Transferring organelles into native neurons: A disease-modifying therapy for neurodegenerative disorders

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Anatomy and Cell Biology
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Abstract

Currently, there are no disease-modifying therapies to counter the progression of neurodegenerative diseases that are associated with mitochondrial dysfunction in the early stages. In this study, we have used a novel strategy of cell fusion to transfer mitochondria from one cell to another using fusogens (syncytin 1 and syncytin 2). Syncytins are placental proteins encoded by endogenous retroviral envelope genes that promote cellular fusion. In this study, we have proposed that donor cells engineered to stably express syncytin when cocultured with recipient cells will allow fusion and facilitate the transfer of mitochondria into recipient cells. Syncytin-mediated systems revealed about 16.6-18.5% cell fusion efficiencies in N2a and SH-SY5Y cells. The present work is proof that our strategy of engineering syncytin expression systems allows cell fusion in neurons.

Keywords: neurodegeneration, mitochondria, cell fusion, syncytins, fusion efficiency, heterologous system, engineered, progression, cocultured, mitochondrial transfer
Summary for Lay Audience

Neurodegenerative disorders are slow-progressing, profoundly depressing, and linked with poor quality of life. Environmental variables and age have a role in the course and clinical symptoms. Oxidative stress and the formation of misfolded protein aggregates are frequent and important aspects of neurodegenerative illnesses and result in mitochondrial dysfunction that often accelerates the development of neurodegenerative disorders, including AD and PD. So far, efforts to slow or stop cellular dysfunction by utilizing biologically active chemicals, tropic factors, and viral-mediated gene transfer have been mostly ineffective. Our long-term goal is to transfer mitochondria into dying neurons at an early stage of the disease, as this would slow the progression of the disease while maintaining the neuronal functionality of the damaged brain area. We proposed employing a new technique called cell fusion to introduce mitochondria (and maybe other organelles) from young neurons with the same characteristics into neurons that are experiencing organelle stress and malfunction. Cell fusion is linked to having more than one nucleus, and it is not known if cell functions are compatible, especially in neurons. If mitochondria could be moved to a host cell, it’s still not clear if they would join the existing network of organelles, live for a long time, and work properly. The goal of the current study is to find out if organelles, especially mitochondria, can be fused together and transferred between neurons. We used the placental proteins syncytins that code for endogenous retroviral envelope genes which maintain fusogenic function. In this study, we wanted to determine whether neurons allow the fusion and transfer of mitochondria. We did this by using two stable neuronal cell lines and in vitro methods to keep things simple and avoid the experimental variations that emerge with an in vivo approach. Syncytin-mediated cell
fusion between two different cell types showed about 18-20% of the cells were able to fuse and allow the transfer of mitochondria between the neuronal cells. The results of our research will pave the way for a new way to treat neurodegenerative diseases that will target the mechanisms that cause neuronal damage without affecting normal neuronal communication.
Acknowledgment

To begin with I would like to thank my supervisor Dr. Nagalingam Rajakumar for giving me an excellent opportunity to work on this novel project. It was an absolute pleasure, and I am humbled by your work ethic, great mentorship, and immense amount of knowledge. It was your guidance and support throughout the project which helped me to overcome the obstacles and challenges with the project. Your innovative expertise and dedication to the scientific community have made me a better person both professionally and personally.

I would like to extend my Heartfelt gratitude to my Co-Supervisor, Dr. Stephen J Renaud, for allowing me to work in his lab. I am grateful for the comprehensive learning and guidance throughout the course, which gave me the determination to complete the project on time.

I express my sincere thanks to my advisory committee members Dr. Martin Duennwald and Dr. John Ronald for their kind support, encouragement, honest feedback, and insightful suggestions.

In addition, I'd like to express my deepest gratitude to Dr. Martin Duennwald for providing me with neuronal cells to start my project and for guiding and helping me throughout my work.

Special thanks to Gargi Jaju Bhattad and Mariyan Jeyarajah, for their generosity and the insights they provided on several experiments. You all have been a great support throughout the course and shared your honest opinions, advice, and skills. I would like to take this opportunity to thank all the Renaud lab members who have been great collaborators to work in an enthusiastic and engaging team.
Finally, I would like to thank all my family members and friends for their unwavering support throughout my life and decisions. Your faith and belief have made me a better person and researcher.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone-Marrow-Derived</td>
</tr>
<tr>
<td>CT</td>
<td>Cytotrophoblast</td>
</tr>
<tr>
<td>Cuo</td>
<td>Cumate Expression system</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal Dementia</td>
</tr>
<tr>
<td>FP</td>
<td>Fusion Peptide</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial Cell-Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>hESC</td>
<td>Human Embryonic Stem Cells</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human Embryonic Kidney Cells</td>
</tr>
<tr>
<td>iPSCs</td>
<td>Induced Pluripotent Stem Cells</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>Levo-dopa (dopamine precursor)</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>MSCs</td>
<td>Human Mesenchymal Cells</td>
</tr>
<tr>
<td>mPTP</td>
<td>Mitochondria Permeability Transition Pore</td>
</tr>
<tr>
<td>N2a</td>
<td>Neuro-2a (mouse neuroblastoma cells)</td>
</tr>
<tr>
<td>NMDA</td>
<td>(N-methyl-D-aspartate) Antagonist</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s Disease</td>
</tr>
</tbody>
</table>
qRT-PCR  Quantitative RT PCR
RA  Retinoic Acid
ROS  Reactive Oxygen Species
SH-SY5Y  Human Neuroblastoma Cells
ST  Syncytiotrophoblast
SU  Surface Subunit
TH  Tyrosine Hydroxylase
TM  Transmembrane Subunit
6-OHDA  6 Hydroxy Dopamine
Chapter-1

1 Introduction

1.1 Neurodegenerative Disorders

Neurodegenerative disorders are characterized by the progressive degeneration of neurons leading to disconnection of brain areas and functional deficits. A gradual loss of neuronal cell populations characterizes neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS). In these chronic neurodegenerative disorders, neurons undergo slowly progressing metabolic dysfunction that may last over a decade followed by retraction of terminals leading to loss of synaptic connections and functional circuits. Neurodegenerative disorders also frequently show aggregation of abnormal protein in affected neurons, particularly in critical organelles such as mitochondria, as well as associated metabolic disturbances as a central pathology.

AD is a severely debilitating, progressive, and the most common neurological disorder accounting for 70-80% of all cases of dementia. AD is characterized clinically by the deterioration of cognitive function and alterations in behavior and personality. Cerebral cortical and hippocampal atrophy, the formation of neurofibrillary tangles consisting mostly of hyperphosphorylated forms of the microtubular protein tau, and the development of senile plaques are pathological hallmarks of AD that have been closely linked to synapse loss, neuronal degeneration and ensuing substantial brain shrinkage and debilitation.
PD is the second most common age-related neurodegenerative disorder, characterized by abnormal involuntary movements\(^\text{16}\). The major neurochemical hallmark of PD is the progressive degeneration of dopamine neurons of the substantia nigra pars compacta (SNpc) resulting in decreased dopamine levels in the striatum causing altered neuronal activity in basal ganglia centers\(^\text{16,17}\). Typically, clinical manifestations of PD appear in patients in their sixth decade and by then, more than 80% of SNpc dopamine neurons have degenerated\(^1\). Patients respond to dopamine replacement therapy in the form of L-DOPA/carbidopa combination for 10-15 years of initiating treatment and then develop drug resistance and severely debilitating side effects including dyskinesia and on-off phenomenon\(^\text{18}\). Several recent studies have aimed at finding reliable biomarkers for early detection of PD\(^{19-23}\).

### 1.1.1 Causes of Neurodegeneration

Neurodegenerative disorders are caused by a combination of genetic and environmental factors. Aging, oxidative stress, and accumulation of altered protein aggregates affecting mitochondria, endoplasmic reticulum, and other organelles in dopamine neurons result in a slowly progressing loss of neurons in PD (Figure 1.1)\(^5,7,9,24,25\). Several genetic mutations have been identified as predisposing to and facilitating the development of AD\(^{26-28}\). In AD, mitochondrial dysfunction may occur at the later stage of the disorder\(^\text{13,14}\). A loss of mitochondrial and endoplasmic reticulum function has been proposed for ALS and FTD\(^2\). The dysfunction of mitochondrial electron transport proteins and related enzymes has been linked to the pathophysiology of AD and PD\(^{29-32}\). Current evidence, therefore, point to
mitochondrial and endoplasmic reticular dysfunction as an important mechanism contributing to the pathogenesis of neurodegenerative disorders\textsuperscript{3}.

Figure 1.1 Factors affecting Neurodegenerative disorders
The representative image depicts factors that contribute to neurodegenerative disorders, with a particular focus on PD. Current evidence suggests that environmental factors and genetic risk factors contributing to oxidative stress, excitotoxicity, and mitochondrial
dysfunction in the brain leading to the degeneration of the midbrain dopaminergic neurons, resulting in PD. In PD, the formation of protein aggregates may disturb mitochondrial membrane potential and generate aberrant calcium influx, impaired respiratory enzyme activities, decreased ATP production, and elevated levels of reactive oxygen species (ROS). Moreover, the release of cytochrome C from damaged mitochondria may initiate the activation of apoptotic signaling cascade and the release of caspases, leading to neuronal death. The production of free radicals may also cause damage to cellular macromolecules and organelles by nitrosylation, oxidation, and peroxidation, directly contributing to neuronal injury. (The above image was taken from Barreto et al., 2015).

1.1.2 Current Treatment Strategies for AD and PD

Currently, there is no cure or disease-modifying therapy for neurodegenerative disorders. The treatment strategies are focused on controlling symptoms, and attempts are made to slow the rate of progress of the degenerative process in patients. The primary treatment strategy for AD has thus been to increase acetylcholine levels in the brain by using acetylcholinesterase inhibitors such as donepezil, galantamine, or rivastigmine to increase the levels of acetylcholine in synaptic clefts to counteract the loss of cholinergic terminals/neurons and hence restore cognitive function\textsuperscript{33}. Memantine, an NMDA (N-methyl-D-aspartate) antagonist, plays important roles in synaptic plasticity and neurodegeneration through BDNF and calcium signaling\textsuperscript{34,35}. Memantine has been used in patients with moderate to severe AD\textsuperscript{36}. However, none of these measures provide satisfactory clinical efficacy and are not effective in controlling the course of the disease in AD patients.
On the other hand, in PD patients, the only effective pharmacotherapy for the past 70 years is using L-DOPA, a dopamine precursor, along with the use of a peripheral DOPA-decarboxylase blocker. This form of replacing lost dopamine in basal ganglia centers is effective in controlling motor symptoms in the early stages of the disease\textsuperscript{37}. However, about 10-15 years into the treatment, patients develop debilitating dyskinesia, and many stop responding to L-DOPA treatment\textsuperscript{38}. Some of these patients may then be treated surgically with deep brain stimulation or grafting of fetal neuronal cells\textsuperscript{39,40}. Although deep brain stimulation is effective in certain patients, many are left with uncontrollable motor symptoms and debilitation\textsuperscript{41}. The efficacy of fetal neuronal grafting is very limited as well\textsuperscript{42,43}. At present, there are clinical trials underway to assess the efficacy of using dopamine neurons derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) and transplanting them into the dopamine-depleted striatum of PD patients (Figure 1.2)\textsuperscript{44,45}. None of these strategies, however, have been expected to modify the course of neurodegeneration in PD.
Figure 1.2 Cell replacement therapy for PD

Strategies for generating dopamine neurons from stem cells for transplantation in PD patients. Cell replacement therapy includes using either ESCs or iPSCs, differentiating them into dopaminergic neuronal phenotype and transplanting them into the dopamine-depleted striatum (Image taken from https://atlasofscience.org/stem-cell-therapy-for-parkinsons-disease/).

The main drawback of cell replacement therapy in neurodegenerative disorders is the failure to restore the lost normal afferent connection to neurons. For example, dopaminergic neurons of the substantia nigra normally receive afferent projections from several brain areas including feedback from the striatum, globus pallidus, serotonergic fibers from the raphe nucleus, and fibers from the hippocampus\textsuperscript{46-48}. Grafting cells into the striatum would replace some of the lost dopamine levels in the striatum but not any afferent connections that would normally come to dopamine neurons of the substantia nigra\textsuperscript{49}. Likewise, attempts to place dopamine neuronal grafts in the substantia nigra were unable to send new fibers to the striatum, hence failing to replace lost dopamine in the striatum\textsuperscript{50}. A major obstacle to achieving restoration of brain function by grafting cells in neurodegenerative disorders is therefore failure to achieve normal afferent modulation of grafted neurons while replacing the lost neurochemicals. Similarly, current pharmacological methods such as L-DOPA therapy in PD and anti-cholinesterase in AD, do not restore normal afferent modulation of neurons and restore normal neuronal circuitry. Consequently, none of the existing therapeutic strategies are curative, nor do they modify the course of the disease or fully restore normal brain function.
1.2 Mitochondrial Dysfunction in Neurodegeneration

Mitochondria are the powerhouses of cells. Each mitochondrion is interconnected with other mitochondria within the cell and the network is also connected to other organelles such as rough endoplasmic reticulum and lysosomes\textsuperscript{51,52}. Mitochondria are double membrane organelles with outer and inner membranes that are structurally and functionally distinct from one another. These membranes separate the intermembrane space from the matrix\textsuperscript{53}. The inner mitochondrial membrane houses the respiratory chain and oxidative phosphorylation complexes, which are essential to produce ATP through oxidative phosphorylation, and survival of the cell\textsuperscript{54}. Mitochondria store several molecules including calcium ions, iron, and lipids, which are involved in the synthesis of numerous metabolic intermediaries. Mitochondria also control inflammation and apoptosis in cells\textsuperscript{55,56}. In neurons, mitochondria play crucial roles in neuronal calcium homeostasis, maintaining membrane potential and excitability, synaptic function, neurotransmission, and plasticity. Mitochondrial DNA (mtDNA) encodes 37 genes and mitochondria possess their own transcriptional and translational machinery necessary for the expression of these genes\textsuperscript{57}. Due to their intricate biogenesis, function, and oxidative environment, these organelles are especially susceptible to progressive damage that may occur throughout the lifespan of a cell. Mitochondrial damage also disrupts energy production and metabolism, a decrease in ATP generation, an increase in reactive oxygen species (ROS), and a reduction in calcium buffering, all of which contribute to chronic neuronal dysfunction and may lead to neurodegenerative disorders. Schematics showing various mitochondrial pathways that may be disrupted in PD and various other neurodegenerative disorders are shown in Figures 1.3 and 1.4, respectively.
Figure 1.3 Mitochondrial dysfunction in PD

Representative pathways of mitochondrial dysfunction are implicated in the pathogenesis of PD. Mitochondrial dysfunction associated with PD pathogenesis may be caused by impairment of mitochondrial biogenesis, increased ROS production, compromised trafficking, electron transport chain dysfunction, altered mitochondrial dynamics, calcium imbalance, or a combination of these factors. (Image was taken from Park et al., 2018)\textsuperscript{58}. 
1.2.1 Mitochondrial Abnormalities in Neurodegenerative Disease

Mitochondria are crucial regulators of energy metabolism, neuronal function, and apoptotic pathways. Consequently, mitochondrial dysfunction compromises cellular viability and is a common feature of neurodegenerative diseases such as AD and PD. Neurons are very sensitive to mitochondrial abnormalities due to the essential role of these organelles in calcium buffering and ATP generation. During metabolic stress, various pathogenic pathways are triggered in mitochondria, including the opening of the mitochondria permeability transition pore (mPTP), leakage of cytochrome C into the cytoplasm, and activation of programmed cell death\textsuperscript{54,58}. Reduced mitochondrial ATP, mtDNA content, and mitochondrial protein levels (including cytochrome C enzymes and ATP synthase) have been described in AD and PD, along with increased levels of ROS, and abnormal size, shape, fragmentation, and function of mitochondria\textsuperscript{59-62}. An evolutionarily conserved pathway exists in all cells to monitor the integrity of mitochondria and perform quality control which is referred to as mitophagy. Mitophagy is crucial for degrading and disposing of defective mitochondria and this pathway is defective in AD and PD\textsuperscript{39}. In addition, mutations of mtDNA have been identified in AD and PD patients\textsuperscript{63,65,66}.
Mitochondria in a cell are considered a network, which is dynamic and exhibits complex behaviours. For example, mitochondria are capable of undergoing fusion when two separate mitochondria fuse to form one mitochondrion. Fission occurs when a mitochondrion divides or separates to form two mitochondria. Fission and fusion of mitochondria occur normally, controlled by dedicated sets of genes and proteins, and are essential for the repair, degradation, and recycling of damaged mitochondria and the regeneration of new mitochondria. Fusion allows for the rapid exchange of mitochondrial membranes, mtDNA, and metabolites within a network. Damaged mitochondria within the network can be mitigated and repaired through fusion with healthy
mitochondria by mixing contents. On the other hand, mitochondrial fission allows damaged mitochondria to be separated and eliminated via mitophagy. Abnormalities in genes and proteins controlling mitochondrial fission and fusion mechanisms have been described in several neurodegenerative disorders including AD and PD (Figure 1.5).

Figure 1.5 Mitochondrial dysfunction during aging and age-related disorders

Aging is associated with progressive mitochondrial dysfunction caused by the accumulation of mitochondrial DNA (mtDNA) mutations and increased ROS production, which causes oxidative damage to macromolecules, resulting in decreased respiratory chain activity and ATP production. Mitochondrial fission and fusion are essential for regulating mitochondrial activity, metabolism, and quality control. Age-related changes in
mitochondrial dynamics may limit mitophagy, resulting in the accumulation of damaged or dysfunctional mitochondria in cells\textsuperscript{71}.

### 1.3 Strategies to Restore Mitochondrial Abnormalities

Chemicals that neutralize and/or reduce the production of oxygen radicals that damage mitochondria have been used with either unknown or moderate beneficial effects. For example, a small molecular derivative of benzoquinone, which is structurally related to coenzyme Q10 that promotes mitochondrial ATP synthesis and has antioxidant properties, has been investigated in clinical trials of patients with chronic degenerative optic nerve atrophy caused by genetic mutations of mitochondrial genes\textsuperscript{72}. The potential application of this molecule has been proposed for AD and PD patients\textsuperscript{73,74}. Furthermore, in contrast to chemicals, gene therapy strategies are focused on replacing or correcting the mutated sequence of mtDNA using a wild-type copy of the altered gene. Both nonviral and viral-mediated approaches to deliver a corrected version of a mitochondrial gene have been attempted. Several promising results were reported in preclinical studies for conditions with single mutation\textsuperscript{75-78}. However, in several neurodegenerative conditions where genetic abnormalities are not clear in mitochondria-related genes, such as AD and PD, the potential efficacy of gene therapy is not inconclusive.

In this thesis, I sought to employ a novel strategy of replacing damaged mitochondria as a whole organelle rather than repairing them \textit{in situ}. This possibility was first identified in a co-culture setup when mitochondrial DNA from donor human mesenchymal stem cells (MSCs) was detected in mitochondria-free recipient cells\textsuperscript{79}. The mitochondria-free
recipient cells were generated by exposing human epithelial cells (A549 rho cells) to ethidium bromide, which depletes their mitochondria. These mitochondria-free epithelial cells were then cocultured with healthy MSCs. The transfer of mitochondria from MSCs to recipient cells appeared to restore certain mitochondrial functions. It was also established that mitochondrial uptake was an active transfer process\textsuperscript{79}.

Mitochondria dysfunction can result in cellular damage and death; hence, a rescue mechanism in which transplanting healthy mitochondria has significant potential. Other cell types, including fibroblast and somatic cells, have also demonstrated the ability to transfer/receive mitochondria\textsuperscript{79}. Mitochondrial donation by MSCs is potentially an efficient physiological mechanism in the cell to replace malfunctioning mitochondria, as opposed to mitochondrial biogenesis. Spees et al. (2006) reported as early as 2006, that mitochondrial transfer between normal cells could occur via the formation of tunnel tubes\textsuperscript{80}. Rustom et al. (2004) showed a \textit{de novo} development of several tunnel tubes that facilitated the passage of membrane vesicles and organelles across cells leading to the formation of complex networks between cells\textsuperscript{81}. In addition to tunnel tubes, mitochondrial transfer via gap junctions, microvesicles, and cell fusion has been proposed (Figure 1.6). Most investigations of mitochondrial transfer efficiency have been conducted using \textit{in vitro} co-culture systems where detection of tunnel tube development is more easily detected. Different mechanisms of transfer have been observed under different pathological situations. The proximity of damaged cells and environmental conditions may regulate the mode and efficiency of mitochondrial transfer\textsuperscript{79}. 
Figure 1.6 Possible modes of mitochondrial transfer

Proposed routes of mitochondrial translocation from MSCs to damaged or wounded cells. Included are transfer via intracellular nanotubes, gap junctions, cell fusion, microvesicles, and direct ingestion of mitochondria. Interestingly, and relevant for this thesis, cell fusion may facilitate the transfer of mitochondria from a host cell into a damaged cell. (Image was taken from Paliwal et al., 2018)⁷⁹.
1.4 Cell Fusion

Cell-cell fusion is a highly controlled and complex process that is essential for growth and homeostasis. Cell-cell fusion is poorly understood, although the fusion of viruses to somatic cells has been a topic of intensive research during the past decade. Cell fusion also seems to be involved in development of cancer and in the regeneration of certain types of cells. In humans, cell-cell fusions occur during embryogenesis as well as in mature adults. Several cell types undergo fusion as part of physiological development. During pregnancy, the fusion of cytotrophoblasts produces syncytiotrophoblast, a massive cell with over 10 billion nuclei that serve as the primary feto-maternal interface. Skeletal muscles are made up of bundles of multinucleated myofibers that form by the fusion of mononucleated myoblasts. Myoblast fusion is required for the maintenance, growth, and regeneration of myofibers. During regeneration, skeletal muscle fibers fuse with satellite cells. Macrophages merge to produce multinucleated giant cells with improved phagocytic capabilities in response to injury and antigen stimulation. There is also evidence that cell fusion contributes to tissue repair in adults. For instance, bone-marrow-derived cells may fuse with hepatic cells, nerve cells, and gastrointestinal cells, and it has been hypothesized that such fusions may facilitate in repairing damaged cells.
1.5 Syncytins

Under normal conditions, cell fusion occurs in only a few cell-types in mammals, including gametes, myoblasts, osteoclasts, and placental cytotrophoblast cells. In all cases, fusion is mediated by specific proteins that stimulate close apposition of cell membranes, therefore creating a fusion pore and interrupting the continuity of the lipid bilayers. The best-studied fusion proteins and fusion mechanisms are during the formation of the syncytiotrophoblast of the placenta. The endogenous retroviral proteins, syncytin 1 and syncytin 2, are required for human cytotrophoblast fusion to generate syncytiotrophoblast. This fusogenic restructuring is triggered by interactions of syncytin 1 and syncytin 2 with their receptors, the ubiquitous neutral amino acid transporters ASCT1/2 (syncytin 1 receptor) and major facilitator superfamily domain containing 2A (MFSD2A; syncytin 2 receptor)87.

During evolution, numerous endogenous retroviruses have been incorporated into the DNA of eukaryotic organisms, approximately 10% of the human genome, is comprised of endogenous retrovirus elements. Syncytins are glycoproteins encoded by endogenous retroviral envelope genes embedded in human DNA that have been co-opted for a crucial function in placentation. Two fusogenic syncytins have been identified in humans: syncytin 1 (encoded by ERVW-I) and syncytin 2 (encoded by ERVFRD-I). Syncytin 1 and syncytin 2 are synthesized as inactive precursors, which are then cleaved into functional subunits: surface subunit and transmembrane subunit. The surface subunit is responsible for receptor binding and the transmembrane subunit consists of a fusion peptide that mediates fusion with cells expressing the appropriate receptors88. Although syncytins are typically only expressed in the placenta, syncytin receptors are ubiquitously expressed in many cell-types.
Therefore, induction of syncytin expression (e.g., using plasmid-based systems) has successfully stimulated fusion in a variety of nonplacental cell-types \textit{in vitro}^{87,88}.

1.6 Rationale

Neurodegenerative disorders are common and characterized by slowly progressive pathogenesis. Despite their debilitating course and clinical manifestations, no curative or disease-modifying treatments are currently available. Current strategies aimed at treating neurodegenerative disorders such as AD and PD attempt to restore lost neurochemicals at target areas of the brain without restoring the lost afferent modulation and damaged neuronal circuitry. Dysfunction of mitochondria is an early and crucial abnormality driving degenerative changes in neurons. Attempts to improve mitochondrial function using pharmacological agents and viral-mediated gene transfer have not yet shown promise. Our long-term goal is to transplant mitochondria into degenerating neurons at the early stages, thereby slowing the disease progression and maintaining neuronal circuitry and afferent modulation of the damaged brain area. We propose to efficiently transfer healthy mitochondria to damaged neurons by fusing them with young neurons (differentiated from pluripotent cells) engineered to conditionally express syncytins. This is a novel strategy as a fusion with neurons, a terminally differentiated cell type, has never been attempted before. Therefore, as a proof of principle, the current study aims to determine whether neurons allow the fusion and transfer of mitochondria from a donor cell \textit{in vitro}.

If successful, the current strategy could slow the course of neurodegeneration in these disorders and allow normal functioning of neuronal circuitry and afferent modulation of neurons for an extended period, perhaps several years. This novel disease-modifying
approach could ensure prolonged symptomatic relief and improved quality of life. The current study is designed to test whether engineering syncytin expression in a heterologous system to stimulate neuronal cell fusion will achieve a significant transfer of mitochondria.

1.7 Hypothesis

I hypothesize that donor cells, engineered to conditionally express syncytin 1 or syncytin 2, are capable of fusing with recipient neuronal cells and transferring mitochondria.

1.8 Aim

My aim is to determine whether neuronal cell lines, N2a and SH-SY5Y, engineered to conditionally express syncytin 1 or syncytin 2, allow fusion and transport of mitochondria into cocultured recipient neuronal cells in vitro while maintaining normal phenotype.

1.9 Specific Objectives

Current studies (as represented in Figure 1.8):

1. To stably over-express syncytin 1 and syncytin 2 in neuronal cell lines N2a and SH-SY5Y.
2. To clone syncytin 1 and/or syncytin 2 into an inducible expression system and to determine fusion efficiency in neuronal cells.
3. To determine the expression of fusogens and transfer of organelles in cocultured neurons in pilot studies.
Pilot concurrent studies for future experiments

In order to further develop these cellular models as relevant systems for PD, I piloted several other experiments. Specifically, I differentiated human ESCs and SH-SY5Y cells into dopaminergic neurons so that they can be used as appropriate donor cells in future experiments. The efficiency of differentiation into dopaminergic neurons is reported in this thesis. These cellular models will be advantageous in future studies to test the efficiency of cell fusion and mitochondrial transfer using a relevant neuronal cell type.

Objectives:

1) To confirm that syncytin-mediated cell fusion allows the transfer of organelles between cocultured neurons.

2) To differentiate and characterize human embryonic stem cells (hESCs) and SH-SY5Y cells into dopaminergic neurons.

3) To stably express syncytin 1 and syncytin 2 in these differentiated neurons.

4) To establish an in vitro model of PD using a population of these differentiated dopaminergic neurons.

5) To determine whether a mitochondrial transfer occurs between engineered dopamine neurons and the PD model of dopaminergic neurons leading to functional recovery in damaged neurons using coculture studies.
Figure 1.7 Diagrammatic representation of the experimental approach
Chapter-2

2. Methods

2.1 Cell Culture and Maintenance

2.1.1 Neuronal cells

All neuronal cells used in this study were cultured in a humidified incubator at 37°C, with an atmosphere consisting of 5% CO₂. Neuro 2a cells (N2a; mouse neuroblastoma cells) were kindly provided by Dr. Martin Duennwald (University of Western Ontario). N2a cells show neuronal and amoeboid stem cell morphology and have been extensively used in studies aimed at evaluating neuronal differentiation, axonal growth, and neurotoxicity\textsuperscript{89-91}.

N2a cells were maintained in Dulbecco’s Modified Eagle Medium- high glucose (DMEM-HG) supplemented with 10% fetal bovine serum (FBS, ThermoFisher Scientific), 100 units/mL penicillin, and 100 µg/ml streptomycin.

SH-SY5Y cells were kindly provided by Dr. Martin Duennwald (University of Western Ontario). The SH-SY5Y cell line is a thrice subcloned cell line derived from the SK-N-SH neuroblastoma cell line, which were originally derived from a bone marrow biopsy of a 4-year-old female patient with neuroblastoma\textsuperscript{92}. SH-SY5Y cells show dopamine neuronal markers and are consequently used in several in vitro studies of PD particularly the role of mitochondrial dysfunction in PD\textsuperscript{93-97}.

SH-SY5Y were maintained in DMEM-HG supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/ml streptomycin.
Both N2a and SH-SY5Y cell lines were passaged when cells reached 70-80% confluence, typically every 3-5 days. To passage cells from a T-25 flask, media were aspirated, and then 1000 µl of 0.05% Trypsin-EDTA were added and incubated for 4 min at 37°C in a humidified incubator. The trypsin was neutralized by adding FBS-containing growth media and triturated 2-3 times. Media containing suspended cells were collected and spun for 5 min at 400g. After centrifugation, the supernatant was aspirated, and the pelleted cells were resuspended in 4 ml basic growth media. 5 ×10⁴ cells (approximately 10% of the cells) were then replated in a T25 flask.

2.1.2 Human Embryonic Kidney Cells

Human embryonic kidney (HEK)-293T cells were obtained from American Type Culture Collection (CRL-3216). HEK-293T cells were originally derived from embryonic kidney cells of an aborted fetus in 1973 and have been used widely for their reliable growth and readily transfectable properties. We employed them as a non-neuronal cell type to serve as a positive control. HEK293T cells were maintained in DMEM-HG medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. As described above for neuronal cells, cells were passaged by light trypsinization prior to reaching confluency and maintained in a humidified cell culture chamber at 37°C and 5% CO₂.
2.2 Overexpression studies of Syncytin1 and Syncytin 2

2.2.1 Transfection

To ectopically express fusogens, cells were transfected with syncytin 2 (encoded in a PLX304 backbone) or syncytin 1 (encoded in a PLentiV6.3 backbone) using Lipofectamine 2000 (ThermoFisher Scientific), according to a protocol provided by the manufacturer with slight modifications. Briefly, the day before transfection, cells were seeded so that they would be 75-85% confluent the next day. Then, 1 h before transfection, basic growth medium was replaced with Opti-MEM (ThermoFisher Scientific) supplemented with 7.5% FBS. Lipofectamine mix and plasmid mix were prepared in 1:1 ratio separately in Opti-MEM, mixed together, and incubated for approximately 10 min at room temperature. The lipofectamine:DNA complexes were then added to cells in a dropwise manner and incubated at 37°C overnight. Media were then removed and replaced with basic growth media, and cells were incubated for up to 48 h.

2.3 Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed thrice in 1×PBS for 5 min each, permeabilized using PBS containing 0.3% Triton-X and 1% bovine serum albumin for 15 min at room temperature, and then subjected to another three washes in 1×PBS for 5 min each. Nonspecific antibody binding was blocked by incubating cells in 10% normal goat serum (ThermoFisher Scientific), and then cells were probed with the primary antibodies overnight at 4°C. Dilutions and catalog numbers of the primary
antibodies used for immunofluorescence are listed in **Table 2.1**. The next day, cells were incubated with Alexa Fluor 555 conjugated anti-mouse or Alexa Fluor 488 conjugated anti-rabbit or Alexa Fluor 646 conjugated anti-goat secondary antibodies (all from ThermoFisher Scientific). Nuclei were counterstained using DAPI. Images were captured using a Zeiss Axio Vert.A1 microscope. GFP (Zeiss Filter Set 38, BP 470/40-FT 495-BP525/50*), DAPI (Zeiss Filter Set 02, G 365-FT 365-BP525/50*), and Texas Red (Zeiss Filter Set 15, BP 546/12-FT 580-LP 590*) filters were used for imaging, and Zeiss Axiocam 506 mono (6MP, high sensitivity, low-light, monochromatic) camera used for fluorescence imaging. Objectives 20x, 40x and 63x magnifications were utilized for imaging.

For the quantification of nuclei in fused cells, images were taken from three randomly selected fields per well in triplicate at 20x magnification. The percent fusion was calculated by counting the total number of nuclei contained within fused cells (lacking clear cell boundaries as demarcated by phalloidin staining) divided by the total number of nuclei and then multiplying by 100.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>CAT #</th>
<th>Company</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>V5</td>
<td>Mouse</td>
<td>80076S</td>
<td>CST</td>
<td>1:200</td>
</tr>
<tr>
<td>Phalloidin</td>
<td>Mouse</td>
<td>8953S</td>
<td>CST</td>
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</tr>
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<td>TH</td>
<td>Rabbit</td>
<td>22595</td>
<td>Abcam</td>
<td>1:200</td>
</tr>
<tr>
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<td>Goat</td>
<td>AF2400-SP</td>
<td>Cedarlane</td>
<td>1:200</td>
</tr>
<tr>
<td>LMX1A</td>
<td>Mouse</td>
<td>50.5A5-s</td>
<td>DSHB</td>
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<tr>
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<td>Goat</td>
<td>AF1979-SP</td>
<td>Cedarlane</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2.1 List of primary antibodies used for immunofluorescence

2.4 Quantitative RT-PCR

For quantitative RT-PCR, RNA was extracted using Ribozol (VWR International), according to the manufacturer’s instructions. RNA was converted into cDNA using reverse transcription (High Capacity cDNA kit, ThermoFisher Scientific), which was then diluted 1:10. Quantitative RT-PCR was performed by amplifying cDNA using Sensifast SYBR Green PCR Master Mix (FroggaBio), and primers listed in Table 2.2. A CFX96 Connect real-time PCR detection system (Bio-Rad Laboratories) was used to detect fluorescence. Cycling conditions involved an initial holding step (95°C for 10 min), followed by 40 cycles of a two-step PCR (95°C for 15 s and 60°C for 1 min), and a dissociation phase. Relative mRNA expression was calculated using the ΔΔCt method. The geometric mean
from three constitutively expressed reference genes (*RNA18SN1, GAPDH, YWHAZ*) was used as reference RNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primers (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ERVW-1</em></td>
<td>CTCCCTAGCAGCAGTAGTCC</td>
<td>TCCAGTGTTCGAAGCTCCT</td>
</tr>
<tr>
<td><em>ERVFRD-1</em></td>
<td>CCAAATTCCCTCCTCCTCCTC</td>
<td>CGGGTGTTAGTTTGGTGT</td>
</tr>
<tr>
<td><em>TH</em></td>
<td>CACCAAGTTTGACCCTGACC</td>
<td>GGAATGGCTCACCCTGCTT</td>
</tr>
<tr>
<td><em>RNA18SN1</em></td>
<td>GCAATTATCCCCCATGAACG</td>
<td>GCCCTCACTAAACCATCCAA</td>
</tr>
<tr>
<td><em>GAPDH</em></td>
<td>TTGAGGCTCAATGAAGGGGT</td>
<td>GAAGGTGAAGGTCGGAGTCA</td>
</tr>
<tr>
<td><em>YWHAZ</em></td>
<td>ATGCAACCAACACATCCTATC</td>
<td>GCATTATTAGGTGCTGTCT</td>
</tr>
<tr>
<td><em>ACTB</em></td>
<td>CCTTGCACATGCCGAG</td>
<td>GCACAGAGCCTCGGCTT</td>
</tr>
</tbody>
</table>

Table 2.2 List of primers used for qRT-PCR analysis

2.5 Cloning of Syncytin 1 and Syncytin 2 into a Cumate Inducible Expression System

Human syncytin 2 and syncytin 1 were cloned into a SPARQ cumate-inducible lentiviral expression system (Fig 2.1; Systembio). Cumate has high dynamic reproducibility and can be finely tuned for inducible and reversible expression. A restriction enzyme cloning strategy was performed to subclone syncytin 1 and syncytin 2 into the SPARQ plasmid. Cloning into the SPARQ vector was conducted with generous support from Dr. Patrick Lajoie (University of Western Ontario). First, primers were designed flanking human
ERVW-1 (from PLentiV6.3) and ERVFRD-1 (from PLX304) with appropriate restriction sites 5’NheI and 3’NotI, and PCR amplified. Conventional PCR was conducted using primers described in Table 2.3. DreamTaq DNA Polymerase (ThermoFisher Scientific) was used to amplify DNA. Cycling conditions involved an initial holding step (95 °C for 3 min), followed by 32 cycles of PCR (95°C for 30 s, 55-63°C for 30 s, and 72°C for 30 s), and a final elongation phase at 72°C for 12 min.

Figure 2.1 Cumate-inducible expression system
Table 2.3 List of components used for PCR amplification

<table>
<thead>
<tr>
<th>S.No</th>
<th>Components</th>
<th>Quantity (25 µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA</td>
<td>2 µl</td>
</tr>
<tr>
<td>2</td>
<td>dNTPs</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>3</td>
<td>Primers (Fwd &amp; Rev)</td>
<td>2 µl</td>
</tr>
<tr>
<td>4</td>
<td>Dream Taq Polymerase</td>
<td>0.125 µl</td>
</tr>
<tr>
<td>5</td>
<td>Dream Taq Buffer</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>6</td>
<td>DNase/RNase-free H₂O</td>
<td>18.9 µl</td>
</tr>
</tbody>
</table>

PCR products were resolved on 1.5% agarose gels, imaged using a ChemiDoc imaging system (Bio-Rad Laboratories) and DNA extracted and purified from the agarose gel using a Geneaid PCR purification kit (FroggaBio). The purified products were restriction digested overnight with 5'Nhe1 and 3'Not1 for syncytin 2 at 37°C and purified products were ligated at room temperature for 2 h. Ligated products were amplified by transforming into NEB Stable Competent E.Coli (High Efficiency; New England Biolabs) according to the manufacturer’s instructions. Bacteria were lysed and plasmids were extracted using a Geneaid mini-prep plasmid kit (FroggaBio). Verification of the correct incorporation of syncytin 1 and syncytin 2 was determined by sequencing.
<table>
<thead>
<tr>
<th>S.No</th>
<th>Restriction Site</th>
<th>Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5’ Nhe1 (PLX304)</td>
<td>GAGCTCTCTTGCTAGCTGTCGG</td>
</tr>
<tr>
<td>2</td>
<td>3’ Not1 (PLX304)</td>
<td>CCACCGGTTAGCGGCGGCTCATTA</td>
</tr>
<tr>
<td>3</td>
<td>5’ Nhe1 (Plenti 6.3)</td>
<td>ACGCCATCCACGCTAGCTTGACCT</td>
</tr>
<tr>
<td>4</td>
<td>3’ Not1 (Plenti 6.3)</td>
<td>AGCACAGTGCGGCGGCGCTCGAGTCT</td>
</tr>
</tbody>
</table>

Table 2.4 List of primers containing restriction enzyme sites used for amplifying ERVW-1 and ERVFRD-1 from Plenti6.3 and PLX304 plasmids, respectively.

2.6 Lentiviral Production and Transduction

Lentiviral plasmids (MD2.G, MDLG/RRE, and RSV-Rev) were used to produce lentivirus. HEK293T cells were transfected using Lipofectamine 2000 (ThermoFisher Scientific) in Opti-MEM medium (ThermoFisher Scientific) with the syncytin 2-cloned SparQ vector and packaging plasmids. The following morning, Opti-MEM containing the transfection mix was removed and replaced with a normal growth medium. Thereafter, culture supernatants containing lentivirus were collected every 24 h for a total of 48 h, centrifuged, and stored at −80°C until use. N2a and SH-SY5Y cells were exposed to lentiviral particles and centrifuged at 1000g for 2 h at room temperature in the presence of hexadimethrine bromide (8 μg/ml, Sigma-Aldrich). After the centrifugation, virus-
containing media were removed and neuronal cells were resuspended in a normal growth medium and plated. After 48 h, transduced cells were selected using puromycin (1 μg/ml, ThermoFisher Scientific) for at least 48 h. A negative control well containing cells not exposed to the virus (and therefore sensitive to puromycin) was used to ensure efficient transduction and selection.

2.7 Cumate inducible expression system

To determine the expression of syncytin 2 and the optimum effective dose of cumate solution, N2a and SH-SY5Y cells transduced with the cumate-inducible syncytin 2 plasmid (N2a-Syn2 and SHSY-Syn2) were seeded at a density of about 5×10⁴ cells and cultured for 24 h. Six different concentrations of cumate solution ranging from 5 to 150 µg/ml were added after 24 h and the cells were cultured for 24 h and analyzed. Two controls were utilized, “C” represents control cells engineered with the cumate-inducible syncytin 2 plasmid but not exposed to cumate solution. “Cuo C” represents an empty cumate vector (lacking syncytin 2) treated with cumate solution (150 µg/ml).

2.8 Statistical Analysis

Statistical comparisons between three or more means were performed using an unpaired student’s t-test or a one-way ANOVA followed by Tukey’s multiple comparison test. All experiments were repeated at least three independent times. Means were considered statistically different if the P-value was less than 0.05. GraphPad Prism 9.0 was used for all graphing and statistical analyses.
2.9. Pilot Studies:

2.9.1 Differentiation of SH-SY5Y cells into Dopaminergic Neurons

I performed pilot experiments to optimize the differentiation of SH-SY5Y human neuroblastoma cells into dopaminergic neurons. Before differentiation, SH-SY5Y cells (passage 13) were cultured and maintained as described in section 2.1.1. On the day of differentiation, the cells were seeded at a density of about $8 \times 10^4$ cells for molecular analyses (western blot and qRT-PCR) and $1 \times 10^4$ cells for immunofluorescence. The medium was changed to DMEM supplemented with 1% FBS, along with Retinoic acid (RA) (10 µM). Media were replaced with fresh differentiation media every 2-3 days. After 5 days, levels of Tyrosine Hydroxylase (TH) were measured using qRT-PCR, immunofluorescence, and western blot as a marker for dopamine neurons.

2.9.2 Characterization and Differentiation of hESCs into Dopaminergic Neurons

Human embryonic stem cells (hESCs) were purchased from WiCell (WA09, P26). To differentiate the cells into dopaminergic neurons, we used a 42-days differentiation protocol first described by Nolbrant et al. (2017). This method yields a large number of high-purity dopamine neurons virtually indistinguishable from fetal mesencephalic dopamine neurons. Before differentiation, ESCs were cultured and maintained in mTeSR$^{\text{plus}}$ (StemMACS, Miltenyi). On day one of differentiation, the media was changed from mTeSR$^{\text{plus}}$ to differentiation medium. The neutralization and patterning were achieved by CHIR99021 (GSK3 inhibitor, (0.8µM) which activates Wnt signaling) and
sonic hedgehog (300ng/ml) until day 8. Fibroblast growth factor 8 b (FGF8b) (100ng/ml), which caudalizes ventral midbrain progenitors, and neurotrophic factors (Brain-derived neurotrophic factor, BDNF (20ng/ml); Ascorbic acid, AA (0.2mM) were added to the cultures to expand the progenitors from day 9 to 16. On day 16, patterned midbrain dopamine neuronal precursor cells were characterized by qRT-PCR and immunofluorescence using LMX1, FOXA2, and OTX2 antibodies. Cells were terminally differentiated into dopaminergic neurons until day 42. Terminally differentiated cells were tested for mature dopamine neuronal markers such as TH, LMX1, and MAP2, and cryopreserved for future experiments. 
Future Study

2. 10 6-OHDA – SH-SY5Y: an in vitro PD Model

Human neuroblastoma (SH-SY5Y) cells exhibit neuronal morphology and are vulnerable to the toxic effects of mitochondrial toxins, ROS, and 6-hydroxydopamine (6-OHDA). They have thus been used extensively as a model of neurotoxicity of relevance to PD. To develop this in vitro PD-like model, cells will be exposed to different concentrations of 6-OHDA and incubate for 24 h at 37°C in a 5% CO₂ atmosphere. In addition, toxicity assays, mitochondrial potential, and ROS levels will be measured in the Parkinson-like cell model. This cellular model will be used for future fusion studies to determine the potential for mitochondria transfer-mediated neuronal recovery.
Chapter- 3

3 Results

3.1 Overexpression of Syncytins

Syncytins are the placental proteins that promote cell fusion and the formation of syncytiotrophoblast, a multinucleated giant syncytia that facilitates the exchange of nutrients between maternal and fetal blood. These placental fusogens are not normally present in other cell types. To determine the expression of placental proteins syncytin 1 and syncytin 2 in neuronal cells, we used two neuronal cell lines SH-SY5Y (Human Neuroblastoma Cells) and N2a (Mouse Neuroblastoma Cells). Easily transfectable HEK293T (Human Embryonic Kidney Cells) were also assessed as a positive control. None of these cells showed evidence of endogenously expressing high levels of the genes encoding syncytins, based on high Cq values when analyzed by qRT-PCR (Fig 3.1). The Cq value is the PCR cycle number at which the sample's reaction curve intersects the threshold line, hence the Cq value indicates how many cycles it took to detect a signal from that sample. Both mouse and human neuronal cells are fast-growing, and our results indicate that they possess good transfection efficiency. We transiently transfected Plenti6.3 - syncytin 1 and PLX304 - syncytin 2 in all three cell lines, incubated them for 48 h, and analyzed the relative expression of fusogens. All three cell lines showed a significant increase in the expression of syncytin 1 and syncytin 2 ($P<0.0001$; Fig 3.1 A, B). N2a and SH-SY5Y cells transfected with the syncytin 1 expression plasmid showed an average of 150 and 655-fold increase in syncytin 1 expression, respectively. For HEK293T, there was an approximate 9.5-fold increased expression in these cells after transfecting the plasmid.
encoding syncytin 1. Similarly, compared to cells transfected with control plasmids, N2a and SH-SY5Y cells transfected with the synctin 2 expression plasmid had a 62 and 380-fold increased expression of syncytin 2, respectively. HEK293T cells showed 6.5 times increased synctin 2 expression. Importantly, when transfecting syncytin 1 or syncytin 2, there was no detectable change in the expression of the other syncytin. Our results indicate that both syncytin 1 and syncytin 2 mRNA were successfully increased in all three types of cells following transfection with the appropriate expression plasmids in comparison to controls.

To determine the expression of syncytins at the protein level, immunofluorescence was performed in all three cell lines transfected with syncytin 1. Syncytin 1 terminus contains a V5 tag. Therefore, levels and localization of syncytin 1 can be detected using V5 staining. For HEK293T cells, we employed red immunofluorescence to label the V5 tag, while green immunofluorescence labeling was used to label N2a and SH-SY5Y cells. Cell nuclei were labeled using DAPI. Our results indicate that HEK293T cells and both neuronal cell types showed increased syncytin 1 expression (Fig 3.2 A). Observation at high magnification revealed that two or more nuclei were found within V5-labeled representative fused cells showing syncytin 1 expression (Fig 3.2 B). Together, the expression of syncytin 1 at the protein level was effective and representative of successful cell fusion. Our attempts to quantify protein levels using western blotting were not successful because we were unable to optimize antibodies that could reliably detect either syncytin 1 protein or V5 tag. Similarly, attempts to label syncytin 2 expression at the protein level were also not successful in our hands, possibly due to a lack of reliable antibodies.
Figure 3.1 Overexpression of syncytin 1 and 2 in neuronal cells

HEK293T, N2a, and SH-SY5Y cells were transfected with Plenti6.3 syncytin 1 and PLX304 syncytin 2, incubated for 48 h, and qRT-PCR was performed to detect the expression of syncytin 1 and syncytin 2 (A and B; N=3, each). A represents syncytin 1 expression in HEK293T, N2a, and SH-SY5Y cells. B indicates syncytin 2 expression in the three cell lines. Cells transfected with an empty PLX304 vector were used as a control. Asterisks (*) are used to denote statistical significance (***, P<0.001; ****, P<0.0001) from controls using one-way ANOVA followed by Tukey’s multiple comparison test. Graphs show the mean +/- standard error of the mean (SEM).
V5
HEK293T  N2a  SH-SY5Y

DAPI/V5

B
HEK293T  N2a  SH-SY5Y
**Figure 3.2 Syncytin 1 overexpression and cell fusion**

The syncytin 1-Plenti6.3 plasmid encodes syncytin 1 fused with a terminal V5 tag. Therefore, immunofluorescence for V5 was used to identify cells transfected with syncytin 1 (A and B). Representative immunofluorescence images depicting V5 staining of syncytin 1-transfected HEK293T cells in red, while transfected N2a and SH-SY5Y cells are shown in green. Panel B shows representative high-magnification images with two or more nuclei with V5 staining. White arrows indicate a cell with two or more nuclei. Scale bars represent 50 µm in panel A and 20 µm in panel B.

### 3.2 Cloning of Syncytins into an Inducible Expression Vector

In order to generate an inducible and reversible expression of fusogens, syncytin 1 and syncytin 2 cDNA within the Plenti6.3 and PLX304 plasmids were subcloned into a cumate-inducible expression system. Cumate has high dynamic reproducibility and it can be finely tuned to be reversible and sensitive based on the dose and timing of cumate exposure. Unfortunately, cloning of syncytin 1 into the cumate-inducible vector was not successful so we proceeded with experiments using cumate-inducible syncytin 2. Cloned syncytin 2 was transiently transfected into HEK293T and N2a cells. Cumate solution was added after 24 h to determine whether expression of syncytin 2 was induced. The cumate vector also contains GFP which is separately translated from syncytin 2 due to an internal ribosome entry site; hence, when cumate is introduced, the gene of interest is expressed, and green fluorescence is emitted. An empty cumate vector was employed as a GFP control (Fig 3.3A). Both HEK293T and N2a cells showed increased GFP expression, demonstrating
that the cumate-inducible promoter was indeed activated after exposure to cumate. Using qRT-PCR, we examined the expression of syncytin 2 in HEK293T and N2a cells (Fig 3.3 B). Both cell lines showed a marked increase in syncytin 2 expression. Compared to control cells exposed to cumate, transfected cells exposed to cumate showed 88 times more syncytin 2 expression in N2a cells, whereas HEK293T showed 1,500-fold increased syncytin 2 expression.
A

HEK293T

UT  Control (No cumate)  Cuo-Syn2 (Cumate)

N2a

UT  Control (No cumate)  Cuo-Syn2 (Cumate)

B

HEK293T

N2a

<table>
<thead>
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<th>Syncytin 2</th>
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<td><img src="N2a.png" alt="Graph" /></td>
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</table>

<table>
<thead>
<tr>
<th>UT</th>
<th>C</th>
<th>Cuo-Syn2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1500</td>
<td>2000</td>
</tr>
</tbody>
</table>

**Significance:** ****
Figure 3.3 Transient expression of Cumate-Syncytin 2

(A) Representative fluorescent images depict GFP fluorescence in HEK293T and N2a cells transfected with the cumate-inducible promoter. An empty cumate vector without the addition of cumate was used as a control for GFP fluorescence. CuO-Syn2 represents the cells transfected with a syncytin 2 engineered cumate vector. (B) Relative expression of syncytin 2 was assessed through qRT-PCR analysis in untransfected cells (UT), cells transfected with an empty cumate vector (C) without the addition of cumate solution, and cells transfected with CuO-Syn2. All cells were treated with 50µg/ml cumate for 36h prior to analysis. (N=3). Asterisks (****) represent significantly different groups (P<0.0001) using one-way ANOVA followed by Tukey’s multiple comparison test. Graphs show the mean +/- standard error of the mean (SEM). Scale bar=50 µm.

3.3 Stable Expression of Cumate-Syn2

To create a stable syncytin 2 engineered cell population and to determine the optimum dose of cumate, we employed lentivirus for transduction, and the transduced cells were selected using puromycin. Using qRT-PCR, the optimal concentration of cumate solution was determined for maximal expression of syncytin 2. Six different concentrations of cumate solution ranging from 5 to 150 µg/ml were used and cells were cultured for 24 h. For this experiment, syncytin 2-transduced cells not receiving cumate solution, and an empty cumate vector provided with cumate solution, were employed as two controls in this study. Cells stably transduced with the cumate-inducible syncytin 2 plasmid showed a significant increase in the expression of syncytin 2 following exposure to cumate in a dose-dependent
manner (P<0.0001; Fig 3.4). Based on these results, 150 µg/ml cumate was used for the remainder of the experiments.

**Figure 3.4: Stable Cumatet-Syn2 engineered cells**

To determine the expression of syncytin 2 and the optimum effective dose of cumate solution, qRT-PCR was performed in both N2a and SH-SY5Y cells transduced with the cumate-inducible syncytin 2 plasmid (N2a-Syn2 and SHSY-Syn2, respectively; N=3). The x-axis shows six different concentrations of cumate solution varying from 5 µg/ml to 150 µg/ml. “C” represents control cells engineered with the cumate-inducible syncytin 2 plasmid but not exposed to cumate. “Cuo C” represents an empty cumate vector (lacking syncytin 2) treated with cumate solution (150 µg/ml). Asterisks (****) represent the groups significantly different (P<0.0001) from control groups identified using one-way ANOVA followed by Tukey’s multiple comparison test. Graphs show the mean +/- standard error of the mean (SEM).
3.4 Cumate ON/OFF Expression

An advantage of using a cumate-inducible promoter is the capacity to temporally control the expression of a target gene, i.e., reduce expression of the target gene after removal of cumate. This may be important for any therapeutic applications to prevent continuous and unwanted expression of specific genes. To determine the kinetics of syncytin 2 expression after the removal of cumate, syncytin 2-transduced N2a and SH-SY5Y cells were cultured in four different conditions. The first condition represents the control cells transduced with the cumate-inducible syncytin 2 plasmid but not exposed to cumate. Cumate was added to the other three conditions (D1, D1R, D1R-3A) on day 0. D1 represents a 36-h exposure to cumate. In D1R cumate was removed after 24 h, followed by culturing for another 24 h without cumate to analyze the syncytin 2 expression after the removal of cumate. In D1R-3A, cumate was removed after 24 h and was added again after 72 h to induce reversible expression. As expected, N2a and SH-SY5Y cells showed increased expression on D1 after exposure to cumate. The expression of syncytin 2 decreased following the removal of cumate (D1R). The expression of syncytin 2 was induced again when the cumate switch was activated after three days.
Figure 3.5: Cumate Switch Inducible and Reversible Expression

To determine the inducible and reversible expression of syncytin 2 using the cumate system, cells were cultured in four different conditions and qRT-PCR was performed. A and B represent the syncytin 2 engineered N2a (N2a-Syn2) and SH-SY5Y (SHSY-Syn2) cells, respectively. D1 represents cells exposed to cumate for 36 h. D1R represents the removal of cumate after 24 h and D1R-3A represents the removal of cumate after 24 h and again added after 72 h. C represents control cells engineered with Cumate-Syn2 without the addition of cumate solution. Asterisks (*) are used to denote statistical significance (**, P<0.01; ***, P<0.001; ****, P<0.0001) represent the group significantly different from the control group determined by one-way ANOVA followed by Tukey’s multiple comparison test. Graphs show the mean +/- standard error of the mean (SEM).
3.5 Determination of fusion efficiency

To determine whether induction of syncytin 2 can stimulate fusion in neuronal cells, control and syncytin 2 transduced N2a and SH-SY5Y cells were stained for cell membrane marker phalloidin in red, and the nucleus was stained with DAPI in blue, and the fusion percentage with and without cumate exposure was determined. Successful activation of the cumate-inducible promoter following the addition of cumate was monitored using GFP. The percentage of fusion was calculated by counting the total number of nuclei contained within fused cells (without phalloidin staining) and dividing it by the total number of nuclei. The fusion rate of N2a cells transduced with syncytin 2 was 16.6%, whereas that of SH-SY5Y cells transduced with syncytin 2 was 18.5%.
Figure 3.6: Quantitative determination of neuronal cell fusion after treating syncytin 2-transduced cells with cumate

To quantify the percentage of cell fusion, fluorescence microscopy was performed in N2a and SHSY-5Y cells stably transduced with the cumate-inducible syncytin 2 plasmid. Panel A shows N2a-syncytin 2 and panel B shows SHSY-syncytin 2 cells. For both cell populations, the cell membrane was stained with phalloidin in red and the nucleus was stained with DAPI in blue. Green fluorescence represents GFP that was induced following cumate exposure. A(i) represents N2a cells transduced with the cumate-inducible syncytin 2 plasmid but not exposed to cumate, whereas A(ii) represents these cells exposed to 150 μg/ml cumate for 2 days. B(i) represents SH-SY5Y cells transduced with the cumate-inducible syncytin 2 plasmid but not exposed to cumate, whereas B(ii) represents these cells exposed to cumate. The white arrow represents multinucleated cells. Graphs in C show the fusion percentage, calculated as the number of nuclei in fused cells (not separated by a distinct cell membrane) divided by the total number of nuclei present (×100%; N=3). C represents control cells not exposed to cumate. Asterisks (*) represent the group that is significantly different (***, P<0.001; ****, P<0.0001) from the control group using an unpaired student’s t-test. Graphs show the mean +/- standard error of the mean (SEM). Scale bar=20 μm.
Pilot Studies

3.6 Differentiation of hESCs into Neurons with a Dopaminergic Phenotype

One of our long-term goals is to decrease the speed of degeneration of nigral dopamine neurons in patients with PD by transplanting organelles from engineered hESCs differentiated into dopaminergic neuronal phenotypes. We propose that transferring healthy mitochondria from young dopaminergic neurons to degenerating dopamine neurons in PD patients at early stages when there is still nigrostriatal circuitry intact would slow the degenerative process, delay disconnection of the circuit, maintain afferent modulation to dopamine neurons and delay the manifestation of debilitating symptoms. The novelty of the proposed strategy is not replacing lost neurochemicals at their site of action as currently attempted but maintaining neuronal circuitry by replacing damaged mitochondria of dopamine neurons (i.e., organelle replacement therapy) via cell fusion. This strategy will ensure the maintenance of normal nigrostriatal circuitry and afferent modulation for an extended period to achieve disease modification and ensure prolonged symptomatic relief and improved quality of life.

As a pilot study, I differentiated hESCs into dopaminergic neuronal phenotypes using a 42-day differentiation protocol. This method prevents differentiation towards endoderm and mesoderm, suppresses pluripotency genes, and provides efficient neuronal differentiation. Fig. 3.7 shows the phenotypic overview of cultures at various differentiation phases from day 2 to day 9. Fig 3.8 shows the phenotype of terminally differentiated dopaminergic cells on days 18, 27, and 36. On day 15, hESCs were fixed and stained for floorplate-derived midbrain dopamine neuronal progenitor markers LMX1, FOXA2, and OTX2. Positive
staining was seen for all three neuronal progenitor markers on day 15 in differentiated hESCs (Fig 3.9). FOXA2 and OTX2 were not detected in undifferentiated hESCs (Fig 3.9). This demonstrates that the hESCs underwent the expected ventral midbrain patterning during differentiation and that terminal differentiation of neural progenitor cells into dopamine neuronal phenotypes was in progress. To ensure proper cell density in the progenitor cultures and avoid cells from being overconfluent, cells were replated between days 16 and 21. Around day 25-30, neuronal morphology was observed, and around day 42, mature dopaminergic neuronal phenotypes were evident (Fig 3.9).
Figure 3.7 hESCs differentiated into a dopaminergic neuronal phenotype

Representative bright field images show the expected phenotype of starting cultures of undifferentiated (B) and differentiated (A) hESCs into dopaminergic neuronal phenotypes and densities of cultures at different stages of differentiation. Scale bar=50 µm.
Figure 3.8 Terminally differentiated dopaminergic neuronal phenotype

Representative bright field images show the expected phenotype of terminally differentiated hESCs into dopaminergic neuronal phenotypes and densities of cultures at different stages of differentiation. Scale bar=50 μm.
Figure 3.9 Characterization of differentiated neural progenitor cells

Representative immunofluorescence images showing LMX1 (green), FOXA2 (red), and OTX2 (red). The nucleus is stained with DAPI in blue. A panel represents undifferentiated (UD) hESCs stained for DAPI, FOXA2, and OTX2. B represents day 15 (D15) differentiated hESCs stained for LMX1 and FOXA2 whereas C represents D15 stained for LMX1 and OTX2. Scale bar=50 µm.
3.7 Characterization and differentiation of SH-SY5Y cells into a dopaminergic neuronal phenotype

In addition, we used SH-SY5Y cells and differentiated them into a dopaminergic neuronal phenotype using retinoic acid (RA). Tyrosine hydroxylase (TH) was used as a marker for mature dopamine neurons. Five days after differentiation, SH-SY5Y cells exhibited a neuron-like phenotype with short neurite development, as seen in Fig. 3.10 (A). The expression of TH was 11-times higher in RA-induced cells than in the undifferentiated group (B) (N=3).
Figure 3.10 Characterization of SHSY-5Y differentiated into a dopaminergic neuronal phenotype

Brightfield images (A) represent undifferentiated and differentiated SH-SY5Y cells depicting dopaminergic neuronal phenotype. (B) represents the relative expression of TH in both differentiated and undifferentiated cells. Asterisks (***) marked above bars represent groups significantly different ($P<0.01$) from the control group using the unpaired student’s t-test. Graphs show the mean +/- standard error of the mean (SEM). Scale bar=50 µm.
Chapter-4

4 Discussion

Neurodegenerative disorders are often slowly progressive, severely debilitating, and associated with both physical and mental disability leading to poor quality of life. Although many neurodegenerative disorders may have a genetic predisposition, environmental factors and aging play an important role in the course and clinical manifestations. Oxidative stress and accumulation of misfolded protein aggregates are the common and central features of neurodegenerative disorders and result in mitochondrial dysfunction that often accelerates the course of neurodegenerative disorders, including AD and PD\textsuperscript{104,105}. Fortunately, cellular abnormalities progress very slowly over decades and therefore provide a window of opportunity to potentially intervene. This is an important window because at this stage of the disorder, underlying neuronal circuits are still intact and therefore afferent modulation of neuronal function is present. To date, strategies to slow or stop cellular dysfunction via supplementing biologically active chemicals, tropic factors, and viral-mediated gene transfer have been mostly ineffective\textsuperscript{72-74}. A few studies suggest that there are several modes of organelle transfer involved in different diseases through tunnel tube formation, microvesicles, gap junctions, cell fusion, and direct ingestion of isolated mitochondria. Interestingly, cell fusion may facilitate the transfer of mitochondria from a host cell into a damaged cell\textsuperscript{79}. In this thesis, we proposed to employ a novel strategy of using cell fusion to transfer mitochondria (and possibly other organelles) from young neurons of the same phenotype into neurons undergoing organelle stress and dysfunction.
The capacity of cell fusion, and the compatibility of polyploidy and cell function in neurons is not known. If mitochondria can be transferred to a host cell, it remains unclear whether they get incorporated into the existing organelle network, survive long-term, and function properly. The current study sought to test whether neurons are amenable to fusion and transfer of organelles, especially mitochondria.

We propose to use well-known cellular fusogens, syncytin 1 and syncytin 2, placental proteins that encode endogenous retroviral envelope genes that maintain fusogenic function. Cell fusion is an intricately orchestrated process in fertilization, growth, and development. A popular molecule mediating fusion between coronavirus and host cell is the spike protein. Viruses use several proteins, including LFA1 and ICAM1 for fusion with host cells. The fusion of ova and spermatozoa is a critical step in fertilization and is mediated by at least four proteins, including TMEM95. During muscle regeneration, muscle satellite cells fuse with damaged muscle fibers to rebuild multinuclear myofibers, and two proteins myomaker and minion are essential for fusion. Among these fusogens, syncytin 1 and syncytin 2 genes are relatively well characterized, more familiar to our laboratory and molecular tools are mostly available, and therefore, we sought to use syncytins as cellular fusogens in our study.

In the current study, we sought to test whether neurons allow fusion and transfer of mitochondria in vitro using two stable neuronal cell lines to provide a proof-of-principle prior to proceeding with more complex approaches such as in vivo methods in animal models. We sought to determine whether neuronal cell lines, N2a and SH-SY5Y, could be engineered to effectively express syncytin 1 and syncytin 2 genes in an inducible manner and allow cell fusion and transfer of mitochondria. The N2a cell line was originally derived...
from mouse neuroblastoma and possesses several neuronal features, including neurofilaments and microtubules, while the SH-SY5Y cell line is from human neuroblastoma. SH-SY5Y cells are commonly used in neurobiological studies, especially in relation to PD because they possess several features of monoaminergic neurons and can be differentiated into dopamine neuronal phenotype. In addition to N2a and SH-SY5Y cells, we also examined the expression of syncytins in easily transfectable HEK293T kidney cells as a positive control.

Initially, we transfected plasmids encoding syncytin 1 and 2 into both the neuronal (N2a & SH-SY5Y) and HEK293T cells, and observed significantly elevated expression of syncytins compared to that of the control group. The major drawback with the expression plasmids containing a ubiquitously-activated promoter used in this experiment is that they will constantly express high levels of the gene of interest, which may lead to undesirable effects. In contrast, the inducible expression system expresses the gene of interest under temporal control. This strategy will be important prior to transitioning to an in vivo model to enable tight temporal control of gene expression. To date, tetracycline/cumate-controlled operator systems are preferred for routine inducible-expression/knockdown experiments due to the easiness of their handling, high efficiency, and negligible side effects. However, different options of configurations, promoters, and activating domains still complicate the selection. The Tet-on and the reverse activator configuration of cumate system are usually prioritized due to their negligible leakiness. The Tet-off configuration and activator configuration of cumate system is preferably selected when experiments need to avoid the presence of tetracycline and cumate in the culture medium. When expression of the endogenous protein is weakly expressed or negligible, the maximum induction of a
particular gene using tetracycline/cumate-controlled operator systems can be up to 100–1,000-fold\textsuperscript{11}. Therefore, in this study, we employed the cumate inducible expression system and engineered syncytin 2. This strategy yielded temporal and reversible induction of syncytin 2 in N2a and SHSY-5Y neuronal cells.

We established a stable population of syncytin 2 engineered cells and identified the optimal dose of cumate for future studies. We used six different concentrations, ranging from 5 \(\mu\)g/ml to 150 \(\mu\)g/ml. Both the syncytin 2 transduced N2a and SH-SY5Y cells exhibited a dose-dependent increase in the expression of syncytin 2. In addition, the inducible and reversible expression of the cumate system was investigated. 36 h cumate-induced cells showed increased syncytin 2 expression in comparison to cells induced with cumate for 24 hours followed by removal of cumate. Removing cumate after 24 h and reintroducing it 72 h later boosted the expression of syncytin 2. Cumate's addition and removal parallel the induction and progressive reduction of syncytin 2 expression presumably through the cumate ON/OFF switch. Our results therefore demonstrate that the cumate ON/OFF switch functions in a reliable manner (i.e., induced and reversed as required). This strategy will be important when we transition to the \textit{in vivo} model to enable temporal control of gene expression.

Fusogenic properties of enveloped viruses and virus-derived proteins may stimulate the production of heterokaryons, hybrid cells, and syncytia. Co-culture of human prostate cancer cells with muscle cells resulted in the upregulation of syncytin 1 and annexin A5 by IL-4 and IL-13\textsuperscript{112}. siRNA-mediated knockdown of annexin A5 expression and likewise blockade of syncytin 1 by a synthetic peptide or shRNA significantly inhibited the
generation of multinucleated PC3 cells and PC3 muscle cell heterokaryons, demonstrating the necessity of syncytin 1 and annexin A5 in fusion of prostate cancer cells.

We investigated stable Cuo-Syn2 protein expression and assessed the fusion efficiency. We found that 16.6% of N2a-Syn2 transduced cells fused, whereas 18.5% of SHSY-Syn2 transduced cells fused. Interestingly, the efficiency of fusion was consistently less than 20%, and we speculate that this could reflect a high level of differentiation seen in neuronal cells that should possess unique properties as a member of a specific neuronal circuit and should naturally resist fusion with other cells. It is important to note that syncytins work with other proteins to facilitate cell fusion, such as receptors on recipient cells, as well as co-modulators, and adapter proteins. In addition, the cells used in this study are neuroblastoma cells. Being cancer cells, they might have suppressed certain signaling intermediaries that might be involved in cell fusion. Despite the ectopic expression of syncytins in neuronal cell lines causing about 18% cell fusion is promising, future studies should investigate the reasons underlying the low fusion rate.

The current study has several limitations. The efficiency of GFP fluorescence in the cumate inducible system was not very effective with the stably transduced cells in comparison to the transiently transfected cells. Further optimization of this strategy or use of a more efficient inducible vector is necessary. Furthermore, we were unable to successfully identify a suitable syncytin 1 or syncytin 2 antibody to confirm the presence of syncytin proteins following exposure of cells to cumate. For transient transfection experiments, the termini of syncytin 1 were fused with a V5 tag, and we used the V5 tag to determine syncytin 1 levels via immunofluorescence. Because transfection efficiency of neuronal cells is low, we used a different strategy of exposing N2a and SH-SY5Y cells to lentiviral
particles and centrifuging at 1000g for 2 hours at room temperature\textsuperscript{98}. This technique allowed viral particles to enter the cell without causing damage to the cells. Puromycin was used to select the transduced cells\textsuperscript{98}. However, we were unable to examine syncytin expression in transduced cells because the V5-tagged termini of syncytins were removed during the restriction digestion process in order to engineer the syncytin gene into the cumate-inducible expression system. As a result, we were unable to examine V5 (and therefore syncytin expression) at the protein level in transduced cells exposed to cumate. Furthermore, an important challenge we faced was to reliably identify fused cells. Although mature neurons do not undergo mitosis, counting nuclei is generally confounded by cells in different stages of the cell cycle. Co-staining for a cell proliferation marker such as Ki67 might help in detecting proliferation-related multinucleation\textsuperscript{119}. A more robust, high throughput strategy to detect fused cells would certainly help in future studies and is currently being investigated in our laboratory.

Our long-term goal is to transplant mitochondria into degenerating neurons at the early stages, thereby slowing the disease progression and maintaining neuronal circuitry and afferent modulation of the damaged brain area. We propose to efficiently transfer healthy mitochondria to damaged neurons by fusing them with young neurons engineered to conditionally express syncytins. This is a novel strategy as a fusion with neurons has never been attempted before. Therefore, in future studies we will attempt to investigate syncytin-mediated cell fusion and organelle transfer by coculturing syncytin-transduced neuronal cells with recipient cells and labeling donor cells with additional green CellTracker and recipient cells with deep red CellTracker to detect cell fusion and organelle transfer. I attempted this experiment in a pilot study, by staining donor cells with MitoTracker green
(to label donor mitochondria), and recipient cells with deep red CellTracker. I expected that the mitochondria transfer from donor to recipient cells could be identified by the presence of yellow-colored cells with two or more nuclei. I observed promising mitochondrial transfer from syncytin 2 engineered donor cells to recipient cells, although as described below there were some challenges with this experimental strategy that still need to be overcome.

MitoTracker green is widely used in cell biological studies of mitochondria and its intensity is believed to represent mitochondrial mass in a cell\textsuperscript{113-115}. A decrease in labeling intensity and diffuse labeling seen in the pilot study may indicate degradation of mitochondria as inferred in studies of mitophagy\textsuperscript{116,117}. Interestingly, conditions that induce depolarization of mitochondria and formation of ROS also decreased the labeling intensity of MitoTracker green suggesting that the labeling could be affected by mitochondrial potential\textsuperscript{118}. There was also interference with the excitation and emission wavelengths of MitoTracker green and CellTracker deep red. As a result, we were unable to stop the bleed-through into the channel. Employing a genetic marker to label donor mitochondria might yield more conclusive results.

Transplanting organelles particularly mitochondria in neurons is a novel strategy to circumvent one of the core mechanisms driving neurodegeneration while preserving normal neuronal circuitry. Our long-term goal is to decrease the speed of degeneration of nigral dopamine neurons in patients with PD by transplanting organelles from engineered human ESCs into native dopamine neurons using cell fusion. This strategy will ensure the maintenance of standard nigrostriatal circuitry and afferent modulation for an extended period to achieve disease modification and ensure prolonged symptomatic relief and
improved quality of life. Interestingly most HERV-related regulatory elements, such as LTRs, and/or LTR–related proteins are produced and active in germ cells, preimplantation embryonic cells, and the placenta\textsuperscript{120-122}. For example, increased HERV-H transcription was detected in human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs)\textsuperscript{122}. Consequently, our aim to employ hESC or hiPSC would be promising in future studies.

In conclusion, transplanting organelles via cell fusion into native dopamine neurons in early stages of degeneration is an original and promising strategy that may not only offer symptomatic alleviation but also lead to disease modification in PD. Our results provide promise that neuronal cells are capable of fusing with grafted engineered cells and accept the transfer of organelles. Therefore, findings from our research will pave the way for an innovative therapeutic strategy in PD and other neurodegenerative disorders to allow prolong maintenance of neuronal circuit and minimize and delay the onset of debilitating symptoms.
Chapter 5

References


Curriculum Vitae

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Education

Masters of Science - Anatomy and Cell Biology
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Scholarships

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Schulich School of Medicine & Dentistry
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Projects and Internships

- Project on “Transferring Organelles into native neurons: A disease-modifying therapy for Neurodegenerative disorders” in University of Western Ontario, London, Ontario, Canada (2020-2022)
- Project on “Differentiation of SHSY5Y Neuroblastoma cells into Dopaminergic neurons” in Stem Cell Facility, AIIMS, New Delhi, India (2018)
• Project on “Isolation and Characterization of Very Small Embryonic like Stem Cells (VSEL) from Cord blood, Mouse bone marrow, and Mouse Ovary in Stem Cell Facility, AIIMS, New Delhi, India (2018)
• Project on “Isolation and Characterization of Human Umbilical Vein Endothelial Cells (HUVEC) from Human Umbilical Cord in Stem Cell Facility, AIIMS, New Delhi, India (2018)
• Invitro Fertilization and Animal cell culture training in Madras Veterinary College (TANUVAS), Chennai, India (2017)
• Project on Biological evaluation of synthesized N- benzoyl Derivatives at SRM University, Chennai, India (2017)
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Related Experience

Graduate Research Student
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Publications


3. Published an abstract in Asian Pacific Association for the Study of the Liver (APASL) conference in *Hepatology International Journal*, March 14-18, 2018 on “Activation of liver X receptor α and β by 22(R)-hydroxycholesterol leads to the generation of dopaminergic neuronal cells from human Mesenchymal Stem Cells”.