the refrigerator,
until administration to rats on the following day.

To prepare cationic liposomes loaded with GDNF, a procedure similar to that for
preparation # 2 was followed. However, GDNF cationic liposomes did not require
filtration following sonication in order to obtain optimal particle sizes. Consequently, the
filtration step in liposomal preparation was omitted. All GDNF loaded liposomes were
prepared so as to achieve 2 µg protein/µl of final solution.
2. Determination of liposomal loading efficiency

The loading efficiency of the liposomes was determined by the indirect method using a BCA Protein Assay kit according to the manufacturer’s instructions. Briefly, following centrifugation of the liposomes, the aqueous supernatant was collected and the residual unincorporated protein was analyzed against a BSA standard curve. The amount of protein in the aqueous phase was subtracted from the total amount of protein added to the liposomal preparation to yield the percentage loading efficiency of the liposomal preparation.

3. Intranasal administration to rats

Experiments were conducted in accordance with the regulations of Northeastern University’s Animal Care and Use Committee and Division of Laboratory Animal Medicine. Male Sprague-Dawley (SD) rats (225g-300g) were anesthetized with a mixture of ketamine/xylazine, at a dose of 80 and 20 mg/kg, respectively, and were placed in a supine position with their noses at an upright 90° angle (Van Den Berg et al., 2002; Gizurarson et al., 2006). Preliminary studies demonstrated that bolus administration of volumes >5 µl /nostril resulted in respiratory distress. Therefore, the administered volume was limited to 5 µl increments, staggered every 4 min, alternating nares for a total of 25 µl per side for the 1 µg/µl preparation. For the 2 µg/µl preparations, 2.5 µl was given per nostril for a total of 12.5 µl per side. For both preparations, the total administered dose was 50 µg of protein per rat. This protein dose was chosen based on review of the literature, which indicated that 50 µg was within the
range of GDNF doses that provided neuroprotection in the rat 6-OHDA model of PD. Consequently, in all the OVAL studies and the single dose GDNF studies, the 50 µg dose of protein was the standard dose. Administration was performed using a 10 µl Hamilton syringe fitted with a 1 cm segment of PE20 tubing (Braintree Scientific). Following intranasal administration, the animals remained in a supine position, with their noses at a 45° angle for 60 min in order to maximize absorption through the nasal mucosa. Figure 35 shows a rat in position for intranasal administration of a dose. Animals were returned to their home cages after recovery from anesthesia.

Figure 35: Method of intranasal administration in rats. Note that the rat’s nose is at a 90° angle. Solution was administered at the opening of the nostrils and allowed to drip down the nasal cavity, in order to maximize contact with nasal epithelium.

4. Transcardial perfusion with 4% paraformaldehyde

Once the specified time points had been reached after intranasal administration, rats were deeply anesthetized with a mixture of ketamine/xylazine and perfused transcardially with 4% paraformaldehyde. Briefly, the rat was placed in a supine
position, and an incision was made in the abdomen at the level of the xiphoid process just below the sternum. The diaphragm was then cut, and the heart was exposed by cutting along the bottom of the ribs and towards the front paws. A needle was then inserted into the left ventricle, and 1 ml of cold heparin (1000 units/ml) was injected to prevent blood clot formation. The needle was removed, and a perfusion needle was then inserted into the same opening in the left ventricle. A cut in the right atrium was then made to allow the blood to drain. Approximately 200-300 ml of cold PBS was infused until the color of the liver changed from maroon to a light tan, indicating that all of the blood had been flushed. Infusion of PBS was then stopped and 300 ml of freshly prepared, cold 4% paraformaldehyde in PBS was infused. Brains and olfactory bulbs were then removed and submerged in cold 4% paraformaldehyde in PBS to post-fix for 2 hours. The brains were transferred to a vial containing 30% sucrose in PBS and refrigerated for 48-72 hours until they sunk to the bottom of the vial. Coronal sections, 25 µm thick, were cut along the rostral to caudal axis of the brain using a cryostat. Brain sections were either placed directly onto slides for fluorescent microscopy, or distributed sequentially into four wells of PBS to be used for immunohistochemical assays (each well containing ~10-12 sections). Brain sections not being used immediately for immunohistochemistry were transferred from PBS to cryoprotectant, and stored at -20°C until the day of the assay. Cryoprotectant is a mixture of 30% sucrose, 30% ethylene glycol, and 1% polyvinylpyrrolidone in distilled water.
5. Distribution of Alexa 488-OVAL in brain after intranasal administration

Rats were intranasally administered 50 µg Alexa 488-OVAL using either preparation # 1 or preparation # 2 at the 1 µg/µl concentration. The animals were sacrificed by transcardial perfusion and their brains post-fixed with 4% paraformaldehyde, as previously described, at either 6 hours or 24 hours after administration. Representative sections were taken at the level of the olfactory bulb, frontal cortex, striatum, midbrain, and posterior regions including the brainstem. Each section was examined by fluorescence microscopy at 40X magnification to localize clusters of green fluorescent deposits that were either scattered throughout the neuropil or localized within cells. To further assess the whether Alexa 488-OVAL deposits in the ventral midbrain colocalized with the dopamine-containing neurons of the substantia nigra and ventral tegmental area, tyrosine hydoxylase (TH) immunohistochemistry was performed on sections from this region.

6. Tyrosine hydroxylase immunohistochemistry

Tyrosine hydroxylase immunohistochemistry was used as a marker for dopamine neurons. Two different means of detection were used in this thesis project. In the studies of AIM # 1, a secondary antibody conjugated to the fluorophore Texas Red was used. In the experiments of AIM # 3, detection was by diaminobenzidine (DAB) in order to permit quantification of the extent of the 6-OHDA lesions. Both assays will be discussed in detail below:
a. Tyrosine hydroxylase fluorescence immunohistochemistry

Free-floating sections of midbrain were first washed 3 times for 10 min each with PBS to remove any traces of cryoprotectant and/or embedding medium (i.e. Neg-50 used for cryostat sectioning). The sections were then blocked with 10% normal goat serum (NGS) in PBS for 60 min to block non-specific binding sites. They were then incubated for 3 hours at room temperature with a 1:500 dilution of a rabbit anti-TH antibody (Chemicon AB152) in 5% NGS. The sections were washed with PBS three times for 10 min, and then incubated for 60 min at room temperature with a 1:100 dilution of goat anti-rabbit secondary antibody coupled to the red fluorophore, Texas Red (Jackson ImmunoResearch Labs). The sections were washed again, mounted on slides, dried, coverslipped with Fluoromount-G, and were then examined by fluorescence microscopy. TH immunolabeling in the ventral midbrain and its possible colocalization with Alexa 488-OVAL was assessed. Figure 36 shows a sample of striatum and SN TH immunohistochemistry using this method.

Figure 36: Tyrosine hydroxylase immunohistochemistry using a secondary antibody coupled to Texas Red. Left panel shows a corpus striatum image, and the right panel shows a midbrain image (20x). Scale bars= 20 µm.
b. Quantitative tyrosine hydroxylase immunohistochemistry

For quantitative TH immunohistochemistry, assays were performed on all groups at the same time, using the same antibody solutions and incubation times in order to maintain uniformity. The single dose treatment groups were assayed in a single batch, and the multiple dose treatment groups were assayed in a single batch at a later time. Free-floating sections of midbrain were washed 3 times for 10 min each with PBS. The brain slices were then pretreated with 0.03% hydrogen peroxide (H2O2) for 30 min to exhaust endogenous tissue peroxidases, followed by 3 washes in PBS for 5 min each. The sections were then blocked with 10% NGS in PBS for 60 min, followed by incubation overnight at 4 °C with a 1:5,000 dilution of a rabbit anti-TH antibody (Chemicon AB152) in 5% NGS. Following this overnight incubation, the sections were washed with PBS three times for 10 min each. The sections were then incubated with a biotinylated anti-rabbit IgG secondary antibody raised in goat (Vector Labs BA-1000; 6 µg/ml) for 1 hour at room temperature and then washed 3 times for 5 min with PBS. The sections were incubated for 1 hour at room temperature with an avidin-biotin-peroxidase complex for antibody signal amplification (Vector Labs, Vectastain Elite ABC kit; PK-6100). The ABC solution was prepared by adding 1 drop of reagent A and 1 drop of reagent B to 5 ml of PBS and allowing it to stand at room temperature for 30 min before use. After the ABC incubation, the brain sections were washed 3 times for 5 min each with PBS. Following these washes, the midbrain sections were transferred to glass vials and incubated for exactly 12 min with 2.5 ml of 3, 3'-diaminobenzidine (DAB), a chromogenic substrate for the peroxidase complex. The peroxidase complex reacts with DAB to yield a permanent brown stain. The DAB solution was prepared in a glass vial
by adding 2 drops of DAB, 1 drop of buffer, and 1 drop of H₂O₂ to 5 ml of distilled water (Vector Lab, peroxidase substrate kit DAB; SK-4100). The vial was protected from light by covering it in foil. After incubation with the DAB solution, the sections were washed three more times with PBS for 5 min each. They were then mounted on slides, dried, and coverslipped using an aqueous mounting medium.

7. **Bioquant® analysis of protein distribution in brain**

Mapping of Alexa-488-OVAL distribution was performed using Bioquant® Nova image analysis software (version 6.90.1) running on a computer interfaced with an Olympus BX51 TRF fluorescence microscope that was equipped with X-Y stage encoders. To map the location of Alexa 488 deposits in brain, a cursor was used to first trace an outline of the section at 2X magnification. The objective was then switched to 40X magnification, and the section was viewed with a green fluorescence filter set. The section was examined for areas containing green fluorescent deposits of Alexa 488. In areas where deposits were observed, a small circle was applied to the Bioquant® image to indicate their location on the section. The circled deposits were retained on the Bioquant image to generate a map showing the distribution of the deposits on each section examined (Figure 37).
Similarly, a conditional frequency analysis was conducted using Bioquant® to determine the proportion of cells in the ventral midbrain which contained Alexa 488 deposits that were also dopamine neurons. This was done by first examining midbrain sections at 40X using the green filter to locate all cells within the substantia nigra and ventral tegmental area that appeared to contain Alexa-488 deposits. These cells were identified with circles on the Bioquant image. Then, without moving the section or adjusting focus, the fluorescence filter was changed to the red filter, and all of the Alexa 488-containing cells were re-examined for the presence of Texas Red, indicating TH immunostaining. Cells co-localizing both Alexa 488 and Texas red were marked with yellow circles in Bioquant. The number of cells with both markers divided by the total number of ventral midbrain cells containing Alexa-488 deposits, multiplied by 100, yielded the percentage of Alexa 488-labeled cells that were dopamine neurons.
8. Ovalbumin immunohistochemistry

OVAL immunohistochemistry was used to verify that the fluorescent deposits in the brain were due to the administered protein. Rats received intranasal administration of 50 µg of plain OVAL in cationic liposomes at a concentration of 2 µg/µl. The animals were sacrificed by perfusion with 4% paraformaldehyde at either 1 hour or 24 hours following administration, and 25 µm coronal sections of brain were collected at the level of the forebrain (striatum) and midbrain (substantia nigra). OVAL immunohistochemistry was carried out using the same method described above in section B.6.a. except that a 1:5000 dilution of a rabbit polyclonal anti-ovalbumin primary antibody (Chemicon AB 1225) was used with a 1:100 dilution of an anti-rabbit secondary antibody coupled to the red fluorophore, Texas Red.

9. Biodistribution of intranasally administered OVAL: Brain versus peripheral tissues

$^{111}$In-OVAL was prepared by conjugating the protein with DTPA succinic anhydride and chelation with $^{111}$In ions. $^{111}$In-OVAL was then purified by gel filtration, dialyzed to remove free $^{111}$In, and then incorporated into both cationic liposomal preparations as previously described. $^{111}$In-OVAL in PBS (no nanoparticles) was used as a control. Rats were intranasally administered a total of 50 µg of the radiolabeled protein using the protocol above. Both liposomal preparations, and the 1 µg/µl and 2 µg/µl concentrations were evaluated. A time course study was conducted to determine the kinetics of OVAL delivery to the brain and peripheral tissues from 1-24 hours after intranasal administration of the dose. Brains, plasma, stomachs, intestines, lungs, and
livers were collected and weighed. $^{111}$In-OVAL uptake was determined by gamma counting. Uptake was expressed as the percentage of the administered dose per gram of tissue after correction for the disintegration half-life of $^{111}$In.

10. GDNF immunohistochemistry

GDNF immunohistochemistry was performed on 25 µm coronal sections of the midbrain and forebrain. Free-floating sections were first washed 3 times for 5 min each with PBS to remove any traces of cryoprotectant, and/or embedding medium. The sections were then blocked for 1 hour in 10% normal donkey serum (NDS) to block non-specific binding sites, followed by incubation overnight at 4°C with a 1:5000 anti-GDNF antibody dilution in 5% NDS (R&D system; AF-212-NA). Following overnight incubation, the sections were washed with PBS 3 times for 5 min each. The sections were then incubated at room temperature for 1 hour with a 1:400 dilution of donkey anti-goat IgG antibody conjugated to Texas Red in 1% NDS (Jackson ImmunoResearch Laboratories, Inc. # 705-075-147). The sections were washed again 3 times with PBS for 5 min each, mounted on slides, dried, and coverslipped with Fluoromount-G. Processed sections were then examined by fluorescence microscopy.

11. Confirmation of GDNF protein integrity by functional ELISA

A GDNF “functional ELISA” assay was developed and optimized to assess the functional integrity of the GDNF protein in the liposomal formulation. The GDNF receptor, GFR $\alpha_1$, was analogous to the “capture antibody”, and the anti-GDNF antibody was used as the “detection antibody”. This functional ELISA was modeled after the
Promega® GDNF ELISA kit. Advice on optimization was provided by R&D systems, the manufacturers of the GFRα1 receptor chimera, and Dr. Mansoor Amiji. This sandwich ELISA was based on the principle that only intact GDNF would be able to bind to its receptor, thereby permitting determination of whether the GDNF protein retained its biological activity through the liposomal preparation process. Briefly, the GFRα1 receptor chimera (R&D systems; 714-GR-100) was diluted to a concentration of 2 µg/ml with Dulbecco’s PBS. ELISA plates (MaxiSorp® Nunc) were then coated with 100 µl per well of the GFRα1 receptor dilution, and incubated covered overnight at room temperature without shaking. The wells were then emptied over a sink, the plate was slapped upside down 3 times on a paper towel to help clear the wells, and they were washed 3 times with Dulbecco’s PBS with 0.05% Tween 20. The ELISA plates were blocked for 1 hour with 300 µl per well of 2.5% bovine serum albumin (BSA) in Dulbecco’s PBS without shaking to block non-specific binding sites. The contents were then emptied and the plate was again slapped upside down 3 times on a paper towel to clear the wells.

Stock GDNF standards were used to generate the GDNF standard curves. The stock solution (2 µg/ml) was aliquoted in advance, 5 µl or 0.01 µg of GDNF per microcentrifuge tube. The liposomal samples used in this assay, on the other hand, had starting GDNF concentrations of 2 µg/µl and 10 µl aliquots were used or nominally 20 µg per sample. The 5 µl GDNF standards were next spiked with 10 µl of plain cationic liposomes (a volume of liposomes equivalent to that in the GDNF liposomal samples included in the assay). Spiking the GDNF standards with plain liposomes was done to normalize the conditions of the assay so as to reduce any interference due to the presence
of phospholipids in the GDNF liposomal samples. The samples and the standards were then treated with 10% Triton X-100 in distilled water and incubated at 37°C for 30 min to lyse the liposomes. Ligands, both samples and standards, were then diluted with 0.5% BSA in Dulbecco’s PBS to achieve final concentrations of 0.75-5 ng/ml, the detection limits of the GFRα1 receptor chimera. Two standard curves were constructed per plate over a range of GDNF concentrations from 0.75-4.5 ng/ml. Each GDNF liposomal preparation was assayed on different plates using the plate design shown on Figure 38. Each well received equal volumes (100 µl) of the appropriate concentrations of either samples or standards according to the plate design shown in Figure 38. The wells on the outer rim of the ELISA plate were not used to maximize plate reader efficiency. Once both the samples and standards had been added to the ELISA plate, the plate was incubated overnight with shaking at 4 °C. Following this overnight incubation, the plate was emptied as previously described, and washed 5 times with Dulbecco’s PBS with 0.05% Tween 20. These washes were followed by an overnight incubation with shaking at 4 °C with a 1:5000 dilution of an anti-GDNF antibody (R&D systems; AF-212-NA) in Dulbecco’s PBS with 0.5% BSA. The well contents were emptied, and the wells were washed 5 times with Dulbecco’s PBS with 0.05% Tween 20. Washes were followed by a 2 hour incubation at room temperature with a 1:5000 dilution of donkey anti-goat secondary antibody conjugated to horseradish peroxidase (HRP; SouthernBiotech, 6420-05); made in Dulbecco’s PBS with 0.5% BSA (100 µl per well). Following this 2 hour incubation, the plate was emptied and washed 5 times with Dulbecco’s PBS with 0.05% Tween 20. Washes were followed by a 15 min incubation with 100 µl/well of SureBlue® TMB microwell peroxidase substrate (1-component; KPL product # 52-00-01). At the
end of this incubation, 100 µl of TMB Stop Solution (KPL product # 50-85-05) was
added to each well. Absorbance was measured at 450 nm on a plate reader within 30 min
of adding the stop solution. The absorbance of the cationic liposomal GDNF
preparations was compared against the GDNF standard curve to identify the amount of
GDNF protein that remained functionally intact in the liposomal preparations. As shown
in Figure 38, each sample was tested six times at each concentration, and a mean
absorbance was obtained at each concentration. Two GDNF standard curves were also
generated on each plate, and a mean absorbance was obtained for each concentration. A
GDNF standard curve was generated from this mean, and a slope and y-intercept were
obtained. The slope and y-intercept were used to determine the amount of protein
detected in liposomal samples at each of the concentrations tested. The following
equation was used to determine the ng amount of protein detected in each sample:

\[
\frac{\text{Observed absorbance at each concentration} - \text{y-intercept of GDNF standard curve}}{\text{Slope of GDNF standard curve}}
\]

Then, the ng amount of GDNF protein detected was divided by the amount expected at
each concentration and multiplied by 100 to obtain the percent GDNF protein detected in
the liposomal sample at each concentration. Finally, the percent GDNF protein detected
from each concentration were added together and then divided by 6 (the total # of
different concentrations used) to obtain the mean percent of GDNF protein detected in
the liposomal preparation.
<table>
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**Figure 38:** ELISA plate design used in the functional ELISA assay per GDNF liposomal preparation. Samples were first treated with 10% Triton X-100 and incubated at 37°C for 30 min. Standards, on the other hand, were first spiked with 10 µl of plain cationic liposomes, and then treated with Triton X-100 as above. Final dilutions of both the samples and standards to 0.75-4.5 ng/ml were made using 0.5% BSA in Dulbecco’s PBS.

12. Partial 6-hydroxydopamine (6-OHDA) lesioning

Partial 6-OHDA lesions of the SN dopamine neurons were generated in rats by modification of an established protocol for creating complete unilateral lesions (White-Cipriano and Waszczak, 2005; Waszczak et al., 2006; White-Cipriano and Waszczak, 2006; Migliore et al., 2007). The goal was to generate lesions that destroyed between 50-80 % of substantia nigra dopamine neurons. Male Sprague-Dawley (SD) rats (200g-275g) 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 animal protocol. The animals were anesthetized by intraperitoneal (I.P.) injections of a mixture of ketamine/xylazine at
a dose of 80 and 20 mg/kg respectively. Thirty min prior to surgery, after the animals had been anesthetized, they received an intraperitoneal injection of desipramine (15 mg/kg), a norepinephrine reuptake inhibitor, in order to minimize the damage to noradrenergic neurons. Additional injections of ketamine (100 mg/ml) were administered in 0.1 ml increments until the toe pinch technique demonstrated insensitivity to pain. The rat’s head was shaved and swabbed with 70% isopropyl alcohol and betadine. The rat was then placed in the stereotaxic instrument. Body temperature was maintained throughout the procedure at 38 °C using a heating pad (Fintronics). A sterile scalpel was used to create a 1-2 cm rostral to caudal incision on the scalp, and to expose the lambdoid suture. Tissue overlying the suture lines was scraped away and the skull was dried using a short application of Super-friendly Air-it. A drill (Dremel®) was then used to create a 3.0 mm burr hole at the following stereotaxic coordinates: +1.2 mm lateral (left) to lambda and +4.4 mm anterior to the lambdoid suture. The needle of a Hamilton syringe, attached to a piece of PE tubing containing the 6-OHDA solution, was then lowered -8.3 mm ventral to the surface of the skull (Ruskin et al., 2002). This piece of PE tubing was also connected on its other end to a 10 µl Hamilton syringe filled with distilled water. This water-filled hamilton syringe was used to inject 4 µl of 6-OHDA (0.5 µg/µl in 0.1% ascorbic acid in saline; 2 µg 6-OHDA total) into the medial forebrain bundle at a rate of 1 µl/min by displacing the 6-OHDA in the PE tubing with water (Harvard Apparatus Infusion Pump). This fluid displacement method of infusing 6-OHDA was necessary because 6-OHDA is highly corrosive and would otherwise oxidize the barrel of the Hamilton syringe. At the end of the 6-OHDA infusion, the needle was left in place for 15 min and was then removed slowly in order to
minimize tracking of the neurotoxin up the needle track to other regions of the brain. The incision was closed by either using veterinary tissue adhesive (Vetbond®) or by using nylon sutures. The animals were administered a subcutaneous injection of buprenorphine (0.05 mg/kg) to minimize post-operative pain and were monitored for ipsilateral rotational behavior following recovery from anesthesia. The rats were allowed to recover for 3-4 weeks after surgery.

13. Behavioral assessment of the 6-OHDA lesion

All lesioned rats underwent behavioral analysis by using the apomorphine rotation test, a behavioral test used to assess the severity of the striatal dopaminergic deficit. On the day of sacrifice, rats received a single I.P. dose of apomorphine (0.5 mg/kg in 0.1% ascorbic acid in saline). Immediately following administration of the apomorphine dose, the rats were placed in a circular bucket, which was placed inside a plexiglass chamber of a San Diego’s Instruments behavioral activity system and were videotaped for 30 min. Upon completion of the behavioral test, the rats were deeply anesthetized and perfused with 4% paraformaldehyde, as previously described. The videotaped behavior was later examined and rotational behavior was scored as follows: the contralateral rotations were counted at 5 min intervals, and then the total number of contralateral rotations over the whole 30 min session recorded per rat. Ipsilateral rotations were not counted.
14. Bioquant® analysis of the 6-OHDA lesion

Bioquant® Nova version 6.90.1 was used to quantify the extent of the 6-OHDA lesion. Several analyses were performed. First, the overall density of tyrosine hydroxylase immunostaining in the intact vs. the lesioned SN was measured. Second, unbiased stereological cell counts were also performed on both the lesioned and unlesioned sides by using Bioquant®’s stereological tool kit. Finally, dopamine cell area, diameter, and intensity of TH staining per dopamine cell body were measured. These later measures were used to assess whether intranasal GDNF treatments affected the basic morphological characteristics of dopamine neurons. Camera and light source settings (i.e. white balance, red and blue offset, sharpness, brightness, and contrast) were standardized using control tissue, and were held constant throughout all experiments. Furthermore, background corrections were performed for every slide by subtracting background determined on an area of the slide not containing tissue.

a. Substantia nigra tyrosine hydroxylase density measurements

Tyrosine hydroxylase SN density measurements were performed on four evenly spaced sections along the rostral to caudal axis of the midbrain corresponding to the atlas views in Figure 39 (Paxinos and Watson, 1998).
For each section, viewed using the 2x objective, a perimeter was first drawn around the whole unlesioned SN (pars compacta and pars reticulata) using a computer keyboard and mouse (see Figure 40). Then, the brown stain indicating TH immunostaining was examined and a brown-stained area was chosen for the purpose of selecting a standard pixel intensity. This process is called pixel thresholding. This pixel threshold was used by the Bioquant® system to determine integrated optical density of the
staining within the entire unlesioned SN. All pixels within the unlesioned SN that were as dark or darker than the previously selected standards were counted by Bioquant®. The perimeter of the SN was then flipped and copied exactly over the corresponding area on the opposite side of the brain using the Bioquant® software. Then, the integrated optical density of the lesioned SN was measured in the same manner and maintaining the same settings for pixel thresholding used on the unlesioned side. These steps are illustrated in Figure 40. This process was repeated for each of the four sections included in the analysis for each brain.

![Figure 40](image.jpg)

**Figure 40:** Image of a midbrain section showing both the lesioned and unlesioned SN (left; 2x). Middle panel illustrates how the perimeter is drawn around the unlesioned SN, and pixels are thresholded (2x). Right panel shows that the perimeter was flipped and copied exactly on the lesioned SN (2x). Integrated optical density was measured while holding pixel standards constant. Scale bars= 200 µm.

Pixel counts of the lesioned and unlesioned sides of the four representative midbrain sections were exported to Excel and the percent lesion for each section was calculated by dividing the integrated optical density of labeling on the lesioned side by that on the unlesioned side. These values were averaged for the four sections from that brain and multiplied by 100 to obtain the percent TH immunostaining density remaining on the lesioned side. This value was then subtracted from 100 to obtain the percent lesion for each animal.
b. Stereological cell counts and determination of dopamine cell size

Using the same four midbrain sections per brain that were used for determining the percent TH immunostaining density, unbiased dopamine cell counts were also performed. Stereological methods were used to permit determining if intranasal GDNF altered the number of dopamine neurons that survived in the 6-OHDA lesioned SN versus the unlesioned SN. Briefly, a perimeter was drawn around the SN pars compacta under 2x magnification. The Bioquant® grid wizard was then used to generate a 300 µm vs. 300 µm grid. The grid was placed over the drawn perimeter (Figure 41), and the Bioquant® system identified six randomly chosen intersections within the perimeter. The X-Y coordinates of these six intersections designated the counting boxes for stereological counting. X-Y stage encoders were used to position the slide at these locations where cell counts would be performed under 40x magnification. The stage encoders were mounted on the microscope stage which interfaced with the Bioquant® system. The stage was moved to the X and Y coordinates for each 334 µm x 250 µm counting box (size of the Bioquant® image window), and dopamine cell counts, area, diameter, and density were conducted simultaneously. Each dopamine cell body within the 334 µm by 250 µm Bioquant® image window was individually traced and counted (Figure 42). Dopamine cell bodies were only traced and counted if their entire cell body was within the image window. If they were only partially within the counting window, they were not counted. In addition, dopamine cells that were outside the drawn SN pars compacta perimeter, or whose cell bodies were not entirely within the perimeter, were not counted. Dopamine cell counts, area, diameter, and TH density from the six locations on the lesioned and unlesioned sides were exported to Excel. This process was repeated for the four midbrain
sections from each brain to yield a mean dopamine cell number, area, diameter, and TH density for both the lesioned and unlesioned sides.

Figure 41: Coronal brain section showing both lesioned (left) and unlesioned (right) SN’s. The right SN pars compacta was traced in blue, and a red 300 vs. 300 µm grid was superimposed over the structure. The six intersections where cell counts were conducted are shown as yellow x’s. Scale bar= 200 µm.
Figure 42: Bioquant® quantification of dopamine neurons. Dopamine cell bodies were traced, and their cell number, area, diameter, and density were calculated. An example of data generated by this method is shown in the table below the image. Scale bar= 15 µm.

<table>
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<td>877.3</td>
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<tr>
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<td>10</td>
<td>308.26</td>
<td>98.12</td>
<td>973.54</td>
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</table>

The average number of dopamine cells was reported as the number of dopamine neurons per unit of brain volume in mm³. This unit of measure was determined as follows. For each section examined, the total dopamine cell counts of the six determinations per SN pars compacta was divided by 6 to yield the mean number of
dopamine cells per counting box (i.e. Bioquant image window). Then, the volume of a counting box $\mu m^3$ was calculated by taking the image window size of $334 \mu m \times 250 \mu m$ and multiplying by the section thickness of $25 \mu m$ to yield a total volume of $2087500 \mu m^3$ for each counting box. The mean number of dopamine neurons per counting box (from above) was then divided by $2087500 \mu m^3$ to yield the mean number of dopamine neurons per $\mu m^3$ for that SN pars compacta. Finally, this number was converted to the number of dopamine neurons per $mm^3$ by using the conversion $1 mm^3 = 1 \times 10^9 \mu m^3$ as follows:

$$\left(\# \text{ dopamine neurons/}\mu m^3\right) \times \left(1 \times 10^9 \mu m^3/mm^3\right) = \# \text{ dopamine neurons/mm}^3 \text{ for that SN.}$$

Then, the average of four midbrain sections was taken to yield the mean number of dopamine neurons per $mm^3$ for that animal’s SN. This method of determining cell number was adapted from a stereological method described by Hyman et al. (1998).

15. Statistical analyses

Statistical analyses were performed using Graph Pad Prism® version 4.03. Data was presented as mean ± standard error of the mean (SEM). Comparison between multiple groups of data was conducted by one-way analysis of variance (ANOVA), and Tukey’s post-hoc test was used to determine the differences between individual groups. Comparisons between two groups of data were performed by using the unpaired Student’s t-test.
IV. RESULTS

A. Specific AIM # 1

The main goal the first AIM was to develop and test a cationic liposomal formulation suitable for intranasal delivery of proteins to the brain. As proof of principle, chicken ovalbumin (OVAL MW= 45 KDa) was chosen as a model protein because its molecular weight resembles the molecular weight of neurotrophic factors, which range from 5-30 KDa (Mine and Rupa, 2003). Most importantly, OVAL was chosen because its molecular weight resembled GDNF’s molecular weight of 30 KDa (Lin et al., 1993). Two different cationic liposomal preparations for intranasal brain delivery were initially prepared. In both preparations dioleoylphosphatidylcholine (DOPC), cholesterol, and stearylamine were mixed in chloroform at a molar ratio of 50:30:5 and then lyophilized to remove the chloroform and to form a lipid film. The liposomes were then generated upon hydration of the lipid film by adding PBS (with or without protein). Preparation #1 consisted of adding the protein (plain OVAL, Alexa 488-OVAL, or $^{111}$In-OVAL) to preformed liposomes and then vortexing. Preparation #2, on the other hand, consisted of adding the protein to the lipid film during the hydration step. This was followed by sonication, and in some cases, filtration through a 0.2 μm syringe filter to reduce particle sizes to 200-300 nm. Dr. Tushar Vyas, a post-doctoral fellow in Dr. Amiji’s laboratory, prepared the cationic liposomes used in all in vivo OVAL studies of AIM # 1.

1. Characterization of the cationic liposomal preparations for OVAL

Cationic liposomes incorporating OVAL were characterized for particle size, zeta potential (surface charge), and protein loading efficiency immediately after vortexing for
preparation # 1, and immediately after sonication and filtration for preparation # 2. For preparation # 1, Dr. Vyas reported particle sizes of $1215 \pm 40.7$ nm, zeta potential of $36 \pm 2.17$ mV, and a protein loading efficiency of 94% determined by the indirect method (Table 2). For preparation # 2, he reported particle sizes of $121 \pm 0.9$ nm for the dilute preparation (1 µg/µl), and $126 \pm 9.8$ nm for the concentrated preparation (2 µg/µl). Preparation # 2 had zeta potentials of $34 \pm 1.67$ mV for the dilute and $56 \pm 1.24$ mV for the concentrated formulation. The protein loading efficiency for preparation # 2 (2 µg/µl), determined by the indirect method, was reported to be 99%. Loading efficiencies were not performed for the dilute formulation. This data is shown in Table 2.

<table>
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<tr>
<th>Liposome Characteristics</th>
<th>Prep # 1 (1 µg/µl)</th>
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<td>Zeta potential (mV)</td>
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<td>Loading Efficiency (%)</td>
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<td>ND</td>
<td>99</td>
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Table 2: Cationic liposomal OVAL characteristics reported by Dr. Tushar Vyas. Data for particle size and zeta potential was presented as mean ± SEM, n not reported. Loading efficiencies were only conducted once for preparation #1 and preparation # 2 (2µg/µl), and preparation # 2 (1 µg/µl) was not tested.

I have subsequently prepared OVAL liposomes by Dr. Vyas’ method in order to complete the data in Table 2 for preparation # 2. I was unable to duplicate the particle size and zeta potential data reported by Dr. Vyas. After sonication and filtration of preparation # 2 (2 µg/µl), I obtained liposomal particle sizes averaging $299 \pm 26.4$ nm, and zeta potentials of $19 \pm 1.5$ mV (mean ± SEM for 3-6 preparations). I found that it was essential that the liposomes be filtered through a 0.2 µm filter after sonication, in
order to achieve particle sizes in the desired range. In retrospect, the much larger particle sizes Dr. Vyas reported for preparation # 1 may have meant that he did not filter this preparation. I have no explanation for the differences in zeta potential. The zeta potential I obtained for preparation # 2 was consistent over multiple batches of liposomes and I believe is accurate.

Dr. Vyas and I did agree on loading efficiencies. For preparation # 2 concentrated formulation, I obtained protein loading efficiencies of 94.3 ± 0.2% (mean ± SEM for 5 preparations) by the indirect method. All together, the protein loading efficiency data indicated that both preparations, and both the 1 and 2 µg/µl concentrations of preparation # 2, incorporated over 90% of the added protein. Thus, administering either 25 or 50 µl of the concentrated or dilute preparations, respectively, resulted in a total dose of nominally 50 µg of OVAL per rat.

With this initial characterization, both preparations were tested in rats, despite the fact that preparation # 1 had considerably larger particle sizes than preparation # 2. The larger particle sizes of preparation # 1 were expected to make it less likely to be transported via paracellular transport from the nose to the brain. However, its similar cationic surface charge was expected to allow it to persist as long as preparation # 2 at the nasal mucosa and therefore facilitate brain transport. For these reasons, both preparations were evaluated by intranasal administration in rats. The *in vivo* data will be presented below.

In addition to characterizing OVAL liposomes, I also characterized the particle size and zeta potential of empty cationic liposomes. After sonication, empty liposomes had particle sizes of 126 nm, and a zeta potential of 34 mV. It is interesting to note that
prior to sonication empty cationic liposomes had particle sizes of 577 nm, indicating that
sonication for 10 min effectively reduced particle sizes by > 75%. With the addition of
protein, however, liposomal particle sizes increased greatly. Prior to sonication, OVAL
cationic liposomes had particle sizes of 2145 nm, whereas after sonication for 2.5 min
their particle sizes were reduced to ~800 nm. Sonication for longer than 2.5 min would
normally be expected to further decrease particle sizes, but I found that it resulted in
larger particle sizes and precipitation of the protein. For this reason, the OVAL
liposomes I prepared and characterized were sonicated for 2.5 min and were then filtered
to bring particle sizes into the desirable range of <300 nm. I later learned that Dr. Vyas
had also filtered his OVAL liposomes for preparation # 2, thus the smaller particle sizes
reported in Table 2 above.

To further characterize the cationic liposomal formulations, transmission electron
microscopy (TEM) was used to observe the shape and size range of the OVAL loaded
liposomes (Figure 43). This image was made from a sample of preparation # 2 prepared
by Dr. Tushar Vyas. The figure shows OVAL liposomes with consistent particle sizes
between 50-125 nm confirming the data reported in Table 2. In addition, it shows the
relatively spherical shape of the liposomes.
2. Qualitative determination of protein transport to brain

To determine if intranasal administration resulted in detectable protein delivery to the brain, ovalbumin (OVAL) conjugated with the fluorescent marker Alexa 488, was loaded into cationic liposomes and administered intranasally to rats. These studies were meant to be a qualitative assessment of brain transport due to the inherent difficulties with fluorescence quantification. As controls for the Alexa 488-OVAL studies, it was also important to determine the level of background fluorescence in the brains of animals given normal saline, plain liposomes without protein, or liposomes loaded with plain (unlabeled) OVAL (n = 2 for each treatment). All rats were given equivalent volumes and concentrations of liposomes as previously described. The level of background fluorescence in the SN and striatal sections of these brains is shown in Figures 44, 45 and 46. Although there was generalized, low level autofluorescence in sections from rats given saline or plain liposomes, there were no areas of punctuate or cellular fluorescence in any of these sections (Figures 44 and 45).
Figure 44: Substantia nigra (left) and corpus striatum (right) sections of rats intranasally administered normal saline, 24 hour time point (20x). Scale bars = 20 µm.

Figure 45: Substantia nigra section showing only normal levels of autofluorescence following intranasal administration of plain cationic liposomes, 24 hour time point (20x). Scale bar = 20 µm.

Figure 46: Substantia nigra (left) and corpus striatum (right) sections of rats intranasally administered plain OVAL in cationic liposomes, 24 hour time point (20x). Scale bars = 20 µm.
However, in rats given plain OVAL-loaded cationic liposomes, there did seem to be a modest increase in fluorescence above background, suggesting that the protein itself may be fluorescent (Figure 46 B). This may appear to have been a limitation because it might prevent distinguishing between the OVAL fluorescence and the Alexa 488 signal. However, since determination of protein delivery to brain following intranasal administration was the ultimate goal, it is irrelevant whether the fluorescence observed was due to Alexa 488 or to OVAL itself insofar as any signal above tissue background indicated protein deposition in the brain.

In order to determine whether the intranasal route of administration could bypass the blood-brain barrier, the intracarotid and intranasal routes of administration were also compared (Figure 47). In this case, Alexa 488-OVAL was administered in PBS (1 µg/µl) for a total dose of 50 µg either intranasally or intravenously (n = 2 for each route). For intravenous administration, the rat was anesthetized with 2.5% isoflurane using a VetEquip vaporizer. A small incision was made in the carotid artery, and PE10 tubing was inserted. The solution was infused using a Harvard Apparatus infusion pump over 1-2 minutes, followed by a 0.5 ml normal saline flush. The animals were sacrificed by transcardial perfusion with 4% paraformaldehyde 24 hours following administration, and 25 µm coronal sections of brain were collected and examined by fluorescence microscopy.
As shown in Figure 47, both routes of administration resulted in some discrete fluorescent deposits in the striatum. However, intracarotid administration appeared to cause significantly less protein deposition in the brain than the intranasal route of administration. This result suggests that the BBB and/or protein degradation by plasma enzymes prevented the protein from entering the brain from the blood. Nevertheless, the appearance of discrete fluorescent deposits in the striatum of rats receiving intranasal Alexa 488-OVAL in PBS was the first evidence that the protein could be transported across the nasal epithelium into brain, even without nanoparticles.

Next, Alexa 488-OVAL was loaded into the two cationic liposomal preparations and administered intranasally to rats at a 1 µg/µl concentration (50 µl per rat; nominally 50 µg of protein). Control rats received Alexa 488-OVAL in PBS in the same volume. The animals were sacrificed 6 and 24 hours after administration (n = 2 per preparation at each time point). Both liposomal preparations, as well as PBS, were found to effectively deliver the fluorescent protein to the target brain areas, SN and striatum. By 6 hours after
intranasal administration, punctuate fluorescent deposits were found scattered throughout
the neuropil along the rostral-caudal axis of the brain, from the forebrain to the hindbrain,
of all animals that received Alexa 488-OVAL, with or without nanoparticles (Figure 48
A, B, C, D).

Figure 48: Intranasal administration of Alexa 488-OVAL in PBS and in cationic liposomes at the 6 hour
time point. A. Striatum of a rat intranasally administered Alexa 488-OVAL in PBS (20x). B. Substantia
nigra of a rat intranasally administered Alexa 488-OVAL in PBS (20x). C. Striatum of a rat intranasally
administered liposomal Alexa 488-OVAL (20x). D. Substantia nigra of a rat intranasally administered
liposomal Alexa 488-OVAL (20x). Scale bars = 20 µm.
Note that scattered fluorescent deposits were observed as early as 6 hours after intranasal administration in both the striatum and SN of all animals that received Alexa-488-OVAL, with or without the liposomal preparation (Figure 48). Since discrete fluorescent deposits were not observed in these brain regions from rats that received identical volumes of either plain liposomes, saline, or unlabeled OVAL intranasally (Figures 44-46 above), it is unlikely that the deposits observed in the Alexa-488-OVAL-treated rats were simply due to tissue autofluorescence.

Although both liposomal and non-liposomal preparations resulted in qualitatively similar transport to brain at 6 hours, the liposomal protein appeared to persist longer in brain. By 24 hours after intranasal administration, rats that received the liposomal preparation continued to exhibit abundant punctuate fluorescent deposits in both the striatum and SN. Figure 49 shows an example of numerous brightly fluorescent deposits in the ventral-lateral edge of the striatum, along its border with the darker-appearing corpus callosum, at 24 hours after administration of liposomal preparation # 1 with Alexa-488-OVAL (1 µg/µl). Similarly, Figure 50 shows the persistence of bright fluorescent protein deposits in the SN of a rat given preparation # 2 24 hours after treatment. However, in rats that received the same dose of Alexa-488-OVAL in PBS, the deposits were fewer, more sparsely distributed, and less intense by 24 hours after administration in both structures. For example, compare the striatal images for PBS versus liposomal Alexa 488-OVAL at the 24 hour time point (Figures 47 A vs. 49).
**Figure 49:** Intranasally administered liposomal Alexa 488-OVAL (preparation #1). Brain atlas section showing the precise location in the corpus striatum where the image was taken; 24 hour time point (20x). Scale bar = 20 µm.

**Figure 50:** Intranasally administered liposomal Alexa 488-OVAL (preparation #2). Brain atlas section showing the precise location in the SN where the image was taken; 24 hour time point (40x). Scale bar = 10 µm.
The pattern of fluorescence distribution also differed between the SN and striatum. In the striatum, the widespread punctate fluorescent deposits did not appear to be localized within discrete cellular structures at either time point, whereas in the SN, the deposits appeared be concentrated within cells by 24 hours after administration of the 1 µg/µl liposomal preparation (Figures 51 A and B). Furthermore, the cellular uptake in the SN was time dependent, insofar as it was not observed at 6 hours after administration of the 1 µg/µl liposomal preparation (Figure 48 D), nor in the rats that received Alexa 488-OVAL in PBS (no nanoparticles; Figure 48 B).

![Figure 51: Intranasal administration of liposomal Alexa 488-OVAL (preparation # 2; 1µg/µl) showing a different pattern of fluorescence distribution. Cellular uptake in the SN is shown in two different animals at the 24 hour time point, and under different magnifications (A = 20x; B = 40x). Scale bars = 20 µm.](image)

Finally, the time course of the cellular uptake of Alexa 488-OVAL in the SN appeared to dependent on the concentration and volume of the intranasal dose of the liposomal protein. When the 50 µg dose was administered in the more concentrated preparation (2µg/µl), cellular uptake was observed by the 6 hour time point in the SN (Figure 52 A, B). These findings suggested that the more concentrated liposomal
preparation resulted in higher uptake and/or faster transport of the fluorescent protein in brain than the more dilute preparation.

Figure 52: Intranasal administration of Alexa 488-OVAL in liposomal preparation # 2 (2 µg/µl). Substantia nigra sections (80x), 6 hour time point. Scale bars = 10 µm.

3. Cellular uptake of Alexa 488-OVAL in ventral midbrain

To determine if at least some of the cells taking up the fluorescent protein were dopamine neurons, tyrosine hydroxylase (TH) immunohistochemistry was conducted on midbrain sections from rats intranasally administered preparation # 2 liposomal Alexa 488-OVAL (1µg/µl; 50 µg) 24 hours previously. A secondary antibody coupled with the red fluorophore, Texas Red, was used to label TH positive neurons. Figure 53 shows the presence of both green and red fluorescence in the same neurons, yielding yellow in the merged image. This result indicates that Alexa 488-OVAL was taken up in part by dopamine neurons. However, uptake of Alexa 488-OVAL was not specific to dopamine neurons since other non-TH-positive cells in the ventral midbrain also accumulated the label (Figure 54).
Figure 53: Intranasally administered preparation #2 liposomal Alexa 488-OVAL is taken up intracellularly in the SN, at least in part, by dopamine neurons; 24 hour time point (40x). Scale bars = 10 µm.

Figure 54: Intranasally administered preparation #2 liposomal Alexa 488-OVAL is taken up intracellularly in the SN, but not always by TH positive dopamine neurons, 24 hour time point (40x). Scale bar = 10 µm.

To determine what percentage of cells that took up the fluorescent protein were also dopamine neurons, a conditional frequency analysis was conducted using Bioquant® image analysis software. All cells containing green Alexa 488-OVAL deposits were first located and marked in Bioquant® using red circles. Then the field was viewed using the red fluorescent filter, and wherever Alexa 488-OVAL was present within a TH...
immunoreactive cell, that cell was marked with a yellow circle as shown in Figure 55. A total of 383 Alexa 488 labeled cells were examined (n=2 rats per time point). The proportion of Alexa 488-OVAL-labeled cells in the ventral midbrain that exhibited TH immunolabeling was approximately 52% for both the 6 and the 24 hour time points.

**Figure 55:** Alexa-488 OVAL (2 µg/µl) deposits in the SN (40x) marked with red circles (left panel), and colocalized areas of Alexa-488 OVAL with TH in the SN (40x) marked with yellow circles (right panel). Scale bars= 10 µm.

4. **Mapping of Alexa 488-OVAL distribution in brain**

The distribution of the labeled protein in brain was assessed using Bioquant® image analysis software in rats intranasally administered the 2 µg/µl Alexa 488-OVAL preparation and sacrificed 24 hours later. Representative coronal sections from the olfactory bulb, frontal cortex, striatum, midbrain, and posterior regions were examined for fluorescent protein deposits. A compilation of these images, arranged from rostral to caudal, is shown in Figure 56. Discrete fluorescent deposits were found dispersed throughout the cortex at all rostral-caudal levels, but they were most abundant in the rostral (frontal) cortex and adjacent olfactory bulbs. Deposits were also seen throughout the striatum, in the medial and ventral areas of midbrain (including the SN and ventral midbrain).
tegmental area (VTA), and throughout the pons. The clustered appearance of the deposits in the figure does not necessarily indicate that the label was distributed in discrete clusters, or that it was restricted to the areas shown in the figure. Indeed, the surrounding areas typically had a similar distribution of the fluorescent protein but may not have been included in the Bioquant analysis of that section. This mapping study demonstrated that intranasal administration of liposomal proteins such as Alexa 488-OVAL results in a widespread distribution of the administered protein along the rostral to caudal axis of the brain within 24 hours of administration.

**Figure 56:** Mapping of Alexa 488-OVAL in the rat brain following intranasal administration (2x). Rostral to caudal representative images of the olfactory bulb, frontal cortex, striatum, midbrain, and a posterior section at the level of the pons (Paxinos and Watson, 1998).
5. **Determination of protein integrity**

To determine whether the fluorescent deposits corresponded to actual protein deposits in brain, OVAL immunohistochemistry was performed on striatum and SN sections from rats intranasally administered cationic liposomes loaded with unlabeled OVAL (2 µg/µl) 24 hours prior to sacrifice. As shown in Figure 57, OVAL immunoreactivity was detectable in both the SN and the striatum. The pattern of labeling was similar to that observed after intranasal administration of Alexa 488-OVAL-loaded liposomes, i.e. the immunofluorescence appeared to be widespread in the striatum without obvious cellular localization, and it appeared to be concentrated within cells in the SN. This pattern suggests that the Alexa 488 distribution observed in the previous studies was likely to reflect deposits of the protein, and not just the label, in brain. Furthermore, the protein’s integrity, as indicated by its recognition by an OVAL-specific antibody, appeared to have been preserved throughout the process of liposomal preparation, uptake at the nasal epithelium, and transport in brain.

![Figure 57:](image)

*Figure 57: Ovalbumin immunohistochemistry. A. Substantia nigra section showing intracellular deposits (20x). B. Corpus striatum section showing widespread protein deposition (20x). Scale bars= 40 µm.*
6. Quantitative determination of protein transport to brain

A time course study was conducted to determine the kinetics of OVAL delivery to the brain and to assess its peripheral distribution. In the first set of experiments, 50 µg $^{111}$In-OVAL was intranasally administered to rats at a concentration of 1 µg/µl in either cationic liposomes (preparation # 1 and # 2) or in PBS, and the rats were sacrificed at 6 and 24 hours following administration (n= 6 per time point). Results indicated that preparation # 2 resulted in higher brain OVAL levels than preparation #1 or control (no liposomes) at both time points (Figure 58 C, D). Since liposomal preparation # 1 was less effective in delivering the protein payload to brain, preparation # 2 was used exclusively from that point forward. Additional studies were carried out to evaluate preparation # 2 at earlier time points, i.e. 1 hour (n= 4) and 4 hours (n= 4) after intranasal administration, and to provide a clearer picture of the temporal characteristics of OVAL delivery to the brain. Brains, plasma, stomach, intestines, lungs, and livers were collected and $^{111}$In-OVAL uptake was determined in each tissue by gamma counting. Tissue concentrations were expressed as the percentage of the administered dose per gram of tissue wet weight. The highest brain concentration of $^{111}$In-OVAL for both liposomal and non-liposomal preparations occurred at the earliest time point, 1 hour after administration, and levels declined steadily over time (Figure 58 A). At the 1 hour time point there was no difference between the control solution ($^{111}$In-OVAL in PBS) and the cationic liposomal preparation (Figure 58 A; P= 0.9060; Student’s t-test). At all remaining time points, the liposomal $^{111}$In-OVAL preparation yielded higher brain levels of the radiolabeled protein than the non-liposomal control (Figure 58 B, C, D). This difference was significant at the 4 hour (P= 0.0468; Student’s t-test) and 24 hour time...
points ($P < 0.01$; one-way ANOVA; Figure 58 B, D), but not at the 6 hour time point (one-way ANOVA; $P = 0.1435$; Figure 58 C). This data suggested that, although cationic liposomes did not appear to increase the initial brain concentrations of OVAL, they did prolong the presence of OVAL in the brain. They further showed that preparation # 2 was superior to preparation # 1, thereby justifying use of only preparation # 2 in the remaining studies.
Figure 58: Brain $^{111}$In-OVAL levels as percent of administered dose per gram of tissue. A. The highest $^{111}$In-OVAL brain levels were achieved at the 1 hour time point, however no statistical significance was seen between the liposomal and non-liposomal groups ($P = 0.9060$). B. At the 4 hour time point, the liposomal preparation was shown to be superior to $^{111}$In-OVAL in PBS. C. The 6 hour time point showed a trend towards preparation # 2 having higher brain levels than preparation # 1 or $^{111}$In-OVAL in PBS. However, this was not statistically significant ($P = 0.1435$). D. At the 24 hour time point, it became clear that preparation # 2 was superior to preparation #1 or control at retaining the $^{111}$In-OVAL in brain longer. Student’s t-test was performed on 1 hour and 4 hour time points. One-way ANOVA was performed on the 6 hour and 24 hour time points.
Examination of the peripheral tissues revealed that the highest percentage of the administered dose of $^{111}$In-OVAL was found in the stomach and, to a lesser extent, the intestines at all time points (Figure 59 and 60). This was true for both the control protein solution in PBS (Figure 60) as well as the liposomal preparation (Figure 59). This data suggested that most of the intranasal dose (given in a total volume of 50 µl) was being swallowed. Subsequently, a more concentrated form of both the PBS and liposomal preparations (2µg $^{111}$In-OVAL /µl) was prepared so that half of the initial volume, 25 µl total, could be administered. A second biodistribution study was carried out to determine if the same dose of protein in a smaller volume might increase retention of the liposomal preparation in the nasal cavity, reduce the percentage of the dose being swallowed, and increase protein delivery to the brain.

Figure 59: $^{111}$Indium OVAL cationic liposomal preparation # 2 (1 µg/µl) peripheral biodistribution.
Paralleling the previous study, rats were given 50 µg of a 2 µg/µl preparation of $^{111}$In-OVAL in cationic liposomes or PBS. Following intranasal administration, the animals were sacrificed at 1 hour, 4 hours, 6 hours, and 24 hours (n= 6 per time point). At all time points, significantly higher brain transport of $^{111}$In-OVAL was observed with the concentrated cationic liposomal preparation compared to the non-liposomal preparation in PBS. The concentrated preparation also delivered a higher percentage of the administered dose to brain than the more dilute (1µg/µl) liposomal preparation (0.2% vs. 0.077% respectively; Figures 61 A and 58 A). As previously noted with the lower concentration solutions, the highest brain levels of $^{111}$In-OVAL still occurred at the earliest time point, the 1 hour time point, suggesting rapid protein transport from the nose to the brain (Figure 63 A). But in contrast to the results obtained with the 1µg/µl concentration of $^{111}$In-OVAL, the 2 µg/µl cationic liposomal formulation provided
significantly higher brain delivery of the radiolabeled protein at the 1 hour, 4 hour, 6 hour, and 24 hour time points.

**Figure 61:** Time course study using intranasally administered $^{111}$In-OVAL (2 µg/µl) in PBS solution (control), or in cationic liposomes (prep #2). Brain levels at 1 hour, 4 hours, 6 hours, and 24 hours after intranasal administration. Data is presented as mean ± SEM. Statistical significance was determined using the Student t-test.
Biodistribution of the labeled protein to peripheral tissues paralleled that observed with the more dilute preparation in that the largest percentage of the administered dose was still found in the stomach and the intestines at all time points. However, the percentage of dose in these and other peripheral tissues was 4-5-fold less at all time points than with the dilute preparations (Figures 59 and 62). These results confirmed that the smaller intranasal volume resulted in a higher uptake in brain and a lower percentage of dose in peripheral tissues. They further revealed that only negligible systemic absorption of the protein occurs by the nasal route since only a small percentage of the dose was recovered in plasma and in the liver at all time points.

Figure 62: $^{111}$Indium OVAL cationic liposomal preparation # 2 (2 µg/µl) peripheral biodistribution.
B. Specific AIM # 2

1. Characterization of cationic liposomal GDNF

Based on the results obtained in the OVAL studies of AIM 1, a cationic liposomal preparation of GDNF was made using preparation # 2 at a concentration of 2 µg of GDNF/µl. Cationic liposomes incorporating GDNF were characterized for particle size, zeta potential (surface charge), and protein loading efficiency. As shown in Table 3, GDNF cationic liposomes had particle sizes averaging 158 ± 26 nm, zeta potentials of 32 ± 8 mV, and protein loading efficiencies of 95 ± 3%. These values were all in the optimal range for an intranasal formulation. The 95% loading efficiency indicated that the cationic liposomal preparation incorporated nearly all of the GDNF protein. Thus, administering 25 µl of the cationic liposomal formulation of GDNF (2 µg/µl) to rats was equivalent to administering a ~50 µg dose of GDNF protein. In addition, GDNF cationic liposomes had particle sizes that were even smaller than those obtained with OVAL. This smaller particle size may facilitate paracellular transport at the olfactory epithelium.
<table>
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<tr>
<td>9</td>
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</table>

**Mean ± SEM**  
158 ± 26  
32 ± 8  
95 ± 3

**Table 3:** Characteristics of GDNF cationic liposomes.

The protein loading efficiency of the liposomes reported above was calculated by using the indirect method. In this method, the liposomes were centrifuged to separate the protein loaded liposomes from the unassociated protein, and a BCA protein assay was conducted on the residual aqueous portion containing the unassociated protein. Using this approach, the loading efficiency was ~95%. Attempts were also made to lyse the GDNF loaded cationic liposomes to perform a more direct measurement of liposomal loading efficiency. The GDNF loaded cationic liposomes were first lysed with 10% Triton X-100, and a protein assay carried out on the lyzed preparation. This assay yielded a protein loading efficiency of 22.6%. It was then hypothesized that the lipids in the liposomal preparation might interfere with the measurement of the protein against a
BSA standard curve. Therefore, a BSA standard curve was generated in the presence of added liposomes and compared to a plain BSA standard curve. As shown in Figure 63, the liposomal and non-liposomal BSA standard curves were superimposable. However, when using a liposomal BSA standard curve and lysing the GDNF loaded cationic liposomes with Triton X-100, the protein loading efficiency of the liposomes was determined to be only 18.6%.

![Comparison of a plain BSA and liposomal BSA standard curve.](image)

Figure 63: Comparison of a plain BSA and liposomal BSA standard curve.

Next it was hypothesized that Triton X-100 was interfering with the BCA protein assay. Therefore, a plain BSA standard curve was compared to a standard curve of liposomal BSA lysed with 10% Triton X-100. As shown in Figure 64, Triton X-100 does not significantly interfere with the basic characteristics of the BSA standard curve (i.e. slope and y-intercept).
Further attempts were made to lyse the GDNF loaded liposomes with several different lysis agents. RIPA lysis buffer, methanol, and 100% ethanol, were used to lyse GDNF cationic liposomes, and these yielded protein loading efficiencies of 16.2%, 8.7%, and 49.9% respectively. For comparison purposes, un-lysed GDNF-loaded cationic liposomes were also assayed against a liposomal BSA standard curve. This resulted in only 8.5% protein detection. These experiments indicated that a strong denaturing reagent such as 100% ethanol was required to directly assess GDNF protein loading efficiencies and to obtain results even close to those gathered by the indirect method. As a result of these experiments, it was hypothesized that the GDNF protein forms a stable complex with the liposomes or their constituent lipids, such that at least part of the GDNF protein molecule was unavailable to react with the BCA reagent. It was concluded that the indirect method provides a better estimate of loading efficiencies. All remaining
GDNF cationic liposomal loading efficiencies were therefore performed by the indirect method.

2. Determination of GDNF integrity in cationic liposomes

In order to determine if GDNF protein integrity was conserved in our stock supply of the protein, as well as following liposomal preparation, two approaches were taken. The first utilized a GDNF sandwich ELISA. To test the possibility that the Amgen GDNF supply had degraded, a sandwich ELISA was performed according to the manufacturer’s specifications (Promega). Amgen’s GDNF was diluted with PBS to the same concentrations as the Promega GDNF provided in the kit, and two standard curves generated. As shown in Figure 65, standard curves generated for both GDNF samples had similar slopes, and the lines almost overlap. This data indicated that there was no significant degradation of the stock GDNF protein used to generate the liposomal preparations, at least insofar as the protein was detectable by an antibody to GDNF. However, a better test of its biological integrity is a “functional ELISA”, and this was the second approach taken.
Figure 65: Comparison between Amgen’s and Promega’s GDNF as assed by sandwich ELISA.

The idea behind this “functional” ELISA was that only intact GDNF would bind to its receptor, thereby permitting determination of whether the GDNF protein remained intact through the liposomal preparation process, which includes sonication. Three different liposomal GDNF preparations were assayed immediately after preparation, and after lysing them with 10% Triton X-100. The average amount of GDNF protein detected in these three liposomal preparations was 82.6%, 79.7%, and 99.4% of the total GDNF quantity added. The mean was 87.2 ± 6.1%. This data suggested that almost 90% of the GDNF protein present in the GDNF liposomal preparations remained functionally intact. As previously stated, GDNF cationic liposomes have a protein loading efficiency of ~95%. By functional ELISA, ~90% of the quantity added remained functionally intact, in good agreement with the loading efficiency data. This means that with administration of a nominal dose of 50 µg per rat, the animal actually received ~ 45 µg of functionally intact GDNF protein.
3. Qualitative detection of GDNF transport and protein integrity in brain

The development of a protocol for GDNF immunohistochemistry required several steps. Briefly, a dilution study was conducted first using varying dilutions of the primary anti-GDNF antibody raised in goat (R&D systems AF-212-NA) and a fixed concentration (1:100) of the secondary antibody, a donkey anti-goat IgG conjugated with a Texas Red fluorophore (TxR; Jackson Immunoresearch Labs). The following concentrations of anti-GDNF antibody were used: 1:100, 1:500, 1:1000, 1:2000, 1:5000, and 1:8000. Based on the primary antibody dilution studies, it was concluded that the 1:5000 dilution of anti-GDNF antibody resulted in the best GDNF immunohistochemical images (Figure 66).

![Substantia nigra GDNF immunohistochemistry using a 1:5000 dilution of anti-GDNF antibodies and a 1:100 dilution of a TxR conjugated secondary antibody (40x), liposomal GDNF treated rat. Scale bar = 20 µm.](image)

Figure 66: Substantia nigra GDNF immunohistochemistry using a 1:5000 dilution of anti-GDNF antibodies and a 1:100 dilution of a TxR conjugated secondary antibody (40x), liposomal GDNF treated rat. Scale bar = 20 µm.

In addition, an antibody elimination control was performed in which the anti-GDNF primary antibody was omitted while maintaining all other conditions in the assay. Primary antibody deletion did not yield any staining (data not shown). Furthermore, in a different set of sections the secondary antibody was omitted (no secondary control), while maintaining all other conditions in the assay. These studies brought to light a peculiar problem. The “no secondary antibody control” sections did exhibit significant
“labeling” in two different animals that received intranasal liposomal GDNF, and in assays conducted on two different days (Figure 67). It was not clear why this labeling was observed since the “no primary antibody control” group did not exhibit evidence of labeling.

![Image](image.png)

**Figure 67:** Midbrain GDNF immunohistochemistry using a 1:5000 dilution of anti-GDNF antibody and no secondary antibody (no fluorophore; 40x). Scale bar = 20 µm.

To further optimize the GDNF immunohistochemical assay, a dilution series for the secondary antibody (donkey anti-goat TxR conjugated antibody) was also performed. The anti-GDNF primary antibody was kept constant at a 1:5000 dilution, and the following concentrations of the donkey anti-goat TxR conjugated antibody were used: 1:100, 1:200, 1:300, and 1:400. From these experiments, it was concluded that the 1:400 dilution of the secondary antibody resulted in the best GDNF immunohistochemical images. Subsequent GDNF immunohistochemical assays were conducted with a 1:5000 anti-GDNF antibody, and 1:400 secondary antibody concentrations. Representative images of GDNF immunoreactivity (IR) from midbrain, striatum, and cortex of rats receiving intranasal PBS or liposomal GDNF are shown in Figures 68, 69, and 70 respectively.
Figure 68: GDNF-IR in midbrain using 1:5000 anti-GDNF primary, and 1:400 secondary antibody. A. Intranasal PBS treated control, 24 hour time point (40x). Scale bar = 20 µm. B. Intranasal cationic liposomal GDNF treated rat, 24 hour time point (80x). Scale bar = 10 µm.

Figure 69: GDNF-IR in the striatum using 1:5000 anti-GDNF primary, and 1:400 secondary antibody. A. Intranasal PBS treated control, 24 hour time point (40x). B. Intranasal cationic liposomal GDNF treated rat, 6 hour time point (40x). Scale bars = 20 µm.
These experiments demonstrated the presence of GDNF-IR in the target brain areas of both PBS-treated control and GDNF-treated rats. Since GDNF is endogenously expressed in the brain, and since there is a 93% sequence homology between human and rat GDNF (Lin et al., 1993), differentiation between administered rhGDNF and the rat’s endogenous form was not possible using immunohistochemistry, i.e. the anti-GDNF antibody recognized both the rat and the administered recombinant human GDNF. In the substantia nigra, where endogenous GDNF expression is relatively abundant (Figure 68 A), there was no obvious difference in staining intensity between control and GDNF-treated rats. However in the striatum, where GDNF expression is normally not so abundant, there appeared to be qualitatively more GDNF immunoreactivity in the GDNF-treated rat compared to a PBS-treated control (Figure 69 A, B). This suggested that the intranasally delivered GDNF protein may have reached the striatum and achieved levels high enough to be detectable by immunohistochemistry. Nevertheless, it was clear that this approach would not permit quantification of how much GDNF was delivered to the
sites of interest, the striatum and SN. Future studies using $^{125}$I-GDNF will be necessary to quantify the amount of GDNF that actually reaches the brain and to assess its regional distribution.

C. Specific AIM # 3

The main goal of this AIM was to assess whether intranasally administered GDNF was neuroprotective against a 6-OHDA lesion in a rat model of PD. This AIM consisted of two studies. The first study was a single dose study to determine if a single (50 µg in 25 µl) dose of intranasally administered GDNF protected against a 6-OHDA lesion, and to assess whether there was an advantage to the liposomal formulation. GDNF in PBS (n = 6), GDNF in cationic liposomes (n = 11), and plain cationic liposomes (n = 9) were administered intranasally to rats 1 hour prior to the 6-OHDA lesion. The animals were allowed to recover for 3-4 weeks and were then sacrificed by cardiac perfusion with 4% paraformaldehyde.

In addition, a non-surgical control was added (n = 5) to assess the weight gain of normal rats of the same age as the lesioned rats over a 3-4 week period. These animals did not receive surgery to generate a 6-OHDA lesion. They were merely administered plain cationic liposomes intranasally.

The second study involved multiple doses of GDNF in PBS (n = 8), GDNF in cationic liposomes (n = 7), and plain liposomes (n = 9) given at 1 day intervals: 1 day prior to a 6-OHDA lesion, 1 hour prior to a lesion, and then 1 day after a lesion. The dose administered was 50 µg in 25 µl per administration, for a total dose of 150 µg of GDNF over 3 days. The rats were allowed to recover for 3-4 weeks, and then they were
sacrificed by perfusion (as above). For the plain cationic liposome group, three animals were administered multiple doses as described above. A Student’s t-test comparing TH immunostaining densities in the SN of rats receiving single and multiple doses of plain cationic liposomes revealed no significant difference between the two groups (P = 0.1374). Therefore, these groups were pooled into one plain liposome control group for a total of 9 animals.

1. **Intranasal GDNF promotes weight gain during post-surgical recovery from a 6-OHDA lesion**

   In both the acute and multiple dosing studies, rats were weighed on the day of their surgery to generate a 6-OHDA lesion, and again on the day of sacrifice. The amount of weight gain in grams was calculated to determine if intranasal GDNF affected the post-surgical recovery of these animals. A more rapid recovery may indicate a neuroprotective effect of GDNF on midbrain dopamine neurons since loss of these neurons reduces reward-motivated behaviors such as eating and drinking. As shown in Figures 71 and 72, intranasal GDNF treatments prevented the suppression of weight gain in 6-OHDA lesioned rats. In the single dose study, only the GDNF in PBS group achieved a significant increase in body weight when compared with the plain liposome group (P = 0.0010 by Student’s t-test; Figure 71). The liposomal GDNF group also achieved a weight gain similar to that of the non-surgical control group, although the result did not reach significance (Student’s t-test).
In the multiple dosing study, only the liposomal GDNF group achieved a significant increase in body weight when compared to the plain liposome group (P<0.05 by one-way ANOVA; Figure 72). These studies indicated that intranasal GDNF treatments prevented the suppression of eating and drinking behavior that normally follows damage to midbrain dopamine pathways.

**Figure 71:** Weight gain differences following a partial 6-OHDA lesion in the acute dosing study. Significance assessed by Student’s t-test.
Figure 72: Weight gain differences following a partial 6-OHDA lesion in the multiple dosing study. Significance assessed by one-way ANOVA and Tukey’s multiple comparison post-hoc test.

2. Intranasal GDNF exerts a neuroprotective effect in the 6-OHDA lesioned SN
   a. Intranasal GDNF increased overall TH immunostaining in the lesioned SN

   The neuroprotective effect of intranasal GDNF in the 6-OHDA lesioned SN was assessed in several ways. The first analysis considered the overall density of TH-positive cell bodies and dendritic fibers in the SN. In control lesioned rats given plain liposomes, the average percent lesion was $75 \pm 7\%$, indicating about a 75% loss of TH staining in the lesioned versus intact SN. Overall, the GDNF treatments prevented the decrease in TH immunostaining density in the 6-OHDA lesioned SN in both the single and multiple dosing studies. Representative images of the ventral midbrain of rats that received plain liposomes, GDNF in PBS, liposomal GDNF, and three doses of liposomal GDNF are shown in Figure 73. In each image, the lesioned SN is on the left. Note the higher TH staining density in the GDNF-treated animals. The inset (center) shows a higher power
(40x) view of the SN from a liposomal GDNF-treated rat. This animal had an unusually dense cluster of TH-positive neurons extending deep into the ventral SN.

**Figure 73:** Representative ventral midbrain images of rats that received plain cationic liposomes, GDNF in PBS, liposomal GDNF, and three doses of liposomal GDNF as indicated (2x). Scale bar = 30 µm.

Despite the similar extent of neuroprotection observed in both GDNF-treated groups, only the GDNF in PBS group achieved a significant increase in the integrated optical density (IOD) of TH immunostaining in the lesioned SN in the single dosing study (P<0.05 by one-way ANOVA; Figure 74). In the multiple dosing study, both GDNF in PBS and liposomal GDNF provided a significant neuroprotection against the lesion (Figure 75). Moreover, there was a slight advantage to the liposomal preparation, insofar as this group had a higher significance (P<0.001) than the GDNF in PBS group (P<0.01) as assessed by one-way ANOVA. This suggested that multiple doses of liposomal GDNF afforded a slightly greater neuroprotection, with slightly less variability, than the multiple doses of GDNF in PBS.
Figure 74: Percent substantia nigra 6-OHDA lesions in the acute dosing study as assessed by the overall density of TH staining in the SN. Significance assessed by one-way ANOVA and Tukey’s multiple comparison post-hoc test.

Figure 75: Percent substantia nigra 6-OHDA lesions in the multiple dosing study as assessed by the overall density of TH staining in the SN. Significance assessed by one-way ANOVA and Tukey’s multiple comparison post-hoc test.

In addition, a comparison of the efficacy of single versus multiple intranasal doses of GDNF revealed that three 50 µg doses of GDNF, in PBS or liposomes, provided a
greater degree of neuroprotection than the respective single 50 µg doses of GDNF in PBS or liposomes (Figure 76). Whereas single doses of GDNF in PBS reduced lesion severity from 75 ± 7% in controls to an average of 41 ± 8%, three doses of GDNF in PBS further reduced the lesion to an average of 34 ± 8%, a slight but non-significant improvement in efficacy (P = 0.5016; Student’s t-test). The advantage of multiple doses was even more apparent in the liposomal GDNF-treated groups. For instance, three doses of liposomal GDNF reduced lesion severity to 27 ± 7% although single doses reduced lesion severity to only 54 ± 9% (P = 0.05; Student’s t-test). Thus, multiple doses of the liposomal preparation may have afforded a cumulative neuroprotective effect, or it may have introduced GDNF at a more optimal time point relative to the lesion.

![Figure 76: Percent substantia nigra 6-OHDA lesion comparison between single dosing and multiple dosing intranasal GDNF.](image-url)
b. Intranasal GDNF increased the average number of dopamine cell bodies in the lesioned SN

A second method for assessing whether intranasal GDNF treatments afforded a neuroprotective effect against the 6-OHDA lesion was provided by dopamine cell counts. The numbers of dopamine neurons/mm³ were determined in both the lesioned and unlesioned SN using an unbiased stereological cell counting method in Bioquant®. The results revealed an increase in dopamine cell number in the lesioned SN of rats given single or multiple doses of intranasal GDNF (Figure 77). However, the increases were significant only for the GDNF in PBS (P = 0.0375), multiple dosing liposomal GDNF (P = 0.006), and multiple dosing GDNF in PBS (P = 0.0368) groups (by Student’s t-test when compared to the plain liposomes groups). One-way ANOVA, on the other hand, resulted in a P value of 0.2226, i.e. no significant differences between groups. Thus, the statistical evidence for neuroprotection using dopamine cell counts was somewhat less robust than for TH immunostaining density. Nevertheless, both measures taken together indicate that intranasal administration of GDNF, in PBS as well as in liposomes, given as a single dose or in multiple doses, provides significant protection against a 6-OHDA lesion in rats.
3. Intranasal GDNF increased the average number of dopamine cell bodies in the unlesioned SN

Dopamine cell counts were also taken on the unlesioned side of the brain in rats that received the various intranasal preparations. The results of this analysis showed that intranasal GDNF also exerted a neurotrophic effect in that it increased the average number of dopamine neurons/mm³ in the unlesioned SN (Figure 78). This effect achieved significance in the single dose liposomal GDNF group versus plain liposome group ($P < 0.05$ by one-way ANOVA), and between the single dose liposomal GDNF group and the multiple dose GDNF in PBS group ($P < 0.05$ by one-way ANOVA). The importance of the latter is unclear since it involved comparing two unrelated groups, i.e. the single dose liposomal GDNF group and the multiple dose GDNF in PBS group. In addition, it was interesting to note that the neurotrophic effect appeared to diminish after
multiple doses of GDNF. Perhaps this decrease in neurotrophic activity was a result of GDNF receptor down regulation. Nevertheless, this data did demonstrate that intranasal GDNF reaches its sites of action, the striatum and SN, at least in quantities sufficient to be neuroprotective and neurotrophic.

![Average Number of SN Dopamine Neurons: Unlesioned Side](image)

**Figure 78:** Average number of SN dopamine neurons in the unlesioned side. Significance assessed by one-way ANOVA and Tukey’s multiple comparison post-hoc test.

4. **Intranasal GDNF did not alter dopamine cell area, diameter, or TH density in either the lesioned or unlesioned SN**

As a measurement of GDNF’s neurotrophic effects, the basic morphological features of dopamine cells such as size, shape, and TH density were also assessed by using Bioquant®. Individual dopamine cell bodies in each stereological counting window were traced under 40x magnification to obtain these measurements. The area of dopamine cell bodies was not changed by intranasal GDNF treatments on either the
lesioned (P= 0.4039; Figure 79 A) or the unlesioned side (P= 0.3028; Figure 79 B) as assessed by one-way ANOVA.

![Average Dopamine Neuronal Area: Lesioned Side](image1)

![Average Dopamine Neuronal Area: Unlesioned Side](image2)

**Figure 79:** Average dopamine neuronal area in the lesioned (A) and unlesioned (B) substantia nigra. Significance assessed by one-way ANOVA.

Similarly, the average diameter of dopamine cell bodies was also not affected by intranasal GDNF treatments in either the lesioned (P= 0.4051; Figure 80 A) or unlesioned SN (P= 0.3913; Figure 80 B) as assessed by one-way ANOVA.
Figure 80: Average dopamine neuronal diameter in the lesioned (A) and unlesioned (B) substantia nigra. Significance assessed by one-way ANOVA.

Lastly, intranasal GDNF did not change the density of TH immunostaining in SN dopamine cell bodies on either the lesioned (P= 0.5647; Figure 81 A) or unlesioned sides (P= 0.5421; Figure 81 B), as determined by one-way ANOVA. Collectively, these results indicated that intranasal GDNF exerts both a neuroprotective and neurotrophic effect in unilateral 6-OHDA lesioned rats. The neurotrophic effect of intranasal GDNF was only demonstrated in one of the measures examined, the dopamine cell count measure. In addition, these results indicated that the liposomal GDNF formulation was only marginally more effective than GDNF in PBS, and only in the multiple dosing paradigm of the neuroprotection study, and in the single dose paradigm in the neurotrophism study in the unlesioned SN.
5. Intranasal GDNF did not significantly reduce behavioral evidence of the 6-OHDA lesion

Behavioral studies were conducted on all lesioned rats in this thesis by administering a single I.P. dose of apomorphine (0.5 mg/kg), a direct-acting dopamine agonist, on the day of sacrifice. Apomorphine typically induces contralateral rotation or turning behavior in unilateral 6-OHDA lesioned animals. A higher number of apomorphine-induced contralateral rotations corresponds to a greater 6-OHDA lesion. Although there was a trend towards a decrease in the number of apomorphine-induced contralateral rotations in the intranasal GDNF groups, consistent with a neuroprotective effect in this model, the results did not achieve statistical significance as determined by one-way ANOVA tests (P = 0.3472 in the acute dosing, and P = 0.2830 in the multiple dosing groups; Figures 82 and 83 respectively). The failure to reach significance...
probably resulted from the high level of variability in this behavioral response in the partial 6-OHDA-lesioned rat.

**Figure 82:** Apomorphine induced contralateral rotations in the single dose study. The groups were not different as assessed by one-way ANOVA.

**Figure 83:** Apomorphine induced contralateral rotations in the multiple dose study. The groups were not different as assessed by one-way ANOVA.
V. DISCUSSION

The blood-brain barrier has been an insurmountable obstacle to the development of new CNS therapeutics, impeding clinical use of otherwise promising proteins and growth factors in the treatment of neurodegenerative disorders such as Parkinson’s disease. This thesis examined a novel approach for delivery of such proteins to the brain, i.e. the intranasal route of administration. It further evaluated the feasibility of a novel formulation, cationic liposomes, for intranasal administration of proteins to brain. Intranasal administration can bypass the blood-brain barrier, and deliver therapeutically relevant concentrations of proteins and neurotrophic factors directly to the brain. Brain delivery following intranasal administration has been previously demonstrated in rodents, non-human primates, and in humans (Liu et al., 2001b; Born et al., 2002; Dufes et al., 2003; Ross et al., 2004; Thorne et al., 2004; Zhao et al., 2004; De et al., 2005; Ma et al., 2007; Thorne et al., 2008). However, to our knowledge, administration of a liposomal formulation for intranasal delivery of proteins has only been demonstrated in a single study by Law et al. (2001) in rodents.

As proof of principle, this project first examined encapsulation of a model protein, OVAL, in a cationic liposomal preparation to determine if it provided higher nose-to-brain transport than a non-liposomal solution of the protein in PBS. The second phase of this research evaluated intranasal delivery of GDNF, a growth factor with powerful regenerative effects on dopamine neurons, in a rat model of Parkinson’s disease. Intranasal administration of GDNF, and the use of cationic liposomes as the delivery system, have not been previously attempted in any CNS disease model.
A. Cationic liposomes deliver ovalbumin to brain after intranasal administration

Delivery of the model protein, OVAL, was assessed qualitatively by fluorescence histological examination using Alexa 488-OVAL, and quantitatively through gamma counting by using $^{111}$In-OVAL. By both methods, intranasally administered OVAL was shown to be delivered to the target brain areas affected by Parkinson’s disease, the SN and striatum. In qualitative studies, intranasally delivered Alexa 488-OVAL resulted in widespread brain distribution, as early as 6 hours following administration for both liposomal and non-liposomal preparations (Figure 48). However, the labeled protein appeared to persist longer in brain after liposomal delivery, as evidenced by a greater number of fluorescent deposits still visible at 24 hours after administration (Figure 49 and 50). In addition, only the liposomal Alexa 488-OVAL resulted in apparent cellular uptake in the midbrain, after 6 hours for the concentrated (2 µg/µl) preparation, and after 24 hours for the 1 µg/µl preparations (Figure 52 and 51 respectively). Approximately 50% of the cells taking up the fluorescent protein were dopamine neurons, as demonstrated by TH immunohistochemistry (Figure 55). This suggests a non-specific mechanism of protein uptake. Assuming that the liposomes reach the midbrain intact, adsorptive endocytosis is likely to be the mechanism involved in the intracellular uptake of cationic liposomes and their cargo (Friend et al., 1996; Miller et al., 1998; Briane et al., 2002). However, a question yet to be answered is if the cationic liposomes reach the brain intact, or if they release their cargo and only the protein is present in brain.

At first, two different cationic liposomal preparations for intranasal delivery of OVAL to the brain were generated and characterized. These liposomal preparations had identical lipid composition and proportions (DOPC, cholesterol, and stearylamine), but
they differed in preparation technique. Preparation # 1 consisted of adding the protein, in this case ovalbumin, to preformed liposomes. This method of preparation does not subject the protein to sonication, which may result in extremely high temperatures and strong denaturing forces. Preparation # 2, on the other hand, involved addition of the protein during the hydration step during liposomal formation followed by sonication. Adding the protein during the hydration step of liposomal formation was expected to result in better encapsulation of the protein, and potentially smaller liposomal particle sizes. However, a potential limitation of this method of liposomal preparation was that the protein might become denatured and precipitate if subjected to sonication for an extended period of time. As a matter of fact, the main obstacle encountered in optimizing the OVAL liposomal preparation # 2 was that extending the sonication time to obtain smaller particle sizes did result in denaturing of the protein and precipitation. Multiple attempts were made to determine the length of sonication time that would result in the smallest liposomal particle sizes without causing the OVAL protein to precipitate. Finally, it was determined that the maximal sonication time of OVAL loaded cationic liposomes that avoided precipitation was 2.5 min. This sonication time resulted in particle sizes that were approximately 800 nm, which is 3-4 times larger than the liposomal particle sizes we originally proposed for intranasal delivery to the brain (i.e. 200-300 nm). Since longer sonication resulted in protein precipitation, it was determined that preparation # 2 would be filtered through a 0.2 µm syringe filter to further lower the liposomal particle sizes. While this did yield an average particle size of about 300 nm immediately after sonication, filtration was only a temporary measure since OVAL-loaded liposomes tended to agglomerate over time and form much larger particle sizes.
within hours. It has subsequently been assumed that the particle sizes of the liposomes used in the \textit{in vivo} studies, which were made by Dr. Vyas, were likely much larger than 300 nm.

**B. Preparation \# 2 was shown to be superior to preparation \# 1**

Preparation \# 2 was chosen over preparation \# 1 early in the course of this project due to its smaller particle sizes, and higher OVAL brain delivery following intranasal administration. Higher OVAL brain delivery was determined by gamma counting following intranasal $^{111}$In-OVAL administration. However, in retrospect, perhaps preparation \# 1 was abandoned prematurely. Dr. Vyas reported that preparation \# 1 OVAL liposomes had particle sizes of approximately 1200 nm, and preparation \# 2 had particle sizes of only 200-300 nm. However, since preparation \# 2 had to be filtered to obtain those lower particle sizes, it is conceivable that preparation \#1 would have yielded this smaller size range had it been filtered. The use of filtration for preparation \# 2, but probably not preparation \#1, was brought to our attention well after all of the OVAL studies had been completed. Perhaps if preparation \# 1 had also been filtered, it might have resulted in higher delivery of OVAL to brain after intranasal administration via paracellular transport across the olfactory epithelium. This remains an unanswered question of this project.

**C. Intranasal administration of OVAL results in rapid brain transport**

A time course study conducted using $^{111}$In-OVAL in PBS, or in cationic liposomes, demonstrated that nose-to-brain transport occurs very rapidly, within 1 hour
of intranasal administration (Figure 58 A). In addition, the highest brain levels were achieved at the earliest time point, 1 hour, and thereafter levels steadily declined over time (Figure 58 A, B, C, D). This rapid uptake indicated that intranasal administration of \textsuperscript{111}In-OVAL probably undergoes paracellular transport at the level of the olfactory epithelium. Transcellular or axonal mechanisms of nose-to-brain transport are unlikely since they would require longer transit times to reach the brain (Graff and Pollack, 2005).

These time course studies also indicated that the cationic liposomal preparation resulted in longer retention of the protein in brain. This could be a potential advantage of a cationic liposomal formulation of GDNF for intranasal administration, assuming a time course similar to that for OVAL. Longer brain retention would presumably allow longer availability of the protein at the site of action. Furthermore, if this treatment strategy were ever to be used in humans, longer brain retention could potentially result in less frequent intranasal administrations, greater therapeutic compliance (or patient adherence), and less irritation to the nasal mucosa.

D. Administration of a smaller volume of a more concentrated liposomal formulation resulted in higher brain levels

Biodistribution studies using 1 µg/µl \textsuperscript{111}In-OVAL, in PBS or in cationic liposomes, demonstrated that the highest percentage of the administered dose following intranasal administration was found in the stomach and intestines (Figures 59 and 60). This indicated that most of the administered dose was being swallowed by the animals. Consequently, a more concentrated form of \textsuperscript{111}In-OVAL (2 µg/µl) was intranasally administered to rats in either PBS or in cationic liposomes, and another biodistribution
study was performed. The highest concentrations of $^{111}$In-OVAL were still found in the stomach and intestines, for both the PBS controls and the cationic liposomal formulation. However, these concentrations were 4-5-fold lower than those obtained with the dilute preparation (Figures 59 and 62). Furthermore, the concentrated form of liposomal preparation # 2 resulted in higher protein levels in brain than the control (PBS) and the dilute liposomal preparation, with the highest levels occurring at the 1 hr time point (Figure 61). Both of these findings taken together indicated that reducing the volume of administration resulted in less of the $^{111}$In-OVAL being swallowed, and more being available for brain transport at the olfactory epithelium.

In addition, these biodistribution studies also demonstrated that very little of the intranasally administered protein undergoes peripheral distribution, since almost undetectable amounts of $^{111}$In-OVAL were found in plasma and in the liver. Extrapolating this to nasal delivery of GDNF, low levels of GDNF protein in the periphery should minimize potential side effects in non-target peripheral organs. Therefore, it was decided to move forward with a concentrated form of preparation # 2 (2 µg/µl) to generate a similar cationic liposomal preparation of GDNF for intranasal delivery to the brain.

E. GDNF cationic liposomes had ideal particle sizes and did not require filtration

Association and/or encapsulation of the GDNF protein to cationic liposomal preparation # 2 resulted in ideal particle sizes (<200 nm), which did not require filtration of the liposomal preparation. Multiple concerns existed initially about the ability of the GDNF protein to withstand sonication. Of particular concern was GDNF’s inter-dimer
disulfide bond which, if broken, destroys GDNF’s neurotrophic activity (Lin et al., 1994). However, several forms of evidence suggested that GDNF was better able to withstand sonication than OVAL. First, GDNF never precipitated as a result of sonication, unlike OVAL. The cationic liposomal GDNF preparation was sonicated for 3-7 min, depending on the strength of the sonicator, but even at the longest sonication time no precipitation was observed visually, and no increases in liposomal particle sizes were detected. Other evidence that the protein remained functionally intact after liposomal preparation will be discussed below.

**F. GDNF cationic liposomes had similar protein loading efficiencies as OVAL**

*cationic liposomes*

The protein loading efficiencies of cationic liposomes incorporating GDNF were measured by the indirect method and resulted in similar protein loading efficiencies as the liposomal OVAL preparations (93-95 %). This indicated that most of the GDNF was incorporated in the cationic liposomes, and that intranasal administration of 25 µl of this liposomal preparation (2 µg/µl) resulted in a nominal dose of 50 µg per rat.

In addition, attempts were made to lyse the GDNF loaded cationic liposomes to perform a more direct measurement of protein loading efficiency. However, it was shown that only a strong lysis agent, such as 100% ethanol, resulted in protein loading efficiencies that even approximated those obtained with the indirect method. This suggested that the GDNF protein formed a stable complex with the cationic liposomes, such that at least part of the GDNF protein was unavailable to react with the BCA reagent of the protein assay. This strong association of the GDNF protein with the cationic
liposomes raises a concern in this project, as well as potential problem in the therapeutic
use of such a formulation. The possibility exists that the GDNF protein may not be fully
released from the liposomes, limiting its availability to bind to its receptors. Failure to
release the protein would effectively reduce the bioactive dose at target sites in brain, and
decrease efficacy of the formulation in functional assays. It is possible that such a loss of
efficacy may have resulted in the failure of the liposomal GDNF formulation to exert a
significant advantage over GDNF in PBS in the 6-OHDA model.

G. GDNF remained functionally intact after liposomal preparation

The concern that the GDNF protein would not be able to withstand sonication
during liposomal preparation led to the development of a functional ELISA assay to
determine GDNF protein integrity in cationic liposomes. The principle of this assay was
that only the intact GDNF protein would be able to bind to its receptor, GFRα1. Results
indicated that approximately 90% of the GDNF added to the formulation remained
functionally intact following liposomal preparation. Previous studies showed that GDNF
cationic liposomes have a protein loading efficiency of ~95 %. So, the results of the
functional ELISA are in good agreement with this value, and suggest that almost all of
the protein added and incorporated in liposomes retained biological activity. Thus,
intranasal administration of GDNF cationic liposomes, at a nominal dose of 50 µg, would
actually result in delivery of 90-95% of this amount, or ~45 µg of functionally intact
GDNF protein. Thus, the liposomal dose was somewhat smaller than the dose of GDNF
in PBS. Consequently, the controls received a slightly higher total dose, i.e. the full 50
µg. This may also contribute to the inability of the GDNF cationic liposomal formulation
to achieve a significant advantage over GDNF in PBS. While a difference of 5 µg of GDNF protein, or 15 µg in the multiple dosing studies, may not appear to be significant, it may have been functionally important in these studies. For instance, GDNF has been shown to have an EC$_{50}$ of only 1 pM on dopamine neurons in midbrain cultures (Lin et al., 1994), so a difference of 15 µg, or even 5 µg, might be relevant and may help explain the results obtained in these studies.

A separate but related issue is whether the GDNF delivered in liposomes remained functionally intact in brain. In order to determine if GDNF protein integrity was conserved in brain tissue following intranasal administration, it was originally proposed that a functional ELISA would be performed on brain homogenates taken from GDNF-treated rats. However, several factors indicated that a functional ELISA on brain homogenates might not be feasible, so it was decided not to pursue these experiments. One of these factors was a personal communication with Dr. William Frey II at the Society for Neuroscience meeting in November of 2007. Dr. Frey stated that determining neurotrophin levels in brain following intranasal administration was a challenge. Neurotrophins bind tightly to their receptors in vivo, such that tissue processing for ELISA determinations results in their incomplete release. For instance, a study conducted on nerve growth factor (NGF) levels in rat brain using ELISA demonstrated that previous reports had underestimated the levels, and that this was due to incomplete release from NGF receptors (Fawcett et al., 1999). In this study, the authors employed multiple freeze-thaw cycles of the brain homogenate to release bound NGF in order to make it available for ELISA quantification. Unfortunately, multiple freeze-thaw cycles often result in the denaturing of proteins, which would defeat the purpose of performing a
GDNF functional ELISA (i.e. denatured GDNF would not bind the GFRα1 receptor). Another method that is often used for neurotrophin extraction and quantification is acidification followed by neutralization (www.promega.com; Rush and Zhou, 2001). This protocol uses 1 N hydrochloric acid to acidify the brain homogenate to a pH of 3-4, followed by 1 N sodium hydroxide to neutralize it back to a pH of 7 prior to the ELISA assay. Unfortunately, this method would also denature the GDNF protein, making it unsuitable for a functional ELISA.

Another factor that indicated that a functional ELISA on brain homogenates might not be feasible was the results of the $^{111}$In-OVAL studies. As previously reported, only small fractions of the administered dose reach the brain following intranasal administration. Assuming that intranasal administration of GDNF results in similar brain levels, the highest brain level achievable would be 0.2% of the administered dose per gram of brain tissue (Figure 61 A). These GDNF levels may fall below the limits of detection of the GFRα1 receptor chimera used in this assay (lower limit = 0.75 ng/ml), especially if some of the GDNF protein remained associated with its receptors or became internalized and degraded by proteases.

Although these factors discouraged an attempt to measure changes in brain levels of GDNF in this thesis, the GDNF functional ELISA may be performed at a later date to see if some of these obstacles may be overcome.
H. GDNF cationic liposomes are delivered to brain following intranasal administration

In qualitative studies, cationic liposomal GDNF was shown to be delivered to the brain following intranasal administration by using GDNF immunohistochemistry. However, immunohistochemical determination of the delivered protein was limited due to the presence of endogenous GDNF protein. Since human and rat GDNF have a 93% sequence homology (Lin et al., 1993), the anti-GDNF antibody could not distinguish between the delivered rhGDNF protein and the endogenous rat GDNF. This was particularly true in the SN, where abundant endogenous GDNF expression was undistinguishable from the administered protein (Figure 68). However, in the striatum, where endogenous GDNF protein expression is limited, there was qualitatively more GDNF immunoreactivity in the cationic liposomal GDNF treated group when compared to the PBS controls. This was also true in the cortex, and in the fibers of the corpus callosum (Figure 69). These studies indicated two things. First, cationic liposomal GDNF is delivered to the brain after intranasal administration. Second, the delivered GDNF protein found in brain was at least sufficiently intact to be recognized by its antibody. These studies did not, however, indicate the amount or regional distribution of the administered GDNF in brain. These determinations will require GDNF radiolabeling and autoradiography studies.

I. A unilateral, partial 6-OHDA lesion model was generated and standardized

The development of a consistent, partial, medial forebrain bundle 6-OHDA lesion was a major undertaking of this project. The standard protocol in Dr. Barbara
Waszczak’s laboratory calls for a complete (> 90 %) unilateral 6-OHDA lesion (White-Cipriano and Waszczak, 2005; Waszczak et al., 2006; White-Cipriano and Waszczak, 2006). However, in this project the goal was to generate a partial 6-OHDA lesion where 50-80% of SN dopamine neurons were destroyed on one side of the brain while leaving the other SN intact. The reason for this decision was that GDNF can only protect damaged or dying dopamine neurons, and rescue them from destruction. GDNF has not been shown to induce neurogenesis in the SN. It is even controversial whether dopaminergic neurons of the SN can even undergo neurogenesis at all. Consequently, generating a 6-OHDA lesion that destroys > 90 % of SN dopamine neurons may result in a model that is too severe to be able to be overcome by GDNF treatments. In fact, one of the main criticisms of the Amgen clinical trial was that it employed PD patients in the advanced stages of the disease, when SN dopamine neurons were mostly destroyed before the initiation of GDNF treatments. If intranasal GDNF were to become a PD treatment in humans, early stage PD patients would gain the greatest benefit from this treatment because they would still have some dopamine neurons that could be rescued.

As a result, considerable effort was employed to generate a partial 6-OHDA lesion. The main approach was to maintain the same medial forebrain bundle stereotaxic coordinates while varying the dose and volume of the 6-OHDA administered. Ultimately, a 0.5 µg/µl concentration and a 4 µl volume (total dose of 2 µg), of 6-OHDA were chosen to generate the partial lesion. This protocol yielded an average loss of TH immunostaining density in the lesioned SN of 75 ± 7%.
J. Intranasal GDNF promoted weight gain during post-surgical recovery from a 6-OHDA lesion

Damage to the midbrain dopaminergic pathways usually results in loss of appetite and weight loss (or lack of weight gain over time) in the 6-OHDA lesioned animals. This is even more pronounced in the bilateral 6-OHDA lesion model, and may even result in the animal’s death. However, intranasally administered GDNF counteracted this normal response to the lesion and promoted weight gain during the post-surgical recovery period following the 6-OHDA lesion (Figure 71). In the acute dosing study, animals that were intranasally administered GDNF achieved weight gains that were comparable to the weight gains observed in the plain cationic liposome controls that did not have surgery. This effect achieved significance in the intranasal GDNF in PBS treated group.

In the multiple dosing study, on the other hand, it was the liposomal GDNF group that achieved significant weight gains when compared with the 6-OHDA-lesioned rats that received plain cationic liposomes (Figure 72). These studies provided one form of evidence that that intranasal administration of GDNF was neuroprotective against a partial 6-OHDA lesion.

K. Intranasal GDNF increased overall TH immunostaining in the lesioned SN

In the first of several measures of neuroprotection, tyrosine hydroxylase immunostaining density in the lesioned SN was compared with that of the unlesioned SN to generate a percent 6-OHDA lesion for each rat. Intranasal GDNF treatments resulted in a decrease in the average severity of the lesion compared to plain cationic liposomes (Figure 74). In the acute dosing study, this achieved significance in the GDNF in PBS
treatment group. Although it did not achieve significance, the cationic liposomal GDNF treatment group also exhibited a 25% decrease in the average size of a SN 6-OHDA lesion. However, due to considerable variability in the liposomal GDNF-treated group, with four animals out of eleven exhibiting lesions as severe as those seen in the control group, the neuroprotective effect did not reach significance for this treatment group. These results make it clear that a single intranasal administration of GDNF in humans is unlikely to result in significant, lasting neuroprotection and relief from PD symptoms. Repeated doses at regular intervals are more likely to be effective. Thus, a multiple dosing study was also attempted.

In the multiple dosing study, both GDNF in PBS and liposomal GDNF significantly decreased the average severity of the 6-OHDA lesion (Figure 75). These results were less variable, and clearly demonstrated a significant neuroprotective effect of multiple doses versus a single intranasal dose of GDNF. However, statistical analysis revealed that multiple doses of GDNF in PBS and GDNF in cationic liposomes were similarly efficacious, with only a slight but non-significant further reduction in lesion severity afforded by the liposomal preparation. These results suggested that intranasal administration of GDNF in cationic liposomes had no clear advantage over administration the GDNF protein in PBS for this parameter.

L. Intranasal GDNF increased the average number of dopamine cell bodies in the lesioned SN

A second measure of neuroprotection examined in these studies was the number of dopamine neurons surviving in the lesioned SN. Intranasal GDNF treatments were
also found to be neuroprotective by this measure as indicated by an increased average number of SN dopamine neurons in the lesioned SN, presumably by rescuing them from destruction by the neurotoxin. The increase in dopamine cells per mm$^3$ was significant in rats that received a single dose of GDNF in PBS, as well as in rats that received multiple doses of liposomal GDNF and GDNF in PBS. The fact that intranasal GDNF neuroprotection was better demonstrated in the multiple dosing groups indicates again that a single dose of GDNF will likely not be sufficient to arrest the progression of PD. Multiple intranasal administrations of GDNF may be required to arrest disease progression and prolong quality of life. This thesis did not address the frequency, or doses of GDNF that would need to be administered intranasally to arrest a chronic and progressive disease process.

M. Intranasal GDNF increased the average number of dopamine cell bodies in the unlesioned SN

In addition to performing dopamine cell counts in the lesioned SN, counts were also taken in the unlesioned SN. Since this side was not exposed to the neurotoxin, but it did receive exposure to intranasally administered GDNF, any increases in the number dopamine neurons in the unlesioned SN would be an indication of a neurotrophic effect. Other measures of GDNF’s neurotrophic effects might be increases in dopamine cell size, TH content or dopamine content. Such changes have been demonstrated in vitro and in vivo (Lin et al., 1994; Bowenkamp et al., 1995; Kearns and Gash, 1995; Kearns et al., 1997; Aoi et al., 2000a; Grondin et al., 2002; Ai et al., 2003; Oo et al., 2003). Stereological cell counts in the unlesioned SN in this study revealed that intranasal
GDNF treatments exerted a neurotrophic effect by increasing the apparent number of immunodetectable neurons per mm$^3$. This does not mean that more dopamine cells were generated as a result of these treatments. As previously stated, GDNF has not been shown to induce neurogenesis of midbrain dopamine neurons as determined by bromodeoxyuridine (Brdu) incorporation studies (Chen et al., 2005). A more likely explanation for the increased number of cells is that dopamine neurons with naturally low levels of TH expression, which are normally obscured by the background staining of dendrites in the SN pars compacta, increase their production of TH and become immunodetectable. A higher TH staining density in these cells may make them more visible, allowing for their quantification.

This neurotrophic effect achieved significance in the animals that received a single dose of liposomal GDNF. However, it should also be noted that both of the acute dosing groups, GDNF in PBS and liposomal GDNF, demonstrated higher numbers of dopamine neurons in the unlesioned SN than the animals that received multiple doses of GDNF (Figure 78). This difference between the single and multiple dosing studies achieved significance between the acute dosing liposomal GDNF, and the multiple dosing GDNF in PBS groups according to post hoc analysis of the ANOVA result. However, since different treatments and time points were compared, the statistical difference between these unrelated groups in the ANOVA analysis is not particularly meaningful. At this time, the significance of the diminished neurotropic effect after multiple doses of GDNF is unclear. One possibility is that multiple intranasal doses of GDNF may result in down-regulation of GFRα1 receptor expression, causing a return to basal levels of TH expression and loss of the apparent neurotrophic effect in the multiple
dosing groups. It is also possible that the increased dopamine cell number in the single
dose GDNF treatment group might have been an erroneous result, despite its statistical
significance. However, arguing against this possibility was a similar finding of an
increase in dopamine cell number per mm$^3$ in the SN of unlesioned rats given the same
intranasal dose of liposomal GDNF (personal communication, Robin Ortiz). This finding
corroborates the result observed on the unlesioned side in this study, and it suggests that a
single dose of liposomal GDNF does in fact exert a neurotrophic effect on SN dopamine
neurons.

N. Intranasal GDNF did not affect dopamine neuronal area, diameter, or TH
density in either the lesioned or unlesioned SN

Intranasal administration of GDNF did not result in neurotrophic effects that
altered the basic morphological features of dopamine neurons. In other words, dopamine
neuronal area, diameter, and TH density of individual cells were not altered in either the
lesioned or unlesioned SN. This data indicates that intranasal GDNF may protect
dopamine neurons from the neurotoxic effects of 6-OHDA without altering the basic
functional status of the existing, healthy neurons. If true, this may be an important
advantage of this approach. According to these results, intranasally administered GDNF
may not result in unwanted neurotrophic effects in brain areas other than those affected
by PD. This is particularly important because intranasal administration of proteins is not
a targeted delivery approach, and results in widespread distribution of the protein
throughout the brain (Thorne et al., 2004; Thorne et al., 2008). Presumably, GDNF will
exert effects only in areas of the brain where its receptors are present, and it should be
devoid of effect in other brain areas. However, given that there is some cross-activation between the different GFRα receptors (Treanor et al., 1996), some effects may be observed in non-target structures in the brain that are exposed to the protein during its transport along the olfactory and trigeminal pathways identified by Thorne and Frey (Thorne et al., 1995; Thorne et al., 2004). More studies are necessary to determine if intranasal GDNF administration may result in unwanted side effects at non-target brain areas and peripheral organ systems. One area in particular that should be examined for possible neurotrophic effects of intranasal GDNF is the ventral tegmental area. This area is just medial to the SN in ventral midbrain, and it is the location of the mesolimbic and mesocortical dopamine cell groups. It has been postulated that a neurotrophic effect on these populations of dopamine neurons may induce psychiatric side effects in PD patients. Thus, it will be important in future studies to assess whether there are changes in dopamine cell number or TH staining density in this region.

O. Intranasal GDNF did not significantly reduce behavioral evidence of the 6-OHDA lesion

Unilateral 6-OHDA lesions generate imbalances in dopaminergic transmission between the lesioned and unlesioned basal ganglia circuitries. This imbalance results in supersensitivity of dopamine receptors in the lesioned striatum (Truong et al., 2006). Stimulation of these dopamine receptors on the lesioned side by a direct dopamine agonist, like apomorphine, results in contralateral rotation behavior. In general, the greater the 6-OHDA lesion, the more contralateral turns the animal will make following apomorphine administration. This project attempted to use this behavioral test to
demonstrate protection against a 6-OHDA lesion. The results, however, indicated that intranasal GDNF does not significantly reduce contralateral turning behavior in rats following apomorphine administration. In the multiple dosing study, intranasal GDNF did decrease the number of contralateral turns in both the GDNF in PBS and liposomal groups. However, the high variability within the groups made it impossible to obtain statistical significance. This raises the question of why there was such high variability within the groups.

There has long been controversy in the field about whether this behavioral method is a reliable indicator of the extent of the lesion in partial 6-OHDA lesioned rats, i.e. rats with dopamine cell losses between 50-70% (Hefti et al., 1980; Melamed et al., 1982; Truong et al., 2006). However, Truong et al. did demonstrate that apomorphine-induced turning behavior could be positively correlated with graded 6-OHDA lesions of the medial forebrain bundle in rats. Perhaps, in this project the high variability observed was due to incomplete development of dopamine receptor supersensitivity. Truong et al. conducted their apomorphine behavioral studies 16-24 weeks after the 6-OHDA lesion surgery, whereas in this thesis, apomorphine rotational behavior was assessed 3-4 weeks after generating the 6-OHDA lesion. This time period may have been too short to develop sufficient dopamine receptor supersensitivity on the lesioned side to mediate a robust rotational response.
VI. FUTURE DIRECTIONS

The main goal of this thesis was to determine whether intranasal GDNF was neuroprotective against a 6-OHDA lesion in rats. The results of this work indicated that this goal was accomplished, and that intranasal GDNF does show therapeutic efficacy in a rat model of PD. These results encourage further development and testing of this approach in other PD models. However, there are still many unanswered questions, and several concerns that need to be addressed before this strategy is attempted in humans. The first unanswered questions are whether or not the cationic liposomes reach the brain intact, and if they release their cargo prior to brain transport or only after the protein is present in brain. One way to answer this question is to conjugate a fluorophore with GDNF and encapsulate the protein in liposomes labeled with a different fluorophore. This study has already been planned. GDNF will be conjugated with Texas Red and will then be incorporated in FITC labeled liposomes. Colocalization of both fluorophores would indicate that both the liposomes and the GDNF protein are present in brain. In addition, brain distribution of the delivered GDNF protein could also be determined by this method.

Another unanswered question is how much of the GDNF protein actually reaches the brain following intranasal administration. Conjugation of GDNF with the radioactive tracer $^{125}$I should provide a quantitative method to determine the amount of delivered GDNF that actually reaches the brain. In addition, autoradiography using $^{125}$I-GDNF should also allow determination of the regional distribution of $^{125}$I-GDNF in brain.

Another concern raised in this thesis was that cationic liposomes may not fully release the GDNF protein in vivo, resulting in the failure of the liposomal formulation to
demonstrate a significant advantage over the GDNF in PBS controls. Altering the liposomal formulation by modifying the proportion of the constituent lipids may result in more favorable liposomal release characteristics. In addition, it has been suggested that substituting DOPE (dioleoylphosphatidylethanolamine) for DOPC might improve release characteristics (Dr. Robert Campbell, personal communication). DOPE, another neutral lipid typically used in the formation of cationic liposomes, is typically used in cationic liposomal preparations to facilitate adsptive endocytosis, and to help destabilize the liposomal membrane (Lv et al., 2006). On the contrary, DOPC forms a more stable bilayer liposomal conformation (Lv et al., 2006). Consequently, using DOPE as the neutral lipid of the cationic liposomal formulation may result in better GDNF protein release characteristics.

Another concern raised in this thesis was that non-equivalent GDNF doses were administered to the liposomal GDNF groups and the GDNF in PBS groups. GDNF cationic liposomes incorporated approximately 95% of the GDNF added during liposomal preparation. In addition, the process of liposomal preparation may have resulted in some GDNF protein degradation as shown by the functional ELISA assay results. This assay suggested that approximately 90% of the GDNF added to the liposomal preparation remained functionally intact. These two assays therefore suggested that the GDNF doses that were administered to rats were as much as 5 and 15µg lower than the non-liposomal groups for the acute and multiple dosing liposomal GDNF groups, respectively. Any future intranasal GDNF studies should take this difference into account, and the intranasal liposomal GDNF dose should be adjusted accordingly.
In addition, further intranasal GDNF studies should include longer time points than those used in this thesis. Here, animals were sacrificed 3-4 weeks following the surgery to generate a 6-OHDA lesion. This time point may have been insufficient to detect any neuroprotective effects exerted by intranasal GDNF in the apomorphine rotation behavioral studies. Inclusion of a 6 week and 8 week time point following the 6-OHDA lesion may better reveal the long term behavioral effects of intranasal GDNF.

Another concern raised by this thesis was whether or not intranasal GDNF in cationic liposomes results in nasal toxicity and non-target organ effects. A preliminary toxicology study, conducted in collaboration with Drs. Eunice Stagliola and Robert Schatz, revealed no significant changes in a number of biochemical measures of nasal toxicity after an acute exposure, but this study should be repeated to determine if multiple doses of cationic liposomes damage or destroy the olfactory epithelial cells in the nasal cavity. Furthermore, an immunological study should be conducted to address whether or not intranasal GDNF results in the formation of anti-GDNF antibodies, and if so, whether the liposomal preparation minimizes this effect by at least partially encapsulating the GDNF protein. Additionally, a longer multiple dosing study should be conducted not only to determine therapeutic efficacy of intranasal GDNF, but also to address the concern that GDNF treatments may result in Purkinje cell loss in the cerebellum, as was reported in monkeys given extremely high doses of GDNF (Hovland et al., 2007).

Finally, intranasal GDNF should be evaluated for other therapeutic indications, such as the treatment of drug addiction. Several reports have demonstrated that GDNF treatments can decrease drug seeking behavior in rodents (Green-Sadan et al., 2003; Green-Sadan et al., 2005; Yan et al., 2007). Currently, GDNF treatment for drug...
addiction is only in the experimental stages since it would be impractical, or unsafe, to administer GDNF via intracerebral infusions in this patient population. If GDNF does have efficacy in reducing drug-seeking behavior, intranasal administration would make its use for treatment of drug addiction a viable possibility, since it would be non-invasive and simple to administer.
VII. CONCLUSIONS

Intranasal administration of GDNF results in significant neuroprotection against a 6-OHDA lesion in rats and has great potential for the treatment of Parkinson’s disease. Intranasal administration is an effective and non-invasive method to transport proteins and neurotrophic factors to the brain. Although the GDNF cationic liposomal formulation was not shown to have an advantage over intranasal administration of the protein in PBS in this project, it is still worth exploring a cationic liposomal formulation for intranasal delivery of proteins to the brain. This thesis succeeded in establishing proof-of-principle, and it should be used as a guide for the further development of this treatment strategy and delivery system. Ultimately, the intranasal route may allow GDNF to be administered non-invasively, rather than directly into the brain, vastly simplifying its use in humans. Such an approach could finally move GDNF from an experimental treatment to a clinical reality, thereby providing the first therapy ever to arrest the progression of Parkinson’s disease and not merely alleviate its symptoms.
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