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A Comparison of PM-NATO3's Influence on Neural Progenitors and Mature Dopamine Neurons

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A Comparison of PM-NATO3's Influence on Neural Progenitors and Mature Dopamine Neurons

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Abstract

This thesis presents significant findings regarding the role of PM-Nato3 in its interaction with developing neurons in the context of Parkinson's disease (PD) and regenerative medicine. We investigated the effects of PM-Nato3 on dopamine (DA) neurogenesis under different culture conditions, both in vitro and in vivo. In the standard dopaminergic culture condition, PM-Nato3 potentially increased the speed of DA neuron production but did not significantly increase the yield of DA neurons. In a minimal culture condition, there was no notable difference between the control and PM-Nato3 conditions, suggesting minimal impact on DA neurogenesis. In vivo studies using a mouse model revealed that PM-Nato3 did not elevate neuroprotective transcription factors (Foxa2, En1, Nurr1) but showed a modest increase in TH mRNA expression, indicating potential effects on dopaminergic neurons. These findings provide insights into the complex relationship between PM-Nato3 and neuronal development. Further research is needed to understand the underlying molecular mechanisms and signaling pathways involved. This knowledge could pave the way for more effective treatments and regenerative strategies for PD, establishing a foundation for future investigations into PM-Nato3, contributing to advancements in Parkinson's disease research and regenerative medicine.

Table of Contents

List of Figures

Abbreviations

AAV- adeno-associated virus
Ab- antibody
bHLH- basic Helix-Loop-Helix
CNS- central nervous system
DA- dopaminergic
DMEM- Dulbecco's Modified Eagle Medium
dNTP- Deoxyribonucleotide triphosphate
DOX- doxycycline
DTT- Dithiothreitol
En1 - Engrailed Homeobox 1
EOPD- early-onset Parkinson’s Disease
Foxa2- Forkhead box protein A2
FP- floor plate
GAPDH- Glyceraldehyde 3-phosphate dehydrogenase
GID- graft-induced dyskinesia
hESC- human embryonic stem cell
iPSCs- induced pluripotent stem cells
Lmx1a- LIM homeobox transcription factor 1, alpha,
MOI- multiplicity of infection
MPTP- 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NGN2- Neurogenin 2
Nurr1- nuclear receptor related 1 protein
PD- Parkinson’s Disease
PITX3- Pituitary homeobox 3
Shh- Sonic hedgehog gene
SNpc- substantia nigra pars compacta
SSIV- SuperScript IV
TH- tyrosine hydroxylase

Chapter 1: Introduction

Introduction

Since Parkinson's Disease (PD) is the second most common neurodegenerative disease found globally, there is a great deal of emphasis on understanding its mechanisms as well as creating effective clinical treatments. It is typically diagnosed in patients in their 60s and affects roughly 1% of the world's population (Kalia & Lang, 2015). PD is caused by a degeneration of the substantia nigra, a portion of the midbrain that contains a majority of the dopaminergic (DA) neurons in the brain (Davie, 2008). As the disease progresses, patients tend to exhibit symptoms ranging from motor function loss to psychiatric disturbances due to the loss of dopamine. Ultimately, the end stages of PD result in patient death.

Currently, there are a limited number of surgical procedures that are used to overcome disease progression in PD, including deep brain stimulation (DBS) and pallidotomy, which uses an electrical probe to damage the globus pallidus or basal ganglia (Kumar et al., 1998). Because most pharmacologic interventions have proven to have a greater focus on symptom-mitigation instead of symptom alleviation, pursuing therapies in the stem cell field seems to be promising.

Replacing dopamine by elevating production of the neurotransmitter in the dwindling population of dopamine neurons of Parkinson's patients, while effective for a short while, creates an inevitable dopamine resistance, meaning other avenues must be pursued (Müller, 2020). This is especially relevant when discussing cell replacement therapies in future treatments. It may be that providing a cell based source of dopamine, replacing the neurons that have been lost to disease, could

be a beneficial therapeutic approach. Studies are currently underway to determine the viability of this treatment in humans and whether this would serve as a method of symptom management or ultimately cure the disease.

For cell replacement therapies, one approach is to convert stem cells and neural progenitors into dopamine neurons that can be transplanted into patients. During embryonic development, there are multiple factors that contribute to the creation of dopamine neurons. These pro-dopaminergic genes promote differentiation into mature DA neurons.

This research concentrates on one transcription factor known to play a role in dopamine neurogenesis, called NATO3 (also known as Ferd3L). NATO3 is endogenously produced during embryonic development and is necessary to regulate DA progenitor cells (Ono et al., 2010). This covers the use of a mutant form of the NATO3 gene created by our lab, coined "PM-NATO3" that has been shown in our unpublished data to promote the expression of key dopaminergic genes in neural progenitors in vivo.

Some genes that are critical for dopamine neurogenesis can also serve roles later in the life cycle of the cell, including EN1, FOXA2, and NURR1. If utilized at a key point prior to the neurons' maturity, genes related to dopamine neurogenesis can not only produce neurons that synthesize the much-needed dopamine, but contribute to their overall health and protection as well (Artegiani & Calegari, 2012).

Because PM-NATO3 promotes the expression of some genes that have been shown to be essential for the protection of mature dopamine neurons against oxidative stress, one part of this study

focuses on the action of PM-Nato3 expression in mature neurons (Peterson et al., 2019). If there is a connection between this gene and the various transcription factors that not only produce dopaminergic neurons early in development, but also help maintain them later in life, there is further potential for clinical relevance in the treatment of PD.

Interestingly, the same set of genes that help promote the genesis of dopamine neurons can also serve to protect those neurons later in the lifetime of the neuron. Nurr1, for example, is essential for the viability of mature neurons in gene knockout and knockdown studies (Beiki et al., 2022), and this same gene is sufficient to promote the differentiation of dopamine neurons from stem cells in the appropriate culture conditions (Beiki et al., 2022).

Other genes, such as Foxa2 are necessary for supporting the genesis of dopamine neurons and the long term viability of mature dopamine neurons (Kittappa et al., 2007). Engrailed-1, or En1 has been found to protect mature DA neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a mitochondrial complex I toxin used to model PD in mice (Alvarez-Fischer et al., 2011). Indeed, a recent publication (2022) shows that Nato3 is necessary for the maintenance of dopamine neuron mitochondrial function and protects against oxidative stress in mice and drosophila (Miozzo et al., 2022).

Given the breadth that some genes can play in dopamine neuron genesis and survival (neuroprotection) we use the term pro-dopaminergic in this thesis to include genes that serve both of these functions.

Purpose

The treatment of PD is reliant on supplying appropriate amounts of dopamine at the right time and frequency (Ovallath & Sulthana, 2017). It may be that cell replacement therapies are not as effective as protecting the patient's remaining neurons. The cellular substrate for these distinct therapeutic approaches are either human embryonic stem cells or the mature dopamine neurons in the substantia nigra.

The consideration of PM-NATO3 action in different cellular substrates such as progenitor cells and mature neurons is because its action could serve to promote neurogenesis in progenitor cells, or neuroprotection in mature cells. However, it is known that progenitor cells can be more responsive to genetic manipulation and extrinsic factors than mature differentiated cells due to restriction of cell fate that occurs across the developmental timeline (Fischer & Morin, 2021). Mechanistically, this has been attributed to chromatin silencing, in addition to a change in the factors present to permit transcriptional activation (Beisel & Paro, 2011). Comparison between cell types (differentiated/mature and progenitor cells) can help clarify this effect. Additionally, examining the role of environmental factors (in vivo compared to in vitro) can help discern factors that may be important for the pro-dopaminergic action of PM-Nato3.

Scope

This project examines two distinct populations of cells and their response to PM-Nato3: progenitor cells and mature dopaminergic neurons. These experiments are pieces within a larger body of work, including unpublished data.

In particular, we tested the efficacy of PM-Nato3 to drive pro-dopaminergic gene expression in human embryonic stem cells in two different culture conditions:

1) To answer the question if PM-Nato3 can increase or accelerate the rate of DA neurogenesis by human embryonic stem cells (hESC) in the presence of a standard dopaminergic culture condition available as a commercial kit. We tested the effect of expressing PM-Nato3 in a hESC cell line that is undergoing dopaminergic differentiation with culture conditions known to drive dopamine neuron differentiation (called the standard condition) in hESCs. We monitored the expression of key markers of dopamine neuron differentiation over the course of differentiation, with exposure to the standard condition as the control and the addition of PM-Nato3 expression (Standard + PM-Nato3) as the experimental condition.

2) To answer the question if PM-Nato3 is sufficient to drive DA neurogenesis in presence of a proneuronal condition, a more restricted culture condition (the minimal condition) with PM-Nato3 expression with a second proneural gene called Neurogenin-2 (NGN2) being simultaneously expressed. This condition does not include expensive and labile media supplements that are included in the standard dopaminergic condition described above. Dopamine neurogenesis differentiation was monitored by measuring key markers of differentiation, comparing the effect of PM-Nato3 + NGN2 expression relative to the expression of NGN2 alone.

3) To test the efficacy of PM-Nato3 to drive pro-dopaminergic genes in mature neurons in vivo, we tested if PM-Nato3 can preserve the expression of TH, En1, Foxa2 and Nurr1 in an animal model of PD neurodegeneration (the En1 haplosufficient mouse model). We used qPCR to extend already completed ICC analysis of Tyrosine Hydroxylase expression in these mice by examining a broader array of pro-dopaminergic genes in the PM-Nato3 treated nigral tissue, specifically Th, Nurr1, Foxa2, and En1.

Combined, these experiments could provide insight for future therapeutic interventions, specifically cell replacement therapy, using hESC cultures. Alternatively, further determination of Nato3's role and its influence on neuroprotection could aid in future gene therapies by genetically expressing PM-Nato3 in mature DA neurons in vivo. Even if there are not significant results supporting clinical usage, PM-Nato3 also has the potential in commercialization for future research endeavors.

Based on preliminary data, it can be assumed that PM-Nato3 induces increased expression of the transcription factors FoxA2 and En1 in vivo in early embryonic neural progenitors when using the embryonic chick model system (see Fig 5). Foxa2 and En1 are considered specific molecular markers for early neurodevelopment in DA neurons (Domanskyi et al., 2014). It was also assumed that the in vitro environment is not the same between differentiated and progenitor cells and cannot be considered a perfect direct comparison. This is due to the fact that culture needs vary for the different cell types and the dependence on different extrinsic and intrinsic factors to survive.

Another important factor was that the markers for DA neurogenesis used in this thesis, including EN1, FOXA2, NURR1, and Tyrosine Hydroxylase (TH) (Ásgrímsdóttir & Arenas, 2020)

identify DA -like neurons. Bona-fide DA neurons that can be used in cell replacement therapy have a host of genetic and electrophysiological characteristics that are beyond the scope of this project to characterize. Should our interventions yield promising results of identifying candidate DA neurons, further study would be needed to fully characterize these cells and determine their viability as a substrate for cell replacement therapy. (Tiklová et al., 2020).

Hypothesis

- PM-NATO3 regulation of DA related genes is restricted to early progenitors and has a more restricted impact on mature DA neurons.
    - 1) In particular we hypothesize that PM-NATO3 will increase the speed of DA neurogenesis and number of DA neurons of hESC cultured in the presence of DA differentiation factors (standard condition).
    - 2) We also hypothesize that PM-NATO3 will be sufficient to specify dopaminergic differentiation by neurons arising in hESC treated with the pro-neuronal gene (Neurogenin2) in the minimal culture condition.
- 3) PM-Nato3 will drive the expression of pro-dopaminergic genes, such as Foxa2 and En1, in mature neural cells in vivo.

Significance

The main purpose of this study is to determine the role of PM-Nato3 in both developing and mature cells. This will add to the current literature surrounding the influence of Nato3 on dopamine

producing cells. Also, it will hopefully produce future clinical relevance in disease treatment for Parkinson's Disease (PD) and other illnesses that cause DA neurons to deteriorate. By contributing to the current literature, future researchers could ideally use this information alongside recent advancements in cellular replacement therapy or neuroprotection of mature neurons from PD conditions.

Chapter 2: Review of Literature

Overview: Parkinson's Disease

Symptoms

The most telltale sign that a patient may be developing PD is a distinct "pill rolling tremor" that appears in the upper extremities at rest. Often, the patient is unaware of his or her tendency to touch and move their index finger and thumb together, and eventually the motor dysfunction continues throughout the limb (Sveinbjornsdottir, 2016). Roughly 80% of PD patients suffer from an extremity tremor, with pill-rolling motions being the most common. Eventually, the patient will struggle to grasp objects firmly or have control of their repetitive, jerking motions.

There is also a noted slowness to patients' voluntary movements and most voluntary movements will take a longer time to initiate, which is also known as bradykinesia. The patient will also experience muscular rigidity when trying to complete these movements. This causes difficulties when ambulating and hand eye coordination (Jankovic, 2008). Eventually, performance of any simultaneous movement will be impaired by bradykinesia, which causes the patient to lose their ability to complete multiple motor activities at the same time.

Another frequently found symptom is the loss of postural reflexes, which is one of the more disabling symptoms of the disease. These are automatic movements that help maintain posture and balance. It is also considered part of the clinical criteria for an official diagnosis (Jankovic, 2008). The two primary components of these reflexes that are impacted are orientation and stabilization. When orientation is disrupted, the patient will typically have stooped posture (Benatru et al., 2008).

There are also prominent non-motor symptoms associated with PD (Pfeiffer, 2016). This includes multiple psychological symptoms and sleep disorders, which manifest at different points of the disease's progression. For example, there is a noted decline in the sensory pathways for taste and smell, which onsets decades prior to the motor symptoms (Jankovic, 2008). Other symptoms, such as aphasia, do not occur until later in the disease's progression.

Hallucinations and delusions will typically occur in 50% of patients diagnosed with PD. These often are mild to moderate in severity, but there are rarely instances of auditory hallucinations. General psychosis and its symptoms have a prevalence from 26-83% (Han et al., 2018). The cognitive impairments in these patients are similar to those suffering from dementia, leading to a decrease in the quality of life significantly while PD is simultaneously damaging the body.

Theories of Pathogenesis

The primary method for determining Parkinson Disease diagnosis is the Braak Staging Hypothesis, which focuses primarily on the neurodegeneration. It is completed post-mortem during an autopsy, so the extent of PD can be fully assessed (Braak et al., 2003). There are a total of six distinct stages of the disease. The initial stage is marked by neuronal death in the medulla and the brainstem (Braak et al., 2003). There are also noted changes in the olfactory system of the patient; however, the patient is considered pre-symptomatic.

The second and third stages are both also considered pre-symptomatic. In stage two, the locus coeruleus, a principal site of norepinephrine production, and the dorsal nucleus, which provides parasympathetic motor innervation., are damaged (Braak et al., 2003). By the third stage, there is a

marked loss of DA neurons in the substantia nigra- particularly in the pars compacta, also known as the SNpc. When the neurons within the SNpc die, DA neurons lose the ability to send their signals to the striatum. After this, the patient is typically symptomatic and there is increased degeneration of the dopaminergic neurons throughout the substantia nigra, and eventually the rest of the cortex (Braak et al., 2003).

The exact cause of this degeneration has multiple theories behind it. This includes the formation of intraneuronal Lewy bodies from the excessive accumulation of a protein called alpha-synuclein and the potential dysfunction of multiple transcription factors and second messengers.

Alpha-synuclein is a neuronal protein that is associated with neurodegenerative disorders- particularly, Alzheimer's Disease and PD (Kim et al., 2014). It makes up 1-2% of all proteins within the central nervous system (CNS) and while its primary function has not been established, it plays a role in synaptic transmission, calcium transport, protein phosphorylation and dopamine release (Ellis et al., 2005).

Eventually, alpha-synuclein can misfold into beta-pleated sheets, which have a greater structural stability than the original form and are more difficult to reverse. The stable beta-pleated sheets expose the hydrophobic regions, causing the proteins to aggregate (Benskey et al., 2016). Neuronal cells have difficulties removing these sheets, causing dysfunction in nigrostriatal dopamine release (Villar-Piqué et al., 2016). This phenomenon is thought to contribute to many of the associated motor deficits.

Another hypothesis regarding the origins of degeneration is a deficit in the genes that regulate DA neurogenesis. There are multiple contributing regulatory genes in the formation of DA neurons,

and dysfunction in these genes can contribute to future dopamine production. In one study, it was found that knocking out genes such as Foxa2 gene in mouse models show age-related asymmetric loss of DA neurons (Domanskyi et al., 2014). There are also noted locomotor symptoms that mirror those of Parkinson's Disease. Similar results have been found when modifying other transcription factors, such as Nato3 (Andersson et al., 2006).

The literature suggests that not all transcription factor genes have equal contribution to PD, despite their role in neurogenesis. An example, MSX1, is a major upstream regulator of the DA subtype when the cell undergoes specification. However, it was found that in a cohort of 202 patients with PD, there was no identified mutation in the MSX1 gene (Deng et al., 2009). This suggests that mutations or dysregulation in the MSX1 gene do not play a significant role in the development and progression of PD.

Only a subset of PD patients seems to have any genetic contribution to disease onset: Early Onset Parkinson's Disease, or EOPD. Roughly 5-10% of patients diagnosed with PD are in this early group and often display different symptoms after diagnosis than elderly patients (Mehanna et al., 2014). The gene PARK2, which is one of the many mutations to the parkin protein, has been found to contribute to autosomal recessive inheritance of PD for people under the age of 40, but only 15% of sporadic cases (Bonifati, 2012).

Impact of Dopamine

Dopamine is a neuromodulatory molecule that plays a role in multiple facets of brain function, including motivational salience, attention, reward, and pleasure (Berridge, 2007). It is also

responsible for maintaining smooth, controlled movements. Produced in the substantia nigra, ventral tegmental area (VTA), and hypothalamus of the brain, dopamine is equally vital in both basic and complex human functions (Juárez Olguín et al., 2016).

The receptors that DA functions with can be divided into two separate categories: D1-like and D2-like. The D1-like receptors include DRD1 and DRD5, which function in memory, learning, and overall, excitatory neuronal transmission (Mishra et al., 2018). D2-like receptors mediate inhibitory transmission overall. The DRD2 receptor inhibits adenylyl cyclase activity, DRD3 decreases lewy body and alpha synuclein aggregation by initiating clearance, and DRD4 contributes to post-synaptic dopamine action(Ptáček et al., 2011). Because of these various receptors, dopamine is not considered solely excitatory or inhibitory, but as a neuromodulator.

Current Interventions: Pharmaceuticals

Current pharmaceutical interventions aim to mitigate the loss of dopamine by increasing dopamine levels in the brains of PD patients. The most used drug in current PD treatment is levodopa, or L-DOPA, which is an amino acid that is the precursor to catecholamines. Because levodopa is capable of crossing the blood-brain barrier, but dopamine itself is unable to do so, it is used to replenish the diminished concentration of dopamine in PD patients (LeWitt, 2015). Carbidopa is also given concurrently during treatment to inhibit the peripheral metabolism of the levodopa, allowing more to cross into the CNS, the primary target of PD. This increase in dopamine availability in the substantia nigra may help alleviate the symptoms for a given amount of time (Müller, 2020).

Since it was first introduced to the market in the 1960s, it was considered the gold standard for treatment; however, it has been known to have multiple serious acute and chronic side effects (Tambasco et al., 2018). This includes dose failure due to nausea, emotional distress, hallucinations, sleep disturbances, and GI dysfunction. It also has complications with its bioavailability, a substantially short half-life, and a high rate of dose failure due to drug resistance (Tambasco et al., 2018). There is also a noted dose-by-dose variability in the plasma concentrations of patients, leading to further problems in the pharmacokinetics (LeWitt, 2015). The combination of negative side effects, pharmacokinetics, its use for motor symptom mitigation, and lack of restorative properties urge researchers to pursue other treatment options.

Dopamine Neurogenesis

During embryogenesis, the notochord receives signaling from sonic hedgehog (Shh) which is a signaling molecule, that creates a structure on the ventral midline of the neural tube in the midbrain to the caudal spinal cord (Yu et al., 2013). The floor plate (FP) region gives rise to the progenitor cells that produce dopamine, which constitute about 75% of DA neurons in a fully developed brain. (Ang, 2006).

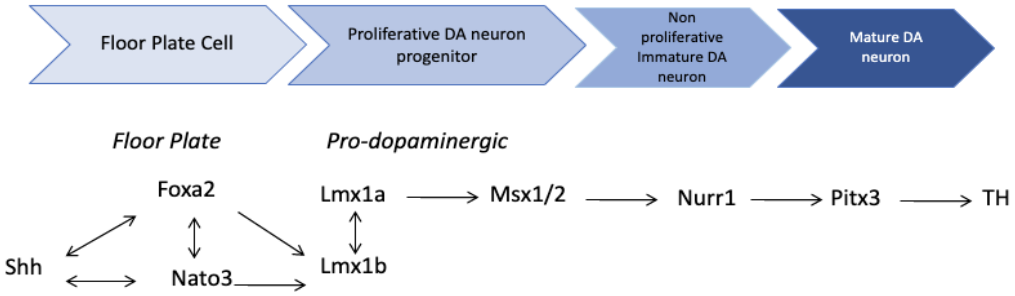

Figure 1: This shows the progression of a DA neuron from a floor plate cell till it reaches maturity and how the influential transcription factors work in tandem during early development and proliferation. Specifically, it shows the interplay between Nato3 and other early transcription factors in the floor plate cell.

Various types of transcription factors and signaling molecules are expressed at distinct times in development in a highly coordinated process. Once neurulation occurs, there are initial transcription factors that are activated to differentiate these FP cells into DA neurons. This includes, Shh (sonic hedgehog), Nato3, and Foxa2 (Wang et al., 2020, p. 20). This process allows for the specification of the mDA progenitor cells. Shh induces Foxa2, which in turn regulates Lmx1a and Lmx1b, which are respectively expressed medially and laterally on the FP. The data suggests that both Lmx1a and Lmx1b are sufficient to induce the generation of mDA neurons through forced expression in the midbrain (Ono et al., 2007). Both Foxa2 and Lmbx1a/b are understood to be consistently expressed throughout the rest of the cell's development.

While the cell is considered an mDA neuron progenitor cell, MSX1/2 (muscle segment homeobox homolog 1 and 2) and EN1 (Engrailed-1 homeoprotein) are also expressed at relatively high levels until the progenitor cell has fully matured. MSX1 is regulated by Lmx1a (Wang et al., 2020). After En1 is expressed in the midbrain, it becomes highly restricted to the mDA neurons of the SNpc and the VTA during the rest of development (Rekaik et al., 2015). Once fully expressed, En1 is considered a postmitotic marker of dopaminergic neurons.

It is in this postmitotic, but still relatively immature state that the mDA neuron expresses an orphan nuclear receptor, or Nurr1. Findings indicate that it is vital in late survival and differentiation, but does not play as important of a role in early development (Wang et al., 2020). Specifically, it facilitates protein expression that is key to the differentiation into various dopamine receptor

phenotypes (Hegarty et al., 2013). Another post-mitotic transcription factor that is expressed in differentiated mDA neurons is Paired-like homeobox protein, or Pitx3, which is induced by Nurr1 with mutual regulation by En1 as well (Veenvliet & Smidt, 2014).

Nurr1 also works concurrently with Pitx3, to induce tyrosine hydroxylase (TH) in a mature neuron. In mice with an absence of Nurr1, the expression of TH was non-existent during the development of those mice (Saucedo-Cardenas et al., 1998). The presence of Nurr1 has also shown to increase the number of TH producing cells in the midbrain as well (Wallén-Mackenzie et al., 2003). TH is the rate-limiting enzyme that is responsible for the biosynthesis of catecholamines. If it is successfully produced, it will convert tyrosine into L-DOPA, which will later on become dopamine that will be used in various signaling pathways (Daubner et al., 2011).

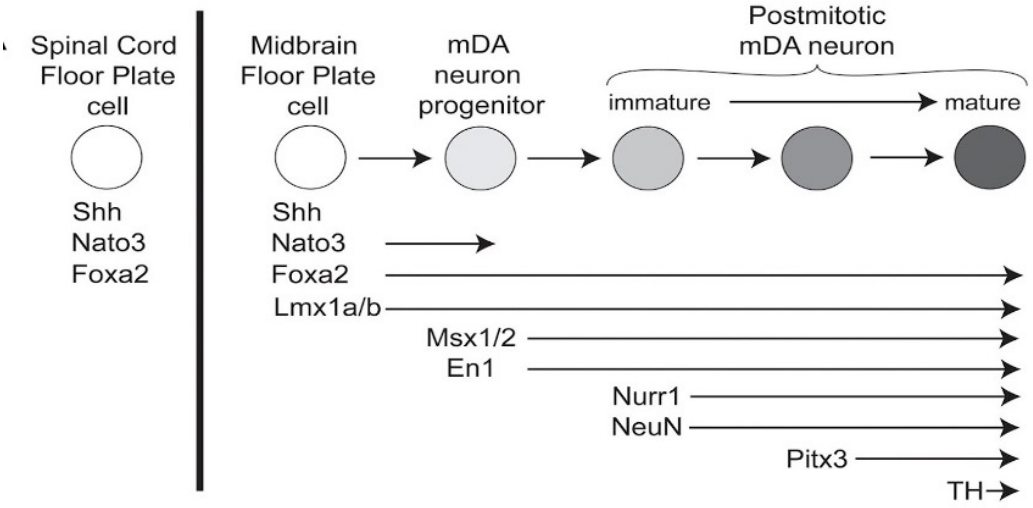

Figure 2: The progression of differentiation into a mature DA neuron. The stem cell shown in the photo progresses to a DA neuron progenitor and a post-mitotic, mature dopaminergic neuron. Key factors and genes including Foxa2, Lmx1a/b, Nato3, Shh, and the enzyme tyrosine hydroxylase (TH) are induced throughout the process of neuronal development.

Neuroprotection

The preservation of both structure and function in mature neurons is primarily the CNS. The primary goal is to preserve neuronal integrity and ideally, prolong the life of the cell by staving off neurodegenerative factors from various stressors that contribute to disease (Casson et al., 2012). Despite the multiple differences in disease presentation, the known mechanisms of degeneration are similar throughout these multiple pathologies (Seidl & Potashkin, 2011). This includes oxidative stress, protein buildup, inflammatory changes, and a lack of oxygen and glucose being sent to the CNS.

Increased oxidative stress in mammals leads to an increase in mitochondrial dysfunction, causing oxidative phosphorylation to become disrupted. In turn, this leads to the release of free radicals, opening up potential damage to DNA and malformation of proteins (Blaudin de Thé et al., 2016). Ultimately, this will decrease the quality of life for the neuron because it becomes more susceptible to degeneration and death.

There are multiple neuroprotective factors that stave off the onset of PD. Mouse models in previous studies have indicated that En1/2, Foxa1/2, Nurr1, Lmx1a/b, and Pitx3 are regulators of adult DA neuron maintenance (Blaudin de Thé et al., 2016). For example, in Lmx1a/b knockout studies, the data suggests that mature DA neurons have a marked loss in the VTA compared to the control, indicating the transcription factor's vital role in cellular maintenance (Blaudin de Thé et al., 2016). Another study shows that loss of one En1 allele in mice leads to increased numbers of phosphorylated serine alpha-synuclein in the SN and VTA (Chatterjee et al., 2019). Because of the

presence of alpha-synuclein in known cortical areas associated with PD, it can be inferred that En1 contributes to its prevention.

In multiple PD models, the delivery of these transcription factors have been shown to be neuron protective due to a lowered risk of PD in their presence. Mechanistically, these transcription factors have shown to be useful in transcriptional and translational regulation as well as DNA repair and metabolic regulation (Blaudin de Thé et al., 2016). Previous studies attempting to protect neurons in the substantia nigra from degeneration have not been successful looking at individual methods or pathways; however, a multifaceted approach to providing neuroprotection may be more effective (Espay et al., 2017).

Importantly, a recent paper (Miozzo et al., 2022) illustrated that the loss of the Nato3 (Fer2) homolog in drosophila resulted in loss of dopamine neurons and motor dysfunction by oxidative stress. They also demonstrated that the loss of Nato3 in mature DA neurons in mouse models also resulted in the expression of genes indicating oxidative stress. These animals also showed motor dysfunction akin to that seen in other animal models of PD (Miozzo et al., 2022). This newfound data links Nato3 with neuronal survival in mature neurons and the action of PM-Nato3 to promote the expression of pro-dopaminergic factors may illustrate a critical target gene for neuroprotective therapy.

Therapeutic Intervention: Cell Replacement Therapy

Cell Replacement Therapy (CRT) is a method that aims to replenish the dopaminergic neurons lost in the progression of PD with new, fully functional dopaminergic neurons. This opens

up the possibility of it not only impacting the extent of symptom alleviation but could also potentially have curative properties in PD treatment.

Recent advancements in stem cell research have allowed human pluripotent stem cells (hPSCs) as a potentially valuable resource for cell replacement therapy (Ásgrímsdóttir & Arenas, 2020). Using hPSCs is particularly appealing because it allows the restoration of lost cells and will be less impacted by catching PD in later stages or in more rapidly progressing cases. It also would be able to potentially address the non-motor symptoms created by PD as well, such as dysphagia, insomnia, incontinence, and fatigue.

There have been successful clinical trials using cell replacement therapy and many of them include tissue from a human fetal ventral midbrain, or VM, which is rich in dopaminergic neuroblasts. One study found that VM tissue was successfully used for cell transplantation, with five cases of transplants surviving longer than 10 years (Lindvall & Björklund, 2011). Another clinical trial with two PD patients showed that long term symptomatic relief after receiving intra-striatal grafts of human fetal VM tissue. The patients not only showed significant motor improvements after the graft, but both patients discontinued pharmacologic dopamine replacement therapy and continued to stay off the medication (Kefalopoulou et al., 2014).

These results were found in patients who experienced EOPD, with their respective onsets at 39 and 42 years old, which provides hope for an otherwise devastating diagnostic outcome. It is also consistent with other mammalian models as well. The data suggests that the best clinical outcomes have been obtained with fetal ventral mesencephalic allografts (Barker et al., 2015) and in rodent models, recovery from PD-like symptoms was observed after four weeks (Perez-Bouza et al., 2017).

Other clinical trials are underway using induced pluripotent stem cells (iPSCs), which are derived from either skin or blood cells and regressed back to a pluripotent state, enabling potential neurodevelopment (*Induced Pluripotent Stem Cells (IPS) | UCLA Broad Stem Cell Center*, n.d.). While use of different types of stem cell-based technologies is hopeful and many long-term results have not been defined yet, there have been problems found in the literature. There was a significant problem with dopamine release from the transplant tissue exceeding normal levels. This eventually led to dyskinesia, which undoubtedly decreases quality of life for the patients in treatment (Henchcliffe & Parmar, 2018).

For many years, this limited investigation of cell replacement therapies, but with the review of prior data, the benefit of transplantation in younger PD patients suggested significant heterogeneity in the response to treatment. Thus, new studies have begun to investigate transplantation in humans, including using human fetal tissue and dopamine neurons derived from induced pluripotent stem cells (iPSCs).

The first clinical trial in humans showed the transplant of iPSCs in the brain of a PD patient. It was noted that symptoms of PD stabilized between 18-24 months after the stem cells were implanted (Schweitzer et al., 2020). Because the current treatments are in early stages of trials, there are still many questions regarding efficacy and scope of this approach; however, it is a hopeful alternative to those who are struggling with traditional methods of treating PD.

Role of PM-Nato3

Preliminary Data

Neural Progenitors: In Vitro

In vitro data with hESC indicates that expression of PM-Nato3 can induce the expression of several key dopaminergic markers in the presence of minimal neuronal media and differentiation conditions (3N, also see Fig 7 for an outline of culture conditions). In order to transiently express PM-Nato3 using the TET on an inducible promoter system. This system employs the tetracycline transactivator (tTA) protein to bind to an operator only if it becomes bound to tetracycline or one of its derivatives (doxycycline). Introducing doxycycline, in turn, will initiate gene transcription (Das et al., 2016).

The stem cell line was generated by Dr. DeLano-Taylor prior to the start of this project and is called PM-Nato3.. Interestingly these cells did not halt cell proliferation, so while the expression of these key factors were important for the expression of the markers EN1 (A) and TH (B). Notably, these cells did show signs of neuronal maturation (morphological changes) (C) and expression neuronal marker TuJ1 and midbrain marker OTX2 (D). Nonetheless, there are still exogenous factors that need to be identified for appropriate differentiation into mature dopamine neurons. In order to test this, we will look at the constitutive expression of PM-Nato3 in the presence of exogenous factors (called the standard condition) to hopefully accelerate the production of DA neuron numbers and maturation in the hESC line.

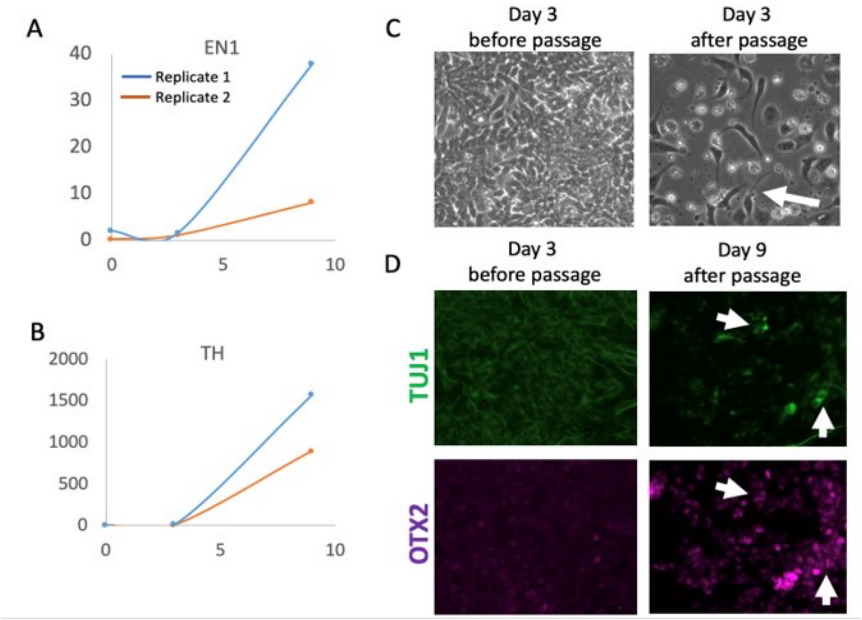

Figure 3: PM-Nato3 alone can induce DA neuron marker expression without requiring expensive labile supplements (minimal condition). Shown above are cells grown in E8 media then passed to 3N media for Days 3-9. **(A,B)** Induction of EN1 and TH mRNA, two key markers for DA neurons (n=2). The blue line represents the treated group while the red line represents the control group. **(C)** Appearance of cells with neuronal morphology on Day 3 (n=3). (D) Expression of midbrain marker OTX2 with early neuronal marker TUJ1 on Day 9 (n=1).

Neural Progenitors: In Vivo

The promise of PM-Nato3 to promote dopaminergic neurons from progenitors is underscored in the analysis of the action of PM-Nato3 action in neural progenitors in the enriched environment of the embryonic developing nervous system. In the in vivo preliminary data illustrated in Figure 4, the neural tube of the chick embryo was injected with PM-Nato3 during the single-layer stage (HH 10-12; (Krull, 2004). Afterwards, multiple genetic markers were monitored to show DA neurogenesis. In ovo electroporation of T101E/S140D in the embryonic chick induced expression of Foxa2, Lmx1b, Nurr1, and En1 in both the midbrain and telencephalon.

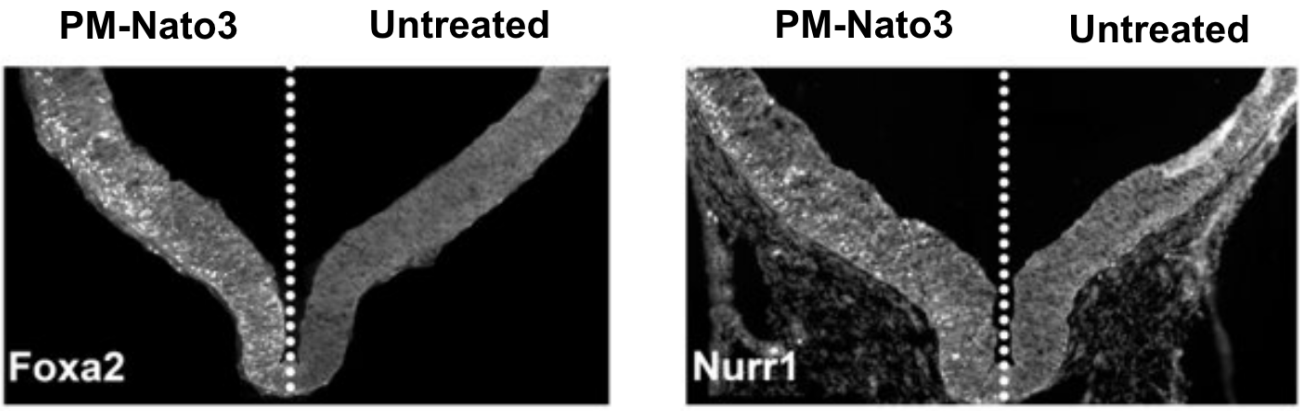

Figure 4: PM-Nato3 induced rostral midbrain expression of Nurr1 and Foxa2. PM Nato3 induces rostral expression that is not seen in the rostral midbrain when observing the wild-type Nato3. This action was seen broadly in CNS for some transcription factors (Foxa2) and more rostral for others (En1, Nurr1).

Another set of these genes were upregulated in progenitors treated with PM-Nato3 overspression in the spinal cord (Shh, Foxa2, and Lmx1b.) This data indicates that PM-Nato3 can potentially induce the expression of key DA markers in a broad array of stem cell populations, from hESC in vitro to neural progenitors in vivo (Figure 4). Since the data shows that PM-Nato3 has the capacity to express pro-dopaminergic genes in progenitor cells in vivo using the in ovo model system, it is notable that this experimental condition provides the extrinsic factors that could help coordinate this expression (by virtue of being in the in vivo condition). This thesis will address whether PM-Nato3 overexpression can promote DA neurogenic genes from hESC in an enriched extrinsic factor, like the promotion of DA neurogenic genes in progenitors in the vivo condition. This would ideally provide insight on neurogenesis and neuron maintenance that can be used in future cellular replacement therapies.

Mature Neurons: In Vitro

The unpublished data show that cell lines derived from differentiated, mature cells are less responsive to PM-Nato3 in vitro than hESCs. SN4741 cells, which are a mouse cell line made by immortalizing TH expressing cells derived from mouse embryos, were tested. LUHMES cells, a cell line derived from human mesencephalic cells, were tested as well. These cells were immortalized by a v-myc transgene under the control of a tet-off system, meaning that they are able to differentiate into a postmitotic neuron after the introduction of tetracycline (Edwards & Bloom, 2019).

They were then maintained in a proliferation medium (Ghosh et al., 2016). In both the SN4741 mouse cell line and the LUHMES human cell line, PM-Nato3 was overexpressed, and the impact of gene expression was assayed by qPCR, but the induction of gene expression was modest and restricted to solely Foxa2 (2-fold; data not shown). Compared to the induction of markers in Figure 4 (hESC + PM-Nato3 no supp). These data suggest that older, more mature cells may not respond to PM-Nato3 as much as progenitor cells. Indeed, gene silencing and chromosomal condensation and packaging shift dramatically as cells mature from the progenitor, multipotent state to the differentiated state.

This assessment is qualified by the very different conditions the cells are maintained, but culture conditions between cell types is difficult to match completely due to the distinct metabolic needs of the cells. The upregulation of dopaminergic factors in mature cells holds relevance for neuroprotection, which is why we were investigating if PM-Nato3 had an impact on mature cell types.

Mature Neurons: In Vivo

PM-Nato3 has exhibited inconsistent responses when introduced to neural progenitors or stem cells in the in vitro setting, which may point to the necessity of an enriched environment with all of the growth factors working together to differentiate DA neurons. Additionally, there are similar findings when looking at the mature cell response to PM-Nato3. This has led prior researchers in this lab to look at in vivo models of neural stem cells and mature neurons to demystify the answers because the environment could have significant contributions to DA neurogenesis and survival.

Since the response of differentiated mature cells (glia or neurons) to PM-Nato3 expression in vivo has been partially characterized, this project will help complete this effort. In collaboration with Patrik Brundin, previous workers in the lab designed a bicistronic AAV2/9 vector expressing PM-Nato3 and IRES-GFP and overexpressed varying doses of PM-Nato3 in 2-month-old C57B6/J mice.

After one-month, injected mouse tissue was harvested for analysis. Since there is anatomical symmetry, a comparison can be made between the substantia nigra tissue treated with PM-Nato3 (ipsilateral to injection) relative to the substantia nigra tissue that was not treated with PM-Nato3 (contralateral to injection).

The ICC analysis indicated that there was no change in the number of TH positive cells between the affected region and contralateral control. Nonetheless, other neuroprotective factors, such as En1, Foxa2, Nurr1 or quantification of levels of TH expression, were not assayed. It is conceivable that some of these factors are elevated while others are not.

If PM-Nato3 induces the expression of these factors in mature neurons in vivo, it would illustrate that these cells have the capacity to express putative neuroprotective factors in the enriched in

vivo environment, in a way that PM-Nato3 was not able to induce in the more refined environment in vitro (with SN4741 or LUHMES cells). This thesis will also complete the analysis of the mouse samples and compare the results to new findings.

Potential Role in Replacement Therapy

NATO3 is a basic Helix-Loop-Helix (bHLH) transcription factor that has shown to be a key regulator of DA progenitor cell development. It is introduced during the floor plate stage of the progenitor cell and continues throughout the progenitor cell stage. It is vital in the regulation of EN1, Lmx1a, and NURR1 expression (Niu et al., 2018). It also plays factors in maturation of the FP cells as well (Ono et al., 2010). Recent data using chick embryos have found that overexpression of Nato3 in a developing chick increases the activity of genes associated with DA neurogenesis, including Foxa2, Lmx1b, Shh, and Nurr1 (Peterson et al., 2019). The findings of this study also suggested that there was an increase in the mRNA expression of TH as well.

The expression of Nato3 has been found to be elevated during specification and expansion, as seen above in Figure 4. This includes findings using phosphomimetic PM-Nato3, a novel, modified mouse Nato3 gene that mirrors the function of a phosphorylated endogenous NATO3. It has a modified phospho-acceptor residue on (S140) to an amino acid with a negative charge on the side chain. This is essentially a glutamate. Preliminary data suggests that the expression of this gene promotes the expression of the aforementioned transcription factors, specifically En1/2, Nurr1, Lmx1a/b and Foxa2.

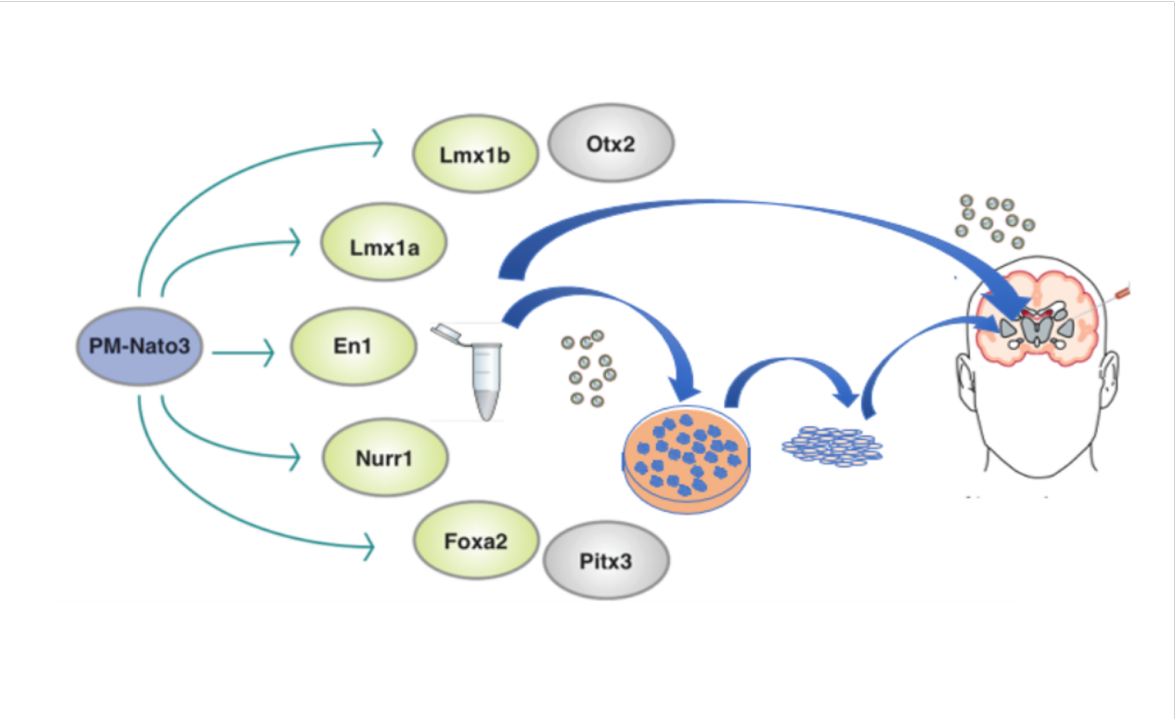

Figure 5: Pictured above is a schematic of how PM-Nato3 could potentially be utilized in cell replacement therapies. Using PM-Nato3's ability to upregulate these other transcription factors (shown in green), a hESC culture could potentially be created and inserted into the target areas of the midbrain where DA neurons are lost. Alternatively, PM-Nato3 could be presented directly into the brain of patients to reprogram cells residing near cells that have lost DA input (the target cells of the SNpc, known collectively as the striatum. Thus, PM-Nato3 could induce neurogenesis in cells residing in the mature brain.

With any stem cell replacement therapy, there are potential complications that arise from the use of immunosuppressants and the potential of tissue rejection (Henchcliffe & Parmar, 2018). . One of the unique potential concerns about using cellular replacement therapy for PD and other neurological diseases is the development of off-medication-state graft-induced dyskinesia (GID). It is theorized that this may be due to an uneven distribution of DA neurons after the transplant has been completed and running (Henchcliffe & Parmar, 2018).

Chapter 3: Methods for Experimentation

Experimental Design

Effect of PM-Nato3 on neural progenitor cells in vitro:

We predict that PM-Nato3 is sufficient to direct the differentiation of inducible neural hESCs to a dopaminergic fate in the presence of a broad pro-neural signal. Inducible neural hESC (also called iNeurons) can be differentiated by transient expression of the proneural gene NEUROG2 (Sheta et al., 2022). These cells typically differentiate into excitatory glutaminergic neurons, but it can effectively induce rapid differentiation in multipopulational neurons. In this experiment, we co-expressed PM-NATO3 with the proneural gene NEUROG2 and monitored the expression of key dopamine neuron transcription factors. This is the third time this condition has been tested and previously collected data will be included in the final analysis for comparison.

In order to induce the transient co-expression of both PM-Nato3 and NEUROG2, we used the TET on system. This uses the tetracycline transactivator (tTA) protein to bind to an operator only if it becomes bound to tetracycline or one of its derivatives (doxycycline). Introducing doxycycline, in turn, will initiate gene transcription (Das et al., 2016). The stem cell line was generated by Dr. DeLano-Taylor prior to the start of this project was called PM-Nato33 T101E/S140D (mouse).

We then harvested at Days 0, 3, 9, 14, and 20. The goal of the experiment was to capture later, more mature samples at Day 30 and 35, but COVID-19 restrictions shut down the laboratory of the collaborators we were working with to conduct the culture of the hESCs. The samples that were collected were kept in trizol until the analysis could be completed as part of this thesis. We then

isolated the mRNA and generated cDNA for qPCRanalysis to measure the expression of key dopaminergic genes.

This approach will allow the detection of genes GAPDH, TH, NATO3, NURR1, FOXA2, and EN1 and their changes in expression relative to Day 0. GAPDH is used as a reference gene for comparison of expression because the preliminary data has established that it is robustly expressed in the cell culture during each time point in development. The differences between cells that have PM-Nato3 expression in tandem with NEUROG2 relative to those that only have NEUROG2 .

It may be that PM-Nato3 is not sufficient to drive complete DA differentiation and maturation from H9 stem cells, even in the presence of the proneural gene NEUROG2. However preliminary data show that PM-Nato3 upregulates some key dopaminergic genes. There is a standard DA culture condition of differentiating hESC into DA neurons that is lengthy (35 days) and has variable and low yield (20-80% bonafide DA neurons defined as TH+/MAP2+).

We hypothesize that expressing PM-Nato3 in the presence of the standard DA culture conditions will introduce pro-dopaminergic genes earlier and support differentiation in less than 35 days or have greater yield (more % DA neurons in culture) than the standard culture condition. Thus, PM-Nato3 may work to promote dopaminergic expression with more precise DA neuron differentiation signals than simply the pro-neural gene NEUROG2.

In order to do this, we chose two platforms, lentiviral expression and mRNA transfection. The lentiviral vector was designed and then transduced into PM-Nato3. The mRNA vector was created to demonstrate phasic expression of PM-Nato3 to potentially mimic natural development of the neurons.

mRNA Vector Generation

RNA transfection of hESC with PM-Nato3 follows published protocols (Xue et al., 2019) and conducted by the University of Michigan human embryonic stem cell core facility. For this project, the scope of work is to generate the mRNA to conduct PM-Nato3 expression in culture conditions that include dopaminergic supplements. PM-Nato3 was generated using T7n mediated transcription of the PM-Nato3 coding sequence. This sequence has been closed into a vector (pcDNA3.0+T7) that contains the T7 promoter and a poly(A) tail. PM-NATO3 mRNA transfection was conducted using Stem-In cationic lipids.

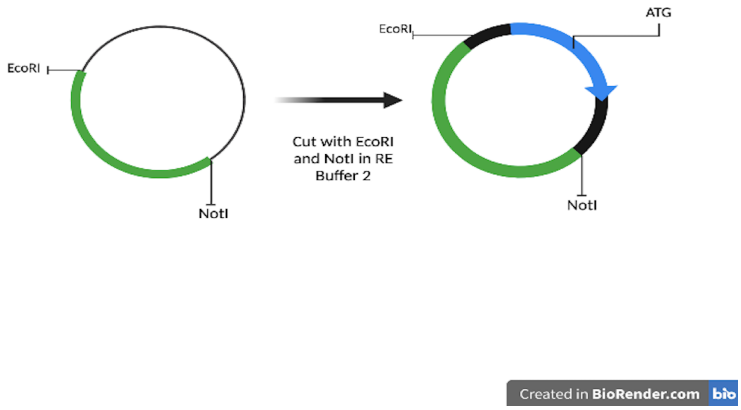

Figure 6: The image above shows the process of cutting the vector for linearization and visualizing the two cut sites. This image was created with BioRender.com for the purpose of being used in this thesis.

The vector needed to be prepared and later linearized for in vitro transcription. The vector was first cut with EcoR1 HF and NotI and placed in R_ Buffer 2. It was then stored for 2 hours at 37 °C. Then, it was transferred to 1% gel and heat inactivated at 65°C for 20 minutes. Afterwards, it was frozen at -20°C. The now linearized vector was isolated on 1% agarose gel with TAE buffer. 5 µl of loading dye was placed into each well. It was then run for 20 minutes at 120°C.

After we generated the mRNA for transcription in hESC, we delivered them to our collaborators at the University of Michigan. They transfected hESC cells that werethen pre-treated with the RNA transfection agent mRESR1 + Ri media for 1-6 hours, which was used to promote cell survival. Typically, the cells should appear to have spiky edges around colonies with Ri treatment to ensure that they are developing into neurons properly. The plate layouts and calculations were prepared prior to the start. 10 μM working concentrations of PM-Nato3mRNA:EGFPRNA duplex were then prepared by diluting each in TE. All dilutions were then kept on ice.

Then, working concentrations of donor plasmid at 1 μg/μL were also prepared by diluting in TE. the destination 6-well plate by aspirating and discarding the excess Matrigel liquid and adding 4 mL of RT mTeSR1 + Ri media to each well. Keep plate with media in the incubator at 37℃ and 5% CO2 until ready to plate cells after the transfection procedure. Working concentrations of Cas9 protein can be stored at -20℃ for up to 2 weeks and the researchers avoid multiple freeze-thaw cycles because less than two is recommended to maintain stability and lead to more robust results.

Lentiviral Vector Generation

In addition to expressing PM-Nato3 in vitro in the presence of media that is cultured in the presence of supplements, this project's scope of work generated a lentiviral vector to express PM-Nato3 in the hESCs. The vector will be generated by cloning the PM-NATO3 coding sequence into the NotI site in the multiple cloning site of pLentiLox RSV vector, which expresses Puromycin resistance gene as well as PM-NATO3.

The lentiviral vector was chosen for the hESC portion of the experiment because lentiviruses have the capability to integrate into the genome in a stable manner because it is an RNA virus (Davis, n.d.). Conversely, adeno-associated viruses, or AAVs, do not function similarly because they are single-stranded DNA viruses and function better in situations where producing at a high viral titer is needed (Zheng et al., 2018). The choice in viral vector for both types of samples has still been shown to be translatable for results in prior work. The vector design was submitted to the University of Michigan's Biomedical Research Core facilities for production of active lentiviral particles.

Culture Conditions: Standard Culture

Culturing of hESC was conducted with our collaborators at the University of Michigan. The initial media chosen for these conditions is E8 media, named after its eight components, including the DMEM/F12. This is a feeder-free medium that was formulated specifically for the growth and expansion of human stem cells and unlike others, which contain over twenty components, E8 contains only eight ingredients (Chen et al., 2011). It was purchased from ThermoFischer Scientific because prior results suggest that this media is a consistent and robust way to support stem cell differentiation and growth while maintaining quality control. Ideally, this will help definitively create dopaminergic neurons

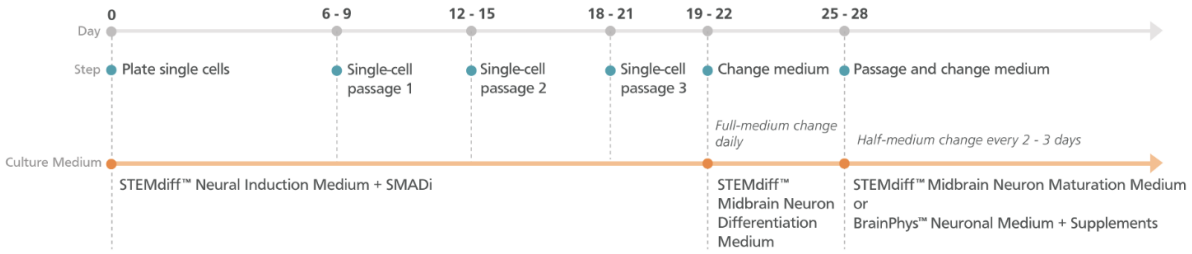

Figure 7: This shows the development of the neurons including the cell passages and each medium changes by day. These neurons were kept only to Day 20 and brought back to the DeLano-Taylor lab for analysis. It includes Puromycin selected PM-NATO3 for stem cell tech differentiation, while the regular H9 line will have no puromycin treatment.

Culture Conditions: Minimal Culture

The medium used for cell development under minimal conditions was a 3N medium, which is a 50-50 mix of Dulbecco's Modified Eagle Medium DMEM/F12, N2 (GIBCO), 5 μg/ml Insulin, 1mM L-Glutamine, 100 μm non-essential amino acids, 100 μM 2-mercaptoethanol, 50 U/ml Penicillin and 50 mg/ml Streptomycin; The B27 medium includes neurobasal (Invitrogen), B27 with or without vitamin A (GIBCO), 200 mM Glutamine, 50 U/ml Penicillin and 50 mg/ml Streptomycin. The 3N medium was supplemented with either 1 μm Dorsomorphin (Tocris) or 500 ng/ml mouse Noggin-CF chimera (R&D Systems), and 10 μm SB431542 (Tocris) to inhibit TGFβ signaling during neural induction 19.

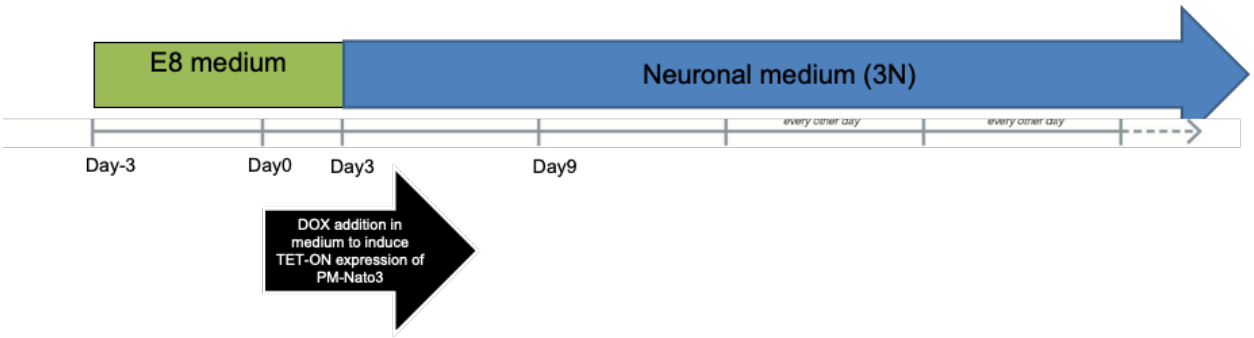

Figure 8: The figure above shows the timeline of cells being plated originally in the Minimal Medium condition. The E8 medium and eventual use of the 3N neuronal medium to induce differentiation into neurons. The doxycycline (DOX) is added to the medium to induce expression of PM-Nato3 by the cells. The TET-ON system uses DOX administration to activate the expression of PM-Nato3.

Cell Culture

Lentiviral treatment: After designing the lentiviral vector with input from the University of Michigan Biomedical Research Core Facilities the lentivirus was be applied to the hESC by the UM Human Embryonic Stem Cell Core Facility with hESC that are plated at $1x10^6$ cells/cm^2. This was completed on a 12 well cell culture plate that is coated with GFR-Matrigel and plated as clusters. Then, they were split one time and then selected for viability under Puromycin selection and the expression of PM-NATO3 will then be determined using quantitative PCR (qPCR) and immunocytochemistry (ICC). After confirmation of expression, these cells were cultured in supplemented media.

Effect of PM-Nato3 on Mature Neurons In Vivo:

We predict that PM-Nato3 will have a modest effect on dopaminergic gene expression in mature neurons in vivo. Presumably, these cells have undergone differentiation and gene silencing that is associated with the mature status of neurons relative to progenitors. In the absence of the rich milieu associated with the conditions of early development and neurogenesis, sole expression of the PM-Nato3 gene alone is not likely to be sufficient to induce expression of pro-neural genes.

Vector and Induction Strategy

To test the effect of PM-Nato3 on survival of dopamine neurons in a PD mouse model we administered PM-Nato3 using an adenovirus vector with bicistronic AAV2/9 and IRES-GFP was generated with our collaborator Patrik Brundin.

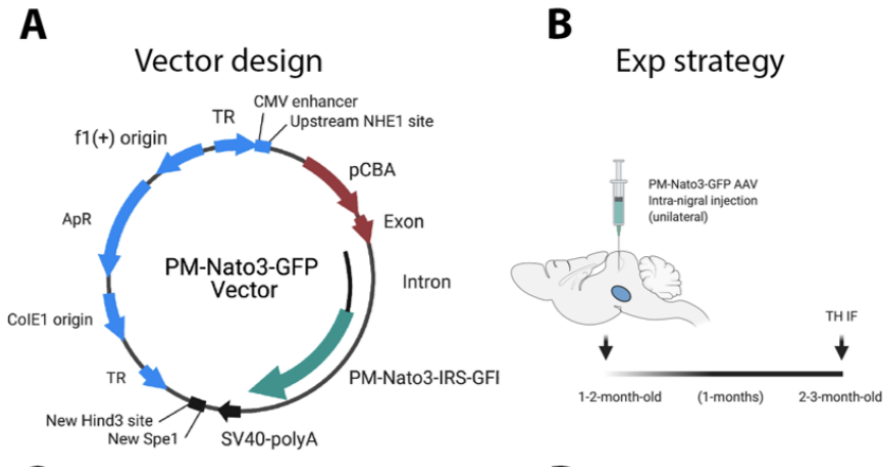

Figure 9 : (A) This demonstrates the design of the PM-Nato3 GFP Vector. It is a bicistronic AAV2/9 vector expressing PM-Nato3 and IRES-GFP. (B) The schematic above illustrates the timeline between the intranigral injection of the vector in the SNpc tissue until the eventual harvest post-injection.

The mice were injected with increasing concentrations of the vector expressing PM-Nato3 and GFP 1x109-13 (IU/ml). Injected mice were then followed for 1-month after which tissue was harvested for analysis. This means that they were allowed to express the construct for 2-3 months, allowing weeks of development into mature neurons.

A total of seven (n=7) SNpc were checked, and the immunocytochemistry was already completed prior to the start of this project; however, qPCR analysis of pro-dopaminergic genes was needed to complete the analysis. The tissue was then isolated in Trizol, the cDNA library for the samples were created, and then the samples were tested for the mouse homologs for the genes: Gapdh, Th, Nurr1, Nato3, Foxa2, and En1.

Analysis

RNA Isolation and cDNA Generation

For the nigral tissue, first the tissue was added to 100 µl of Trizol, then it was broken up with a disposable homogenizer (#H1001-50 from Zymo Research). Next, 200 µl of Trizol was added and was with a 1 µl pipette. The solution was then spun for one minute then further broken up with the homogenizer. It was then triturated again with the 1µl pipette 6-8 times until the clumps of tissue were no longer visible and eventually spun again.

The supernatant was transferred into a fresh tube and added to another 200µl of trizol for a total of 500µl. Afterwards, 500µl of EtOH was added and then loaded into a column and spun. It was then pre-washed twice with 400 µl of trizol, spun again and washed for 2 minutes again with more trizol. The solution was then eluted with 15 µl of H_2O and speed vacuumed for 5 minutes.

For the hESC samples, the RNA isolation was completed in a similar manner. 500 µl was taken from the hESC sample, and placed in an Eppendorf tube, then 500 µl of EtOH was added and vortexed. It was then loaded into a column and spun again for 1 minute. Then, it was prewashed twice with 400 µl before spinning again. The supernatant was then discarded after each pre-wash. 700 µl of the wash was added and spun again for two minutes.

cDNA was then generated from both types of samples for later qPCR analysis to monitor the expression of key dopaminergic genes. The RNA primer mixed was first created, combining 1 µl of the primer, 1µl of Deoxyribonucleotide triphosphate (dNTP), and 4 µl of H_2O. The 6 µl was then added

into PCR tubes. The superscript reaction mixed was created with 4 μl of buffer, 1 μl of DTT, 1 μl of RNA inhibitor and 1 μl of (SSIV).

qPCR Analysis

For both the hESC and mouse SNpc samples, quantitative reverse transcription PCR was completed. The cDNA library was created. All the cDNA was stored in a -20℃ freezer before use.

For all types of samples, standard TaqMan PCR protocol was used. This method was chosen because the specific hybridization between probe and target that is required to generate the signal reduces false positives and it requires no post-PCR processing allowing for reliable results(*TaqMan® vs. SYBR® Green Chemistries*, n.d.). First, the cDNA mix was created using 10 μl of TaqMan Fast Advanced Master Mix, 3 μl of RNA-free H_2O and 2 μl of the cDNA. It was plated as a triplicate for each primer. The probe mix used 4 μl of RNA-free H_2O and 1 μl of the primer matching the corresponding transcription factor. The primers used for this portion of the experiment were Hs02786624_g1 for GAPDH, Hs00165941_m1 for TH, Hs00541737_s1 for FERD3L (NATO3), Hs05036278 for NURR1, Hs00428691_m1 for FOXA2 and Hs00154977_m1 for EN1.

When plating the murine samples for qPCR, the left-sided neuron cDNA mix was plated first to avoid cross contamination with the right side, which had over-expressed Nato3. For both human and murine samples, GAPDH was used as a reference gene because its expression should remain relatively constant regardless of condition or cell type (Zainuddin et al., 2010).

The primers used for the murine samples were as follows: Mm999999915_m1 for Gapdh, Mm00447546_m1 for Th, Mm00443060_m1 for Nr4a2 (Nurr1), Mm01294538_m1 for Ferd3l (Nato3), Mm00453413_s1 for Foxa2 and Mm00438709_s1 for En1. The samples were then run using the MXPro3000x and the data was then compiled to compare the ipsilateral and contralateral side of each mouse brain sample.

Immunocytochemistry Analysis

Antibody Staining

To quantify the number of TH+ neurons (TH+/TuJ1+) at the final day of differentiation (Day 35), antibody staining was completed on fixed by the UMHGESC with 4% paraformaldehyde, which cross-links and maintains structural integrity of the cells. Upon receipt of the samples from our collaborators the cells were then permeabilized for 20 minutes in 1x PBS with 0.7% Triton x100. The blocking buffer was created with 5% Goat normal serum, 1% BSA in 1xPBS with 0.5% Tween-20 for 1 hour at room temperature.

To visualize the mature DA neurons, the nuclear marker DAPI was used as a counterstain to visualize the nucleus, and the key marker for mature dopaminergic neurons, Tyrosine Hydroxylase (TH) was used to determine TH expression on neurons (MABXXXX). Neurons were visualized with Beta III tubulin (a pan-neuronal marker also called TuJ1) (XXXXX).

Then, the primary antibody was put into the solution with the blocking buffer overnight and stored at 4℃. The samples were removed from storage and washed gently with PBS three times for

three minutes each. They were kept in a four well tissue culture plate. They then underwent secondary antibody (Ab) staining for an hour at room temperature and then washed three more times with 1xPBS for 3 minutes each. The nuclei were then stained with DAPI for 5 minutes at RT and washed one more time with PBS for another 3 minutes. It was then stored in 1xPBS and analyzed one week after the ICC protocol was started.

Imaging and Cell Counting

The analysis was completed using NIS (Nikon Imaging system) capture on a Nikon Ti Eclipse inverted microscope. Images were overlaid using Adobe Photoshop Creative Suite 5.0 software on the processor. DAPI was then stained to observe the nucleus of each neuron and TUJ1 was stained in order to establish the progenitors were growing properly into neurons because it is a widely established CNS marker (Lee et al., 2005). Once the clearest image of neurons with little to no background artifacts were found, the pictures were taken under three different filters to show the luminescence, and therefore expression. The images were then overlaid using Adobe Photoshop and the clearest pictures were included in both this document and the formal presentation.

To quantify the results, 10 visual fields from each experimental condition (PM-NATO3 Lentivirus in Standard Medium, compared to Standard medium alone) were captured and counting of TuJ1+ cells, TH+ cells and TuJ1+/TH+ double positive cells was conducted blindly by two different members of the laboratory. The total number of the cells were then expressed as a percentage of the TuJ1 cells. Notably, very few TuJ1- cells were seen, indicating that differentiation into neurons was completed at Day 35 in vitro.

Chapter 4: Results

In Vitro- Implications of Current Data (Lentiviral Expression)

1. Can PM-NATO3 increase or accelerate the rate of DA neurogenesis in the presence of a standard, dopaminergic culture condition?

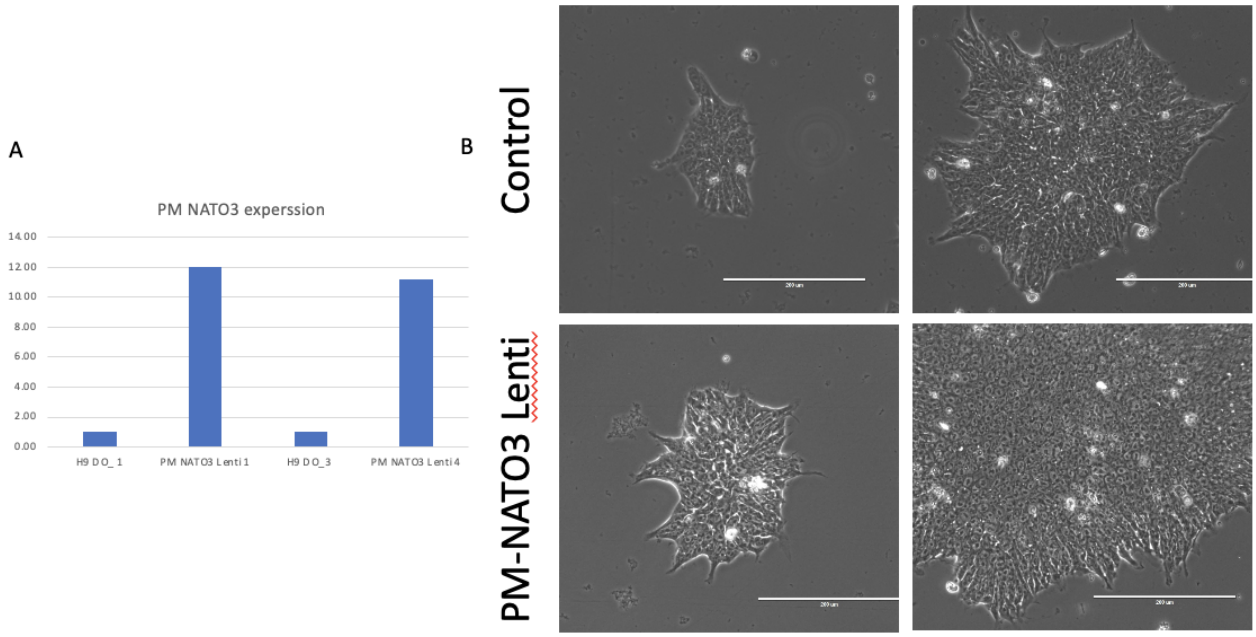

Figure 10: (A) This represents two identical cell lines. The H9 line represents the untreated control condition, while the PM-NATO3 treated lines are on the right of each respective line. Both showed a tenfold increase. **(B)** Phase photos show the morphology of the cell lines. The photos show cell line D0_1 and D0_3 at the top, respectively, and PM-NATO3 Lenti 1/PM-NATO3 Lenti 4, respectively.

Varying concentrations of lentiviral vector showed greatest selection at 1:100 dilution (data not shown), Fig. 10 shows the expression of a single lentiviral clone that was selected and passaged on separate days (PM-NATO3 Lenti 1 and PM-NATO3 Lenti 4 represent different passages of the same

lentiviral infected clone). The consistent expression of PM-NATO3 over two distinct passages illustrates the stability and consistency of PM-NATO3 in this cell line. Other lentiviral infected clones were also selected and passed to be used in future experiments, showing similar or greater levels of PM-NATO3 expression.

When looking at the morphology of the cells, there does not appear to be any significant differences between the two lines. There is no evidence of change in the cell number, which shows that this method did not induce over-proliferation of the cells. Also, based on the general appearance of the cells, there does not appear to be a difference in cell type.

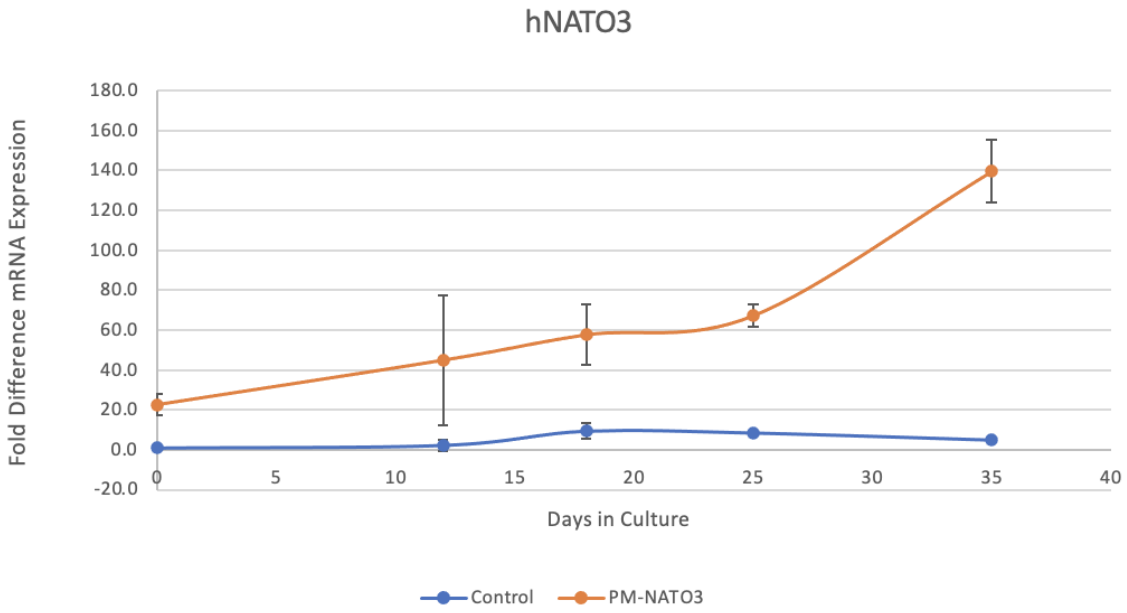

Figure 11: This shows the level of NATO3 overexpression in the cell lines. The blue line shows the control while the orange line shows the PM-NATO3 treated cells.

There is an increase in expression over differentiation for PM-NATO3. It also shows some fluctuations in endogenous NATO3 in the H9 cell line while in the standard media condition, which is to be expected. A one way ANOVA was completed and revealed that there was a statistically

significant difference the fold difference of mRNA expression between the two groups (F(1,8) = [9.516], p = 0.015).

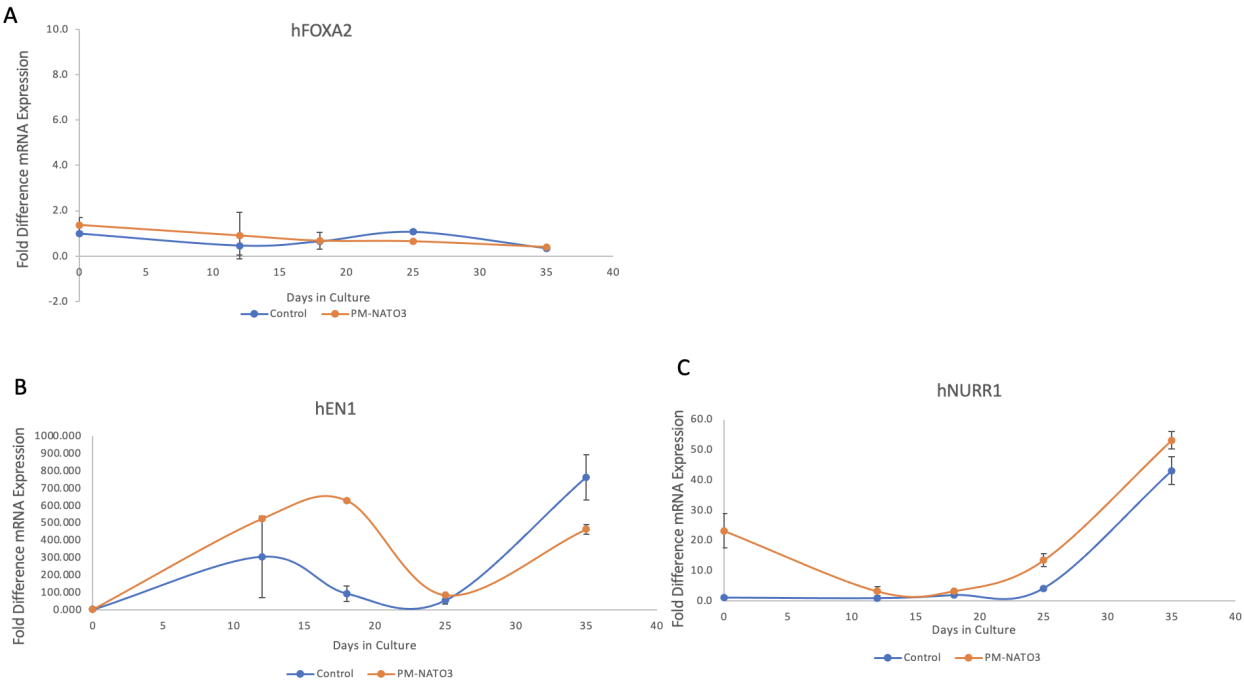

Figure 12: Expression of three key maturation markers over the 35 days. Blue lines indicate the control group while the orange lines indicate the cells treated with PM-NATO3. **(A)** Fold mRNA expression for FOXA2 **(B)** Fold mRNA expression for EN1 **(C)** Fold mRNA expression for NURR1.

When looking at the key maturation markers, FOXA2 did not increase in either condition and the CT values remained relatively low in Fig 12A. EN1 appeared to have higher CT values at the earlier phases (Days 12-18); however, it drops by a 34 fold count by Day 20 (Fig 12B). NURR1 showed that it was consistently higher at Day 25 through Day 35; however this was proven to not be statistically significant. .

A one-way ANOVA was performed to compare the effect of PM-NATO3 treatment on mRNA expression for each marker. None of these markers showed any significant difference in expression after the analysis was completed.

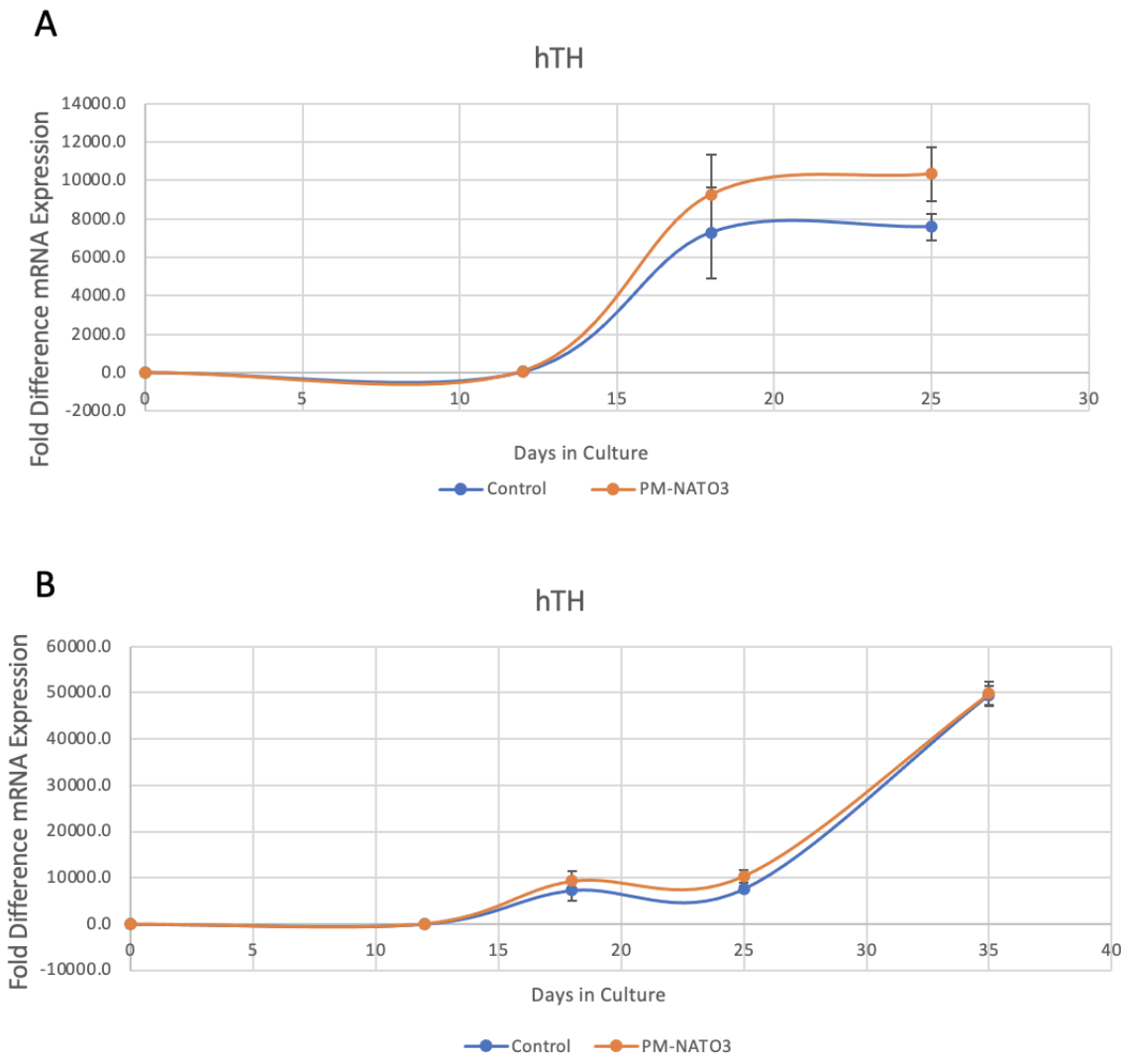

Figure 13: TH expression over the first 25 and 35 days of development. The blue line indicates the control group, and the orange line indicates the cells treated with PM-NATO3**. (A)** Tyrosine hydroxylase showed a modest increase in expression significantly between Days 18 through 25. **(B)** The increased effect shown on Day 25 diminishes over the following 10 Days by Day 35.

Since qPCR cannot discriminate the expression in a single cell cell but rather a population of cells, a question that remains is that we would like to know if the total number of TH+ cells was different between the two conditions. To test this, we completed an ICC analysis of the sample. The

ICC results did not show any significant difference in the number of TH positive neurons expressed in the cells compared to the control with no lentiviral induction of PM-NATO3.

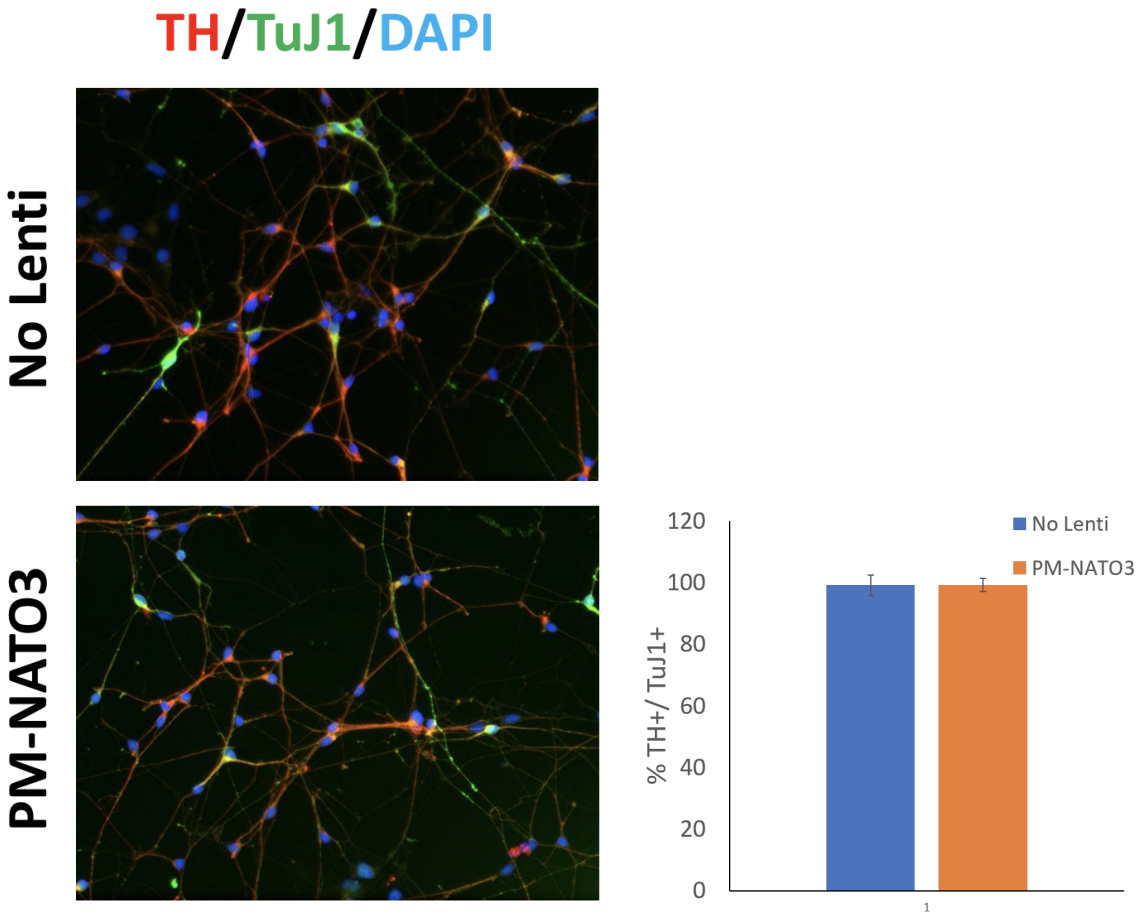

Figure 14: Staining shows no difference in expression of TH and TuJ1 in the control group compared to the group treated with PM-NATO3. Cell morphology also shows no change.

The DA maturation medium showed normal expression of TH+ cells being expressed as cited by the commercialization kit. 99% of the TuJ1 cells also showed that they were TH+. PM-NATO3 did not show any significant difference when tested at the last day of differentiation. If there are no significant changes in expression in the standard condition, one question that remains is if the proposed pro-dopaminergic action of PM-NATO3 could drive DA neurogenesis in minimal culture conditions.

2. Can PM-NATO3 sufficiently drive neurogenesis in a proneural (minimal) condition?

The next question that requires attention is the efficacy of PM-NATO3 driving neurogenesis in a minimal condition. The minimal condition includes media supplements that are sufficient to promote cell cycle exit and cell survival and has been shown to sustain hESC differentiation into glutaminergic neurons when they overexpress the pro-neural gene NGN2.

We employed an inducible promoter that drives the expression of either NGN2 in the control condition and NGN2 and PM-NATO3 in the experimental condition. To activate expression, Doxycycline was added to the media for 9 days during differentiation as described in the methods section above. We used qPCR to monitor the level of mRNA expression of the four key dopaminergic genes over the course of differentiation. These relative differences were compared to the first day of culture in the Ngn2 alone control condition.

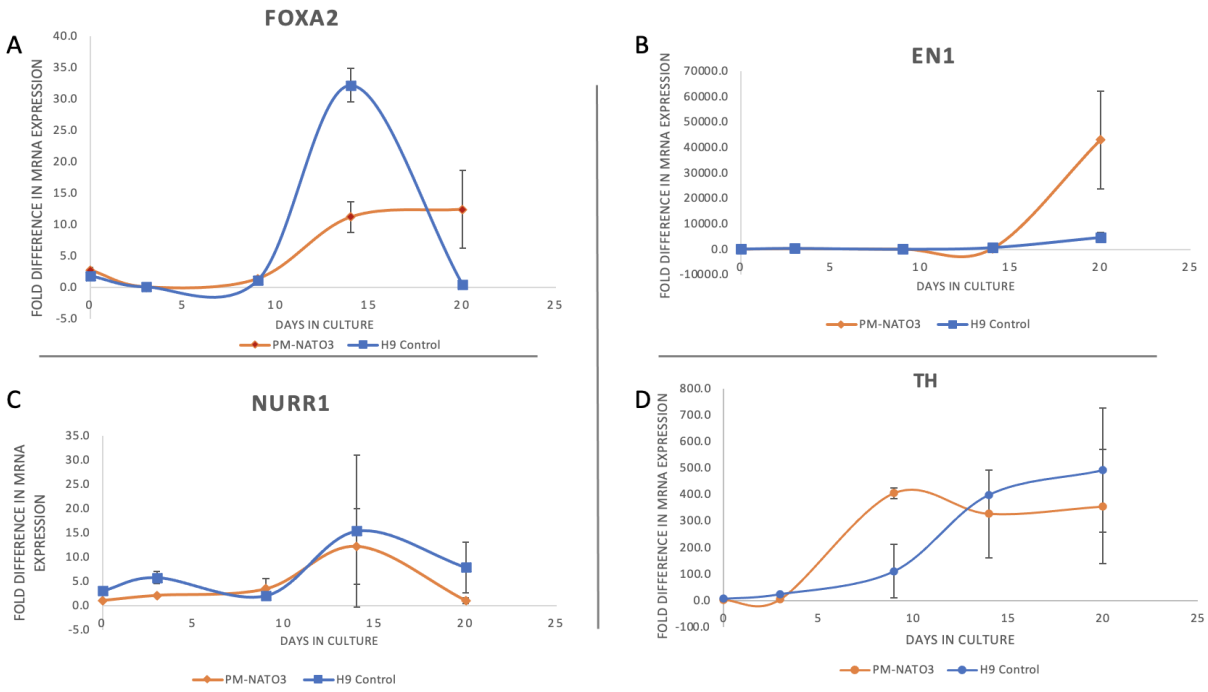

Figure 15: This shows the fold mRNA expression of the four key dopaminergic maturation markers in NGN2 cell line compared to NGN2+PM-NATO3, which appear here in the order they are involved in neurodevelopment. The blue lines indicate the H9 control group over the 20 days while the orange lines indicate the NGN2 and PM-NATO3 condition. **(A)** Fold mRNA expression for FOXA2 **(B)** Fold mRNA expression for EN1 **(C)** Fold mRNA expression for NURR1 **(D)** Fold mRNA expression for TH.

When looking at the comparison between the NGN2-treated cell line and the cell line treated with NGN2 and PM-NATO3, we did not find a significant change in the expression of most of the key markers for dopamine neurogenesis in the PM-NATO3 + NGN2 overexpression condition, relative to the NGN2 condition alone. However, when looking at EN1 in the +PM-NATO3 group, there was an approximately 9-fold increase seen by D20. Due to the COVID-19 pandemic-related shutdowns, these RNA samples had to be frozen at the 20-day time point for later analysis. As a result, the analysis was unable to be carried out for the full intended duration of 35 days. A single factor ANOVA was

completed for all four of the markers. For all four of the markers, none of their statistical tests demonstrated any significance. For example, the ANOVA for EN1 revealed that there was no statistically significant difference in fold induction of mRNA expression between the control and treatment groups ($F(1,8) = [0.76]$, $p = 0.4071$.)

3. Can PM-Nato3 Sufficiently Drive Dopaminergic Factors In Vivo?

The final question is addressed in this continuation of the in vivo study that was started in this lab prior to this thesis beginning. It aims to understand if PM-Nato3, while in an enriched environment of mature cells, drives dopamine neuroprotection.

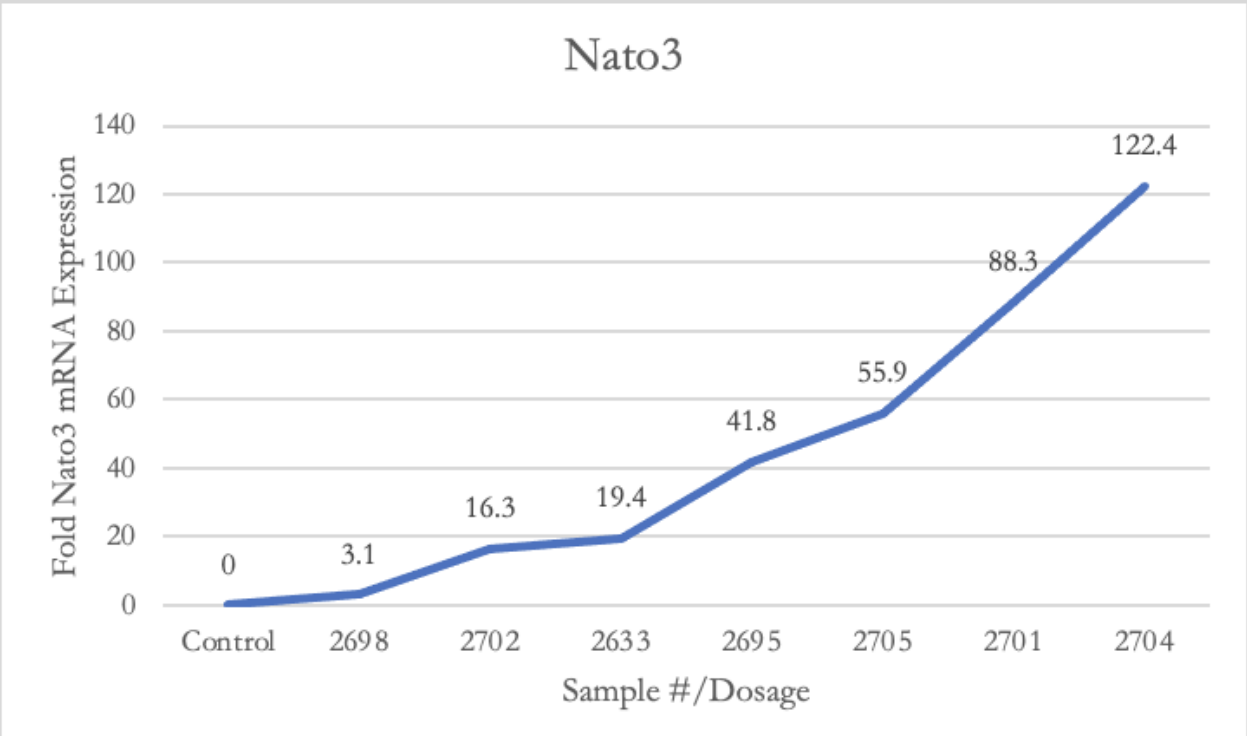

Figure 16: This shows the dosage of PM-Nato3 injected into the mice matched with the sample number. This image functions illustrate the development of a potential dose response curve for some of the transcription factors that were tested.

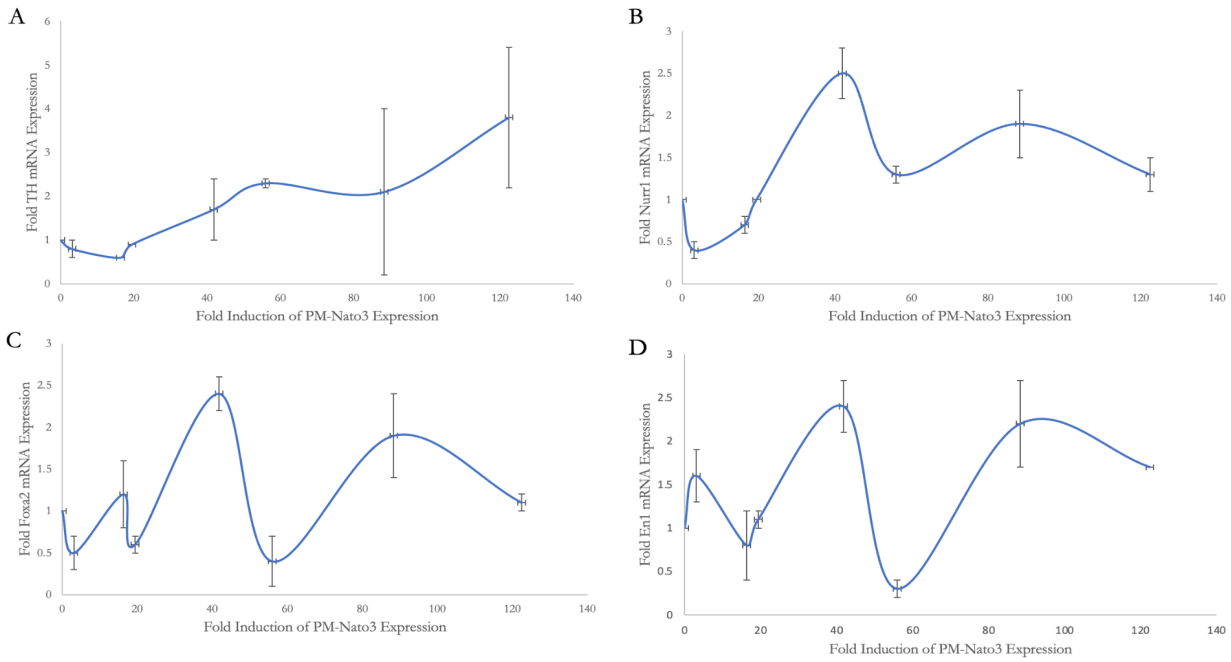

Figure 17: All four of the graphs above show the fold mRNA expression of four transcription factors associated with DA development compared to the fold induction of Nato3 that was expressed in the sample. **(A)** The fold induction of TH mRNA present in the cells. **(B)** The fold induction of the Nurr1 expression. **(C)**The fold induction of Foxa2 mRNA expression. **(D)** The fold of En1 mRNA expression.

When looking at RNA expression of all four transcription factors, it seems to oscillate in fold for Nurr1, Foxa2, and En1. The only transcription factor that appears to have a significant increase in the murine samples is TH and the effect is roughly four-fold. Simple linear regression was used to test if dosage of PM-Nato3, which is indicated by fold induction of PM-Nato3, significantly predicted mRNA expression for each of the major markers.

The overall regression was statistically significant when looking at TH expression in these mouse samples ($R2 = 0.88$, $F(1, 6) = 45.59$, $p = 0.0005$). However, there was not any findings that the other markers were impacted by the increase in PM-Nato3 fold induction in the mice. For example, it

was found that PM-Nato3 expression did not significantly predict Foxa2 expression ($R^2=0.083$ $F(1,6) = 0.452$, $p = 0.531$).

Chapter 5: Discussion

Principal Findings

1. Can PM-NATO3 increase or accelerate the rate of DA neurogenesis in the presence of a standard, dopaminergic culture condition?

The experimental conditions employed were adequate for assessing the rate of dopamine neurogenesis. Figure 10 demonstrates a successful over-expression of NATO3 at all time points. Interestingly, the expression of NATO3 in the PM-NATO3 treated group was increasing throughout the differentiation process. There are two potential explanations for this observation. Firstly, it is possible that there is a reduction in the number of cells that exhibit lower levels of PM-NATO3 expression, thus influencing the percentage of surviving PM-NATO3 positive cells until day 35.

However, there was no change in the total cell count between the control and lentiviral conditions, which would be expected if there was significant cell death. Alternatively, the induction of PM-NATO3 via lentivirus may be amplifying an existing phenomenon. In the control group, endogenous NATO3 expression doubled, and the cell line treated with PM-NATO3 showed a similar increase. This could be influenced by the stability of the mRNA present following lentiviral induction of the hESC.

Overall, there were little to no changes in the expression of most of the key markers for dopamine neurogenesis in PM-NATO3 + NGN2 overexpression condition, relative to NGN2 condition alone. This may be due to the modest level of PM-NATO3 overexpression (13X at Day 0 in

Fig 11, 20X at Day 35 in Fig 11). Nonetheless, some subtle effects that may prove to be statistically significant if replicated with multiple differentiations.

Does PM-Nato overexpression accelerate the speed of DA neuron maturation hESC?

An interesting observation is that the expression of FOXA2 (Fig 13A) remained unchanged during hESC differentiation under the control condition. Similarly, there was no increase in FOXA2 expression upon exposure to PM-NATO3. The attempts to analyze the FOXA2 protein using ICC at Day 35 were unsuccessful. However, according to additional research conducted by another student in the Delano-Taylor lab as part of their thesis, the expected increase in FOXA2 expression over time was observed. Hence, it is possible that an unidentified and distinctive factor in this process disrupted the expression of FOXA2. This distinction should be considered as we replicate these results.

The effect of PM-NATO3 on EN1 appears to be bimodal, with an increase observed in the treatment group as early as day 12 and the increase continues until day 25 (Figure 13B). Previous research has established that the overexpression of PM-NATO3 in vivo leads to an increase in EN1 expression in the midbrain (Figure 4a). Notably, the capacity of PM-NATO3 to drive EN1 expression is restricted to the midbrain.

Overexpression of PM-NATO3 in the spinal cord is not sufficient to drive EN1 expression (data not shown). This suggests that midbrain specific exogenous factors could potentiate the effect of PM-NATO3. This is in keeping with the increase of expression seen early in differentiation in Fig 13B.

Based on the preliminary data, this study aimed to examine the effects of PM-NATO3 in minimal conditions without the presence of exogenous midbrain factors. Considering this, it was

expected that PM-NATO3 expression in the presence of exogenous factors would exhibit a synergistic effect, as these factors are known to drive EN1 expression in conjunction with PM-NATO3. Previous studies have shown that other transcription factors, such as NURR1, have shown to have synergistic effects when combined with a realistic microenvironment in vitro (Beiki et al., 2022).

However, the data did not demonstrate a summative effect in later phases of differentiation as initially anticipated. One possibility is that the standard conditions used on those later days in the study already promote EN1 expression to a significant extent, and therefore, the additional action of PM-NATO3 does not further elevate its expression. It is plausible that the transcriptional pathways activated by PM-NATO3, such as LMX1A or FOXA2, may overlap with those activated by the standard conditions, both contributing to an increase in EN1 expression. This potential overlap could explain the absence of a synergistic effect observed in the data at day 25 and 35 in Figure 14.

To better understand the mechanisms underlying the relationship between PM-NATO3 and EN1 expression, further investigation is warranted. By exploring the specific transcriptional pathways influenced by PM-NATO3 and elucidating how they interact with the standard conditions, we potentially gain insights into the regulatory mechanisms governing EN1 expression and its modulation by PM-NATO3.

There was a modest increase of expression of NURR1 at Day 25 in the PM-NATO3 condition; this follows the peak of expression in the PM condition with EN1 at D18 (Fig 14). This sequence of transcriptional regulation, with EN1 preceding (and indeed helping to promote the expression of) NURR1, mirrors the endogenous DA neuron maturation (see Figure 1).

When looking at TH expression, there was a subtle increase of expression at Day 25, which was lost by the time the sample reached Day 35. This could indicate that maturation of hESC could be accelerated with a modest expression of PM-NATO3. This elevation of TH expression at D25 coincides with the elevation of NURR1 and EN1, suggesting that even modest levels of PM-NATO3, when introduced, could accelerate the maturation of hESC.

Does PM-NATO3 overexpression increase the yield of DA neurons?

As noted, before there was no significant difference in the number of TH+ cells counted in the ICC analysis. This may be due to a ceiling effect, or a situation where the variable being measured can no longer increase or show further improvement beyond a certain point. If the cells are already expressing high levels of TH at their baseline, then any increase in TH would seem insignificant. A potential solution to this would be to observe at earlier time points in the ICC to see any potential changes in the staining results. However, the qPCR data suggests that the magnitude of the effect on TH expression is small at day 25 at about a three-fold difference (Figure 13).

2. Can PM-NATO3 sufficiently drive neurogenesis in a proneural (minimal) condition?

The experimental conditions were not sufficient to fully address our question but offers some insight into future study. Although the data set examined in this study reveals that certain human embryonic stem cells (hESCs) did not reach full maturation by Day 30 or 35 due to COVID-19 restrictions halting the experiments, there are encouraging trends worth noting. Specifically, when

analyzing the PM-NATO3 +NGN2 condition, it was observed that the EN1 levels were higher (Fig. 15B) relative to NGN2 alone.

This finding aligns with the preliminary data (Fig. 3A), indicating that PM-NATO3 may have the potential to enhance EN1 expression under minimal media conditions. However, the differences observed in other maturation markers were not as significant and may not yield statistically significant results.

TH expression was lower in the PM-NATO3 +NGN2 condition compared to NGN2 alone, this may be due to the early stages of hESC differentiation (D20). TH expression increases several tens of thousands-fold in standard differentiation conditions at later stages of maturation (see Fig 14), so these fluctuations seen in NGN2 vs PM-NATO3 (increase of ~500 fold) conditions are likely to be modest.

To gain further clarity on these observations, the project requires replication, and this is currently underway as part of another student's thesis. Additional investigation is needed to capture the later stages of maturation in this condition. This raises an intriguing question: If EN1 levels are higher in the PM-NATO3 condition at Day 20, does it establish a favorable environment for more effective maturation by Day 35?

Therefore, the ongoing work beyond the scope of this thesis aims to address these uncertainties and provide a more comprehensive understanding of the impact of the PM-NATO3 condition on hESC maturation. By conducting repeated experiments and investigating the later stages of maturation, the project aims to shed light on the potential long-term effects of EN1 expression and determine if it serves as a crucial factor in promoting effective maturation in hESCs.

3. Can PM-Nato3 Sufficiently Drive Dopaminergic Factors In Vivo?

Prior work with En1 haploinsufficient mice showed that PM-Nato3 overexpression was achieved and ICC analysis indicated that there was no change in the number of TH positive cells between the affected region treated with PM-Nato3 adenovirus and the contralateral control. Our question was to determine if other neuroprotective factors, such as En1, Foxa2, Nurr1 were affected, in addition to quantification of levels of TH expression.

We analyzed the animals to determine the dose of PM-Nato3 overexpression (see figure 16) and then aligned the response of each of the four factors (see Figure 17A-D). shows that none of the neuroprotective factors (En1, Foxa2, Nurr1) were elevated, oscillating in a non-dose dependent manner.

There are multiple factors that could contribute to this phenomenon. One important fact to note is that the C57B6/J mice that were used in this project were fully mature because the tissue was harvested when they were at the three month mark (Jackson et al., 2017). Because the mice used in this project were past the early developmental milestones, we may have missed the window of time that PM-Nato3 may have significant impact on the expression of En1, Foxa2, and Nurr1.

Future studies should complete the stereotaxic surgery and analyze RNA expression closer to one month of age or earlier to provide insight on the expression of these pro-dopaminergic genes in the En1 haploinsufficient mice.

Remarkably, in Figure 18, the expression of TH (Tyrosine Hydroxylase) showed an intriguing dose-response relationship. While the number of TH+ cells in the immunocytochemistry (ICC) did not exhibit any significant changes, it is possible that the cells appearing as TH+ displayed an elevated

amount of TH. Prior studies did not assess the brightness of the TH ICC signal, but the quantification of TH mRNA in Figure 17 could provide support for this hypothesis.

Despite this interesting finding related to TH expression, our investigation into neuroprotective factors with PM-NATO3 overexpression did not reveal an overall increase. It appears that other factors or pathways may be influencing the observed effects, warranting further exploration and research.

Limitations

The present study acknowledges certain limitations that are inherent to its methodology and scope. This includes issues due to COVID-19 shutdowns, lentiviral optimization, and cytotoxicity. These limitations should be considered when interpreting the findings and understanding the implications of this research.

A huge roadblock with the previous projects that this thesis was contingent on is due to COVID-19 shutdowns. Both the minimal media condition and the in vivo data was halted and re-assessed at a later date-which could have had an impact on the results.

Another limitation of our study is the relatively modest level of PM-NATO3 expression observed in the lentiviral data. To address this limitation, it is important to further investigate higher levels of PM-NATO3 expression in additional cell lines treated with lentivirus. This can be achieved by making adjustments to the viral titer, the multiplicity of infection (MOI), and the transduction duration. Specifically, increasing the MOI can facilitate a higher number of viral particles infecting the target cells, thereby leading to enhanced expression levels of PM-NATO3.

By implementing these modifications, we can potentially optimize the lentiviral transduction process, resulting in increased PM-NATO3 expression in the treated cell lines. This will allow us to visualize the effects of PM-NATO3 more readily and gain a better understanding of its impact.

Another potential problem could be related to cytotoxicity. Some samples, specifically mice that were treated with high titers of PM-Nato3, had high (over 30) cycle threshold values for GAPDH. This could be indicative of cytotoxicity at certain doses of PM-Nato3 being introduced, which would in turn guide future researchers into what limitations are needed when increasing the titer or dosage of PM-Nato3.

By acknowledging these constraints, the aim is to provide a comprehensive and transparent account of the boundaries, potential biases, and areas for future investigation. Despite these limitations, the study's outcomes still contribute valuable insights to the field, and efforts have been made to mitigate any potential impact on the validity and generalizability of the results.

Future Directions

There is indeed a natural progression for this research that should be pursued. This involves exploring alternative media formulations, in addition to E8, to identify the most suitable conditions for the natural growth cycle of dopamine (DA) neurons. By altering the culture media, researchers can potentially optimize the expression of various genetic markers at the appropriate developmental stages. This optimization is crucial for understanding the precise timing and progression of DA neuron differentiation.

Furthermore, extending the study to longer-term development, beyond the 40-day mark, would provide valuable insights into the sustained effects of PM-Nato3 on the differentiation of DA neurons and their subsequent maturation into different receptor types. Analyzing the longer-term impacts would offer a more comprehensive understanding of the influence of PM-Nato3 over time. By implementing these suggestions, researchers can gain a deeper understanding of the optimal culture conditions for DA neuron growth and assess the extended effects of PM-Nato3 on their differentiation.

Also, there are multiple transcription factors that were not included in this specific analysis that are part of DA neuron development in the SNpc and VTA. Specifically, Lmx1a, Lmx1b, and Pitx3, could all be of value in further analysis. Other studies could include further qPCR monitoring of Hes1, another bHLH transcription factor that suppresses proneural gene expression and is often inhibited by the presence of NATO3 (Hegarty et al., 2013).

It should also be noted that brain derived neurotrophic factor, or BDNF, which is often expressed when the midbrain DA neurons are found to have potential neuroprotective factors (Razgado-Hernandez et al., 2015). This has been shown to be especially protective against PD when BDNF is overexpressed, so monitoring it after PM-Nato3 is introduced to the cells could further solidify the prospective role of NATO3 in neuroprotection.

Conclusions

The results presented in this thesis hold significant implications for comprehending the interaction between PM-Nato3 and developing neurons. Initially, we investigated whether PM-Nato3

influenced the rate of DA neurogenesis in the presence of a standard dopaminergic culture condition. Our findings indicated a potential acceleration in the production speed of DA neurons when observing the increases in two of the targeted transcription factors, EN1 and NURR1 as well as the later increase in the presence of the enzyme TH. However, there was no substantial increase in the yield of DA neurons generated under this supplemented media.

Furthermore, the investigation into the impact of PM-Nato3 on DA neurogenesis under a minimal culture condition presents an intriguing contrast to the results obtained in the standard culture setting. The absence of significant changes between the control condition and the PM-Nato3-induced condition, as evidenced by the data, prompts further exploration into the specific conditions and mechanisms that mediate the interaction between PM-Nato3 and the developing dopaminergic neurons.

Moreover, we investigated whether PM-Nato3 could effectively induce dopaminergic factors in vivo using a mouse model. The results indicate that none of the neuroprotective transcription factors (Foxa2, En1, and Nurr1) exhibited elevated levels. However, there was a modest increase in TH mRNA expression. This finding, however, should be interpreted cautiously, considering the potential influence of the murine sample's age at the time of tissue harvesting. The data suggests that mature cells are not as responsive to the actions of PM-Nato3 as progenitor cells, both in vitro (Fig 4 in preliminary data) and in vivo (Fig 17).

Taken together, these comprehensive findings provide a rich foundation upon which future research can build. The findings presented in this thesis lay the groundwork for further research and advancements in the realm of Parkinson's disease and regenerative medicine. The elucidation of PM-

Nato3's role in neuronal development deepens our understanding of the underlying mechanisms involved in Parkinson's disease pathogenesis. Armed with this knowledge, researchers can now focus on further unraveling the precise molecular pathways and signaling cascades influenced by PM-Nato3, and explore potential therapeutic interventions that can harness its benefits.

References

Alvarez-Fischer, D., Fuchs, J., Castagner, F., Stettler, O., Massiani-Beaudoin, O., Moya, K. L., Bouillot, C., Oertel, W. H., Lombès, A., Faigle, W., Joshi, R. L., Hartmann, A., & Prochiantz, A. (2011). Engrailed protects mouse midbrain dopaminergic neurons against mitochondrial complex I insults. *Nature Neuroscience*, *14*(10), 1260–1266. https://doi.org/10.1038/nn.2916

Andersson, E., Jensen, J. B., Parmar, M., Guillemot, F., & Björklund, A. (2006). Development of the mesencephalic dopaminergic neuron system is compromised in the absence of neurogenin 2. *Development (Cambridge, England)*, *133*(3), 507–516. https://doi.org/10.1242/dev.02224

Ang, S.-L. (2006). Transcriptional control of midbrain dopaminergic neuron development. *Development*, *133*(18), 3499–3506. https://doi.org/10.1242/dev.02501

Artegiani, B., & Calegari, F. (2012). Age-related cognitive decline: Can neural stem cells help us? *Aging*, *4*(3), 176–186. https://doi.org/10.18632/aging.100446

Ásgrímsdóttir, E. S., & Arenas, E. (2020). Midbrain Dopaminergic Neuron Development at the Single Cell Level: In vivo and in Stem Cells. *Frontiers in Cell and Developmental Biology*, *8*. https://www.frontiersin.org/article/10.3389/fcell.2020.00463

Barker, R. A., Drouin-Ouellet, J., & Parmar, M. (2015). Cell-based therapies for Parkinson disease—Past insights and future potential. *Nature Reviews Neurology*, *11*(9), Article 9. https://doi.org/10.1038/nrneurol.2015.123

Beiki, R., Khaghani, M., Esmaeili, F., & Dehghanian, F. (2022). Synergistic Effects of Combined Nurr1 Overexpression and Natural Inducers on the More Efficient Production of Dopaminergic Neuron-Like Cells From Stem Cells. *Frontiers in Cellular Neuroscience*, *15*.

https://www.frontiersin.org/articles/10.3389/fncel.2021.803272

Beisel, C., & Paro, R. (2011). Silencing chromatin: Comparing modes and mechanisms. *Nature Reviews Genetics*, *12*(2), Article 2. https://doi.org/10.1038/nrg2932

Benatru, I., Vaugoyeau, M., & Azulay, J.-P. (2008). Postural disorders in Parkinson's disease. *Neurophysiologie Clinique/Clinical Neurophysiology*, *38*(6), 459–465. https://doi.org/10.1016/j.neucli.2008.07.006

Benskey, M. J., Perez, R. G., & Manfredsson, F. P. (2016). The contribution of alpha synuclein to neuronal survival and function – Implications for Parkinson's disease. *Journal of Neurochemistry*, *137*(3), 331–359. https://doi.org/10.1111/jnc.13570

Berridge, K. C. (2007). The debate over dopamine's role in reward: The case for incentive salience. *Psychopharmacology*, *191*(3), 391–431. https://doi.org/10.1007/s00213-006-0578-x

Blaudin de Thé, F.-X., Rekaik, H., Prochiantz, A., Fuchs, J., & Joshi, R. L. (2016). Neuroprotective Transcription Factors in Animal Models of Parkinson Disease. *Neural Plasticity*, *2016*, 1–11. https://doi.org/10.1155/2016/6097107

Bonifati, V. (2012). Autosomal recessive parkinsonism. *Parkinsonism & Related Disorders*, *18*, S4–S6. https://doi.org/10.1016/S1353-8020(11)70004-9

Braak, H., Del Tredici, K., Rüb, U., de Vos, R. A. I., Jansen Steur, E. N. H., & Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiology of Aging*, *24*(2), 197–211. https://doi.org/10.1016/s0197-4580(02)00065-9

Casson, R. J., Chidlow, G., Ebneter, A., Wood, J. P., Crowston, J., & Goldberg, I. (2012). Translational neuroprotection research in glaucoma: A review of definitions and principles. *Clinical & Experimental Ophthalmology*, *40*(4), 350–357. https://doi.org/10.1111/j.1442-9071.2011.02563.x

Chatterjee, D., Sanchez, D. S., Quansah, E., Rey, N. L., George, S., Becker, K., Madaj, Z., Steiner, J. A., Ma, J.,

Escobar Galvis, M. L., Kordower, J. H., & Brundin, P. (2019). Loss of One Engrailed1 Allele Enhances Induced α-Synucleinopathy. *Journal of Parkinson's Disease*, *9*(2), 315–326. https://doi.org/10.3233/JPD-191590

Chen, G., Gulbranson, D. R., Hou, Z., Bolin, J. M., Ruotti, V., Probasco, M. D., Smuga-Otto, K., Howden, S. E., Diol, N. R., Propson, N. E., Wagner, R., Lee, G. O., Antosiewicz-Bourget, J., Teng, J. M. C., & Thomson, J. A. (2011). Chemically defined conditions for human iPS cell derivation and culture. *Nature Methods*, *8*(5), 424–429. https://doi.org/10.1038/nmeth.1593

Das, A. T., Tenenbaum, L., & Berkhout, B. (2016). Tet-On Systems For Doxycycline-inducible Gene Expression. *Current Gene Therapy*, *16*(3), 156–167. https://doi.org/10.2174/1566523216666160524144041

Daubner, S. C., Le, T., & Wang, S. (2011). Tyrosine Hydroxylase and Regulation of Dopamine Synthesis. *Archives of Biochemistry and Biophysics*, *508*(1), 1–12. https://doi.org/10.1016/j.abb.2010.12.017

Davie, C. A. (2008). A review of Parkinson's disease. *British Medical Bulletin*, *86*, 109–127. https://doi.org/10.1093/bmb/ldn013

Davis, E. (n.d.). *Choosing Between Lentivirus and Adeno-associated Virus For DNA Delivery*.

Deng, H., Zhu, S. H., Le, W. D., Yang, H. R., Lv, H. W., Xu, H. B., Xie, W. J., & Jankovic, J. (2009). Examination of the MSX1 gene in patients with Parkinson's disease. *Acta Neurologica Scandinavica*, *120*(6), 442–444. https://doi.org/10.1111/j.1600-0404.2009.01271.x

Domanskyi, A., Alter, H., Vogt, M. A., Gass, P., & Vinnikov, I. A. (2014). Transcription factors Foxa1 and Foxa2 are required for adult dopamine neurons maintenance. *Frontiers in Cellular Neuroscience*, *8*. https://www.frontiersin.org/article/10.3389/fncel.2014.00275

Edwards, T. G., & Bloom, D. C. (2019). Lund Human Mesencephalic (LUHMES) Neuronal Cell Line

Supports Herpes Simplex Virus 1 Latency In Vitro. *Journal of Virology*, *93*(6), e02210-18. https://doi.org/10.1128/JVI.02210-18

Ellis, C. E., Murphy, E. J., Mitchell, D. C., Golovko, M. Y., Scaglia, F., Barceló-Coblijn, G. C., & Nussbaum, R. L. (2005). Mitochondrial lipid abnormality and electron transport chain impairment in mice lacking alpha-synuclein. *Molecular and Cellular Biology*, *25*(22), 10190–10201. https://doi.org/10.1128/MCB.25.22.10190-10201.2005

Espay, A. J., Brundin, P., & Lang, A. E. (2017). Precision medicine for disease modification in Parkinson disease. *Nature Reviews Neurology*, *13*(2), Article 2. https://doi.org/10.1038/nrneurol.2016.196

Fischer, E., & Morin, X. (2021). Fate restrictions in embryonic neural progenitors. *Current Opinion in Neurobiology*, *66*, 178–185. https://doi.org/10.1016/j.conb.2020.10.014

Ghosh, A., Tyson, T., George, S., Hildebrandt, E. N., Steiner, J. A., Madaj, Z., Schulz, E., Machiela, E., McDonald, W. G., Escobar Galvis, M. L., Kordower, J. H., Van Raamsdonk, J. M., Colca, J. R., & Brundin, P. (2016). Mitochondrial pyruvate carrier regulates autophagy, inflammation, and neurodegeneration in experimental models of Parkinson's disease. *Science Translational Medicine*, *8*(368), 368ra174-368ra174. https://doi.org/10.1126/scitranslmed.aag2210

Han, J. W., Ahn, Y. D., Kim, W.-S., Shin, C. M., Jeong, S. J., Song, Y. S., Bae, Y. J., & Kim, J.-M. (2018). Psychiatric Manifestation in Patients with Parkinson's Disease. *Journal of Korean Medical Science*, *33*(47), e300. https://doi.org/10.3346/jkms.2018.33.e300

Hegarty, S. V., Sullivan, A. M., & O'Keeffe, G. W. (2013). Midbrain dopaminergic neurons: A review of the molecular circuitry that regulates their development. *Developmental Biology*, *379*(2), 123–138. https://doi.org/10.1016/j.ydbio.2013.04.014

Henchcliffe, C., & Parmar, M. (2018). Repairing the Brain: Cell Replacement Using Stem Cell-Based

Technologies. *Journal of Parkinson's Disease*, *8*(Suppl 1), S131–S137. https://doi.org/10.3233/JPD-181488

Induced Pluripotent Stem Cells (iPS) | UCLA Broad Stem Cell Center. (n.d.). Retrieved March 6, 2022, from https://stemcell.ucla.edu/induced-pluripotent-stem-cells

Jackson, S. J., Andrews, N., Ball, D., Bellantuono, I., Gray, J., Hachoumi, L., Holmes, A., Latcham, J., Petrie, A., Potter, P., Rice, A., Ritchie, A., Stewart, M., Strepka, C., Yeoman, M., & Chapman, K. (2017). Does age matter? The impact of rodent age on study outcomes. *Laboratory Animals*, *51*(2), 160–169. https://doi.org/10.1177/0023677216653984

Jankovic, J. (2008). Parkinson's disease: Clinical features and diagnosis. *Journal of Neurology, Neurosurgery, and Psychiatry*, *79*(4), 368–376. https://doi.org/10.1136/jnnp.2007.131045

Juárez Olguín, H., Calderón Guzmán, D., Hernández García, E., & Barragán Mejía, G. (2016). The Role of Dopamine and Its Dysfunction as a Consequence of Oxidative Stress. *Oxidative Medicine and Cellular Longevity*, *2016*, 9730467. https://doi.org/10.1155/2016/9730467

Kalia, L. V., & Lang, A. E. (2015). Parkinson's disease. *The Lancet*, *386*(9996), 896–912. https://doi.org/10.1016/S0140-6736(14)61393-3

Kefalopoulou, Z., Politis, M., Piccini, P., Mencacci, N., Bhatia, K., Jahanshahi, M., Widner, H., Rehncrona, S., Brundin, P., Björklund, A., Lindvall, O., Limousin, P., Quinn, N., & Foltynie, T. (2014). Long-term clinical outcome of fetal cell transplantation for Parkinson disease: Two case reports. *JAMA Neurology*, *71*(1), 83–87. https://doi.org/10.1001/jamaneurol.2013.4749

Kim, W. S., Kågedal, K., & Halliday, G. M. (2014). Alpha-synuclein biology in Lewy body diseases. *Alzheimer's Research & Therapy*, *6*(5), 73. https://doi.org/10.1186/s13195-014-0073-2

Kittappa, R., Chang, W. W., Awatramani, R. B., & McKay, R. D. G. (2007). The foxa2 gene controls the birth

and spontaneous degeneration of dopamine neurons in old age. *PLoS Biology*, *5*(12), e325. https://doi.org/10.1371/journal.pbio.0050325

Krull, C. E. (2004). A primer on using in ovo electroporation to analyze gene function. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, *229*(3), 433–439. https://doi.org/10.1002/dvdy.10473

Kumar, R., Lozano, A. M., Montgomery, E., & Lang, A. E. (1998). Pallidotomy and deep brain stimulation of the pallidum and subthalamic nucleus in advanced Parkinson's disease. *Movement Disorders: Official Journal of the Movement Disorder Society*, *13 Suppl 1*, 73–82.

Lee, S., Choi, K., Ahn, H., Song, K., Choe, J., & Lee, I. (2005). TuJ1 (class III beta-tubulin) expression suggests dynamic redistribution of follicular dendritic cells in lymphoid tissue. *European Journal of Cell Biology*, *84*(2–3), 453–459. https://doi.org/10.1016/j.ejcb.2004.11.001

LeWitt, P. A. (2015). Levodopa therapy for Parkinson's disease: Pharmacokinetics and pharmacodynamics. *Movement Disorders: Official Journal of the Movement Disorder Society*, *30*(1), 64–72. https://doi.org/10.1002/mds.26082

Lindvall, O., & Björklund, A. (2011). Cell Therapeutics in Parkinson's Disease. *Neurotherapeutics*, *8*(4), 539–548. https://doi.org/10.1007/s13311-011-0069-6

Mehanna, R., Moore, S., Hou, J. G., Sarwar, A. I., & Lai, E. C. (2014). Comparing clinical features of young onset, middle onset and late onset Parkinson's disease. *Parkinsonism & Related Disorders*, *20*(5), 530–534. https://doi.org/10.1016/j.parkreldis.2014.02.013

Miozzo, F., Valencia-Alarcón, E. P., Stickley, L., Majcin Dorcikova, M., Petrelli, F., Tas, D., Loncle, N., Nikonenko, I., Bou Dib, P., & Nagoshi, E. (2022). Maintenance of mitochondrial integrity in midbrain dopaminergic neurons governed by a conserved developmental transcription factor. *Nature*

Communications, *13*, 1426. https://doi.org/10.1038/s41467-022-29075-0

Mishra, A., Singh, S., & Shukla, S. (2018). Physiological and Functional Basis of Dopamine Receptors and Their Role in Neurogenesis: Possible Implication for Parkinson's disease. *Journal of Experimental Neuroscience*, *12*, 1179069518779829. https://doi.org/10.1177/1179069518779829

Müller, T. (2020). Pharmacokinetics and pharmacodynamics of levodopa/carbidopa cotherapies for Parkinson's disease. *Expert Opinion on Drug Metabolism & Toxicology*, *16*(5), 403–414. https://doi.org/10.1080/17425255.2020.1750596

Niu, W., Zang, T., Wang, L.-L., Zou, Y., & Zhang, C.-L. (2018). Phenotypic Reprogramming of Striatal Neurons into Dopaminergic Neuron-like Cells in the Adult Mouse Brain. *Stem Cell Reports*, *11*(5), 1156–1170. https://doi.org/10.1016/j.stemcr.2018.09.004

Ono, Y., Nakatani, T., Minaki, Y., & Kumai, M. (2010). The basic helix-loop-helix transcription factor Nato3 controls neurogenic activity in mesencephalic floor plate cells. *Development (Cambridge, England)*, *137*(11), 1897–1906. https://doi.org/10.1242/dev.042572

Ono, Y., Nakatani, T., Sakamoto, Y., Mizuhara, E., Minaki, Y., Kumai, M., Hamaguchi, A., Nishimura, M., Inoue, Y., Hayashi, H., Takahashi, J., & Imai, T. (2007). Differences in neurogenic potential in floor plate cells along an anteroposterior location: Midbrain dopaminergic neurons originate from mesencephalic floor plate cells. *Development (Cambridge, England)*, *134*(17), 3213–3225. https://doi.org/10.1242/dev.02879

Ovallath, S., & Sulthana, B. (2017). Levodopa: History and Therapeutic Applications. *Annals of Indian Academy of Neurology*, *20*(3), 185–189. https://doi.org/10.4103/aian.AIAN_241_17

Perez-Bouza, A., Di Santo, S., Seiler, S., Meyer, M., Andereggen, L., Huber, A., Guzman, R., & Widmer, H. R. (2017). Simultaneous Transplantation of Fetal Ventral Mesencephalic Tissue and Encapsulated

Genetically Modified Cells Releasing GDNF in a Hemi-Parkinsonian Rat Model of Parkinson's Disease. *Cell Transplantation*, *26*(9), 1572–1581. https://doi.org/10.1177/0963689717721202

Peterson, D. J., Marckini, D. N., Straight, J. L., King, E. M., Johnson, W., Sarah, S. S., Chowdhary, P. K., & DeLano-Taylor, M. K. (2019). The Basic Helix-Loop-Helix Gene Nato3 Drives Expression of Dopaminergic Neuron Transcription Factors in Neural Progenitors. *Neuroscience*, *421*, 176–191. https://doi.org/10.1016/j.neuroscience.2019.09.003

Pfeiffer, R. F. (2016). Non-motor symptoms in Parkinson's disease. *Parkinsonism & Related Disorders*, *22*, S119–S122. https://doi.org/10.1016/j.parkreldis.2015.09.004

Ptáček, R., Kuželová, H., & Stefano, G. B. (2011). Dopamine D4 receptor gene DRD4 and its association with psychiatric disorders. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, *17*(9), RA215–RA220. https://doi.org/10.12659/MSM.881925

Razgado-Hernandez, L. F., Espadas-Alvarez, A. J., Reyna-Velazquez, P., Sierra-Sanchez, A., Anaya-Martinez, V., Jimenez-Estrada, I., Bannon, M. J., Martinez-Fong, D., & Aceves-Ruiz, J. (2015). The transfection of BDNF to dopamine neurons potentiates the effect of dopamine D3 receptor agonist recovering the striatal innervation, dendritic spines and motor behavior in an aged rat model of Parkinson's disease. *PloS One*, *10*(2), e0117391. https://doi.org/10.1371/journal.pone.0117391

Rekaik, H., Blaudin de Thé, F.-X., Prochiantz, A., Fuchs, J., & Joshi, R. L. (2015). Dissecting the role of Engrailed in adult dopaminergic neurons: Insights into Parkinson disease pathogenesis. *FEBS Letters*, *589*(24 Pt A), 3786–3794. https://doi.org/10.1016/j.febslet.2015.10.002

Schweitzer, J. S., Song, B., Herrington, T. M., Park, T.-Y., Lee, N., Ko, S., Jeon, J., Cha, Y., Kim, K., Li, Q., Henchcliffe, C., Kaplitt, M., Neff, C., Rapalino, O., Seo, H., Lee, I.-H., Kim, J., Kim, T., Petsko, G. A., … Kim, K.-S. (2020). Personalized iPSC-Derived Dopamine Progenitor Cells for Parkinson's

Disease. *The New England Journal of Medicine*, *382*(20), 1926–1932. https://doi.org/10.1056/NEJMoa1915872

Seidl, S. E., & Potashkin, J. A. (2011). The Promise of Neuroprotective Agents in Parkinson's Disease. *Frontiers in Neurology*, *2*, 68. https://doi.org/10.3389/fneur.2011.00068

Sheta, R., Teixeira, M., Idi, W., Pierre, M., de Rus Jacquet, A., Emond, V., Zorca, C. E., Vanderperre, B., Durcan, T. M., Fon, E. A., Calon, F., Chahine, M., & Oueslati, A. (2022). Combining NGN2 programming and dopaminergic patterning for a rapid and efficient generation of hiPSC-derived midbrain neurons. *Scientific Reports*, *12*(1), Article 1. https://doi.org/10.1038/s41598-022-22158-4

Sveinbjornsdottir, S. (2016). The clinical symptoms of Parkinson's disease. *Journal of Neurochemistry*, *139 Suppl 1*, 318–324. https://doi.org/10.1111/jnc.13691

Tambasco, N., Romoli, M., & Calabresi, P. (2018). Levodopa in Parkinson's Disease: Current Status and Future Developments. *Current Neuropharmacology*, *16*(8), 1239–1252. https://doi.org/10.2174/1570159X15666170510143821

TaqMan® vs. SYBR® Green Chemistries. (n.d.). Retrieved August 26, 2022, from https://www.biosyn.com/tew/taqman-vs-sybr-green-chemistries.aspx

Tiklová, K., Nolbrant, S., Fiorenzano, A., Björklund, Å. K., Sharma, Y., Heuer, A., Gillberg, L., Hoban, D. B., Cardoso, T., Adler, A. F., Birtele, M., Lundén-Miguel, H., Volakakis, N., Kirkeby, A., Perlmann, T., & Parmar, M. (2020). Single cell transcriptomics identifies stem cell-derived graft composition in a model of Parkinson's disease. *Nature Communications*, *11*(1), Article 1. https://doi.org/10.1038/s41467-020-16225-5

Veenvliet, J. V., & Smidt, M. P. (2014). Molecular mechanisms of dopaminergic subset specification: Fundamental aspects and clinical perspectives. *Cellular and Molecular Life Sciences*, *71*(24), 4703–

4727. https://doi.org/10.1007/s00018-014-1681-5

Villar-Piqué, A., Lopes da Fonseca, T., & Outeiro, T. F. (2016). Structure, function and toxicity of alpha-synuclein: The Bermuda triangle in synucleinopathies. *Journal of Neurochemistry*, *139*(S1), 240–255. https://doi.org/10.1111/jnc.13249

Wallén-Mackenzie, Å., Mata de Urquiza, A., Petersson, S., Rodriguez, F. J., Friling, S., Wagner, J., Ordentlich, P., Lengqvist, J., Heyman, R. A., Arenas, E., & Perlmann, T. (2003). Nurr1-RXR heterodimers mediate RXR ligand-induced signaling in neuronal cells. *Genes & Development*, *17*(24), 3036–3047. https://doi.org/10.1101/gad.276003

Wang, M., Ling, K.-H., Tan, J. J., & Lu, C.-B. (2020). Development and Differentiation of Midbrain Dopaminergic Neuron: From Bench to Bedside. *Cells*, *9*(6), Article 6. https://doi.org/10.3390/cells9061489

Xue, Y., Zhan, X., Sun, S., Karuppagounder, S. S., Xia, S., Dawson, V. L., Dawson, T. M., Laterra, J., Zhang, J., & Ying, M. (2019). Synthetic mRNAs Drive Highly Efficient iPS Cell Differentiation to Dopaminergic Neurons. *Stem Cells Translational Medicine*, *8*(2), 112–123. https://doi.org/10.1002/sctm.18-0036

Yu, K., McGlynn, S., & Matise, M. P. (2013). Floor plate-derived sonic hedgehog regulates glial and ependymal cell fates in the developing spinal cord. *Development*, *140*(7), 1594–1604. https://doi.org/10.1242/dev.090845

Zainuddin, A., Chua, K. H., Rahim, N. A., & Makpol, S. (2010). Effect of experimental treatment on GAPDH mRNA expression as a housekeeping gene in human diploid fibroblasts. *BMC Molecular Biology*, *11*, 59. https://doi.org/10.1186/1471-2199-11-59

Zheng, C., Wang, S., Bai, Y., Luo, T., Wang, J., Dai, C., Guo, B., Luo, S., Wang, D., Yang, Y., & Wang, Y.

(2018). Lentiviral Vectors and Adeno-Associated Virus Vectors: Useful Tools for Gene Transfer in Pain Research. *Anatomical Record (Hoboken, N.j. : 2007)*, *301*(5), 825–836. https://doi.org/10.1002/ar.23723