Trans-Nasal Mucosal Delivery of BDNF AntagoNAT Oligonucleotides Using Heterotopic Mucosal Engrafting for Parkinson’s Disease

Thesis presented

By

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ABSTRACT

Chronic neurodegenerative diseases, such as Alzheimer’s disease (AD) and Parkinson’s diseases (PD) are currently the major health care challenge around the world [1]. The currently used levodopa therapy leads to motor complications like dyskinesias. Invasive techniques like deep brain stimulation used for treating PD leads to nerve cell damage in patients. Delivering drugs in the brain is limited by the presence of the blood brain barrier (BBB). Despite many new developments in the field of CNS drug delivery, overcoming the BBB continues to pose a serious challenge.

Parkinson’s disease is characterized by the loss of nigrostriatal dopaminergic neurons in turn reducing dopamine levels in brain. Brain-derived neurotrophic factor (BDNF) is a high molecular weight protein, responsible for dopaminergic neuron survival [2]. However, the therapeutic applications of BDNF in CNS diseases are limited by the BBB, its complex structure, off-target toxicity, immunogenicity and post-translational modifications. BDNF AntagoNATs (AT’s) are small oligonucleotides that can up regulate endogenous BDNF protein expression by inhibiting the natural antisense transcripts (NAT’s) and could be a potential therapy for neurodegenerative diseases like AD and PD. The AT’s are highly locus specific and are highly stable structures that can improve the uptake of these oligonucleotides in cells.

Despite several advantages and significant promise of AT’s, they cannot pass through the BBB. In my doctoral project we have successfully developed an innovative endonasal heterotopic mucosal grafting technique that can provide a permanent way of delivering high molecular weight therapeutics like proteins and oligonucleotides to the brain bypassing the BBB. This method is based on human endoscopic skull base surgeries which are currently in practice in the clinic.

Using the endonasal mucosal grafting technique, we successfully showed that a model protein like ovalbumin (45 kDa) can be delivered to the brain in spite of its high molecular weight. We utilized a liposome-in gel system to protect the protein from degradation and to allow to
sustained drug release. The results of this study were published in *PLOS-1* in December 2018 [3]. Regarding BDNF AT’s, we have successfully shown the liposomes encapsulating BDNF AT’s can up regulate the BDNF mRNA and protein levels in rat schwannoma cells and our results also demonstrate the grafts are capable of delivering therapeutic levels of AT’s in rat brain which further resulted in significant protein up regulation in striatum and substantia nigra. We have also shown that the liposomes encapsulating BDNF AT’s are capable of protecting dopaminergic neurons in a 6-hydroxydopamine rat model of PD further confirming the therapeutic effect of trans-nasal delivery of BDNF AT’s. A manuscript comprising of the results from this study is currently in review in the journal *Science Advances*.

Considering one of the limitations of the mucosal engrafting model being that it allows the delivery of therapeutics from the top of the head in a rat model instead of through the nose, we developed an olfactory depot surgery rat model which is closer to mimicking the proposed drug delivery strategy in humans. In this particular model, our aim was to create a drug depot using a liposome-in-gel system right above the nasal grafts in a rat and deliver the drug directly through it. We have successfully shown that the depot surgery model can also deliver therapeutic amounts of BDNF AT’s in rat brain.

As a platform technique these results suggest that the surgical techniques for CNS drug delivery developed during the course of my thesis project could be potentially used to deliver other high molecular weight therapeutics to the brain by passing the BBB completely which could open door to additional treatment options for CNS diseases.

**References**


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OBJECTIVES AND SPECIFIC AIMS

Parkinson’s disease (PD) is an age-related profoundly debilitating neurodegenerative disorder that affects millions of people worldwide. The current clinical treatments available for treating Parkinson’s disease mainly include dopaminergic medications, which mainly help in replenishing the dopamine in brain or mimicking the action of dopamine. The most commonly used dopaminergic agonist is levodopa, however long-term levodopa therapy is associated with motor complications like dyskinesias. Most of these medications are given orally to PD patients, which reduces the therapeutic efficacy of these drugs as the BBB prevents the drugs from entering the brain. Apart from medications, surgical procedures like deep brain stimulation are used for treatment of PD, however, are only utilized if the patient is exhausted of the medical treatment. These surgical procedures are invasive and can damage healthy brain tissue and nerve cells of patients. Hence there is a need of new therapeutic treatments for Parkinson’s diseases.

The BBB is a tightly regulated barrier in the central nervous system. Dysfunction of the BBB may be involved in the progression of neurological diseases like Parkinson’s disease and this affects the drug efficacy and the drug permeability. The BBB, formed by tight junctions between capillary endothelial cells, limits the delivery of drugs to the brain. Despite of many attempts that have been made for developing invasive as well as non-invasive drug delivery targeted to CNS tissues, getting past the BBB continues to pose a serious challenge. Hence there is a need of novel therapies and drug delivery techniques, which can overcome the BBB for effective treatment of Parkinson’s disease.

The main objective of the doctoral thesis project was to investigate a novel trans-mucosal drug delivery technique for penetrating the BBB and delivering high molecular weight therapeutics directly to the brain bypassing the BBB. We employed a cationic liposomal system for protecting the target molecule from proteolytic and enzymatic degradation and for a slow and sustained release to the CNS. Our strategy was to create a trans-nasal window
(craniotomy) in the BBB in rats and repair it with use of a nasal mucosal graft from a donor rat. Positioning of the graft on the craniotomy resulted in the construction of an internal reservoir directly above the graft. The reservoir was then be used to deliver target therapeutic molecule directly to the brain. Apart from the mucosal engrafting technique we also developed an olfactory depot surgery model which helped in delivering the therapeutics to the brain through the nose instead from the top of the rat head which is closer to the proposed drug delivery strategy in humans.

Brain-derived neurotrophic factor (BDNF) is a high molecular weight protein that mediates brain plasticity and in particular, supports the survival of dopaminergic neurons. Parkinson’s disease is characterized by death of dopaminergic neurons in the substantia nigra pars compacta region of midbrain, in turn affecting the nigrostriatal dopaminergic pathway, which reduces the striatal dopaminergic levels. BDNF is one of the most intensely studied targets and has shown reversal of PD symptoms. However, the clinical translation faces the problems of recombinant BDNF protein manufacturing and delivery through BBB owing to its high molecular weight (14 kda). Also, the complexity of translational and posttranslational regulation of BDNF creates challenges for development of BDNF recombinant therapy.

The challenges mentioned above in recombinant BDNF therapy could be avoided by designing therapies that target natural antisense transcripts (NAT’s). NAT’s are a subclass of endogenous non-coding RNA’s, which specifically inhibit the expression of many disease related proteins by epigenetic mechanisms. Inhibiting these NAT’s by using synthetic oligonucleotide-based compounds (AntagoNAT’s) leads to up regulation of the endogenous target protein. BDNF-AS is one such NAT present endogenously that suppresses BDNF production. Hence it can be targeted using BDNF AntagoNAT to up regulate BDNF expression in neurodegenerative diseases. AntagoNAT’s being single stranded oligonucleotides are negatively charged and hence cannot penetrate the BBB and are susceptible to degradation. Hence, we employed cationic liposomal system to successfully deliver the AT’s to the brain.
The overall hypothesis for my thesis project was that “the novel trans-nasal mucosal drug delivery technique along with a cationic liposomal system will lead to a direct, sustained delivery of the BDNF AntagoNAT’s to the CNS, bypassing the BBB. To test the above hypothesis, six specific aims of the thesis project were:

**Specific aim 1**: *In vivo* qualitative and quantitative evaluation of delivery of Cy5-labeled ovalbumin and unlabeled ovalbumin in saline and cationic liposome-in-gel (LiG) in rat brain using the mucosal engrafting procedure.

**Specific aim 2**: Qualitative and quantitative evaluation of delivery of Cy5-labeled AntagoNAT (AT) and unlabeled active BDNF AntagoNAT (AT) in saline and cationic liposomes in RT4-D6P2T rat schwannoma cells.

**Specific aim 3**: Qualitative and quantitative evaluation of delivery of Cy5-labeled AntagoNAT (AT) and unlabeled active BDNF AntagoNAT (AT) in saline and cationic liposomes in rats using trans-nasal mucosal engrafting technique.

**Specific aim 4a**: Detection and evaluation of BDNF protein levels in different genetic and toxic models of PD.

**Specific aim 4b**: Development of rat PD model using rotenone microspheres and subsequent evaluation of PD biomarkers in the model.

**Specific aim 5a**: Development and characterization of 6-hydroxydopamine induced toxic rat model of PD.
Specific aim 5b: Therapeutic evaluation of neuroprotective effects of BDNF AT liposomes in a rat 6-hydroxydopamine model of PD.

Specific aim 6: Olfactory mucosal depot rat model development as an alternative to heterotopic mucosal engrafting surgery and subsequent evaluation of BDNF AT delivery using a liposome-in-gel formulation
CHAPTER 1: The Challenge of Developing Disease Modifying Biological Treatment for Chronic Neurodegenerative Diseases

1. Chronic Neurodegenerative Diseases

The number of individuals affected by neurodegenerative diseases such as Parkinson’s disease (PD) is expected to increase in the coming years; patients with these diseases represent a major disease burden associated with much human suffering and staggering economic costs required for developing cures[1]. According to the National Institute for Neurological Disorders and Stroke (NINDS), about 50,000 new cases of neurodegeneration are reported in the US each year. In industrialized countries, about 4% of individuals over 60 years of age suffer from Parkinson’s disease. In the US, it is estimated that the number of aged people suffering from neurodegeneration will double by 2030 [1].

The patients suffering from neurodegenerative diseases like Parkinson’s disease and their caregivers experience immense social and economic burden. According to the NINDS, the estimated economic cost of Parkinson’s disease exceeds about 6 billion dollars every year. However, therapeutic strategies available to date remain symptomatic only, and are not disease-modifying [2]. Parkinson’s disease is characterized by substantial loss of dopaminergic neurons in the substantia nigra pars compacta region of the midbrain, and the associated loss of dopamine in the striatum [3]. Patients with Parkinson’s disease may also exhibit Lewy body inclusions, composed of alpha synuclein and ubiquitin in the brain [3]. Hence the therapies for Parkinson’s disease include the restoration of the lost dopamine in the dorsal striatum, which further reduces the presence of lewy bodies. For example, levodopa (a pro-dopamine drug) is a widely used therapeutic for treating Parkinson’s disease – and while levodopa alleviates symptomatic motor defects, the diseases worsens with time, and patients develop severe dyskinesias [4].

Over the last few decades, much research has focused on unraveling the therapeutic targets for treating neurodegenerative diseases and on the development of effective therapies [2]. However, despite the tremendous investment in this field, few drugs have reached the market
[5], primarily on account of the blood brain barrier (BBB) and blood cerebrospinal fluid barrier (BCSFB), which poses an enormous challenge: almost 98% of drug candidates fail to pass the BBB, due to its tight composition [6]. Large protein based therapies including antibodies, growth factors have demonstrated significant potential in treating various neurodegenerative diseases like Parkinson’s disease (PD), Alzheimer’s disease (AD) [7]. A recent study demonstrated the clinical efficacy of adacanumab (Biogen), a monoclonal antibody in patients with AD [8], (PRIME; ClinicalTrials.gov Identifier NCT0247780). However high molecular weight proteins delivered systemically face a number of challenges like rapid serum clearance, proteolytic degradation and BBB as mentioned above [6]. Currently no effective permanent strategies exist that are capable of overcoming the BBB and delivering therapeutics to the neural tissue [6]. There is an urgent need to design novel drug delivery strategies that can bypass the BBB and that have the potential to transform the therapies for treating neurodegenerative disorders.

2. Disease Modifying Biological Therapeutics

2.1 Neurotrophic Factors

Neurotrophic factors also called neurotrophins are a group of growth factors consisting of the Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4 (NT4)[9]. The neurotrophic factors are responsible for growth, maintenance and differentiation of neurons during the development of vertebrate nervous system. Each of the neurotrophins bind to their respective tyrosine kinase receptors to activate survival responses. For example, NGF binds to TrkA, BDNF and NT4 bind to TrkB and NT3 binds to TrkC. Along with cell survival pathways, the neurotrophins can also elicit apoptotic pathways by binding to P75NTR receptors[9].

Due to their critical role in protecting neurons, many neurotrophins are implicated in neurodegenerative disease. Many of these diseases like PD an AD are associated with altered levels of neurotrophins [9]. As most of the literature has explored BDNF for its role in
neurogenerative disease, we have explored BDNF as a therapeutic target for treating PD for my thesis project.

Various neurotrophic factors are currently under study for various neuronal disorders (Table 1.1).

<table>
<thead>
<tr>
<th>Diseases</th>
<th>Neurotrophic Factors</th>
<th>Target neurons</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>NGF and BDNF</td>
<td>Motor neurons</td>
<td>Recruiting for Phase 1 and Phase 2</td>
</tr>
<tr>
<td>Parkinson’s disease</td>
<td>GDNF/neurturin</td>
<td>Striatal neurons</td>
<td>Some phase 1 complete, ongoing in phase 1 and phase 2</td>
</tr>
<tr>
<td>Huntington’s disease</td>
<td>BDNF</td>
<td>Striatal neurons</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Alzheimer’s disease</td>
<td>NGF and BDNF</td>
<td>Cholinergic neurons, entorhinal neurons</td>
<td>Ongoing phase 1</td>
</tr>
<tr>
<td>Down syndrome</td>
<td>NGF</td>
<td>Cholinergic neurons</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Spinal cord injury</td>
<td>BDNF and NT3</td>
<td>Site of injury</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Obesity</td>
<td>BDNF</td>
<td>Hypothalamus</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Lysosomal storage disorders</td>
<td>BDNF</td>
<td>Various in CNS</td>
<td>Pre-clinical</td>
</tr>
<tr>
<td>Sensory neuropathies</td>
<td>NGF</td>
<td>Sensory and sympathetic neurons</td>
<td>Phase 2 completed</td>
</tr>
<tr>
<td>Supranuclear palsy</td>
<td>GDNF</td>
<td>Various in CNS</td>
<td>Phase 2 completed</td>
</tr>
</tbody>
</table>

Table 1.1: An overview of various neurotrophic factors currently under clinical trials for treating various neurodegenerative diseases [9].

2.2 Genetic Therapies

Gene therapies for neurodegenerative diseases involve administration of genes that can potentially alter the expression of several proteins that are responsible for neuroprotection and neurorestoration [10]. The main advantage of gene therapies over proteins is that gene therapy does not require chronic re-administration and genes can alter levels of proteins at endogenous levels. Gene therapies can also overcome several limitations of protein therapy like post
translational modification, limited bioavailability[10]. As genes are highly negatively charged molecules, a carrier system would help in efficient delivery to end target organ. Various carriers like viral vectors and nanoparticle systems can be used to deliver genes to specific target site. Apart from carriers, neurosurgical delivery of genes is a gold standard platform to delivery genes for treating CNS diseases. The neurosurgical delivery systems include convection-enhanced delivery, stereotactic infusion pumps [10].

2.3. Cell Therapies

Cell based therapies for neurodegenerative diseases mainly include the use of stem cells for treating various disorders. The embryonic stem cells are deriving from the inner mass of the blastocyst and they can proliferate indefinitely [11]. This property of stem cells makes them pluripotent and they can differentiate in cells of three germ layers namely ectoderm, mesoderm and endoderm [11]. Even though the embryonic stem cells (ESC’s) are considered highly important for treating various CNS diseases, the isolation of ESC’s raises lot of concerns as they are extracted from human embryos. Also once transplanted in patients, they face a risk of rejection [11].

These limitations have been overcome by using alternative methods for isolating or generating ESC’s [11]. These new methods generate pluripotent ESC’s from differentiated adult somatic tissue. This can be achieved by various methods like nuclear transfer, cell fusion or reprogramming of cells [11]. In 2006, Yamananka group published a paper where they induced the expression of four transcription factors Oct3/4, Sox2, c-Myc and Klf4 (OSKM factors) in mouse fibroblasts which resulted in generation of embryonic stem cells also called as induced pluripotent stem cells (iPSC’s) [12]. These iPSC’s are similar to the embryonic stem cells in various properties like proliferation, differentiation and teratoma formation. As the method of generating these cells is not controversial, the iPSC’s are now being widely explored for various applications like therapeutic screening, disease models [13].
Regarding neurodegenerative diseases, various efforts have been directed towards generating different lineages of neuronal cells from ESC’s and iPSC’s. Using this technology, the differentiated somatic cells from various diseased patients can be used to induce the pluripotent transcription factors in turn generating iPSC’s which can be further differentiated into neuronal cells of interest (e.g. dopaminergic cells for Parkinson’s disease) [11, 13]. As the neurons are differentiated from patient specific iPSC’s, they provide an important tool to model specific phenotypes of various CNS diseases [13]. This has increased the effectiveness of developing human in vitro models which help in overcoming he current discrepancies in various CNS disease in vitro models. This technology has been applied to various CNS diseases like Parkinson’s disease, Alzheimer’s disease and Huntington’s disease to generate disease specific iPSC’s and utilize them to study disease phenotypes and screen various therapeutic molecules [11, 13]. This technology gas also opened doors to genetic modifications of the iPSC’s using CRISPR/cas9 resulting in generation of isogenic mutated cell lined which can be used to study mutation specific pathologies of certain diseases.
Figure 1. 1: Diagrammatic representation of derivation of iPSC’s from differentiated human somatic tissue (fibroblast cells from skin):

The iPSC’s can be generated from somatic cells of a diseased patient carrying certain mutation or abnormality and can be further differentiated into neurons of interest which can serve as a screening model of various therapeutics [11].

3. Role of the Blood-Brain Barrier in Limiting Access of Biological Therapies in the CNS

The BBB is a complex dynamic barrier that is present in all animals with a complex CNS and it creates a unique extracellular fluid environment within the CNS[5]. The BBB protects the brain from invading organisms, bacteria, viruses and toxic substances[14]. The BBB also prevents
the transport of drugs from blood to brain allowing essential molecules to pass through which have molecular weigh less than 500 Daltons[6]. The impermeability of the BBB to most CNS therapeutics is the major reason for why most drugs do not reach the market, and understanding the physiology of the barrier would greatly help in mitigating this problem[14].

The BBB comprises endothelial cells that are connected to each other via tight junctions. The endothelial cells line the blood vessels and are surrounded by other cells, including astrocytes, microglia and pericytes[5]. Together these cells and their junctions form the “Neurovascular Unit”, which blocks permeation of small ions and hydrophilic molecules via the paracellular pathway[15]. The tight junctions that connect the adjacent endothelial cells are composed of various transmembrane proteins such as the occludins, claudins, junctional adhesion molecules, cingulins, and AF-6, which collectively strengthen the tight junctions and prevent the entry of polar molecules through aqueous pores into the brain[16]

Several transporters - GLUT-1, LAT-1, transferrin receptors, and lipoprotein receptors are expressed on endothelial cells which allow the entry of essential nutrients[17]. Several efflux transporters like p-gp, MDR1 also inhibit several therapeutic agents from crossing the BBB[17]. Adsorptive-mediated transcytosis and receptor-mediated transcytosis regulate the transfer of proteins and large molecules based on their charge and receptor specificity, respectively. These barrier systems together comprise the “physical barrier” whereas the “chemical barrier” is composed of extracellular and intracellular enzymes which metabolize various toxic substances in transit to the brain[17]. The movement of leukocytes across the BBB is regulated by the endothelial and immune cells which together comprise of the “immune barrier”[17]. The presence of all the barriers mentioned above prevents therapeutic molecules from passing through the BBB and limits the clinical translation of several therapeutic molecules. This barrier system has driven researchers to resort to new strategies like intranasal and trans nasal delivery for delivering drugs to the CNS
Figure 1.2: Blood-brain barrier structure and transport:
A) Diagrammatic representation of the “Neurovascular Unit”. B) Potential transport routes for permeation and transport across the BBB [17]

4. CNS Drug Delivery Strategies

4.1. Invasive Delivery Strategies

The severity of neurological diseases and the significant limitations in delivering therapeutics to CNS tissues have resulted in extensive research targeted at bypassing the BBB. The major strategies currently being explored for enhancing CNS drug delivery can be broadly categorized as invasive and non-invasive (Figure 1.3). Invasive strategies involve the physical disruption of the BBB, and direct delivery of drug molecules into the CSF and the brain parenchyma. This approach includes use of stereotactically administered intraventricular, intrathecal, intracerebral injections [18]. An advantage of direct injection of molecules into the brain means that the drugs do not need to be modified. Major disadvantages of the invasive technique include the risk of brain trauma and infections [18]. An invasive catheter based system that continuously delivers drugs to the brain through a reservoir, and is programmable, have been extensively tested in clinical trials [19].
4.2 Non-Invasive Delivery Strategies

Non-invasive strategies involve chemical or biological modification of drug molecules, and leverage the various receptors and transporters present on the endothelial cells of the BBB to deliver drugs to the brain parenchyma, thus preventing injury or trauma [20]. The chemical modifications primarily include use of prodrug and lipophilic analogs, which improve solubility and lipophilicity, and enhance BBB permeability. An example is the use of levodopa[20]. Biological approaches also include the conjugation of drug molecules to an antibody or ligand – examples include lectins, or sugars that can directly bind to the receptors and transporters present on the cells of the BBB and that undergo AMT (adsorptive mediated transcytosis) and RMT (receptor mediated transcytosis)[20]. Other approaches use viral vectors like the adenovirus and herpes simplex virus, as well as colloidal drug carriers like liposomes, micelles, and polymeric nanoparticles, to deliver drugs across the BBB [18].

Figure 1. 3: Current strategies for delivering drugs to the central nervous system (CNS) [18].
Intranasal drug administration has also been explored as a non-invasive strategy this method takes advantage of the connections between the nasal mucosa and the brain to bypass the BBB[21]. Biological therapeutics can gain rapid direct access to the CNS along the olfactory nerve pathways. The drug molecules after coming in contact with the nasal mucosal can be transported either through perivascular channels in lamina propria or via extracellular and intracellular mechanisms across the olfactory receptor neurons and trigeminal nerves (Figure 1.4) [21]. The drug molecules can then reach the olfactory bulbs via the axons of the olfactory receptor neurons after passing the cribriform plate [21]. Hence intranasal administration allows direct access into the CNS thereby offering several advantages like patient compliance, reduced systemic exposure and better bioavailability over other invasive strategies available[22]. However intranasal administration also faces certain limitations like variable drug contact with the olfactory mucosa in different patients resulting in variable and poor drug distribution, mucociliary clearance, narrow “internal valve” in the anterior nose resulting in variable drug absorption[23]. Also, the results from rodent models have failed to clinically translate due to deficit of olfactory mucosa in humans relative to rodents. There is also a significant deficit in the understanding of physiology of targets in CNS diseases which has resulted in identification of wrong pharmacological targets [24-27].

Focused ultrasound is also a non-invasive technique that is being studied for its potential to deliver therapeutics across the BBB [28]. While each of these strategies is promising, they require extensive manipulations, are short-lived, and difficult to scale up to meet the needs of the growing population affected by neural disorders.
5. References


CHAPTER 2: Trans-nasal mucosal delivery of Brain-derived neurotrophic factor (BDNF) AntagoNAT’s (AT’s) as a potential therapeutic approach in treating neurodegenerative diseases

1. BDNF as a Promising Therapeutic Agent for Parkinson’s Diseases

Neurotrophic factors, also referred to as neurotrophins are a group of growth factors that have important roles in the development of the vertebrate CNS. The neurotrophin family consists of various growth factors, including Brain Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT3), neurotrophin 4 (NT-4), and Nerve Growth Factor (NGF) [9]. BDNF protects existing neurons, promotes the growth and differentiation of new neurons, and mediates neuronal plasticity. BDNF also exhibits a neuroprotective effect under disease conditions like neurotoxicity, cerebral ischemia, hypoglycemia. BDNF protein and mRNA is present in almost all brain areas including cortex, olfactory bulbs, hippocampus, basal forebrain and spinal cord [30].

BDNF is synthesized as a 32-35 kDa precursor analog protein in the endoplasmic reticulum called as “pro BDNF” (Figure 2.1 A). The precursor analog moves through the golgi apparatus and trans-golgi network [31]. Further the lipid raft associated sorting receptor carboxy peptidase E (CPE) sorts the pro BDNF and transports it into activity dependent secretion by post-synaptic dendrites (Figure 2.1 B) [31, 32]. The pro BDNF is then cleaved by a convertase enzyme to the mature form of BDNF (mBDNF) which has molecular weight of 13-14 kDa (Figure 2.1 A). The pro BDNF essentially interacts with p75NTR receptors and the mature form interacts with tyrosine kinase TrkB receptors[32]. Upon activation of TrkB receptors by mature BDNF, several downstream pathways are activated that regulates cell survival, proliferation and neurogenesis. These pathways mainly include RAS/MAPK/ERK pathway, IRS-1/PI3K/AKT pathway and PLC/DAG/IP3 pathway. Whereas activation of P75NTR receptors by pro BDNF activates several cell apoptosis pathways [31].

Neurodegenerative diseases like Parkinson’s disease and Alzheimer’s disease are associated with low levels of BDNF in the brain [9]. BDNF is required for the normal growth and
functioning of dopaminergic neurons in the Substantia Nigra pars compacta, which is affected in Parkinson’s disease[33]. A recent study has documented that PD patients with altered cognitive functions have altered levels of BDNF in the brain [34]. Another recent study on PD patients showed that serum levels of BDNF are low in PD patients [35]. Given its functions and implications in neurodegenerative diseases like PD and AD, it has been argued that delivery of these factors could restore brain functions and thus help in treating neurodegeneration. However delivery of these growth factors still remains a challenge due to their high molecular weight, polar nature and presence of BBB [9]. Hence this has impeded the clinical translation of neurotrophin delivery to the brain. Hence there a need for an alternative drug delivery strategy that could effectively delivery these growth factors to the brain.

Figure 2. 1: BDNF production from its precursor and its transport:

(A) The human BDNF gene forms the preproBDNF which is then converted to proBDNF (32 kDa). The proBDNF is then cleaved by proteases into mature form of BDNF (13-14 kDa) which binds to TrkB receptors and pro-peptide form (17 kDa) which binds to P75 receptors [30]. B) The
proBDNF passes through the endoplasmic reticulum and golgi apparatus after which the carboxy peptidase E (CPE) sorts it into activity dependent secretion [31].

1.2 Challenges in Clinical Translation of BDNF Delivery for Treating Neurodegenerative Diseases

Biopharmaceutical drugs like recombinant proteins have the advantage of high specificity and potency as compared to small molecules [36]. The structural complexity of these macromolecules is responsible for making them specific for a target. Owing to their complex structure, biopharmaceutical drugs like recombinant proteins (BDNF) are highly flexible and are sensitive to environmental conditions like moisture, temperature, pH, surface interaction and contaminants [36]. Any conformational change in the protein structure could affect its therapeutic activity. Being of biological origin, these macromolecules can be easily contaminated with other biological impurities like viruses and can undergo translational changes during the production or after the final product has been formulated. The translational and structural changes in the recombinant proteins can be responsible for immunogenic responses, which can lead to serious safety issues and loss of therapeutic activity [36]. This makes them one of the most challenging macromolecules to formulate and deliver. The high molecular weights of these molecules (for example: BDNF- 14 kDa) makes them impermeable across biological barriers like the skin, mucosal membranes, blood brain barrier, cell membranes[37]. Further production of recombinant proteins faces several challenges of loss of expression of gene of interest and post translational processing.

The BDNF gene has a highly complex structure consisting of 9 functional promoters, various transcripts, pre protein processing and a regulatory natural antisense transcript (NAT) called BDNF-AS [38]. The promoter and various splicing mechanisms give rise to several BDNF transcripts making it difficult for recombinant protein manufacturing that can exactly mimic the
endogenous protein [38]. Hence this makes it challenging to develop a recombinant BDNF therapy.

2. Therapies Targeting Natural Anti-sense Transcripts (NAT’S) as a Potential Therapy for Treating Parkinson’s Disease

Historically most of the research has been targeted towards developing inhibitors that can down regulate a particular target rather than exploring agents that could up regulate the expression of a particular target. For many targets like the growth factors and transcription factors, up regulation of gene would be important as up regulation can help in alleviating certain disorders [39]. For example, in neurodegenerative diseases, up regulation of certain genes like BDNF, GDNF could help in alleviating these disorders [40]. Several strategies are available that can up regulate gene expression like enzyme replacement therapy, gene therapy, artificial transcription factors, DNA modulators and so on. However all these strategies have their own limitations, for example enzyme replacement therapy requires long term administration and is not curative and gives rise to immunogenic response. On the other hand gene therapy can also lead to an immune responses and viral vector associated toxicities [39]. Hence there is a need for novel approaches that could effectively up regulate the target of interest with less adverse effects. A recent development in this field has been targeting the long non-coding RNA’s (IncRNA’s) for up regulation of certain genes of interest [39].

The IncRNA’s are present in human genome and help in regulating gene expression. Work by the International FANTOM consortium has revealed that about 70 % genome is transcribed out of which only 20 % is actually translated giving rise to a large amount of non-coding RNA transcripts (IncRNA’s) [39]. One of the classes of these IncRNA’s is the natural antisense transcript (NAT). A NAT is mainly transcribed in the opposite direction and often overlaps the sense RNA. After transcription by RNA polymerase, both the sense and the antisense mRNA form duplexes which inhibits sense mRNA transcription and protein translation.
The NAT for BDNF is called BDNF-AS and it represses the expression of sense mRNA through histone modifications (PCR2 AND LSD1) which in turn down regulates BDNF expression [39]. Hence inhibition of these NAT’s can in turn lead to up regulation of gene (transcriptional de-repression) and protein expression. This can be achieved by designing single stranded oligonucleotides that can specifically block the interaction of these NAT’s with the sense mRNA’s or degrade the NAT’s. These specific inhibitors of the NAT’s are called as AntagoNAT’s (AT’s) [41].

Figure 2.2: Sense RNA - antisense RNA duplex formation:

The antisense RNA transcription takes place in a direction opposite to the sense mRNA transcription resulting in exactly complementary sequences for sense and antisense mRNA transcripts. Hence the antisense mRNA transcript forms a duplex with sense mRNA thereby inhibiting the mRNA transcription and protein translation [42].

A recent study published in Nature in 2012 showed that the BDNF AT could up regulate the RNA as well as protein levels of BNDF in various regions of the brain following 4 weeks of administration. The up regulation of BDNF showed an increase in neuronal growth and survival [43]. Hence based on the fact the antagoNAT’s are widely expressed throughout the human genome and that their inhibition using specific antagoNAT’s can up regulate gene and protein expression, AT based therapies could be applied for treatment of various neurodegenerative disorders like Parkinson’s disease [43]. The AT’s work by either forming a duplex with NAT thereby inhibiting its activity or by degrading the NAT by RNase H activity (Figure 2.3).
Figure 2. 3: Mechanism of action of AntagoNAT’s (AT’s):

A natural antisense transcript (NAT) can inhibit the sense mRNA transcription by histone modification. However an AntagoNAT (AT) can inhibit NAT by forming a duplex with it as they
have complementary sequences or by RNAse H activity through which it can degrade the NAT [39].

2.1 Advantages and Challenges for Developing AntagoNAT Based Therapies

Up regulation of certain genes and proteins by targeting NAT’s can help in up regulating endogenous protein in a natural environment. Also the antagoNAT’s are locus specific and can up regulate specific genes thereby reducing off target toxicity [41]. Also, another advantage is that the less abundance of these NAT’s in the genome would require a lower dose of AT’s to inhibit them.

One of the disadvantages of AT’s is that inhibiting NAT may give rise to certain compensatory mechanisms which could overcome the effects of antagoNAT’s. AT’s can also hybridize on or off target, which can lead to off target toxicity [39]. Also, effective strategies to deliver the AT’s remains to be a major hurdle in clinical translation of this approach. AT’s being highly negatively charged are impermeable to biological membranes like the blood brain barrier and are highly susceptible to degradation by DNase and RNase. Several delivery strategies like intracerebroventricular, intrathecical and intracranial delivery have been explored for direct delivery of AT’s to the CNS, however these strategies are highly invasive and can damage the surrounding brain tissue [39]. Hence there is a need for an alternative strategy for effective delivery of the antagoNAT’s to the brain for treating neurodegenerative disorders like Parkinson’s disease.

3. Trans-Nasal Mucosal Drug Delivery Strategy for Delivering BDNF AT’s to the Brain

3.1 Catheter Based Systems for Delivering Drugs to the Brain

The commonly used method for administering these proteins into the brain is by directly injecting them into CNS tissue; however, this is an invasive procedure and the protein distribution is limited by the rate of diffusion. Intrathecal and intraventricular injections are less invasive and
can reach a wide area as compared to intraparenchymal drug delivery [44]. Intraventricular administration of BDNF has been studied in rats and monkeys, but is limited by the ependymal lining of the ventricles [45]. Drug delivery to the brain through these routes can be better achieved with use of catheters to chronically administer the neurotrophins over time; however, this approach is greatly influenced by the placement and stability of the catheter[46]. Also, some disease conditions may affect catheter placement and 4 to 9 % rates of infection have been reported in patients with brain catheters. It has also been reported that intrathecal catheter placement for administering BDNF affected the therapeutic delivery, suggesting that although the intrathecal and intraventricular routes each offers the advantage of direct injection of growth factors to the brain, challenges related to brain trauma and catheter infections and stability have not yet been resolved. These findings highlight a critical need for developing drug delivery strategies that minimize brain traumas and achieve chronic, direct delivery of growth factors and other proteins to the brain [46].

3.2 Advantages and Disadvantages of Trans-nasal Drug Delivery to the Brain

The previously described limitations for direct application of drugs and proteins to the brain have driven research to explore new pathways for delivering therapeutics to the brain. The trans-nasal pathway is one such pathway, which has the advantage of direct delivery to brain tissue, bypassing the blood brain barrier (BBB) along the olfactory nerves, avoiding first pass metabolism in systemic circulation, and thereby leading to fewer side effects and better therapeutic effects. The highly vascularized surface area of the nasal mucosa is responsible for better drug absorption, and quick onset of pharmacological activity [47]. However, the pathway also has some disadvantages related to nasal mucociliary clearance, and drug absorption from the nasal mucosa. The rate of absorption of drugs from the nasal mucosa depends on the molecular weight and partition coefficient of the drug; also, the high protease and peptidase activity in nasal mucosa rapidly degrades proteins and growth factors, thus limiting the amount of these drugs that can be
presented to the brain tissue itself [47]. We plan to overcome these disadvantages of nasal delivery by developing a new trans-nasal drug delivery approach to overcome these obstacles. An improved alternative trans-nasal drug delivery approach will likely be of great use in transforming current treatments for neurological disorders, and also in opening doors to innovative techniques for bypassing the BBB.

3.3 Minimally Invasive Endoscopic Skull-base Surgical Procotol

Endoscopic skull-base surgery is an innovative procedure that is used to remove brain lesions and tumors – the technique employs an endoscope to reach the base of the skull base through the nose and sinuses [48, 49] (Figure 2.4 A). Access to tumors and lesions in the brain requires removal of intervening non-neural tissue to reach the relevant neural tissue - this tissue includes the nasal mucosa, dura, arachnoid membrane and bone. Removal of this intervening tissue creates a large window at the base of the skull that allows direct communication between CSF space, brain, and the nasal cavity [48]. Once the surgery is completed, this window must be reconstructed (Figure 2.4 B and 2.4 C). Over the past years, the tissue opening has been reconstructed with use of nasal mucosal grafts, which are water-tight and immunocompetent, and which separate the nasal compartment from the brain tissue. For our purposes, nasal mucosal grafts are almost 1000 times more permeable than the BBB, and can be utilized for the delivery of high and low molecular weight proteins to the CNS [48].

Human nasal mucosa normally forms a barrier between the external environment and body tissues, and often comes in contact with the vast amount of external secretions and microbes[50]. The nasal mucosa consists of a single layer of epithelial cells, which are covered by mucus and antimicrobial products, and has its own adaptive and innate immune systems. These defense mechanisms of the nasal mucosa confer safety on the skull-base procedures and block postsurgical infections. Also, as the surgery does not involve any external incisions, the patients can
recover within less than 24 hours with less long-term side effects. As this surgical technique has proven to be safe, it is performed throughout the world using endoscopic instruments [51].

We propose to use an innovative CNS drug delivery strategy that utilizes heterotopic mucosal grafts based on the endoscopic skull-base reconstruction to bypass the BBB and delivery therapeutic proteins and drugs directly to the brain [50].

Figure 2. 4: Diagrammatic representation of human endoscopic skull base surgery and its transition to an extra cranial rodent graft model:

A) Diagrammatic representation of human endoscopic skull base surgery where an endoscope is inserted through the nasal cavity for removing tumors by getting rid of the underlying dura and bone creating a cranial window. B) Normal skull base anatomy showing an intact BBB, which prevents the diffusion of the liposome-in-gel formulation. The mucosal graft (in red) implanted in the sphenoid sinus thereby creating a semipermeable pathway into the brain. Graft-brain interface enabling free diffusion of the formulation into the brain across the mucosal graft [52] C) An overview of the human olfactory mucosa with the location of sphenoid sinus.
3.4 Development of an Extra-cranial Rodent Graft Model

In order to leverage the inherent advantages of trans-nasal delivery while overcoming its limitations, our group has developed a technique to create a semi-permeable mucosal conduit to the CNS in a rodent model using established endoscopic skull base surgical techniques (Figure 2.5). The rodent model was developed by creating a cranial window (craniotomy) using a surgical incision on top of the rat head. The craniotomy was performed on rat head instead of nose due to the small surface area available in rodents [23]. The rodent model was developed by creating a cranial window by removing the underlying dura and bone and hence exposing the underlying brain tissue similar to human endoscopic skull base surgeries [23]. The cranial window was then repaired using an isolated mucosal graft from a donor rat. No graft rejection was observed as the donor graft was from the same species of rats. A 200 µl sterile propylene reservoir was placed over the implanted graft to deliver the target therapeutic molecule. The main objective of the developed rodent model was to mimic the human endoscopic skull base surgeries and further study the trans nasal drug delivery through the implanted nasal graft into the brain bypassing the BBB[23].
4.Liposomes as Drug Carriers for CNS Delivery

Biological therapeutics such as growth factors, oligonucleotides are highly susceptible to degradation by proteases, DNase's and Ranse's; hence CNS delivery of such drugs requires a system to protect the growth factors from such degradation. Various colloidal carriers, including polymeric nanoparticles, micelles, and liposomes, have been explored as vehicles for the delivery of various therapeutics like proteins, biologics and nucleic acids.

Liposomes are colloidal carriers that can encapsulate proteins and nucleic acids of interest [53]. They are highly lipophilic nanoparticles that protect the payload from degradation and can be engineered to provide a sustained release [53]. Better delivery through the nasal mucosa is achieved with use of a Pluronic F-127 gel-based system, which reduces the mucociliary clearance of the drug by adhering to the mucus and thus supporting sustained release [54]. We have utilized a liposome-in-gel system for delivering protein and a cationic liposomal system for oligonucleotide therapeutics to the brain via the heterotopic mucosal grafting technique. The gel being viscous at
room temperature will allow a sustained and slow delivery of the cargo (BDNF protein and AntagoNAT) into the brain. This will also enable repeated dosing as needed enabling controlled drug delivery (Figure 2.6).

The liposomes will help in protecting the payload from degradation, which will increase the uptake and distribution of the therapeutic in the brain. As the AT’s are highly negative in charge, a cationic liposome will result in a better encapsulation and delivery to the brain.

![Pluronic gel at 4°C](image1) ![Pluronic gel at room temperature](image2)

Figure 2.6: Phase transition property of pluronic F-127 gel:

The pluronic gel is liquid at 4°C and forms a gel at room temperature making it easy to deliver.

5. References


CHAPTER 3: Direct CNS delivery of ovalbumin using thermosensitive Liposome-in-gel (LiG) carrier by heterotopic mucosal engrafting

1. Introduction

Based on human endoscopic skull-based surgeries explained above, we developed an extra cranial rodent graft model (as explained in Chapter 2). Ovalbumin was used as a model protein for the study. Ovalbumin is a high molecular weight protein (45 kDa), which would serve to be a good candidate to study the uptake of high molecular weight therapeutics in the brain using the surgical model. Cy5-labeled ovalbumin was used for qualitative uptake to look at the distribution of the protein in different parts of rat brain using microscopy. The total unlabeled ovalbumin delivered to the brain was quantified using an ELISA.

For a successful clinical adoption of drug delivery systems, a carrier platform that can protect the cargo from proteolytic degradation and prolong mucosal residence time is required. Hence, we utilized liposomal system for this study as liposomes are lipophilic carriers and can protect the cargo and increase the uptake. In this study we evaluated both the anionic and cationic liposomes encapsulating ovalbumin and selected the one that resulted in the maximum uptake in rat brain. We also utilized a thermosensitive liposome-in-gel (LiG) system to improve the residence time and improve transmucosal diffusion.

The main objective of this pilot study was to test the feasibility of a LiG system to deliver high molecular weight therapeutics like ovalbumin to the brain using our innovative rodent extra cranial mucosal graft model.

2. Materials and Methods

2.1 Study design

We used an innovative heterotopic mucosal engrafting technique for delivering a representative high molecular weight protein (e.g., ovalbumin, 45 kDa) to the rat brain in male Sprague-Dawley rats (Figure 3.1). After engraftment (described under surgical methods), the
mucosa was initially exposed to Cy5-labeled ovalbumin and ovalbumin in saline. The uptake of the Cy5-labeled ovalbumin in solution was quantitatively determined after 72 hours using an ICYTE imaging cytometer (CompuCyte Corp., Cambridge, MA). The uptake of ovalbumin in solution in the rat brain was then determined after 48 and 72 hours quantitatively using commercially available ovalbumin specific (Biomatik) Elisa kit. A thermosensitive LiG formulation was then designed and formulated to provide sustained delivery to the rat brain over 72 hours. The uptake of Cy5-labeled ovalbumin LiG and ovalbumin LiG was determined after 72 hours using similar techniques as mentioned above.

2.2 Surgical Methods

Ethics Statement

All the animal procedures were approved by the Northeastern University Institutional Animal Care and Use Committee (Protocol number: 180101-R) that was approved in January 2018. For mucosal permeability studies, de-identified human septal mucosal tissue samples were obtained from Mass Eye and Ear Infirmary (MEEI). An Investigational Review Board (IRB) at MEEI has approved the protocol for harvesting the tissue samples in May 2018. Northeastern University IRB has also approved protocol number 16-06-03 to assess mucosal permeability with the de-identified tissue obtained from MEEI in May 2018.

Donor graft

The Sprague-Dawley rats used in the study were ordered from Charles River, Kingston, MA, USA. All surgeries were performed under isoflurane anesthesia and all efforts were made to minimize suffering. All the rats were housed in a climate-controlled room on a 12/12-hour light/dark cycle and were provided with food and water ad libitum. All the surgeries were performed during the rat’s light cycle. For the heterotopic mucosal engrafting procedure, a donor rat was used to harvest the nasal septum according to the methods previously described in [1-3]. Briefly, a donor rat was euthanized by carbon dioxide and surgical scissors were used to remove
the skin from the nasal dorsum. A surgical drill and scissors were used to remove and isolate a unilateral septal mucoperichondrial graft, which was stored in saline solution for a maximum of two hours.

**Graft implantation**

The experimental rat (250-300 grams) was anesthetized using isoflurane placed in a stereotaxic frame under an operating microscope. The surgical site was sterilized using povidone iodine and alcohol swabs. A sagittal incision was made from the level of midorbit to the occiput using a scalpel. Bilateral skin flaps were then elevated exposing the pericranium. A scalpel was used to clear the pericranium from the intended craniotomy site (1.5 mm anterior-posterior and 2 mm medial-lateral to bregma). A surgical drill was used to create a 3 mm craniotomy leaving the underlying dura intact (Figure 3.1 A). The underlying dura and arachnoid were then removed leaving the underlying pia matter undisrupted. The harvested graft from the donor rat was then implanted over the craniotomy such that the basolateral membrane of the graft faced the exposed pia matter. A piece of sterile nitrile was placed over the graft to prevent adhesion to surrounding tissue. The skin flaps were the sutured back and the rat was left to engraft for 3 days. After 3 days the skin flaps were again reflected carefully without disrupting the underlying implanted mucosal graft. The graft was then inspected using a dissection microscope to ensure viability and circumferential engraftment (Figure 3.1 B).

**Reservoir placement**

A 250 µl polypropylene reservoir was placed over the mucosal graft such that the reservoir had good contact with the surrounding skull. The reservoir was attached to the skull using cyanoacrylate and tested for leaks using sterile saline. After implantation of a screw (Morris Precision screws and parts – 000x 3/32 Flat self-tap screws), dental cement from Stoelting was applied to the skull to fix the reservoir (Figure 3.1 C).
Figure 3. 1: Rat model of heterotrophic mucosal engrafting technique:

(A) A 3mm craniotomy was outlined using a surgical drill at bregma. (B) The healed implanted mucosal graft 3 days after the engrafting. (C) A propylene reservoir placed and secured over the graft for efficient drug delivery [4].

2.3 Cy5-Labeling of Ovalbumin

Chicken egg ovalbumin (Sigma Aldrich) and sulfo Cy5-NHS ester dye (Lumiprobe) was used for labeling ovalbumin with Cy5. The ratio of ovalbumin and Cy5 required for labeling was calculated according to the manufacturer's protocol. Briefly, ovalbumin and Cy5 were dissolved in saline and stirred overnight in the dark. After 24 hours, the ovalbumin Cy5 conjugate solution was dialyzed (12000-14000 Da dialysis membrane) for 18 hours in 1X PBS followed by overnight dialysis in deionized water to remove unconjugated protein and dye. The conjugate solution was freeze-dried overnight and utilized for dosing animals.

2.4 Dosing of Unlabeled and Labeled Ovalbumin in Rat Brain

For Cy5-labeled ovalbumin dosing 6 μg dissolved in 60 μl saline (n=1) was placed in the reservoir. The rats were sacrificed after 72 hours. For ovalbumin dosing alone, 50 μg of ovalbumin in 50 μl of saline (n=2) was placed in the reservoir. The rats dosed were sacrificed after 48 and 72 hours. Among the Cy5-labeled ovalbumin group, brains were flash frozen and placed in OCT solution at -80°C. The brain was then sliced into 50-micron sections using a cryotome and
observed using an ICYTE imaging cytometer (CompuCyte Corp., Cambridge, MA) for quantitative uptake of Cy5-labeled ovalbumin. Among the ovalbumin group, brains were divided into 4 equal parts using a rat brain coronal precision brain slicer (Braintree Scientific, Braintree, MA) starting from anterior to the posterior part of the brain. Each part was weighed and homogenized in tissue lysis buffer (50 mM Tris-HCl, pH 7.5, 50 nM NaCl and 0.5 % v/v IGEPAL CA-640) containing the EDTA-free Protease inhibitor cocktail tablet (Sigma-Aldrich, St. Louis, MO). Homogenate from each section was centrifuged at 14,000 g for 30 minutes and the supernatant was collected, and the ovalbumin uptake was quantified by enzyme linked immunosorbent assay (ELISA, Biomatik, Wilmington, DE).

2.5 Preparation of Cationic and Anionic Liposomes

The cationic lipid film was prepared using DOTAP (1,2-dioleoyl-3-trimethylammonium-propane chloride salt) a cationic lipid, cholesterol (stabilizer), and DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) a neutral lipid in a 5:3:5 molar ratio. For the lipid film, a stock concentration of each lipid was made in chloroform and 1ml of each lipid was added to a Sigma-Aldrich ST/NS14/20 10 ml round-bottom flask attached to a Rotavap (IKA works Inc. Wilmington, NC-28405, Model RV 10 C S99), and allowed to rotate at 100 rpm in a water bath at room temperature (RT). An anionic lipid film was formed using 2.37 mg DPPC (1,2-dipalmitoyl-sn-glycero-3-phosphocholine) a neutral lipid, 2.35 mg cholesterol (stabilizer) and 0.9 mg 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N [amino (polyethylene glycol)-2000] ammonium salt (DSPE-PEG 2000) dissolved in 5 ml chloroform in a Sigma-Aldrich ST/NS14/20 10 ml round-bottom flask attached to a Rotavap, and allowed to rotate at 100 rpm in a water bath at room temperature. Following chloroform evaporation, the thin lipid film at the bottom of the flask was dried overnight in vacuum to remove the solvent, and the film (cationic and anionic) was subsequently hydrated with 250 ug/ml of Cy5-labeled ovalbumin and ovalbumin solution in saline as starting concentration, followed by additional vortexing for 1 minute. After hydration of the lipid
film, the liposomal preparation was placed on ice for two minutes, vortexed, placed in a water bath at 37 degrees for two minutes, and vortexed again. Five such freeze-thaw cycles were performed.

The liposomal preparation was then probe-sonicated for five minutes on ice. The mixture was ultracentrifuged in a Beckman-Coulter ultracentrifuge at 100,000 rpm for 1 hour to separate the protein-encapsulated liposomes from unencapsulated protein. The pelleted liposomes were suspended in saline to get a liposomal solution, extruded through 800nm, 400nm, and 200 nm membranes using an extruder (Avanti Lipids, Alabaster, AL) and the supernatant was used for further analysis. Encapsulation efficiency was determined with the use of an indirect method. In the indirect method, the amount of protein in the supernatant obtained after ultracentrifugation was measured using the Pierce BCA assay kit (Thermo Fisher Scientific) for ovalbumin and was subtracted from the starting amount of the protein to get the total protein encapsulated in liposomes. Extruded liposomes were characterized for hydrodynamic diameter, polydispersity index (PDI), and surface charge (zeta potential) using a Zetasizer (Nano-ZS90, Malvern Instruments, Inc Westborough MA). Liposomal morphology was determined by the transmission electron microscopy (TEM).

2.6 Preparation of Cy5-Labeled Ovalbumin and Ovalbumin in LiG Formulation

Pluronic F-127 (BASF Corp., Florham Park, NJ) was used to prepare a thermosensitive LiG system. The Pluronic F-127 gel remains liquid at 4°C but turns into a viscous gel at RT. Hence the entire procedure was performed at 4°C. A 30% (w/v) Pluronic F-127 aqueous solution was prepared by slowly adding 3 grams of Pluronic F-127 in 10 ml of saline while stirring continuously at 4°C until completely dissolved. The Pluronic F-127 solution was left at 4°C to remove air bubbles and the Cy5-labeled ovalbumin encapsulated in anionic or cationic liposomes as well as unlabeled ovalbumin encapsulated in cationic liposomes were added to the solution at 4°C and stirred for 20 minutes to form a homogenous LiG system. The final concentration of either Cy5-
labeled ovalbumin or unlabeled ovalbumin in the LiG was based on the dosing required for the animal experiments.

2.7 Cy5-Labeled Ovalbumin and Ovalbumin in LiG dosing in Rat Brain

For Cy5-labeled ovalbumin anionic and cationic LiG dosing, the liposomes were suspended in 30% (w/v) Pluronic F-127 such that 60 µl cationic and anionic LiG dose contained 6 µg of Cy5-labeled ovalbumin. Cy5-labeled ovalbumin in saline (6 µg in 60 µl saline) and Cy5-labeled ovalbumin in Pluronic F-127 gel (6 µg in 60 µl of 30% Pluronic F-127 gel) were used as controls. For ovalbumin cationic LiG dosing, cationic liposomes were suspended in 30% Pluronic F-127 gel such that 170 µl cationic LiG dose contained 50 µg of ovalbumin (n=2). The rat brains were further processed for qualitative and quantitative uptake as mentioned in section 2.3 above.

2.8 In Vitro Permeability of Ovalbumin in LiG Across Human Nasal Mucosal Tissue

De-identified tissue samples were harvested from patients undergoing endoscopic sinonasal surgery. The mucosa was mounted in a self-contained Ussing’s chamber (Warner Instruments, LLC Hamden, CT 06514, Model U2500) and incubated in transport medium (375 ml HBSS (Hank’s balanced salt solution), 892.5 mg HEPES 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 675 mg D (+) glucose 10 mM) for 10 minutes at 37°C. After equilibrating in the transport medium, the chamber facing the apical side of the mucosa was emptied and was filled with 250 µg of Cy5-labeled ovalbumin in 3 ml 1X PBS or 250 µg of Cy5-labeled ovalbumin liposomes suspended in 1 ml 1X PBS and 2 ml of 30% Pluronic F-127 gel and the basolateral side was filled with 3 ml of transport medium. The nasal mucosa was then incubated for 3 hours with the formulations with a continuous supply of carbogen. After 1, 1.5, 2, 2.5 and 3 hours a 200 µl sample was aliquotted from the basolateral side. Fluorescence was measured using a Biotek plate reader. The apparent permeability coefficient was calculated using the equation

\[ P_{\text{app}} = \frac{(dQ/dt)}{(C_0 \times A)} \]
where \( \frac{dQ}{dt} \) is the transport rate and is defined by the slope obtained from linear regression of the amount (nanograms) transported on the basolateral side. \( C_0 \) is the initial concentration of Cy5-labeled ovalbumin in saline and LiG on the apical side and \( A \) is surface area of the tissue.

3. Results and Discussion

3.1 *In Vivo* Distribution of Cy5-Labeled Unlabeled and Labeled Ovalbumin in Saline

To determine the impact of trans-nasal mucosal delivery on the uptake of high molecular weight proteins, we developed a rat model to mimic the human skull base surgery as described in materials and methods section 2.2. The mucosal engrafting surgery was well tolerated in rats and there was no evidence of infections. Mason’s Trichome staining showed that the implanted grafts were intact, and the craniotomy was completely covered with the graft after day 3 and day 7 (Figure 3.2).

![Figure 3.2](image)

**Figure 3.2:** The intact mucosal graft stained with Mason’s Trichome depicting intact graft with no sign of infections at:

(A) Day 3 and (B) Day 7.

Based on the imaging cytometry and semi-quantified data, the sections from rat brain treated with Cy5-labeled ovalbumin in saline showed high Cy5 fluorescence intensity as compared to untreated rats. (**Figure 3.3 A and 3.3 B**).
Figure 3.3: Measurement of Qualitative uptake of Cy5-labeled ovalbumin in saline in rat brain using microscopy:

The uptake of Cy5-labeled ovalbumin in saline as compared to untreated rats (A) imaging data from ICYTE B) Imaging cytometry quantified data from the four selected regions for untreated and Cy5-labeled ovalbumin in saline treated rats (n=1) using equation total Cy5 intensity = area of Cy5 in selected region in each section * total Cy5 intensity in each selected region in each section

The total uptake of ovalbumin in rat brain was determined by dividing the rat brain into four equal parts using a precision brain slicer (Braintree scientific) (Figure 3.4 A). This was done to determine the uptake of ovalbumin in four different parts of the brain based on their distance from the site of delivery (craniotomy) with part 2 being exactly below the site of delivery and part 4 being farthest from the site of delivery. The results in (Figure 3.4 B) shows that there was a significant uptake of ovalbumin in part 3 for ovalbumin in saline 48 hours as compared to untreated rats confirming maximum uptake near the site of the craniotomy. The results in (Figure 3.4 C) showed that the total ovalbumin uptake (four parts combined) was significantly more at 48 hours as compared to ovalbumin in saline 72 hours and untreated rats. It can be concluded that at longer
time points, such as, by 72 hours the uptake of ovalbumin in the rat brain decreases when compared with 48 hours. This could be attributed to the faster diffusion pattern of the saline formulation when instilled in the reservoir directly above the craniotomy.

Figure 3. 4. Quantitative uptake of ovalbumin in saline in rat brain using mucosal engrafting technique:

(A) Rat brain cut into 4 equal parts for ELISA with part 3 being the craniotomy site. (B) Ovalbumin detected by ELISA for treatment groups: Untreated (n=2), ovalbumin saline 48 hours (n=2), ovalbumin saline 72 hours (n=2) in the 4 isolated parts of the brain. Significant ovalbumin uptake was only found in part 3 where Ovalbumin in saline 48 hours showed significantly greater uptake as compared to untreated rats and ovalbumin in saline 72 hours (** p<0.01; one-way anova with post-hoc tukey’s test). (C) Total ovalbumin (four parts combined) detected by ELISA for treatment groups: Untreated ovalbumin saline 48 hours, ovalbumin saline 72 hours. Ovalbumin in saline (48 hours) showed significantly greater uptake as compared to untreated (**p<0.01, ***p<0.001; one-way anova with post-hoc tukey’s test). Data are represented as mean ± SD (n=2).
3.2 *In Vivo* Distribution of Cy5-Labeled in Anionic and Cationic Liposomes-Containing LiG Formulations

The formulated Cy5-labeled ovalbumin cationic and anionic liposomes and unlabeled ovalbumin cationic liposomes were characterized for their size, polydispersity index, surface charge, and TEM was used for determining the structure of liposomes (*Table 3.1, Figure 3.5 A, Figure 3.5 B, Figure 3.5 C*). A negative stain (uranyl acetate) was used to stain the liposomes.

<table>
<thead>
<tr>
<th>Liposome Sample</th>
<th>Average Size (nm)</th>
<th>Polydispersity index (PDI)</th>
<th>Average charge (mV)</th>
<th>% Encapsulation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anionic Cy5-labeled ovalbumin liposomes</td>
<td>148.9 ± 1.3</td>
<td>0.2 ± 0.08</td>
<td>-1.7 ± 0.01</td>
<td>73.7 ± 13.7</td>
</tr>
<tr>
<td>Cationic Cy5-labeled ovalbumin liposomes</td>
<td>181.8 ± 12.2</td>
<td>0.3 ± 0.05</td>
<td>+30.3 ± 1.2</td>
<td>97 ± 0.2</td>
</tr>
<tr>
<td>Cationic unlabeled ovalbumin liposomes</td>
<td>221.6 ± 22.8</td>
<td>0.2 ± 0.3</td>
<td>+22.7 ± 1.5</td>
<td>85 ± 0.2</td>
</tr>
</tbody>
</table>

*Table 3.1: Characterization data (average size, PDI, surface charge and percent encapsulation efficiency) for anionic Cy5-labeled ovalbumin liposomes, cationic Cy5-labeled ovalbumin and cationic unlabeled ovalbumin liposomes (n=3).*

![Anionic Cy5-labeled ovalbumin liposomes](image1.png) ![Cationic Cy5-labeled ovalbumin liposomes](image2.png) ![Cationic ovalbumin liposomes](image3.png)

*Figure 3.5: Transmission electron microscopy images of formulated liposomes: (A) Anionic Cy5-labeled ovalbumin liposomes (B) cationic Cy5-labeled ovalbumin liposomes (C) and cationic unlabeled ovalbumin liposomes.*

After hardening of the dental cement, the rats were dosed with LiG formulations as mentioned in section 2.6. All the animals were sacrificed after 72 hours and the brains were
processed as mentioned in section 2.3. From Figure 3.6 A and 3.6 B it was observed that Cy5-labeled cationic LiG had the maximum uptake as compared to the anionic LiG and the other control groups. Hence, it was decided to perform further experiments with cationic LiG only.

Figure 3.6: Measurement of Qualitative uptake of Cy5-labeled ovalbumin anionic and cationic LiG in rat brain using microscopy:

A) Imaging cytometry data for ovalbumin in anionic and cationic LiG as compared to controls. B) ICYTE quantified data from the four selected regions for untreated, Cy5-labeled ovalbumin in Pluronic F-127 gel, Cy5-labeled ovalbumin anionic LiG and Cy5-labeled ovalbumin cationic LiG (n=1) using equation total Cy5 intensity = area of Cy5 in selected region in each section * total Cy5 intensity in each selected region in each section
Rats were dosed with cationic ovalbumin LiG as mentioned in section 2.6. The animals in each group were sacrificed after 48 and 72 hours and the uptake were determined using protein specific ELISA. From Figure 3.7 A it can be seen that ovalbumin LiG showed significantly better uptake in parts 2 and 4 as compared to untreated rats again confirming maximum uptake near the site of craniotomy. From Figure 3.7 B it can be seen that there was a significant uptake of ovalbumin cationic LiG in rat brain at 48 as compared to 72 hours which can again be attributed to the fast diffusion pattern of the formulation from the reservoir. However, from Figure 3.7 C ovalbumin LiG resulted in a significant uptake in rat brain at 72 hours as compared to untreated and ovalbumin in saline at 72 hours. Hence it can be concluded that the cationic LiG formulation helps in a time-dependent controlled release as the amount of ovalbumin detected in rat brain at 72 hours for LiG formulation was significantly more than the saline formulation and untreated rats.

Figure 3. 7. Quantitative uptake of ovalbumin cationic LiG in rat brain using mucosal engrafting technique:
(A) Ovalbumin detected by ELISA for treatment groups: Untreated (n=2), ovalbumin LiG 48 hours (n=2), ovalbumin LiG 72 hours (n=2) in the 4 isolated parts of the brain. Ovalbumin LiG 48 hours shows significant uptake in part 2 as compared to untreated (* p<0.05). Ovalbumin LiG 48 hours also shows significant uptake in part 4 as compared to untreated and ovalbumin LiG 72 hours (**) p<0.01; one-way anova with post-hoc tukey’s test). (B) Total Ovalbumin detected (four parts combined) by ELISA for treatment groups: Untreated (n=2), ovalbumin LiG 48 hours (n=2), ovalbumin LiG 72 hours (n=2). Ovalbumin LiG (48 hours) shows significant uptake as compared to untreated and ovalbumin LiG (72 hours) (**) p<0.01; one-way anova with post-hoc tukey’s test). (C) Ovalbumin LiG (72 hours) shows significant uptake as compared to untreated (**) p<0.01) and ovalbumin in saline (72 hours) (* p<0.05 one-way anova with post-hoc tukey’s test). Data are represented as mean ± SD (n=2).

### 3.3 Permeability of Cy5-Labeled Ovalbumin in LiG Through Human Nasal Mucosal Tissue

The uptake of Cy5-labeled ovalbumin through human nasal mucosa was studied using Ussing’s chamber. Sample was collected from the basolateral side of the chamber to measure the fluorescence intensity at defined time-points. Both Cy5-labeled ovalbumin in saline and cationic LiG can permeate through the graft from apical to the basolateral side and there was an increase in the diffusion from time zero to 3 hours though not significant. Also, at 3 hours ovalbumin LiG showed increase in uptake as compared to Cy5-labeled ovalbumin in saline. However no statistical significance was found. It can be observed that the overall significant interaction is sensitive to different patterns of means across time but no individual post hoc tests are significant for any given time point due to lower number of samples used in this study. The Cy5-labeled ovalbumin in saline had an apparent permeability coefficient of $2.6 \times 10^{-5}$ cm/hour and Cy5-labeled ovalbumin LiG had an apparent permeability coefficient of $3.5 \times 10^{-5}$ cm/hour. Hence this shows that the human nasal graft is permeable to both formulations for up to 3 hours (Figure 3.8). The study was conducted up to 3 hours due to ex vivo tissue viability issues.
A significant overall interaction between the timepoints and vehicles (Cy5-labeled ovalbumin in saline and LiG) was obtained (\(^ \ast \), \( p<0.05 \)). No significant difference was found when post hoc paired T tests were applied to determine the difference between vehicles at each time point. Data are represented as mean ± SEM (\( n=2 \)).

Our results from Figure 3.3 and 3.4 demonstrate that high molecular weight proteins (eg. ovalbumin, 45 kDa and Cy5-labeled ovalbumin) can be delivered to the brain in significant amounts as compared to untreated rats, further validating the transmucosal delivery route. Our results from table 3.1 and Figure 3.5 demonstrate that both cationic and anionic liposomes can be formulated using both Cy5-labeled ovalbumin and ovalbumin alone. The cationic liposomes having maximum encapsulation efficiency. The higher encapsulation efficiency for cationic liposomes can be attributed to the electrostatic complexation between the positively charged
liposomes and the negatively charged ovalbumin at pH 7 (isoelectric point for ovalbumin is 4.54) [5]. Our results from Figure 3.6 indicate that the cationic LiG has better uptake than the anionic LiG. Results from Figure 3.7 demonstrate that the encapsulation of the ovalbumin in LiG enhances transmucosal delivery over 72 hours thereby validating this delivery platform and setting the stage for delivery of other high molecular weight protein based neurodegenerative therapies. Results from Figure 3.8 demonstrate that the diffusion through human nasal mucosa approximates that of our rat model. Furthermore, our results show that the use of the LiG carrier does not impair ovalbumin diffusion over short time frames of less than 3 hours. This experiment was not performed for longer time points due to human explant viability issues.

4. Conclusion

We report this study as an exploratory pilot study through which our findings confirm that the described mucosal engrafting technique can be used to deliver representative high molecular weight proteins to the brain by overcoming the limitations of intranasal drug delivery. Our results therefore validate the rat as a novel model of direct transmucosal delivery and open the door to testing biologically active and clinically relevant protein-based agents such as antibodies and neurotrophic factors for the treatment of neurodegenerative disease. Additionally, the use of the LiG delivery platform improved the delivery of our surrogate protein, ovalbumin, emphasizing the effectiveness of LiG formulations to protect the drug from degradation and act as a depot for drug for sustained release.

5. References


CHAPTER 4: *In vitro* evaluation of delivery and transfection efficiency of BDNF AT liposomes in RT4-D6P2T rat schwannoma cells

1. Introduction

After confirming the uptake of ovalbumin using the mucosal engrafting technique, we evaluated the uptake and transfection of BDNF AT’s *in vitro* and *in vivo*. As RT4-D6P2T rat schwannoma cells were previously used by a group at OPKO therapeutics to study the levels of BDNF NAT and BDNF AT transfection efficiency, we selected these cells for all our *in vitro* studies. As described in introduction, AT’s inhibit the natural antisense transcripts (NAT’s) and up regulate endogenous protein expression. Before testing these BDNF AT’s *in vivo* we were interested in confirming the BDNF mRNA and protein up regulation *in vitro* in rat schwannoma cells.

Hence, the main objective of this study was to confirm the therapeutic effectiveness of these AT’s in cells before evaluating them in rodents. For this study, we utilized a cationic liposomal carrier system to encapsulate BDNF AT and evaluated the uptake and transfection efficiency of BDNF AT liposomes as compared to BDNF AT in saline and untreated controls at three different concentrations of 50, 100 and 300 nM and at different time points of 12, 24, 48 and 72 hours. We also evaluated the toxicity of cationic liposomes in cells using a live/dead cell staining assay.

2. Materials and Methods

2.1 Formulation and Characterization of Cy5-labeled AT and Active BDNF-AT Cationic Liposomes

The cationic lipid film was prepared using DOTAP (1,2 dioleoyl-3-trimethylammonium propane (chloride salt)) (a cationic lipid), cholesterol (stabilizer), and DPPC (1,2 Dipalmitoyl-sn-glycero-3-phosphocholine) (a neutral lipid) in a 5:3:5 molar ratio. A stock concentration of each lipid was made in chloroform and 1ml of each lipid was added to a 10 ml round-bottom flask
attached to a Rotavap (IKA RV Control 10) and allowed to rotate at 100 rpm in a water bath at room temperature. When the chloroform had evaporated, the thin lipid film at the bottom of the flask was dried overnight in vacuum to remove solvent. The film was hydrated the next day, with 200 μg/ml of Cy5-labeled AT or active BDNF AT in saline as a starting concentration followed by 1 minute of vortexing. The liposomal preparation was placed on ice for two minutes, vortexed, placed in a water bath at 37°C for two minutes, and vortexed again. Five such freeze thaw cycles were performed. The preparation was then probe-sonicated for 5min on ice. The mixture was ultracentrifuged in a Beckman-Coulter ultracentrifuge at 100,000 rpm for 1 hour to separate the AT encapsulated liposomes from un-encapsulated AT. The pelleted liposomes were suspended in 400 μL of saline and extruded through 800nm, 400nm and 200 nm membranes, using an extruder (Avanti Lipids). The concentrated supernatant was used for further analysis. An indirect method was used to determine the encapsulation efficiency. The amount of AT in the supernatant was measured using nano drop and subtracted from the staring amount giving the total AT encapsulated in liposomes. Extruded liposomes were characterized for size, PDI (Polydispersity index) and charge, using a Zetasizer (Nano-ZS90, Malvern Instruments, Inc Westborough MA). Liposomal morphology was determined using transmission electronic microscopy (TEM). The liposomal particles were negatively stained using 1.5% uranyl acetate. A 300-mesh carbon coated copper grid was applied to 10 μl of liposomal sample (10x diluted). Excess sample was wicked off by briefly touching the grid to a piece of filter paper followed by three brief DI water rinses followed by touching the grid to 1.5% uranyl acetate stain 3 – 5 times and wicking off the excess fluid after each step. Sample grids were viewed using a JEOL, JEM 1010 TEM operated at 80kV.

2.2 Qualitative Evaluation of Cy5-labeled AT Uptake in RT4-D6P2T Rat Schwannoma Cell Line
The RT4-D6P2T rat schwannoma cells (American type culture collection) were incubated at 37°C with 5% CO₂ in DMEM with 10% FBS and 2% antibiotics. All exposures occurred at 80% confluency. Cells were seeded into 6 well plates at a density of 2x10⁵ cells per well and were cultured in 2 ml of FBS free DMEM for 24 hours at 37°C with 5% CO₂. Cells were then incubated with 300 nM of Cy5-BDNF-AT in saline and cationic liposomes for 30 minutes, 4 hours, 8 hours and 12 hours. After incubating the cells with Cy5-labeled AT for specified time points the cells were washed twice with 1X PBS and were fixed with 4% formalin for 10 minutes. A drop of DAPI stain was then added to each slide before placing the coverslips for nuclei staining. After DAPI staining, the coverslip was glued to the slides and the slides were further observed under confocal microscope for determining the uptake in cells. (Zeiss LSM 700 laser scanning confocal microscope, Thornwood, NY, USA, Magnification used – 10X, all images were taken using auto exposure settings from the ZEN 2009 software).

2.3 Quantitative Evaluation of Active BDNF-AT Uptake in RT4-D6P2T Rat Schwannoma Cell Line

Quantitative transfection studies were done in RT4-D6P2T rat schwannoma cells. Cells were seeded as described above and treated with 50 nM, 100 nM and 300 nM of active BDNF-AT in saline and cationic liposomes for 12, 24 and 48 hours. A hybridization assay was developed to detect the concentration of BDNF AT in samples. Cells were washed with 1X PBS, pelleted, and lysed using the RIPA lysis buffer (Thermo Fisher Scientific) containing EDTA-free protease inhibitor cocktail (Millipore Sigma catalog no 11836170001). The cell lysate was then centrifuged at 20,000g for 20min to remove cell debris. The supernatant was then used in the hybridization assay as follows. The capture and detection probes were designed to specifically detect BDNF AT. The capture probe was complementary to the 3’ end and the detection probe was complementary to the 5’ end of the BDNF AT as follows:

**Capture probe** - (5AmMC12/iSp18/iSp18//G*+T*+G*+C*+G*+G*+A*+G)

**Detection probe** - (+G*+G*+T*+C*+T*+C*+C*+T*+A*+T*+G/iSp18/iSp18/iBiodT//3BioTEG)

Where * designates phosphorothioate bond, + designates LNA modifications, 5AMmc12 is a 5’-amino modifier C12m, iSp18 is an internal 18-mer spacer, iBiodT is an internal biotin-dT and 3BioTEG is a 3’ biotin-TEG). The probes were synthesized by Qiagen Inc, Germantown, MD, 20874-1415 (capture probe catalog no: 339412 YCO0070251 and detection probe catalog no: 339412 YCO0070253). The capture and detection probes were reconstituted with nuclease-free water to 5000 pmole/ml. Forty μl of the 5000 pmole/ml capture probe was then added to 19.96 ml of 500 mM Na₂HPO₄ and 1 mM Na₂EDTA (pH 8.5). 150 μl of the capture probe mix was added to each well of a 96-well white Nunc™ plate (Thermo scientific, catalog no 436007) and incubated overnight at 4°C. The next day the coated plate was washed five times with 300 μl/well of wash buffer (1X TBST). After the last wash the plate was incubated with blocking buffer (3% BSA in 1X DPBS) for two hours at room temperature on a plate shaker and washed. Then 150 μl of the detection probe/sample mix was added to each well. The detection probe/sample mix was prepared as follows. First, 200 μl of 5,000 pmole/ml of detection probe was added to 19.8 ml of 4X SSC/0.5% sarkosyl buffer. Then the diluted detection probe (225 μl) and the cell extract sample (25 μl) were annealed in a thin walled V bottom plate using a thermocycler (RT 100 Bio-Rad) at 90°C for 12.5 minutes followed by incubation at 40°C. After addition of annealed product, the coated plates were incubated at 45°C for 2 hours on a plate shaker. Then the plates were washed again and incubated with diluted streptavidin-HRP conjugate (Jackson Immunoresearch) (1:50,000 times diluted with poly HRP buffer (Thermo Fisher Scientific) for 30 minutes at 37°C on a plate shaker. The plate was washed again, and the luminescence was detected immediately by adding 150 μl of the Elisa Femto Solution Mix (Thermo Scientific).

**2.4 Quantification of in vitro BDNF AT Mediated BDNF mRNA Derepression**
After harvesting the BDNF AT exposed RT4-D6P2T cells as previously described, cells were lysed and processed for RNA extraction using Quick-RNA™ MiniPrep kit (Zymo Research). The extracted RNA was used for cDNA synthesis using the SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific). qPCR was then performed using the TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific) according to manufacturer's instructions using the LightCycler 480 Instrument II as a PCR platform. The 18S RNA was used as an internal control.

**2.5 Quantification of in vitro BDNF-AT Mediated BDNF Protein Derepression**

After harvesting the BDNF AT exposed RT4-D6P2T cells as previously described, cells were washed with 1X PBS, pelleted, and lysed using the RIPA lysis buffer (Thermo Fisher Scientific) containing the EDTA-free protease inhibitor cocktail. The cells were incubated with the lysis buffer for 15 minutes and the cell lysate was then centrifuged at 20,000 g for 20 minutes to extract the protein. The supernatant (protein) was then collected and samples were diluted 6x to detect the BDNF levels using a commercially available BDNF ELISA kit (EMD Millipore CYT306).

**2.6 Quantification of in vitro BDNF AT Mediated Cytotoxicity in RT4-D6P2T Rat Schwannoma Cells**

RT4 Schwannoma cells were seeded at a density of 8000 cells per well in a 96 well flat bottom cell culture plate (Denville Scientific Inc) and cultured in 200 µL of growth medium for 24 hours at 37°C with 5% carbon dioxide. After RT4-D6P2T cell exposures to BDNF-AT as described above, the old media was replaced by fresh growth media containing the blue stain (Hoechst 33342 for live cells) and green stain (propidium iodide for dead cells) from the ReadyProbes™ Cell Viability Imaging kit. (Molecular probes). The 96-well plate was then incubated for 15 minutes and each well was imaged using Keyence BZ-X710 All-in-One Fluorescence microscope. The % of viable cells in each well were quantified using ImageJ software.

**3. Results and Discussion**
3.1 Formulation and characterization of Cy5-labeled and active BDNF AT cationic liposomes

Liposomes in the size range of 230-250 nm were obtained which was considered ideal size to dose the cells with. The liposomes were further characterized for size, PDI and charge using zetasizer. The characterization data for the liposomes is as follows:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average size (nm)</th>
<th>Average Polydispersity index (PDI)</th>
<th>Average charge (mV)</th>
<th>% Encapsulation efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy5-labeled AntagoNAT cationic liposomes</td>
<td>239.13 ± 24</td>
<td>0.252 ± 0.06</td>
<td>42.40 ± 5.8</td>
<td>~100%</td>
</tr>
<tr>
<td>BDNF AntagoNAT cationic liposomes</td>
<td>229.44 ± 17.6</td>
<td>0.235 ± 0.04</td>
<td>37.28 ± 3.3</td>
<td>~100%</td>
</tr>
</tbody>
</table>

Table 4.1: Characterization data (average size, PDI, surface charge and percent encapsulation efficiency) for Cy5-labeled AT liposomes and BDNF AT liposomes (n=3).

Figure 4.1: Transmission electron microscopy images for Cy5-labeled and BDNF AT liposomes (Phosphotungstic acid (PTA) negative stain).

3.2 Cy5-labeled and unlabeled active BDNF AT liposomes results in significant uptake in rat schwannoma cells as compared to controls

Using confocal microscopy, it was observed that Cy5-labeled AT liposomes (300 nM) demonstrated uptake of AT in cells as early as 30 minutes after exposure which significantly increased 12 hours after exposure. Whereas as the Cy5-labeled AT in saline started showing
uptake in cells 12 hours after exposure and hence demonstrated a delayed uptake in cells (Figure 4.2). These results were confirmed quantitatively by measuring the uptake of BDNF AT in cells using AT hybridization assay where BDNF AT liposomes resulted in a significant uptake of BDNF AT in cells at all concentrations (50, 100 and 300 nM) starting as early as 12 hours and gradually increasing 48 hours after treatment as compared to BDNF AT in saline control and untreated cells. The BDNF AT in saline control did result in some uptake in cells at all concentrations and time points, however the uptake was not significant compared to untreated cells (Figure 4.2).

![Figure 4.2: Evaluation of in vitro uptake of Cy5-BDNF-AT and BDNF AT cationic liposomes and saline formulation in RT4-D6P2T rat schwannoma cells using confocal microscopy and AT hybridization assay.](image)

Confocal microscopic images of time dependent uptake of vehicle control and Cy5-BDNF-AT at 300nM in RT4-D6P2T rat schwannoma cells demonstrating more rapid and robust transfection in
the liposomal (AT-LIPO) versus saline (AT-SALINE) group (bar = 500µm). Histograms represent quantification of BDNF-AT uptake by dose, time, and formulation in the RT4-D6P2T cell line relative to protein normalized control (NEG represents vehicle control). Note the significant dose dependent increase in uptake efficiency in the liposome relative to the saline and negative control groups (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; two-way ANOVA). Data are presented as mean ± SD (n = 4 - 6).

3.3 BDNF AT liposomes results in significant BDNF mRNA expression and BDNF protein up regulation in rat schwannoma cells

qPCR revealed that the liposomal group demonstrated significantly greater dose and time dependent BDNF transcription than the saline group. BDNF protein levels followed a similar pattern for the 50 and 100 nM dosing conditions while the increased expression among the 300nM conditions was not statistically significant (Figure 4.3).
Figure 4. 3: Evaluation of *in vitro* transfection efficiency of BDNF AT cationic liposomes and saline formulation in RT4-D6P2T rat schwannoma cells using qPCR and BDNF specific ELISA:

Bar graphs of qPCR (top row) and ELISA (bottom row) demonstrating BDNF transcriptional and protein expression in RT4-D6P2T rat schwannoma cells following exposure to vehicle control (NEG), liposomal (AT-LIPO), and saline (AT-SALINE) BDNF-AT demonstrating both a dose and time dependent upregulation among the 50 and 100 nM concentrations (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001; two-way ANOVA). Data are presented as mean ± SD (n = 4 for qPCR analysis and n = 6 for ELISA).

3.4 BDNF AT liposomes do not result in significant cell cytotoxicity

Both BDNF AT liposomes and BDNF AT in saline did show some cytotoxicity 48 hours after treatment, however it was less than 20% for all concentrations (50-300 nM) (Figure 4.4)
Figure 4. 4: Detection of cell viability in RT4-D6P2T rat schwannoma cells after treatment with BDNF AT liposomes (AT-LIPO) and BDNF AT in saline (AT-SALINE) as compared to negative control (NEG):
A-C. Live/dead assay in RT4-D6P2T rat schwannoma cells after exposure to 50-300nM of vehicle control (NEG), liposome encapsulated BDNF-AT (AT-LIPO), and BDNF-AT in saline (AT-SALINE) for 24-48 hours demonstrating greater than 80% viability at the doses and time-points studied. (Data presented as mean ± SD, Student’s t-test).

4. Conclusion

Our *in vitro* data confirmed that our BDNF AT constructs are capable of inducing both BDNF transcription and translation in BDNF NAT-expressing rat schwannoma cells at 48 hours. Furthermore, this model validated that our liposomal formulation was capable of enhancing both transfection and efficacy over the saline vehicle alone and also did not result in significant cytotoxicity. Having demonstrated that BDNF ATs can successfully upregulate BDNF *in vitro*, we next evaluated their *in vivo* distribution and efficacy in healthy naïve rats using the mucosal engrafting technique.
CHAPTER 5: *In vivo* evaluation of trans-nasal mucosal delivery of BDNF AT liposomes in naïve rats using heterotopic mucosal engrafting

1. Introduction

After confirming the effects of BDNF AT on BDNF protein regulation in schwannoma cells, we evaluated the uptake and efficiency of BDNF AT liposomes in vivo using the extra cranial rodent graft model. The goal of this study was to study the safety, distribution, and efficacy of transmucosal BDNF AT delivery in a rodent model.

2. Materials and methods

2.1 Qualitative Evaluation of Transmucosal Cy5-labeled AT and BDNF AT Distribution in the Rat Brain

Cationic liposomes encapsulating Cy5-labeled AT and BDNF AT were formulated and characterized as mentioned in Chapter 4 (section 2.1). Cy5-labeled AT in saline were used as controls. Only Cy5-labeled AT in saline and cationic liposomes were utilized for this study for determining the qualitative uptake of AT *in vivo*. Cy5-labeled AT in saline and cationic liposomes were delivered to the rat brain through the mucosal graft following verification of successful engraftment as described in Chapter 3 (section 2.1). The implanted reservoir was filled with 100µL of Cy5-labeled AT in saline (n=2) and cationic liposomes (n=2) for a total dose of 0.15 mg/kg. Rats were then re-dosed every 72 hours for a total of 3 times. Positive (direct parenchymal dosing) and negative (craniotomy without dura/arachnoid reflection) controls (n=2 per group) were additionally dosed with Cy5-labeled AT in saline at the same concentration and dose regimen. All rats were sacrificed 3 days after the last dose (e.g. 12 days after the initial dose). Rat brains were isolated, flash frozen in acetone and dry ice solution, and placed in OCT solution -80°C. Brains were sliced into 50micron sections using a cryotome and the sections comprising the striatum, hippocampus and substantia nigra were imaged (n =2 sections per sub region) under an epifluorescence microscope (Leica DM IL LED fluorescent microscope (Leica, Buffalo Grove, IL)
(Magnification 20X, exposure 229.4ms, Cy5 filter gain settings for striatum, hippocampus and substantia nigra were 2, 5.5 and 8 respectively). The total Cy5-flourescence intensity for each image was quantified using ImageJ software by employing the equation Integrated density = [area of selected region (fluorescent region) x mean fluorescence of the selected area] – background mean fluorescence.

2.2 Quantitative Evaluation of Transmucosal Active BDNF-AT Distribution in the Rat Brain

Active BDNF-AT in saline and cationic liposomes were delivered to rat brain through the mucosal graft as described in Chapter 3 (section 2.1). The implanted reservoir was filled with 100µL of BDNF AT in saline (n=4) and cationic liposomes (n=4) for a total dose of 0.15 mg/kg. Rats were then re-dosed every 72 hours for a total of 3 times. Positive and negative controls as described above (n=4 per group) were additionally dosed with BDNF AT in saline at the same concentration and dose regimen. After sacrifice, rat brains and olfactory bulbs were isolated and 3mm tissue biopsy punches (Integra miltex) were used to isolate the striatum, hippocampus, substantia nigra and cerebellum both ipsilateral and contralateral to the side of the mucosal graft dosing site. The tissue punches were homogenized in 300 μl ice cold tissue lysis buffer and homogenates were centrifuged at 20,000 g for 20 minutes to extract the total protein. The extracted protein was then used for the BDNF AT hybridization assay as described in Chapter 4 (section 2.3). The detected AT levels were then normalized to the total protein content in the samples measured using a Pierce BCA assay kit (Thermo Fisher scientific).

2.3 Quantification of in vivo BDNF-AT Mediated BDNF Protein Derepression in the Rat Brain

A portion of the extracted protein samples isolated during the BDNF-AT hybridization procedure mentioned above were used to quantify BDNF protein levels in each of the isolated tissue punches and prefrontal and occipital cortical regions in rat brain. BDNF protein
concentrations were determined by commercially available ELISA (EMD Millipore) and normalized to the total protein content in the sample measured using the Pierce BCA assay kit. The data was reported as % of control normalized to protein content.

3. Results

3.1 Cy5-labeled AT liposomes resulted in a better distribution and increased uptake in rat brain as compared to controls

Following sacrifice 12 days after the initial transmucosal dose, the Cy5-labeled AT in saline group demonstrated a general trend towards increased distribution in the hemisphere ipsilateral to the mucosal graft relative to the contralateral side. Conversely, the liposomal Cy5-labeled AT group trended towards improved distribution on the contralateral side. Among the striatum and substantia nigra sub-analyses, the liposomal group demonstrated significantly greater distribution than both the negative and positive control on both sides. Within the hippocampus, the saline group had significantly greater distribution than both the negative control and liposomal group (Figure 5.1).
Figure 5. 1: *In vivo* uptake of Cy5-labeled AT in saline and cationic liposomes in rat brain using epifluorescence microscopy:

A) Fluorescent microscopic images of liposomal (AT-LIPO) and saline (AT-SALINE) formulation of Cy5-BDNF-AT distribution on post-dosing day 12 (scale bar = 500µm demonstrating Cy5 distribution by side and brain end-target sub-region. B. Histograms quantifying Cy5 fluorescence by group, side, and end-target sub-region (* p < 0.05, ** p<0.01, *** p<0.001, **** p<0.0001; two-way ANOVA) demonstrating differential efficiency of the liposomal and saline formulations based on region. Data are presented as mean ± SD (n = 2).

### 3.2 BDNF AT liposomes resulted in an increased uptake and increased BDNF protein expression in rat brain as compared to controls

We next utilized a hybridization assay to quantify the distribution of both the liposomal and saline BDNF-AT formulations in the relevant brain end-target regions. No BDNF AT was
detectable within the negative controls. In general, there was greater BDNF AT in the hemisphere ipsilateral to the mucosal graft regardless of group. There were no significant differences in distribution between liposomal and saline BDNF AT formulations with the exception of the ipsilateral hippocampus where the saline group outperformed the liposomal group (Figure 5.2 A).

In order to test the functional efficacy of the BDNF AT, we then quantified BDNF protein expression in both groups relative to basal expression in control brains. These results demonstrated a trend towards increased expression in all brain sub-regions regardless of side or delivery vehicle although there were no strong correlations between BDNF AT levels and expression. The substantia nigra demonstrated significant BDNF upregulation relative to control bilaterally among both the liposomal and saline formulations (Figure 5.2 B).

Figure 5. 2: *In vivo* transmucosal efficacy of BDNF AT in saline and cationic liposomal formulation in rat brain using AT hybridization assay and BDNF specific ELISA:

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A) Bar graphs quantifying BDNF-AT by hybridization assay in the ipsilateral and contralateral end-target regions in brain B) BDNF-AT derepressed protein expression by side and delivery vehicle group in the same end target sub-regions of the rat brain (* p<0.05, ** p<0.01, *** p<0.001; two-way ANOVA). Data are presented as mean ± SD (n=4).

3.3 BDNF AT liposomes resulted in increased BDNF protein expression in cortical regions of rat brain as compared to controls:

We also determined change in BDNF protein expression in prefrontal cortex and occipital cortical regions of rat brain. Both BDNF AT in saline and liposomes resulted in an increased expression of BDNF protein in end target cortical regions of rat brain (Figure 5.3).

![Figure 5.3: BDNF protein up regulation in cortical regions of rat brain on treatment with BDNF AT liposomes:](image)

Bar graphs quantifying BDNF protein upregulation in ipsilateral and contralateral cortical regions of rat brain among the liposomal (AT-LIPO) and saline (AT-SALINE) groups as compared to
negative control (NEG, * p<0.05, ** p<0.01; two-tailed unpaired Student’s t-test). Data are presented as mean ± SD (n=4).

Having demonstrated that BDNF-ATs could successfully upregulate BDNF in vitro, we next evaluated in vivo distribution and efficacy in healthy rats. As AntagoNATs cannot cross the BBB, we adopted a novel transmucosal delivery strategy previously described by our group [16] which is capable of delivering molecules up to 500kDa directly to the brain. We used complementary Cy5 labeling and hybridization assays to confirm successful AT delivery within critical end-target rat brain regions relevant to PD. Our BDNF ELISA data then verified that this delivery was effective resulting in BDNF protein upregulation within the substantia nigra.

One unexpected finding was that saline outperformed the liposomal formulation in the hippocampus region ipsilateral to the mucosal graft. This finding may be due to differences in fiber tract orientation and density within the hippocampus which facilitated diffusion of the saline solution relative to the denser gray matter within the striatum and substantia nigra. A second interesting result was the lack of strong correlation between BDNF-AT distribution and subsequent BDNF protein upregulation. This is not entirely surprising as the ATs only act to derepress expression and thus rely on intrinsic regulatory pathways to promote transcription and translation which likely differ between brain sub regions. This finding therefore serves to support the concept that ATs may be associated with less toxicity than recombinant protein delivery, as they will only act on cells programmed to express the protein of interest.

4. Conclusion

Our data confirm that ATs can be used to upregulate BDNF expression in vivo within key end target brain regions germane to PD. We have further shown that a common surgical endoscopic endonasal mucosal grafting technique can be adopted to overcome the inability of AT to cross the BBB. Finally, our results demonstrate that liposomal encapsulation enhances both BDNF AT distribution and efficacy within the brain. More generally, given the ease of translation
of these findings into clinical practice, our work suggests that transmucosal oligonucleotide delivery may provide a novel and much needed therapeutic option for patients suffering from both PD and other neurodegenerative diseases.
CHAPTER 6: BDNF levels in chemical toxin-induced and genetically engineered animal models of Parkinson’s disease

1. Introduction

1.1 Animal models of Parkinson’s disease

Parkinson’s disease (PD) is a neurodegenerative disorder that primarily affects the dopaminergic neurons in striatum and substantia nigra also referred to as the nigrostriatal pathway [1]. However apart from this pathway, several other systems have now been implicated in PD which include serotonergic, nor-adrenergic, glutaminergic, cholinergic systems [2]. To gain insights into critical questions about pathophysiology of PD, appropriate animal models of PD are required for screening various therapeutic molecules. Various in vitro and in vivo models have been explored so far and are categorized into two main types – the “toxin” and the “genetic” models of PD [2].

1.2. Toxin models of PD

The two classical toxin models of PD widely used even today are the 6-hydroxydopamine (6-OHDA) and MPTP model of PD. Apart from them, other toxic agents like rotenone, paraquat are also used for developing toxin models of PD [3]. Though the toxin induced models of PD guarantee excessive neurodegeneration in the nigrostriatal pathway, the process occurs rapidly (acute models) instead of slow development for years and the animal models also lack protein inclusions called “Lewy bodies” which are mainly implicated in human PD brain [3]. Following are few of the toxin models of PD with their pathological features they exhibit (Table 6.1).

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Motor behavior</th>
<th>SNc loss</th>
<th>neuron loss</th>
<th>Striatal DA loss</th>
<th>Lewy body/Syn pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP Mice</td>
<td>Reduced locomotion, bradykinesia</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>NO</td>
</tr>
</tbody>
</table>
Table 6.1: Pathological feature of various toxin induced PD rodent models.[3]

<table>
<thead>
<tr>
<th>Animal model</th>
<th>Motor behavior</th>
<th>SNc neuron loss</th>
<th>Striatal DA loss</th>
<th>Lewy body/Syn pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPTP monkeys</td>
<td>Reduced locomotion, altered behavior, tremor and rigidity</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>NO</td>
</tr>
<tr>
<td>6-OHDA rat</td>
<td>Reduced locomotion, altered behavior</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>NO</td>
</tr>
<tr>
<td>Rotenone</td>
<td>Reduced locomotion</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>YES</td>
</tr>
<tr>
<td>Paraquat/maneb</td>
<td>Reduced locomotion</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>YES</td>
</tr>
<tr>
<td>MET/MDMA</td>
<td>Reduced locomotion</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>NP</td>
</tr>
</tbody>
</table>

1.3 Genetic models of PD

Genetic models of PD better represent the underlying mechanisms involved in genetic forms of PD [2]. However, they face the same limitation of not being able to replicate the pathological and behavioral phenotypes observed in humans. Some of the genetic models being currently explored include α-synuclein, LRRK2, PINK1, Parkin and DJ1. Mutations in each of these genes are responsible for pathological features of PD in humans [2].

Among these models, α-synuclein was the first gene linked to dominant type familial PD and is also the main component of “Lewy bodies” which are found in PD brain. Following table summarizes the pathological features observed in various genetic rodent models of PD (Table 6.2) [3].
Table 6. 2: Pathological features in various genetic rodent models of PD [3]

<table>
<thead>
<tr>
<th>PARKIN</th>
<th>No obvious alterations or reduced locomotion</th>
<th>NO</th>
<th>↑</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ-1</td>
<td>Decreased locomotor activity</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

1.4 Implication of brain-derived neurotrophic factor (BDNF) in PD

Based on 20 years of research, BDNF has been implicated in PD due to its role promoting survival of dopaminergic neurons in substantia nigra [4]. Several studies have reported that BDNF plays an important role in survival of dopaminergic neurons \textit{in vitro} and \textit{in vivo}. Also knockout of TrkB receptor in rodents (the target receptor for BDNF) resulted in degeneration of SN dopaminergic neurons and led to accumulation of α-synuclein in the neurons [4]. Also a significant decrease in BDNF mRNA and protein in substantia nigra pars compacta has been reported in post mortem PD brains [5, 6]. A study also reported reduced levels of peripheral BDNF in serum of PD patients showing a direct association between decreased BDNF and PD [7].

In spite of the evidence suggesting decreased BDNF mRNA and protein levels in human PD patients, rodent studies have shown variable results. A few studies have shown that BDNF mRNA, protein levels and TrkB receptor levels did not change in aged rats [8]. However another study looked at BDNF levels in aged and young sparague dawley rats and found out that the BDNF and trkB levels had decreased in aged rats as compared to young rats [9].

Due to the discrepancy in the levels of BDNF protein and mRNA levels in rodent PD brains and aged rodent brains, it was important we first determine BDNF levels in a few established rodent models of PD. Rat brain samples for various rodent PD models like \textbf{PINK1 KO, only rotenone microsphere treated, rotenone + PINK1 KO and LRRK2} models were obtained from
**Dr. Craig Ferris’s lab** at Northeastern University. BDNF levels in different parts of brains for each model was determined using ELISA as below. Appropriate model showing low BDNF protein levels was selected for further studies.

2 Materials and Methods

2.1 Detection of BDNF protein levels in different PD rodent models

The total BDNF protein levels were detected in different PD rat models to determine which of the model has lowest levels of BDNF as compared to control rats. The Long Evans (LE) control rats, rotenone microspheres treated Long Evans rats (PD model), PINK1 KO + rotenone microspheres treated Long Evans rats (PD model) and only PINK1 KO Long Evans rats (PD model) were obtained from Dr. Craig Ferris’s lab at Northeastern University. The rats were sacrificed, and brains were isolated. Tissue biopsy punches (3 mm) were used to isolate striatum, hippocampus, substantia nigra and cerebellum. The tissue samples were then homogenized in 300 µl ice-cold homogenization buffer and the tissue homogenates were then centrifuged at 20,000 g for 20 minutes at 4°C to obtain protein samples. BDNF specific Elisa kit from EMD Millipore (CYT306) was used to measure BDNF levels in the tissue samples.

3. Results and Discussion

From Figure 25 it can be seen that only the PINK1 KO + rotenone treated LE rats showed a significant decrease in BDNF protein levels in substantia nigra and cerebellum as compared to LE control rats. However, the PINK1 KO rat brain samples showed a significant increase in BDNF protein levels as compared to control rats and toxin induced PD models. (Figure 6.1 and 6.2). A slight increase (not statistically significant) in BDNF was also observed in the LRRK2 genetic model (Figure 6.1 and 6.2).
Figure 6.1: BDNF protein levels in different toxin induced and genetic rodent models of PD:

BDNF protein levels detected in striatum, hippocampus, substantia nigra and cerebellum for Long Evans (LE) control rats (n=3), PINK1 KO + rotenone treated LE rats (n=3), rotenone treated LE rats (n=3) and only PINK KO LE rats (n=3). A significant decrease in BDNF protein levels was observed in PINK1 KO + rotenone treated rats as compared to LE control rats (*, p<0.05, **, p<0.005, ***, P<0.001, unpaired student T test). Data are presented as mean ± SD (n=3).
Figure 6. 2: BDNF protein levels in LRRK2 rodent PD model:

BDNF protein levels detected in striatum, cortex, midbrain, cerebellum and brain stem for Long Evans (LE) control rats (n=3) and LRRK2 rats (n=3). No significant changes in BDNF levels were observed compared to control rats. Data are presented as mean ± SD (n=3).

4. Conclusion

From above results it can be concluded that the PINK1 KO + rotenone rats showed a significant decrease in BDNF levels in substantia nigra which supports the fact that Parkinson’s disease involves death of dopaminergic neurons in substantia nigra and affects the nigrostriatal pathway in the brain. The significant increase in BDNF protein levels in the genetic models (PINK1 KO and LRRK2) could be attributed to the activations of compensatory mechanisms to protect dopaminergic neurons. As only genetic models showed an increase in BDNF levels and due to time constraints, only the rotenone model was selected to determine other PD biomarkers in brain and to further study the therapeutic effects of BDNF AT liposomes.
5. References

CHAPTER 7: Development of a rat PD model using rotenone microspheres and subsequent evaluation of Parkinson’s disease biomarkers

1. Introduction

1.1 Rotenone induced models of Parkinson’s disease

Rotenone is a naturally occurring pesticide and was initially used in PD research in 1980’s. Rotenone is a highly lipophilic compound and hence can easily bypass the BBB and does not need any transporters [1]. Rotenone has been implicated in PD and hence many studies have evaluated the pathological features of rotenone induced models of PD in rodents. As it is highly lipophilic, rotenone can be administered systemically and overcomes all the disadvantages of 6-OHDA and MPTP induced models in terms of administration which require intra-cranial stereotaxic injections [1].

It was first demonstrated in 2000 that systemic administration of rotenone can induce hallmarks of PD in rats [2]. The pathological hallmarks induced in rats by rotenone are similar to those observed in humans which include death of dopaminergic neurons in SN and striatum region and loss of noradrenergic neurons [1]. However, as it is administered systemically, the major disadvantage of this model is that it’s not reproducible in terms of the lesions and location and severity of the lesions in different rats. This variability in the model diminishes interest in studying this model further [1].

However, the Scripps research institute reported a study wherein they used subcutaneous injections of rotenone microspheres in rodents and they showed that the microspheres provided a gradual increase in levels of plasma rotenone in the first few weeks which then declined over time [3]. In 2013, another study was reported by Dr. Ferris and group wherein with some modifications to the study reported by Scripps institute, they reproduced the hallmarks of PD in rodents with the rotenone microspheres [4]. For this thesis project we decided to follow the same
rotenone model as reported by [4] and looked at the mRNA levels of several target proteins like BDNF, TH, α-synuclein, Tnf-α and IL-6 and protein levels of TH in striatum and substantia nigra.

2. Materials and methods

2.1 Formulation and characterization of rotenone microspheres

The rotenone microspheres were formulated using emulsion solvent evaporation/extraction method. The rotenone was embedded in a biodegradable polymer of poly(DL-lactide-co-glycolide) (PLGA) from Sigma. About 258 mg of rotenone was dissolved with 403 mg of PLGA in 15 ml of dichloromethane. The solution was then vortexed for 15 minutes at room temperature. This was the organic phase. The organic phase was then poured into 300 ml of ice-cold 4% (w/v) polyvinyl alcohol. The emulsion was then stirred for an hour at maximum speed in hermetic condition. The seal was broken to evaporate the dichloromethane for 3 hours at room temperature. The formed microspheres were collected by centrifugation and were washed with distilled water. A control batch was made without adding rotenone.

2.2 Development of a rotenone PD model by subcutaneous injections of rotenone microspheres

Rotenone microsphere were formulated as mentioned above. Male Sparague Dawley rats (400-500 grams) were utilized for the study. All rats were anesthetized with isoflurane before dosing subcutaneously. The rats were dosed with 100 mg/kg of rotenone microspheres subcutaneously once. All dosed rats were monitored two to three time a week. Rats were divided into two groups wherein one set of rats was sacrificed 30 days after microsphere injection and other set of rats were sacrificed 60 days after microsphere injection. Each group of rats (30 days and 60 days) were further divided into two groups wherein half the rats were used for detecting mRNA levels for Tyrosine hydroxylase (TH), BDNF, Tnf-α, IL-6 and α-synuclein using quantitative
PCR technique. The rest half of the rats were used for detecting TH protein levels using western blot.

2.3 Detection of TH protein levels in PD brains using western blotting technique

For western blotting technique, rats were sacrificed 30 and 60 days after rotenone microsphere injection and the brains were isolated and striatum and substantia nigra was isolated using tissue biopsy punches. The tissues were then homogenized in ice cold RIPA buffer (pierce RIPA buffer catalog no 89900) and the tissue homogenate was centrifuged at 12000 g for 20 minutes at 4°C to extract total protein. Total protein was quantified using pierce BCA assay kit (catalog no 23225) and 50 µg protein of each sample was loaded into each well of 4-12% Bis tris gels and proteins were separated using XCell SureLock mini cell electrophoresis system. The separated proteins were then transferred onto a PVDF membrane using the iBlot from Invitrogen. The PVDF membrane with the transferred protein was blocked in 5% milk in 1X TBST for 1 hour and then the blots were incubated overnight with tyrosine hydroxylase (TH antibody (F-11): catalog no sc-25269, 1:1000) and β-actin (β-actin (C4) antibody: catalog no sc-47778, 1:200) primary antibodies at 4°C on a shaker. The next day the blots were washed with 1X TBST for 45 minutes and were incubated in m-IgGκ BP-HRP (catalog no 516102, 1:1000 for santa cruz primary antibodies) secondary antibodies for 2 hours at room temperature on a shaker. The membranes were then washed using 1X TBST for 45 minutes and the blots were then developed using enhanced chemiluminescent detection reagent (thermo fisher scientific catalog no 34580 and Santa Cruz biotechnology catalog no sc-2048). The blots were then imaged using BioRad molecular imager ChemiDOX™ XRS + imaging system and a semi-quantitative analysis was performed on the blots using ImageJ. Results are expressed as TH protein band intensity normalized to β-actin band intensity.
2.4 Detection of TH, BDNF, α-synuclein, TNF-α and IL-6 mRNA levels in PD brains using quantitative PCR technique

RNA extraction

For quantitative PCR (qPCR), rats were sacrificed 30 and 60 days after rotenone microsphere injection and the brains were isolated and striatum, hippocampus, substantia nigra and cerebellum were isolated using 3 mm tissue biopsy punches.

For RNA extraction, tissue samples were homogenized in 600 µl of trizol and were kept at room temperature for 5 minutes. 120 µl Chloroform was added to each sample and the samples were again kept at room temperature for 3 minutes. The samples were then vortexed and were centrifuged at 12000g for 15 minutes at 4°C. This resulted in separation of organic and aqueous phase. The aqueous phase (top layer) was collected and 300 µl isopropanol was added to aqueous phase. This resulted in RNA precipitation. The samples were then vortexed briefly and kept at room temperature for 10 minutes. The samples were then centrifuged at 12000g for 10 minutes at 4°C. The resulting RNA pellet was then washed with 1 ml 80% ethanol after removing the supernatant. After adding ethanol, the samples were again centrifuged at 7500g for 5 minutes at 4°C. The supernatant was then removed, and the pellet was allowed to air dry. After air drying the RNA sample, it was resuspended in 30-50 µl of DNase and RNase free water. The RNA was then quantified using nanodrop.

cDNA synthesis

cDNA synthesis was performed using the iScript™ reverse transcription supermix from Biorad (catalog no 1708840). Each reaction had 1 µg of extracted RNA and 16 µl of extracted RNA (62.5 ng/µl). The 20 µl cDNA reaction was then run on a thermocycler (BIO-RAD T100 Thermal cycler) using the following protocol:
<table>
<thead>
<tr>
<th>Primes</th>
<th>Time and Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Priming</td>
<td>5 minutes at 25°C</td>
</tr>
<tr>
<td>Reverse transcription</td>
<td>20 minutes at 46°C</td>
</tr>
<tr>
<td>RT inactivation</td>
<td>1 minute at 95°C</td>
</tr>
</tbody>
</table>

The cDNA was further used to run PCR using specific primers.

**qPCR reactions**

Each qPCR reaction for each sample consisted of 5 µl of synthesized cDNA, 5 µl of specific primer mix (forward + reverse primer) and 10 µl of iTaq™ Universal SYBR green supermix from biorad (catalog no 172-5120). The primers for the reaction were ordered from Eurofins genomic LLC. The primer sequences are as follows:

<table>
<thead>
<tr>
<th>Primer Sequence name</th>
<th>Primer Sequence 5’ to 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH_forward primer</td>
<td>CAGCTGGAGGATGTGTCTCA</td>
</tr>
<tr>
<td>TH_reverse primer</td>
<td>GGCATGACGGATGTACTGTG</td>
</tr>
<tr>
<td>BDNF_forward primer</td>
<td>TTTGATGAGACGGGTTCCC</td>
</tr>
<tr>
<td>BDNF_reverse primer</td>
<td>AGGATGTCATCACTCTCTCTCA</td>
</tr>
<tr>
<td>α-synuclein (SNCA)_forward primer</td>
<td>TCAGCCAGAGCCTTTTCAC</td>
</tr>
<tr>
<td>α-synuclein (SNCA)_reverse primer</td>
<td>AGCCACAACCTCCCTCTTTGA</td>
</tr>
<tr>
<td>Tnf-α_forward primer</td>
<td>CGAGATGTGGAACTGGCAGA</td>
</tr>
<tr>
<td>Tnf-α_reverse primer</td>
<td>GCCACGAGCAGAATGAGAA</td>
</tr>
<tr>
<td>IL-6_forward primer</td>
<td>AGTCCGGAGAGGAGACTTCA</td>
</tr>
<tr>
<td>IL-6_reverse primer</td>
<td>CAGTGCATCATCGCTGTTTCAT</td>
</tr>
<tr>
<td>Gapdh_forward primer</td>
<td>CCCCAATGTATCCCTGTTG</td>
</tr>
<tr>
<td>Gapdh_reverse primer</td>
<td>TAGCCAGGATGCCCTTTAG</td>
</tr>
</tbody>
</table>
Each reaction as mentioned above was pipetted in a 96 well PCR plate and the plate was run on Roche LightCycler 480 II according to manufacturer’s instructions. The results were plotted as gene expression normalized to gapdh expression.

3. Results and Discussion

3.1 Rotenone microsphere injection did not result in a significant decrease in mRNA levels for PD biomarkers except for TH

From Figure 7.1 it can be seen that there were no significant changes in TH, BDNF and α-synuclein mRNA levels as compared to untreated rats (baseline levels) 30 days after rotenone microsphere injection. Regarding inflammatory markers Tnf-α and IL-6, there were no significant changes 30 days after rotenone microsphere injection.

Similarly, for rats, sacrificed 60 days after rotenone microsphere injection, no significant changes were observed in TH, BDNF, α-synuclein, Tnf-α and IL-6 mRNA levels (Figure 7.2). However, a significant decrease in TH mRNA levels was observed in the group of rats that were sacrificed 60 days after the injection.
Figure 7.1: Changes in Tyrosine hydroxylase (TH), BDNF, α-synuclein, Tnf-α and IL-6 mRNA levels in 30 days treated rat brain samples:

Tyrosine hydroxylase (TH), BDNF, α-synuclein, Tnf-α and IL-6 mRNA levels detected in striatum, hippocampus, substantia nigra and cerebellum using q-PCR. The results are reported as target mRNA expression normalized to gapdh. No significant differences were observed in rats treated with rotenone microspheres for 30 days as compared to untreated controls. Data are represented as mean ± SD (n=4).
Figure 7. 2: Changes in Tyrosine hydroxylase (TH), BDNF, α- synuclein, Tnf-α and IL-6 mRNA levels in 60 days treated rat brain samples:

Tyrosine hydroxylase (TH), BDNF, α- synuclein, Tnf-α and IL-6 mRNA levels detected in striatum, hippocampus, substantia nigra and cerebellum using q-PCR. The results are reported as target mRNA expression normalized to gapdh. No significant differences were observed in rats treated with rotenone microspheres for 60 days except for TH as compared to untreated controls (* p<0.05; two way anova (p=0.0464)). Data are represented as mean ± SD (n=4).

3.2 Rotenone microsphere injection did not result in a significant decrease in TH protein levels

From Figures 7.3 and 7.4 it can be observed that no significant decrease in TH protein levels was observed in striatum and substantia nigra in rats sacrificed 30 days and 60 days after rotenone injection as compared to untreated controls.
Figure 7.3: Changes in TH protein levels in striatum and substantia nigra in 30 days treated rats:

Representative western blot images and semi-quantified levels of tyrosine hydroxylase protein detected in striatum and substantia nigra of rats treated with rotenone microspheres for 30 days. No significant changes were observed in levels of TH protein in treated rats as compared to controls. Data is represented as mean ± SD (n=4).
Figure 7. 4: Changes in TH protein levels in striatum and substantia nigra in 30 days treated rats:

Representative western blot images and semi-quantified levels of tyrosine hydroxylase protein detected in striatum and substantia nigra of rats treated with rotenone microspheres for 60 days. No significant changes were observed in levels of TH protein in treated rats as compared to controls. Data is represented as mean ± SD (n=4).

4. Conclusion

From the results above it can be concluded that there were no changes in target mRNA levels in rats sacrificed 30 days after injection. However, in the group sacrificed 60 days after rotenone microsphere injection, there were no changes in the target mRNA levels except for that of tyrosine hydroxylase (TH). As a significant decrease in TH mRNA levels was observed in the group sacrificed 60 days after injection, we further assessed TH protein levels in rats
treated with microsphere for 30 and 60 days. TH is an important biomarker in PD rodent models and hence a change in TH mRNA levels was considered positive.

TH western blot analysis revealed that there were no significant changes in TH protein levels in both striatum and substantia nigra in both 30- and 60-day’s time point groups. As no significant changes were observed in TH protein, none of the other bio markers were assessed further for protein levels. As TH which is the most important biomarker for PD did not show any change in striatum and substantia nigra, we did not utilize this model further for therapeutic analysis of BDNF AT liposomes. Based on our results, we would also like to report that we could not replicate the studies mentioned in the previous paper published by Dr. Ferris and group. One of the reasons for this could be the different strain of rats (Sprague Dawley rats) used for my project. Hence, we decided to explore a more acute invasive rat 6-hydroxydopamine PD model for further therapeutic studies.

5. References

CHAPTER 8: Development of 6-hydroxydopamine rat Parkinson’s disease model and subsequent evaluation of biomarkers

1. Introduction

1.1 6-hydroxydopamine (6-OHDA) model of Parkinson’s disease (PD)

Since it was first described in 1959, 6-hydroxydopamine (6-OHDA) has played a vital role in PD research. Being a structural analogue of dopamine and catecholamines, it exerts a toxic effect on dopaminergic neurons by inducing oxidative stress [1, 2]. The 6-OHDA model was first developed in 1968 by Urban Ungerstedt [3] where 6-OHDA was injected bilaterally into substantia nigra region of rat brain. This model subsequently resulted in a high mortality rate which led to the development of a unilaterally injected 6-OHDA rat model [1]. Since then many rodent models have been developed by changing the amount of 6-OHDA injected and by changing the location of injection (either in striatum or substantia nigra). The severity of the lesion can be controlled depending on how many sites the toxin is injected and whether the toxin is injected unilaterally or bilaterally [1].

1.2 Mechanism for 6-OHDA induced neurotoxicity

6-OHDA is not lipophilic and hence it cannot cross the BBB if injected systemically. Hence, the toxin needs to be injected using stereotactic surgery making this an invasive form of administration [1, 2]. Once into the brain, the toxin is taken up by catecholaminergic neurons by means of DAT (dopamine transporter) or NAT (nucleobase-ascorbate transporter). Once inside the neurons, 6-OHDA undergoes degradation by enzymes like MAO-A which results in auto-oxidation. This process generates several toxic species inside the neurons which damages the neurons [1] (Figure 8.1).
Figure 8. 1: 6-OHDA induced neurotoxicity mechanism [1].

6-OHDA is stored in catecholaminergic neurons after being taken up by Dopamine transporter (DAT) or norepinephrine transporter (NAT). Further 6-OHDA gets degraded by monoamine oxidase A (MAO-A) or undergoes self-oxidation which gives rise to several toxic species which can cause neuronal damage. 6-OHDA can also affect the activity of mitochondria by impairing complex 1 which can again lead to neuronal death.

1.3 Advantages and limitations of 6-OHDA PD model
The 6-OHDA model provides a valuable tool to study the therapeutic effects of various drugs as the model responds to classic motor test (rotarod test, apomorphine rotation test) and also causes severe dopaminergic neuronal degeneration in striatum and substantia nigra depending on site of injection. However, the model cannot replicate several other pathological features of PD observed in humans. However, most of the current PD models face the same limitation [1, 2].

For thesis, we decided to go with a unilateral 6-OHDA model with one injection of 6-OHDA in right striatum. We selected striatum as this results in a retrograde transport of the toxin from striatum to substantia nigra, does not result in animal death and also causes degeneration in substantia nigra. We followed the same procedure as described in [4] to develop the unilateral 6-OHDA model and looked at tyrosine hydroxylase (TH) and BDNF protein levels using western blot and at dopaminergic (TH positive) cell death in striatum and substantia nigra using immunohistochemistry.

2 Materials and Methods

2.1 Development of rat 6-OHDA PD Model

Sparague Dawley rats (300-350 grams) were injected with 10 µl of 6-hydroxydopamine (6-OHDA) solution (10 µg/2 µl of 6-OHDA HBr salt (Sigma Aldrich 162957) in 0.1% ascorbic acid in saline) at the rate of 0.66 µl/min using a syringe pump (Harvard apparatus remote/infuse withdraw pump 11 elite nanomite programmable syringe pump (catalog no 70-4507)). The solution was injected into striatum using a 5 µl neuros Hamilton syringe (catalog no 65460-03) at the following co-ordinates: anterior-posterior (AP), - 0.5 mm; medial-lateral (ML), + 2.5 mm; and dorsoventral (DV), - 4.5 mm over 3 minutes. The syringe was kept in place for an additional five minutes after injection before withdrawal. After injection withdrawal, rats were sutured back and allowed to heal. All 6-OHDA treated rats were sacrificed 16 days after injection and rat brains
were analyzed for Tyrosine hydroxylase (TH) and BDNF protein levels using western blot analysis. Qualitative TH expression was also evaluated in rat brain slices using immunohistochemistry.

2.2 Quantification of TH and BDNF protein levels in 6-OHDA PD rat brains using Western Blot

After sacrificing 6-OHDA treated rats, rat brains were isolated, and 3 mm biopsy punches were used to isolate striatum and substantia nigra. The tissues were then homogenized with 220 µl of ice-cold pierce RIPA lysis buffer and the tissue homogenate was centrifuged at 12000 g for 20 minutes at 4°C to extract total protein. Total protein was quantified using pierce BCA assay kit (catalog no 23225) and 50 µg protein of each sample was loaded into each well of 4-12% Bis tris gels and proteins were separated using XCell SureLock mini cell electrophoresis system. The separated proteins were then transferred into PVDF membrane using iBlot from Invitrogen. The PVDF membrane with the transferred protein was blocked in 5% milk in 1X TBST for 1 hour and then the blots were incubated overnight with Tyrosine hydroxylase (TH antibody (F-11): catalog no sc-25269, 1:1000), BDNF ( anti-BDNF antibody (3C11) catalog no ab203573, 5 µg/ml) and β-actin (β-actin (C4) antibody: catalog no sc-47778, 1:200) primary antibodies at 4°C on a shaker. The next day the blots were washed with 1X TBST for 45 minutes and were incubated in m-IgGk BP-HRP (catalog no 516102, 1:1000 for santa cruz primary antibodies) and goat anti-mouse IgG (H+L) HRP secondary antibody ( catalog no ab 97023 for abcam primary antibody) secondary antibodies for 2 hours at room temperature on a shaker. The membranes were then washed using 1X TBST for 45 minutes and the blots were then developed using enhanced chemiluminescent detection reagent (thermo fisher scientific catalog no 34580 and Santa Cruz biotechnology catalog no sc-2048). The blots were then imaged using BioRad molecular imager ChemiDOX™ XRS + imaging system and a semi-quantitative analysis was performed on the blots using ImageJ.
Results are expressed as TH and BDNF protein band intensity normalized to β-actin band intensity.

2.3 Immunohistochemical Analysis of TH Expression in the Rat Brain

Rats were sacrificed 16 days after 6-OHDA injection and were transcardially perfused with 200 ml of 1X PBS and then with 200 ml of 4% paraformaldehyde (Sigma catalog no 1004965000). The brains were then post-fixed with 4% paraformaldehyde for 4 hours and then were transferred into 30% sucrose solution in 1X PBS for 24 hours at 4°C. The brains were then embedded in reservoirs using optimal cutting solution (OCT). The next day rat brains were sectioned into 50 µm thick coronal slices and the brain slices were stored in 0.1% sodium azide in 1X PBS until staining. The sections were washed for 30 minutes in 0.5% triton X-100 and 100 nmole/L glycine in PBS and then in PBS containing Triton X-100 for 1 hour (20 minutes 3 times). The sections were then blocked in UltraCruz blocking reagent (catalog no sc-516214) for 2 hours at room temperature. Sections were incubated overnight in TH primary antibody diluted in blocking reagent (TH antibody (F-11): catalog no sc-25269, 1:50) at 4°C on a shaker. The next day the sections were washed in PBS containing Triton X-100 (20 minutes 3 times) and then were incubated in m-IgGκ BP-CFL 488 secondary antibody (catalog no sc-516175, 1:50) at room temperature. Sections were then washed in PBS containing Triton X-100 (20 minutes 3 times) and in 1X PBS (20 minutes 3 times). Sections were then mounted on glass slides using vectashield mounting medium (Vector Laboratories H-1000). The coverslips were secured in places on the slides using a transparent nail polish. The images were captured using Keyence B2-X710 all-in-one fluorescence microscope.

3. Results

From TH immunohistochemistry results (Figure 8.2 A), it can be seen that there was a significant death of TH positive dopaminergic neurons in both striatum and substantia nigra for 6-
OHDA treated rats (vehicle + 6-OHDA) as compared to the positive control (POS CONTROL). The positive control group contains rats that were not treated with 6-OHDA and was used to determine baseline levels in comparison to 6-OHDA treated rats.

From western blot results (Figure 8.2 B), it can be seen that there was a significant decrease in TH protein levels in both striatum and substantia nigra for 6-OHDA treated (vehicle + 6-OHDA) rats as compared to positive control. A decrease in BDNF protein levels was also observed in both striatum and substantia nigra for 6-OHDA treated (vehicle + 6-OHDA) rats as compared to positive control however this decrease was not statistically significant.

Figure 8.2: 6-OHDA PD model development:

A) Coronal fluorescent IHC images of TH staining in the rat 6-OHDA injury model (ipsilateral to injection, bar = 200 µM) demonstrating death of TH immunopositive cells in both the striatum and substantia nigra following 6-OHDA injection relative to vehicle control (POS control represents baseline expression in uninjured rat). B) TH and BDNF expression by western blot within rat striatum and substantia nigra demonstrating significant decrease in TH levels (****, p<0.0001, *, p<0.05; unpaired student T test, values are normalized to β-actin) and a non-significant trend towards decrease in BDNF levels. Data are represented as mean±SEM (n=6).
Figure 8. 3: Representative western blot images for TH and BDNF in 6-OHDA PD model rats as compared to untreated rats in (A) striatum and (B) substantia nigra

4. Conclusion

From the results above it can be concluded that 6-OHDA rat PD model is effective and does results in significant TH and BDNF protein loss in striatum and substantia nigra leading to death of TH positive dopaminergic neurons. As this model was found to be effective when compared to the rotenone microsphere model, we selected this model to evaluate the therapeutic efficacy of BDNF AT liposomes.

5. References


CHAPTER 9: *In vivo* evaluation of therapeutic efficacy of BDNF AT liposomes in a 6-OHDA rat Parkinson’s disease model

1. Introduction

Based on the studies described in Chapter 8, we successfully developed and characterized the unilateral 6-OHDA rat model of PD. As we achieved sufficient dopaminergic neuronal death and a significant decrease in TH protein levels and a drop in BDNF protein levels in striatum and substantia nigra, we decided to use this model to study the therapeutic effects of BDNF AT liposomes.

BDNF AT liposomes were administered to the rats immediately after 6-OHDA injection by mucosal engrafting (surgical method described in Chapter 3, section 2.2). We evaluated the therapeutic effects of the BDNF AT liposomes by looking at changes in TH and BDNF protein levels in striatum and substantia nigra and qualitative TH cell death in striatum and substantia nigra using immunohistochemistry.

2. Materials and methods

2.1 Rat 6-hydroxydopamine model development and subsequent dosing of BDNF AT liposomes

Following graft inspection 3 days after engrafting, rats were injected with 10 µl of 6-hydroxydopamine (6-OHDA) solution in the right striatum as mentioned in Chapter 8 (section 2.1). After syringe withdrawal, the 250 µl propylene reservoir was implanted as described in the heterotopic grafting procedure Chapter 3 (section 2.1). Rats were then dosed with only saline (vehicle + 6-OHDA) and BDNF AT liposomes (AT-LIPO + 6-OHDA). Untreated rats (POS Control) were used as controls to detect baseline levels. All rats were sacrificed 16 days after 6-OHDA injection.
2.2 Quantification of TH and BDNF protein levels in BDNF AT liposomes treated 6-OHDA rat brains

After sacrificing BDNF AT liposomes and only saline treated 6-OHDA rats, rat brains were isolated, and 3 mm biopsy punches were used to isolate striatum and substantia nigra. The tissues were processed to detect TH and BDNF protein levels using western blot technique as mentioned in Chapter 8 (section 2.2).

2.3. Immunohistochemical Analysis of TH Expression in 6-OHDA rats treated with BDNF AT liposomes

Rats were sacrificed 16 days after 6-OHDA injection, and the brains were processed as mentioned in Chapter 8 (section 2.3) for TH immunohistochemical staining. The images were captured using Keyence B2-X710 all-in-one fluorescence microscope.

3. Results and discussion

3.1 BDNF AT liposomes exert a neuroprotective effect on dopaminergic neurons in a rat 6-OHDA model of PD

In order to determine the therapeutic efficacy of the liposomal BDNF -AT (AT-LIPO), we examined both tyrosine hydroxylase (TH) and BDNF protein expression within the striatum and substantia nigra in an established 6-OHDA rat model of PD (n=6 per group). We demonstrated that AT-LIPO was associated with preservation of both TH and BDNF expression using complimentary western blot and immunohistochemical (IHC) techniques relative to vehicle control. These results suggested that AT-LIPO delivery conferred a neuroprotective effect in the 6-OHDA induced disease state (Figure 9.1 and Figure 9.2).
Figure 9.1: Therapeutic efficacy of AT-LIPO in rat 6-OHDA injury model:

A) Coronal fluorescent IHC images of TH staining in the rat 6-OHDA injury model (ipsilateral to injection, bar = 200 μM) demonstrating qualitative preservation of TH immunopositive cells in both the striatum and substantia nigra following AT-LIPO treatment relative to vehicle control (POS control represents baseline expression in uninjured rat).

B) TH and BDNF expression by western blot within rat striatum demonstrating significant preservation of TH expression following AT-LIPO treatment (**** p<0.0001; unpaired student T test, values are normalized to β-actin) and a non-significant trend towards BDNF preservation.

C) TH and BDNF expression by western blot within rat substantia nigra demonstrating significant preservation of both TH and BDNF expression following AT-LIPO treatment (*, p<0.05) relative to vehicle control. Data are represented as mean±SEM (n=6).
Figure 9. 2: Representative western blot images for TH and BDNF in 6-OHDA PD model rats by treatment group:

in striatum (A) and substantia nigra (B)

4. Conclusion

After dosing the 6-OHDA rats with BDNF AT liposomes, we found that AT-LIPO was capable of preserving TH expression in both the striatum and substantia nigra confirming its therapeutic neuroprotective effect.

Our data confirm that BDNF AntagoNAT’s can be used to upregulate BDNF expression both in vitro and in vivo within key end target brain regions germane to PD and can help is preserving dopaminergic neurons in rat PD model. We have further shown that a common surgical
endoscopic endonasal mucosal grafting technique can be adopted to overcome the inability of AntagoNATs to cross the BBB. More generally, given the ease of translation of these findings into clinical practice, our work suggests that transmucosal oligonucleotide delivery may provide a novel and much needed therapeutic option for patients suffering from both PD and other neurodegenerative diseases.
CHAPTER 10: Olfactory mucosal depot rat model development as an alternative to heterotopic mucosal engrafting surgery and subsequent evaluation of BDNF AT delivery using a liposome-in-gel formulation

1. Introduction

One of the most significant obstacles in the treatment of neurological disease is the blood-brain barrier (BBB) which prevents 98% of all potential neuropharmaceuticals from reaching the central nervous system (CNS) [1]. This impediment has catalyzed a large body of research into the direct trans-nasal pathway which seeks to bypass the BBB by taking advantage of the direct anatomic connection between the CNS and nasal mucosa afforded by the olfactory nerves [2,3].

Though promising, the current generation of topical intranasal delivery strategies suffer from a variety of limitations which hindered progress in the development of a clinically effective platform approach (as described in Chapters 1 and 2).

In order to overcome these limitations, we have developed a novel platform minimally invasive nasal depot (MIND) technique as an alternative to the heterotopic mucosal engrafting technique to provide targeted delivery of an agent to the olfactory mucosa. Using an endoscopic camera system placed within the nostril, doctors are currently able to perform a focused injection within virtually any region of the nasal cavity using only topical anesthesia. When applied to trans-nasal CNS drug delivery, this injection may be adapted to precisely implant a depot within the submucosal space directly adjacent to the olfactory nerves to deliver a reproducible concentration and volume of therapeutic. This approach eliminates essentially all of the drawbacks of traditional topical intranasal techniques including distribution, mucosal residence time, and epithelial permeability.

In order to experimentally validate this clinical technique, we developed a rodent model to mimic the placement of a depot within the olfactory mucosal space. For the thesis project, initially only BDNF AT in gel was delivered using this surgical technique to detect BDNF AT levels and BDNF protein up regulation. The main objective of this pilot study was to evaluate and confirm
the BDNF AT delivery and protein up regulation through this surgery. We also employed direct intranasal delivery to compare the BDNF AT delivery by olfactory mucosal depot surgery.

After confirming BDNF up regulation using this surgical technique, we further utilized a thermosensitive liposomes-in-gel system to create a drug depot over the olfactory mucosa and to enable a sustained delivery of BDNF AT’s to the brain as compared to BDNF AT in gel.

2. Materials and Methods

2.1 Ethics Statement

All the animal procedures were approved by the Northeastern University Institutional Animal Care and Use Committee (Protocol number: 180101-R) that was approved in January 2018. The Sprague-Dawley rats (300-325 grams) used in the study were ordered from Charles River, Kingston, MA, USA. All surgeries were performed under isoflurane anesthesia and all efforts were made to minimize suffering. All the rats were housed in a climate-controlled room on a 12/12-hour light/dark cycle and were provided with food and water ad libitum. All the surgeries were performed during the rat’s light cycle.

2.2 Olfactory mucosal depot surgery

Rats were anesthetized using 2.5% isoflurane and were placed on stereotaxic apparatus. The surgical site was sterilized using povidone iodine and alcohol swabs. A midline sagittal incision was made from the naso-frontal suture line to the nares down to periosteum using a size 10 surgical blade. After the development of bilateral subcutaneous pockets, the nasal periosteum was reflected bilaterally. A high-speed drill was then used to thin the nasal bones to the point where the bone could be removed thereby exposing the basolateral (e.g submucosal) aspect of the olfactory mucosa. The skin incisions were then closed using a running 5-0 nylon running locking stitch. An 18 guage needle was then used to make a separate puncture into this submucosal potential space and inject the formulations (Figure 10.1).
2.3 Quantitative evaluation of BDNF AT delivery and subsequent protein up regulation in rat brain using the Olfactory mucosal depot surgery as compared to intranasal delivery.

Rats were dosed with 0.3 mg/kg BDNF AT in gel using the olfactory depot surgery as mentioned above and with 0.3 mg/kg BDNF AT in saline intranasally. For intranasal delivery, rats were anesthetized using 2.5% isoflurane and were placed in supine position. A 10µl pipette was used to deliver BDNF AT in saline in each nostril (20 µl in total with 10 µl in each nostril ). All rats were re-dosed every 72 hours for a total of 3 times and were sacrificed 3 days after the last dose (e.g 12 days after the initial dose). Brain tissues from the rats were removed and 3 mm tissue biopsy punches were used to isolate olfactory bulbs, striatum, hippocampus, substantia nigra and cerebellum. Ice-cold tissue homogenization buffer was used to homogenize the tissue samples and protein was extracted. AT hybridization assay (explained in chapter 4) was used to determine BDNF AT levels and BDNF specific ELISA (EMD Millipore CYT306) (explained in Chapter 4) was used to determine BDNF protein up regulation.

2.4 Quantitative evaluation of BDNF AT liposomes-in-gel (LiG) delivery in rat brain using the olfactory mucosal depot surgery as compared to BDNF AT in gel.

Figure 10. 1: A diagrammatic representation of the olfactory mucosal depot surgery.
BDNF AT liposomes were formulated and characterized as mentioned in **Chapter 4**. The formulated liposomes were suspended in 30% pluronic F-127 gel as mentioned in **Chapter 3**. Rats were then dosed with 0.15 mg/kg of BDNF AT in gel (100-120 µl) and BDNF AT LiG (150-200 µl) using the novel surgical method. All rats were sacrificed 6, 24, 48 and 72 hours after injection. Rat brains were extracted, and 3 mm tissue biopsy punches were used to isolate striatum, hippocampus, substantia nigra and cerebellum. Olfactory bulbs and liver were also collected along with plasma. AT hybridization assay (explained in Chapter 4) was used to determine BDNF AT levels.

### 3. Results and Discussion

3.1 The Olfactory depot surgery resulted in a significant delivery of BDNF AT’s in rat brain with a significant BDNF protein up regulation.

![Figure 10. 2: In vivo evaluation of BDNF AT delivery and protein up regulation in rat brain using olfactory mucosal depot surgery as compared to intranasal delivery:](image-url)
A) Bar graphs quantifying BDNF AT using hybridization assay in different parts of rat brain delivered by the depot surgery and intranasal delivery (* p<0.05, *** p<0.001; unpaired student T test) B) BDNF AT derepressed protein up regulation by depot surgery and intranasal delivery in the same end target sub-regions of the rat brain (* p<0.05, ** p<0.01, *** p<0.001; unpaired student T test). Data are represented as mean ± SD (n=3).

From Figure 10.2 (A), it can be seen that both the olfactory depot surgery (BDNF AT in gel) and intranasal delivery (BDNF AT in saline) resulted in a significant BDNF AT delivery in different parts of rat brain. However though not significant higher BDNF AT levels were detected by the depot surgery technique as compared to intranasal delivery. Similarly, both delivery techniques resulted in a significant BDNF protein up regulation in different parts of rat brain Figure 10.2 (B). However, the depot surgery resulted in a significantly higher BDNF protein up regulation in substantia nigra as compared to intranasal delivery technique.

Hence as the depot surgery resulted in delivery of detectable levels of BDNF AT in rat brain and also resulted in BDNF protein up regulation, we decided to evaluate the BDNF AT delivery in rat brain using a sustained release LiG formulation. The main objective of the study was to create a sustained release depot system over the exposed olfactory mucosa and deliver the AT’s to the brain efficiently as compared to only BDNF AT in gel.

3.2 The olfactory depot surgery successfully delivered detectable BDNF AT levels in different parts of rat brain with no significant difference between BDNF AT in gel and BDNF AT LiG formulations

BDNF AT levels were detected in different parts of the rat brain using both BDNF AT in gel and LiG formulations. Surprisingly there was no significant difference in the BDNF AT levels detected for both formulations at all time points. This could be attributed to the stable structure of BDNF AT oligonucleotides that protects it from degradation. As expected, higher levels of BDNF AT were detected in olfactory bulbs as compared to other parts of the rat brain. Also, it can be
observed that highest levels of BDNF AT were detected 6 hours after delivery which eventually starts declining after 24 hours and eventually plateaus at 72 hours.

Figure 10.3: \textit{In vivo} quantification of BDNF AT levels in different parts of rat brain at 6,24,48 and 72 hours using the olfactory depot surgery:

Graphs comparing the BDNF AT levels at 6,24,48 and 72 hours using hybridization assay in different parts of rat brain delivered by the depot surgery. There was no significant difference between the AT levels detected using the BDNF AT in gel and LiG formulations.
4. Conclusion

Our data confirms that the novel olfactory mucosal depot surgery can be used to deliver BDNF AT to the brain and eventually upregulate BDNF protein expression. We further show the depot surgery can deliver detectable levels of BDNF AT in rat brain as compared to intranasal drug delivery. We have also successfully shown that BDNF AT’s delivered using the olfactory mucosal depot surgery can results in a significant BDNF protein up regulation in all parts of brain with the exception of substantia nigra where the BDNF protein levels detected were significantly higher than intranasal delivery.

A surprising observation was that there was no significant difference between the BDNF AT’s delivered using BDNF AT in gel and LiG formulation. A similar pattern of diffusion was observed for both formulations. In conclusion, the olfactory depot surgery is a minimally invasive technique that can be used to deliver therapeutic levels of drug molecules to the brain. Moreover, given the ease of clinical translation of this drug delivery technique, it could open doors to new non-invasive methods of drug delivery to the CNS.

5. References

CONCLUDING REMARKS

Currently the development of an effective therapeutic option for neurodegenerative diseases like Parkinson’s disease and Alzheimer’s disease has failed to keep up pace with the considerable advances in our understanding of pathogenesis of these diseases. For example, Levodopa is the only treatment option available for PD patients since its discovery in 1961. This is mainly due to the BBB which limits 98% of all neuropharmaceuticals from reaching the Central Nervous System (CNS). Currently there is no method available that can permanently bypass the BBB. From the currently available drug delivery strategies, intranasal delivery is the only non-invasive method of delivery that takes advantage of direct pathway between the olfactory nerves and brain. However, intranasal delivery has its own limitations that has prevented it from being clinically translated. Hence there is a need of novel platform technologies that can help therapeutics bypass the BBB.

In my thesis project, we have successfully developed a trans-nasal mucosal drug delivery strategy as an alternative to intranasal delivery. We have shown that ovalbumin, a high molecular weight model protein can be successfully delivered to rat brain using this surgical technique (results published in *PLOS1*). Further we also showed that high molecular weight oligonucleotides like AntagoNAT’s can also be delivered to rat brain using this technique which can help up regulating endogenous proteins. For my project we have utilized BDNF AntagoNAT’s (AT’s) and we successfully showed that therapeutic levels of these BDNF AT’s can be delivered to rat brain and that the BDNF protein up regulation due to these AT’s can help protect dopaminergic neurons in a rat 6-hydroxydopmaine (6-OHDA) model of PD (manuscript under review in *Science Advances*). As an alternative method to the trans-nasal mucosal model we also developed an olfactory depot model where we created a depot within the submucosal space directly adjacent to the olfactory nerves to deliver a reproducible concentration and volume of therapeutic. In Conclusion, throughout the course of my thesis project we have successfully developed novel
drug delivery platforms to delivery therapeutics to the CNS by passing the BBB which will open doors to new CNS drug delivery techniques.