THE EFFECTS OF DEVELOPMENTAL EXPOSURE TO THE ORGANOCHLORINE PESTICIDE DIELDRIN ON SUSCEPTIBILITY TO PARKINSON'S DISEASE

By

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ABSTRACT

Parkinson's disease (PD) is the fastest-growing neurological disease worldwide, with increases outpacing aging and occurring most rapidly in recently industrialized areas, suggesting a role of environmental factors. Exposure to the organochlorine pesticide dieldrin is a risk factor for sporadic PD. In a model of increased PD susceptibility, mice exposed to dieldrin during development show a male-specific increased susceptibility to MPTP (a dopaminergic (DAergic) toxicant) as adults, and adult male mice exposed to dieldrin during development show increased susceptibility to synucleinopathy induced by α- synuclein (α-syn) preformed fibrils (PFFs), with dieldrin-induced exacerbation of PFF-induced deficits in motor behavior and dopamine (DA) turnover. We hypothesize that dieldrin-induced epigenetic modification during development causes changes in gene expression and phenotype that persist into adulthood,
altering the sensitivity to Parkinsonian insults and contributing to the development of PD.

Specifically, we hypothesized that alterations in DA handling contribute to the observed changes and assessed vesicular monoamine transporter 2 (VMAT2) function and DA release in this dieldrin/PFF two-hit model. Using a developmental dieldrin/PFF two-hit model, vesicular ^{3}H-DA uptake assays and fast-scan cyclic voltammetry (FSCV) were performed 4 months post-PFF injection. Dieldrin induced an increase in DA release in striatal slices in PFF-injected animals, but no change in VMAT2 activity. These results suggest that developmental dieldrin exposure increases a compensatory response to synucleinopathy-triggered striatal DA loss. These findings are consistent with silent neurotoxicity, where developmental exposure to dieldrin primes the nigrostriatal striatal

system to have an exacerbated response to synucleinopathy in the absence of observable changes in typical markers of nigrostriatal dysfunction and degeneration. The epigenome is a potential mediator of this relationship between developmental exposures, increased neuronal vulnerability, and adult disease. In support of this, we recently identified sex-specific differential methylation patterns in response to developmental dieldrin exposure, suggesting exposure establishes a sex-specific poised epigenetic state early in life that modulates adult susceptibility to neurotoxicity. Candidate genes with developmental dieldrin-induced differential modification include *Nr4a2*, a transcription involved in DAergic development, and *Ephb2*, a receptor tyrosine kinase that regulates axonal guidance during neuronal development. Using a 3D human neurosphere model, we have shown that modification of these candidate genes during proliferation alters the DAergic trajectory of these neurospheres later in differentiation and modifies a key marker of DAergic vulnerability to toxicity. Suggesting that these observed epigenetic modifications to candidate genes, *NR4A2* and *EPHB2* during development alter the DAergic differentiation in developing neurons which may modify susceptibility to toxicity later in life. Overall, data from this project investigates mechanisms in which developmental exposure to dieldrin may induce functional changes in phenotype that alter Parkinson's disease susceptibility.

In loving memory of
Richard A. Souser

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PREFACE

At the time this dissertation was written, Chapter 2 was a published manuscript in the Journal of Toxicological Sciences. Chapters 3 and 4 are being prepared as separate manuscripts. All data for published figures for Chapter 2 are available in the Dryad Data Repository. GraphPad Prism files can be viewed in a free Viewer mode. R is freely available. All projects were preregistered using OSF Registries. Figures were created in BioRender.

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LIST OF ABBREVIATIONS

α-syn	α-Synuclein
5-HT	Serotonin
5mC	5-methylcytosine
5hmC	5-hydroxymethylcytosine
6-OHDA	6-hydroxydopamine
AADC	L-aromatic amino acid decarboxylase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
COMT	Catechol-O-methyltransferase
CpG	Cytosine nucleotides before guanines
D1R	Dopamine 2-like receptor
D2R	Dopamine 1-like receptor
DA	Dopamine
DAergic	Dopaminergic
DAT	Dopamine transporter
DNAMT	DNA methyltransferases
DNT	Developmental neurotoxicity
DOPAC	3,4-Dihydroxyphenylacetic acid
DOPAL	Dihydroxyphenylacetaldehyde
EPHB1	Ephrin type-B-receptor 1
EPHB2	Ephrin type-B-receptor 2
FBS	Fetal bovine serum
FSCV	Fast scan cyclic voltammetry

GABA	Gamma-aminobutyric acid
GBA1	Glucocerebrosidase
GPe	Globus pallidus externus
GPi	Globus pallidus internus
GWAS	Genome-wide association study
HSPG	Heparan sulfate proteoglycan
HVA	Homovanillic acid
iPSC	Induced pluripotent stem cells
L-DOPA	Levodopa
LB	Lewy body
LRRK2	Leucine rich-repeat kinase 2
LUHMES	Lund University Human Mesencephalic Cells
MAO	Monoamine oxidase
MPP+	1-methyl-4-phenyl pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NAC	Non-amyloid-β-component
NE	Norepinephrine
NAM	New approach methodologies
NAc	Nucleus accumbens
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
NR4A2	Nuclear receptor subfamily 4 group A member 2
Nurr1	Nuclear receptor related-1

OC	Organochlorine
pSyn	Phosphorylated α-Synuclein
PD	Parkinson's Disease
PBDEs	polybrominated diphenyl ethers
PCBs	polychlorinated biphenyls
PFF	Pre-formed fibrils
PFOS	Perfluorooctane sulfonic acid
PINK1	Phosphatase and tensin homolog-induced putative kinase 1
POP	Persistent organic pollutant
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
SDS	Sodium dodcyl sulfate
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
STN	Subthalamic nucleus
TBS	Tris-buffered saline
TBS-Tx	Tris-buffered saline-Triton-X
TEM	Transmission electron microscopy
TET	Ten eleven translocase
TH	Tyrosine hydroxylase
VTA	Ventral tegmental area
VMAT2	Vesicular monoamine transporter 2
VSP35	Vacuolar protein-sorter-35

Chapter 1: Introduction

Parkinson's disease: Overview

Demographics

Parkinson's disease (PD) is the most common movement disorder, the second most common neurodegenerative disease, and the fastest-growing neurological disorder worldwide (Dorsey et al. 2007). The primary risk factor for PD is age with an incidence of 47-77 per 100,000 for 45–65-year-olds and 108-212 per 100,000 in the population over 65 years of age (Willis et al. 2022). The prevalence of PD is continuing to rise, and the number of people with PD is expected to double between 2005 and 2030 (Dorsey et al. 2007). Only 5-10% of PD cases are caused by monogenically inherited mutations; the remaining large majority of sporadic (or idiopathic) cases are caused by a complex combination of genetic and environmental factors (Pang et al. 2019) Supporting a role of environmental factors, the greatest increases in PD prevalence are occurring in recently industrialized regions, consistent with a link between compounds associated with industrialization and PD risk (Dorsey et al. 2007).

Clinical symptoms

PD is clinically defined by the cardinal motor symptoms which include bradykinesia or slowed movements, rigidity, resting tremor, and postural instability (Postuma et al. 2015; Postuma et al. 2016). While diagnosis typically occurs after the onset of motor symptoms, it is thought that disease progression begins before these symptoms appear with a long prodromal phase. This prodromal phase precedes the onset of motor symptoms, often by decades, and is characterized by a range of nonmotor symptoms (Postuma and Berg 2016; Heinzel et al. 2019; Armstrong and Okun 2020). These include REM sleep behavior disorder and other sleep disorders, depression, anxiety,

constipation, and other gastrointestinal problems, olfactory loss, cognitive impairment, and autonomic dysfunction (Iranzo et al. 2021; Jo et al. 2021; Miglis et al. 2021; Zhang et al. 2022; Zolfaghari et al. 2022; Barone et al. 2023). Autonomic dysfunction can range from GI disturbances like constipation to urinary urgency, blood pressure variability, and orthostatic hypotension (Armstrong and Okun 2020).

Sex differences

Epidemiological studies suggest that there are sex differences for both the incidence and symptomology of PD, particularly for sporadic PD. In general, there is a higher prevalence of PD in males with a male-to-female ratio of 1.9 (Van Den Eeden et al. 2003). For males, there is 19.0 per 100,000 for an age-adjusted incidence rate, compared to 9.9 per 100,000 for females (Van Den Eeden et al. 2003). Women diagnosed with PD have a 26% lower risk of death compared to men (Schootman 2012). There are also some identified sex-specific differences in genetic risk factors in PD. For example, there is an increased prevalence of specific risk alleles and polymorphisms such as the apolipoprotein E4 allele, Monoamine Oxidase B (MAO-B) allele, and the Rs1113666 GAPDH polymorphism (Raheel et al. 2023). In females, there is a higher prevalence of mutations in the PD-associated gene Leucine rich-repeat kinase 2 (LRRK2) (Raheel et al. 2023). Variants of the LRRK2 mutations result in a sex-specific display of symptoms. For example, females with the G2385R variant tend to have a reduced risk of autonomic dysfunction, while males present a decreased risk of cognitive impairment (Raheel et al. 2023).

In addition, both PD motor and non-motors symptoms present differently between male and female patients. Specifically, women tend to have less severe rigidity and similar

rates of resting tremor and bradykinesia, but higher rates of postural instability, depression, anxiety, sleep disorders, fatigue, pain, restless leg syndrome, tremor, and worse side effects in response to the PD therapeutic Levodopa (L-DOPA) (Scott et al. 2000; Shulman and Bhat 2006; Subramanian et al. 2022). Male patients with PD tend to present motor symptoms earlier and progress more rapidly compared to female patients (Scott et al. 2000; Shulman and Bhat 2006; Subramanian et al. 2022).

Neuropathology

Two pathological hallmarks are required for a definitive PD diagnosis upon post-mortem analysis: the presence of Lewy bodies (LB) that contain α-synuclein (α-syn) and the loss of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc). LBs are intracellular protein inclusions composed primarily of aggregated α-syn. In addition, up to 70 other proteins were identified in LBs, many of which are involved in phosphorylation, mitochondrial, lysosomal, autophagy, and microtubule pathways (Wakabayashi et al. 2007a; Power et al. 2017). Degeneration of DA neurons and the resulting loss of striatal DA underlies the primary motor symptoms through disruption of the basal ganglia.

Degeneration of the nigrostriatal pathway

In a healthy brain, the basal ganglia circuit is responsible for controlling motor movement through an interaction between the nigrostriatal pathway and the thalamocortical circuitry (Figure 1.1A). In a non-parkinsonian state, DAergic neurons from the SNpc innervate the striatum, and they activate both the direct and indirect pathways (Obeso et al. 2000; Mehler-Wex et al. 2006; Wu et al. 2012; Blandini 2014). In the direct pathway, inhibitory GABAergic neurons project from the striatum to the globus

pallidus internal (GPi), reducing the inhibition of the thalamus and further activating the motor cortex to initiate motor movements. In the indirect pathway, GABAergic neurons from the striatum inhibit the globus pallidus external (GPe) which activates inhibitory GABAergic neurons projecting to the subthalamic nuclei (STN) which inhibits the excitatory glutamatergic neurons in the STN. These STN neurons provide excitatory input to the GPi, resulting in more inhibition of the thalamus less activation of the motor cortex, and inhibition of motor movements (Figure 1.1A). Together, the direct and indirect pathways provide opposing signals for the control of movement.

In PD, the nigrostriatal DAergic neurons degenerate leading to progressive loss of DAergic signaling to the striatum. Nigrostriatal terminals are significantly decreased and almost completely diminished in 4 years post-PD diagnosis (Kordower et al. 2013). The

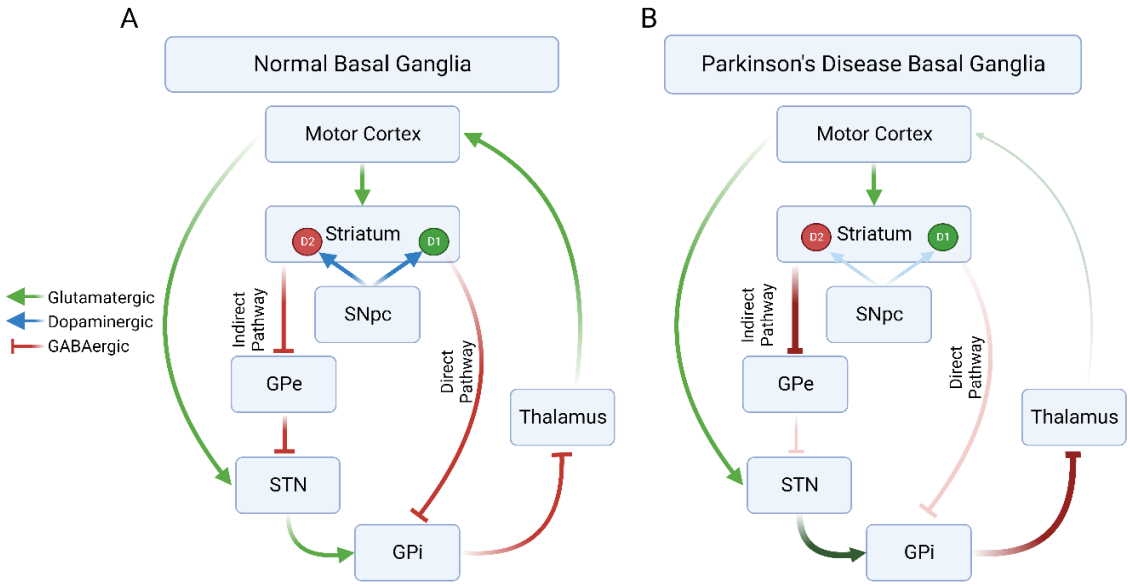

Figure 1.1. Basal ganglia circuitry. A) Basal ganglia circuitry in healthy individuals. B) Basal ganglia in individuals with Parkinson's disease. Made in BioRedner.

nigral cell bodies have also degenerated at this same time point but to a much lesser extent (approximately 50-90% loss). This suggests that nigrostriatal DAergic neuron

terminals innervating the striatum are lost before DAergic cell bodies of the nigra supporting the idea of the dying back hypothesis where dysfunction and the loss of striatal terminals precede loss of cell bodies (Kordower et al. 2013). This leads to decreased signaling through the direct pathway and increased signaling through the indirect pathway. Together, this produces reduced inhibition (via the direct pathway) and increased activation (via the indirect pathway) for the GPi and STN. This increased inhibitory output from the GPi and STN reduces activity in the thalamus and decreases thalamic activation of the motor cortex (Figure 1.1B). Thus, the net outcome of SNpc degeneration in PD is reduced activation of the motor cortex and impairment of motor control. (Obeso et al. 2000; Sharman et al. 2000; Mehler-Wex et al. 2006; Wu et al. 2012; Maiti et al. 2017; Blandini et al.; Riederer et al.).

Lewy bodies

For post-mortem diagnosis of PD, LBs must be found in DAergic neurons of SNpc, but the distribution of LBs is more widespread, particularly in other monoaminergic neurons, as well as other structures in both the central and peripheral nervous systems (Pollanen et al. 1993; Spillantini et al. 1997). The distribution and neuropathological staging of LB progression during PD were well described by Braak and others (Braak et al. 2003; Braak et al. 2004; Braak and Del Tredici 2017).

During the preclinical phase before motor symptom onset, LBs are localized to the olfactory bulbs, dorsal motor nuclei in the vagal and glossopharyngeal nerves in the brainstem, pontine tegmentum (locus coeruleus, magnocellular nucleus of the reticular formation, and lower raphe nuclei). As motor symptoms begin to appear, LB neuropathology can be found in the pedunculopontine nucleus, the cholinergic

magnocellular nuclei of the basal forebrain, the SNpc, the hypothalamus, portions of the thalamus, and begins to appear in the mesocortex. In later stages of the disease, LB pathology extends into neocortical regions and may contribute to cognitive impairments observed in the latest stages of the disease (Braak et al. 2003; Braak et al. 2004; Braak and Del Tredici 2017). LBs have also been found in the peripheral nervous system in sciatic nerves, the enteric nervous system, and throughout the gastrointestinal system, including in the appendix (Beach et al. 2010; Gelpi et al. 2014; Killinger et al. 2018; Killinger and Labrie 2019).

Alpha-synuclein protein

α-syn is the major component of LBs (Spillantini et al. 1997; Spillantini et al. 1998). It is a 140 amino acid protein encoded by the gene SNCA that belongs to the synuclein protein family along with β- and γ-synucleins and is highly expressed in the human brain (Goedert 2001; Marques and Outeiro 2012; Bridi and Hirth 2018). α-syn contains 3 domains: the N-terminal amphipathic region, the hydrophobic non-amyloid-β component (NAC), and the C-terminal acid tail domain (Maries et al. 2003; Bridi and Hirth 2018). The N-terminal amphipathic region mediates binding to phospholipids with a preference for high curvature, resulting in α-syn localization to presynaptic and vesicular membranes (Middleton and Rhoades 2010; Jensen et al. 2011; Pranke et al. 2011; Busch et al. 2014; Xu et al. 2016). The 12 amino acids comprising the NAC domain of α-syn are the key mediators in α-syn aggregation (Biere et al. 2000; Lee et al. 2002; Lee and Lee 2002; Giasson et al. 2003; Jucker and Walker 2013; Jucker and Walker 2018). Notably, β-synuclein lacks the NAC region, and β-synuclein aggregation is not observed (Cheng et al. 2011). The C-terminal acidic tail domain is known to be

important in calcium binding, chaperone activity, and protection against oxidative stress and α-syn aggregation. (Kim et al. 2002; Park et al. 2002; Albani et al. 2004; Chandra et al. 2005; Cheng et al. 2011; Games et al. 2014; Chaari et al. 2016; Sharma and Priya 2017).

In addition to α-syn, which is the major component of LBs, many other types of proteins and lipids were identified within LBs. LBs have a high lipid content, and this is likely because they contain high amounts of membrane-bound structures related to autophagosomes, lysosomes, and lipid membrane fragments (Goldman et al. 1983; Wakabayashi et al. 2007b; Beyer et al. 2009; Shahmoradian et al. 2019). Other components such as mitochondria, protein aggregates, cytoskeletal elements, and vesicles also contribute to LBs (Goldman et al. 1983; Wakabayashi et al. 2007b; Beyer et al. 2009; Shahmoradian et al. 2019).

Parkinson's disease treatments

Currently, there are no disease-modifying therapies to delay or prevent the progress of PD (Armstrong and Okun 2020). However, there are several DA and non-DA-based therapies for the treatment of motor symptoms (Lee and Yankee 2022). Levodopa (L-DOPA) is the gold standard for PD therapy (Armstrong and Okun 2020; Lee and Yankee 2022). There are also nonselective DA agonists that are used to activate D1 and D2 receptors to treat motor symptoms (Armstrong and Okun 2020; Lee and Yankee 2022). Monoamine oxidase inhibitors including COMT inhibitors work to inhibit the metabolism of DA and L-DOPA (Lee and Yankee 2022). These therapeutics are used to increase DA levels, activate DA receptors, or inhibit the metabolism of DA to restore DA and improve motor symptoms of PD (Lee and Yankee 2022). In addition to DA therapies,

Anticholinergic pharmacological treatments have also been used to treat motor symptoms by reducing acetylcholine to treat tremors in PD. These therapies are choline receptor antagonists to restore the homeostasis between DA and acetylcholine which is disrupted in PD (Lee and Yankee 2022). More recently, these anticholinergic treatments have been largely replaced by L-DOPA or other dopaminergic agonist therapies (Armstrong and Okun 2020; Lee and Yankee 2022).

Another treatment option for severe PD that is untreated by traditional pharmacological treatments is deep brain stimulation, which is an implant used to stimulate the basal ganglia via electrical stimulation (Lee and Yankee 2022). Deep brain stimulation can improve both motor and non-motor symptoms and can improve bradykinesia, tremor, and rigidity. However, deep brain stimulation is less effective at treating gait disturbances, balance, and speech impairments. Overall, pharmacological, and surgical treatments are used to diminish motor symptoms and some non-motor symptoms of PD. However, there are no available disease-modifying treatments for preventing or inhibiting the progression of the disease(Lee and Yankee 2022).

Risk factors of Parkinson's disease

Age is the primary risk factor for PD, but there are several genetic and environmental risk factors for the disease (Zaman et al. 2021). Together, these factors implicate multiple molecular mechanisms of PD etiology including mitochondrial dysfunction, oxidative stress, protein aggregation, disrupted protein clearance, and neuroinflammation serving as intrinsic risk factors (Zaman et al. 2021).

Genetics

Only 5-10% of PD cases are caused by inherited monogenic mutations, with the majority (>90%) of sporadic or idiopathic cases arising from a combination of genetic and environmental factors. Genetic cases of PD are caused by highly penetrant, rare mutations that follow Mendelian inheritance patterns. In contrast, sporadic cases are caused by the combination and interaction of common genetic polymorphisms with weak to moderate effect sizes and environmental exposures. Thus, there is a range of genetic variants underlying PD etiology from common polymorphisms with modest effects to rare highly penetrant variants (Blauwendraat et al. 2020).

Autosomal dominant PD genes

SNCA encodes α-syn, which, in addition to being the major component of LBs, is thought to play a role in regulating synaptic vesicles, synaptic plasticity, and regulating lipids (Steece-Collier et al. 2002; Shulman et al. 2011; Klein and Westenberger 2012). It is thought that α-syn is natively unfolded and can fibrillate to assume the β-pleated sheet conformation observed in LBs. While it remains unclear if the fibrils themselves, protofibrils, or other intermediates in the fibrilization pathway, or the loss of soluble α-syn are pathogenic in PD, the accumulation and aggregation of α-syn is a defining feature of most forms of PD.

The most common α-syn point mutations, including A30P, A53T, A53E, E46K, H50Q, and G51D, are located in the N-terminal domain and are associated with early-onset PD (Bridi and Hirth 2018). Multiple mutations within the N-terminal domain and duplication or triplication of the *SNCA* locus can cause familial non-genetically inherited forms of PD, but mutations in *SNCA* are also associated with an increased risk of sporadic PD.

The autosomal-dominant A53T mutation can accelerate this fibrilization process. The missense mutation A30P accelerates the conversion of monomeric α-syn to oligomeric protofibril forms but does not play a role in the formation of fibrils (Conway et al. 2000). Both the A30P and A53T mutations induce neuronal toxicity by disrupting and permeabilizing cell membranes through pore-like formations (Steece-Collier et al. 2002). Duplication and triplication of the *SNCA* locus also cause familial PD and the severity of PD progression in these cases correlates with the number of copies of the SNCA locus (Lill 2016). PD cases caused by SNCA autosomal dominant mutations typically present with a similar clinical phenotype amongst all mutations with varying severity in SNCA multiplications. In the early stages, patients are L-DOPA responsive, however, over time L-DOPA loses effectiveness and patients experience severe rigidity and dementia. SNCA autosomal dominant cases present with widespread α-syn pathology in neurons and glia throughout the brainstem and cerebrum (Puschmann 2013).

In addition to *SNCA*, there are several other autosomal dominant PD-causing genes. In the US and Europe, LRRK2 is the most commonly mutated gene in both familial and sporadic PD (Zimprich et al. 2004; Nuytemans et al. 2010; Funayama et al. 2023). LRRK2 has both kinase and GTPase functions. At least 6 pathogenic mutations in LRRK2 were identified, and the most common LRRK2 mutation is Gly2019Ser which was identified in 1% of idiopathic or sporadic PD cases and 4% in familial (Lill 2016). The clinical phenotype of LRRK2 familial PD is late-onset, L-Dopa responsive (Brice 2005). Interestingly, the pathological characteristics associated with familial LRRK2 PD vary dramatically even between family members. Specifically, some patients show typical PD-associated α-syn containing LBs, some show tau pathology, and other cases

do not present with any α-syn or tau pathology. However, nigrostriatal degeneration is prominent amongst almost all LRRK2 autosomal dominant cases (Brice 2005).

Variants in the Vacuolar protein sorter-35 (VSP35) protein a confirmed causative of autosomal dominant PD (Lill 2016; Funayama et al. 2023). The function of the VPS35 protein is to facilitate the retrograde trafficking of proteins from endosomes to the trans-Golgi network, and the VPS35 variants are thought to result in disrupted vesicular formation, impaired autophagy, and lysosomal function (Lill 2016). The p.Asp620Asn variant was first identified in a Swiss family with late-onset autosomal dominant PD with incomplete penetrance (Sassone et al. 2021). PD cases with the p.Asp620Asn variant presented with a tremor-predominant, slowly progressive, and L-Dopa-responsive form of the disease. Other variants of the VPS35 have since been identified resulting in a similar clinical phenotype (Williams et al. 2017).

Autosomal recessive PD genes

Mutations in the Parkin gene are involved in early onset genetic forms of PD, in particular, Parkin from the PARK2 locus mutant carriers is the most common cause of juvenile forms of PD (Klein and Westenberger 2012). PARK2 encodes the Parkin protein, a ubiquitin-E3-ligase playing a role in ubiquitination and is recruited by the mitochondrial protein, phosphatase, and tensin homolog-induced putative kinase 1(PINK1) protein together promoting selective degradation of damaged mitochondria via mitophagy (Klein and Westenberger 2012; Konovalova et al. 2015). The most frequent autosomal recessive mutation is Parkin, and approximately 8.6% of patients diagnosed with early onset PD at the age of 50 or less have a Parkin mutation. PINK1 mutations

account for 3.7% of PD cases, the majority of mutations are nonsense or missense mutations(Lill 2016).

The least common autosomal recessive gene associated with PD is DJ-1 which occurs in 0.4% of PD cases and is most commonly found with point or structural mutations, and

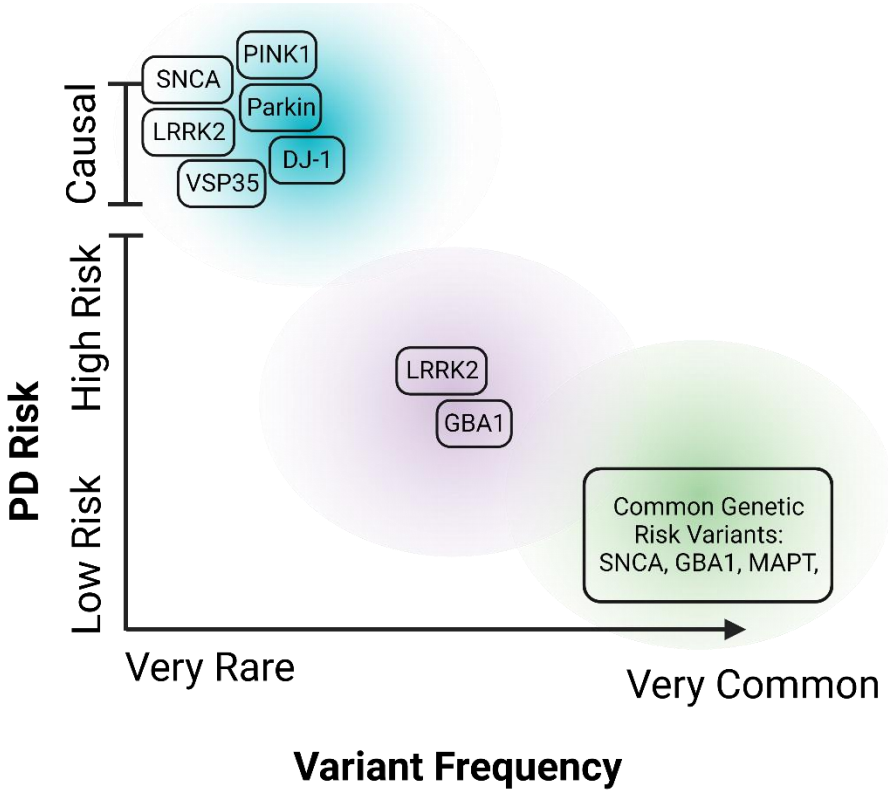

Figure 1.2. Schematic showing PD genetic risk variants. Monogenic autosomal dominant mutations causing PD are listed in blue, and other autosomal recessive mutations are listed in purple and genetic risk variants are in green. Genes shown here are representative of the etiology of PD and does not include every gene, mutation, or variant. Made in BioRedner.

deletions are rare (Lill 2016; Funayama et al. 2023). A more recently identified autosomal recessive gene, DNAJC6 encodes Auxilin, a clathrin-associated protein involved in vesicular trafficking associated with early onset. These autosomal recessive genes have an average age of onset of less than 30 years (Lill 2016).

Genetic Risk Factors for sporadic PD

Candidate gene and genome-wide association studies (GWAS) have identified many common single nucleotide polymorphisms (SNPs) associated with increased risk of PD (Lill 2016). A recent meta-analysis of GWAS studies from European ancestry samples identified 78 genomic regions and 90 independent genome-side risk signals associated with PD (Nalls et al. 2019). The recent meta-analysis by Nalls et al. found SNCA as one of the top hits (consistent with other GWAS) and some of the other top hits include LRRK2, glucocerebrosidase (GBA1), bone marrow stromal cell antigen 1, 3-methylcrotonyl-CoA Carboxylase subunit 1, Transmembrane Protein 175, and Microtubule-associated protein tau. Both LRRK2 and SNCA were identified as genetic risk variants for sporadic PD, and the same mutations can be monogenically inherited and can cause autosomal dominant PD (Satake et al. 2009; Simón-Sánchez et al. 2009). Homozygous GBA1 carriers develop the lysosomal disorder, Gaucher disease; both heterozygotes and homozygotes have an increased risk of PD. GBA1 is a lysosomal protein involved in protein clearance, specifically α-syn protein (Funayama et al. 2023). The risk of PD is higher in those homozygous and heterozygous making GBA1 a major risk factor for PD (Avenali et al. 2020). Tau protein the primary component of neurofibrillary tangles, a pathological hallmark in many forms of dementia, is occasionally found in postmortem brains with PD and can be found in up to 50% of cases with Lewy Body Dementia (Arima et al. 1999; Chin et al. 2020). Genetic risk factors alone are not causative of PD because these variants only show moderate association with PD. Genetic risk factors alone are not causative of PD because these variants only show moderate association with PD. Only a small portion of PD cases are

monogenically inherited, and the remaining proportion of PD cases is due to a combination of common genetic risk variants and environmental factors (Figure 1.2).

Gene-Environment Interactions

As mentioned above, only 5% of cases are due to monogenically inherited mutations. Therefore, interactions between these identified genetic risk factors that are common amongst the population must be interacting with environmental factors resulting in increased risk of the disease. Therefore, gene-environment interaction in PD must be studied to better understand this interaction (Lill 2016). A study completed by Hill-Burns et al. investigated to identify genes that interact with smoking (Hill-Burns et al. 2013). Using genome-wide interaction analysis in nicotine-exposed Drosophila and humans, the gene *SV2C* was identified as a gene-smoking interaction in PD. The *SV2C* gene encodes synaptic vesicle glycoprotein 2C which functions to regulate vesicle stability and trafficking. Nicotine has also been found to increase DA release from synaptic vesicles. Based on these results and the known functions of *SV2C* may play an important role in PD as a gene-smoking interactor (Hill-Burns et al. 2013).

Another study by Hazma et al. identified *GRIN2A* as an interacting gene involved in coffee consumption and reduced risk of PD (Hamza et al. 2011). Here, a genome-wide association and interaction study was completed to test single nucleotide polymorphisms on the effect of smoking and PD risk. Here, the rs4998386 mutation in *GRIN2A* was identified as a top hit. The *GRIN2A* encodes the NMDA-glutamate receptor subunit which is involved in excitatory transmission and was shown to reduce excitotoxicity serving as neuroprotective (Hamza et al. 2011). Although these types of

studies are difficult to replicate, they do provide important insight into gene-environment interactions and their relevance in PD (Lill 2016).

Environmental Risk Factors in Parkinson's Disease

Protective factors

Meta-analysis results show that there are specific factors that serve as a protective factor, reducing the risk of PD. Specifically, it was shown that coffee intake, smoking, and alcohol reduce the risk of PD (Noyce et al. 2012). It has also been found that certain prescription drugs are negatively associated with PD such as calcium channel blockers and anti-inflammatory drugs (Noyce et al. 2012). Other lifestyle factors that are thought to be protective against PD include a diet low in dairy, diets with high antioxidant content, and high physical activity levels (Jiang et al. 2014; Yang et al. 2015; Talebi et al. 2022).

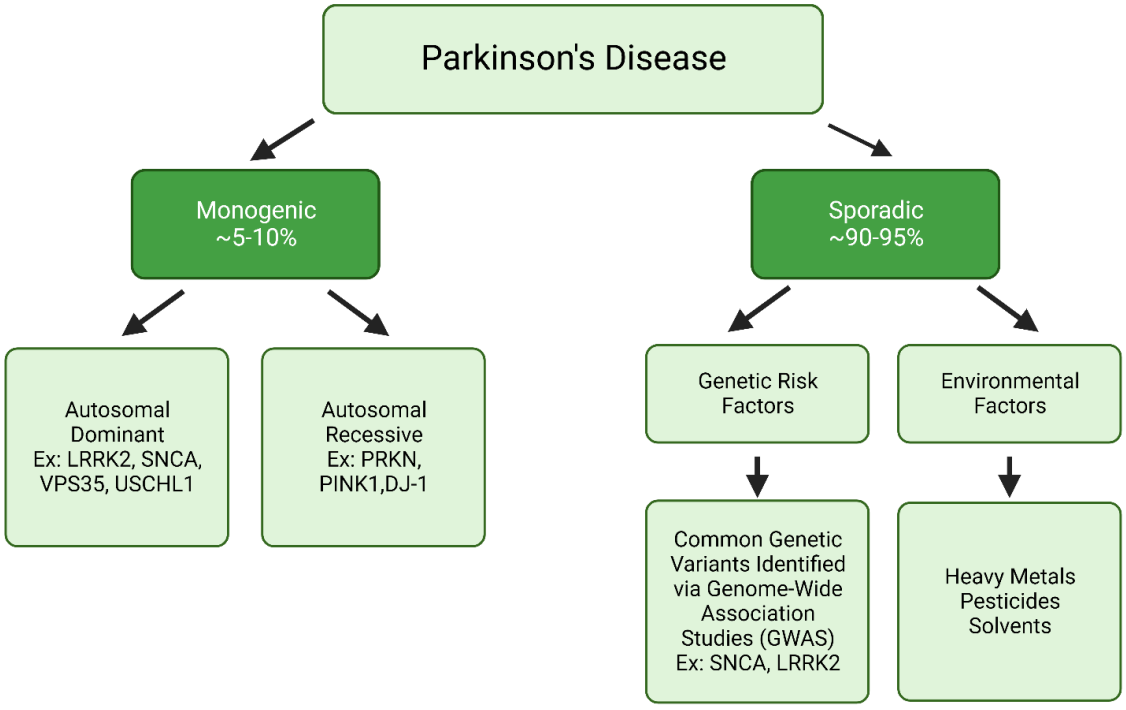

Figure 1.3. Parkinson's disease risk factors. Made in BioRedner.

Environmental risk factors

In addition to this, head injury, constipation, mood disorders, and the use of beta-blockers were also found to be positively associated with PD. Specific environmental factors including rural living, farming/agriculture occupations, drinking well water, and use of pesticides are significantly associated with increased risk of PD (Noyce et al. 2012).

Persistent organic pollutants

Epidemiological studies show an association between exposure to persistent organic pollutants (POPs) and an increased risk of PD (Tanner and Langston 1990; Semchuk et al. 1992; Le Couteur et al. 1999; Priyadarshi et al. 2000; Ritz and Yu 2000; Tanner and Aston 2000; Priyadarshi et al. 2001; Ascherio et al. 2006; Brown et al. 2006; Steenland et al. 2006; Elbaz et al. 2009; Wirdefeldt et al. 2011; Caudle et al. 2012; Freire and Koifman 2012). POPs are a class of pollutants that include industrial chemicals polychlorinated biphenyls (PCBs), polybrominated diphenyl eithers (PBDEs), perfluorooctane sulfonic acid (PFOS), pesticides, and industrial by-products (Alharbi et al. 2018). POPs are known to bio-accumulate into the soil and lipid-rich tissues due to their lipophilic nature, low volatility, and slow degradation. Exposure to POPs can result in various effects on health such as hormonal disruption, cancer, cardiovascular disease, effects on the immune system reproductive harm, and neurological disease and disorders (Alharbi et al. 2018). Case-control studies along with many others have shown that there is an increased risk of PD with POP exposure (Weisskopf et al. 2010).

Industrial toxicants

Industrial contaminants include a broad range of chemicals that can result in exposure via occupational or contaminated food products, and in recent years these chemicals have been linked to PD risk (Steenland et al. 2006; Caudle et al. 2012; Goldman 2014). Many classes of industrial compounds are thought to contribute to PD risk including organohalogens, PCBs, PBDEs, PFOS, metals, nanoparticles, trichloroethylene, and solvents (not all of which are POPs) (Caudle et al. 2012). PCBs have demonstrated a significant association with PD in both human and animal studies by inducing changes in oxidative stress, calcium homeostasis, and DA stasis. PBDEs are associated with PD risk in animal studies with similar mechanisms of action to PCBs. A variety of metals including iron, copper, manganese, lead, and mercury exposure have a significant association showing changes in oxidative stress, and metal-specific effects on α-syn fibrilization, DA homeostasis, mitochondrial dysfunction, or calcium homeostasis. Nanoparticles result in oxidative stress and signs of neuroinflammation in animal studies, but there is no reported association of these effects on humans currently. Solvents such as trichloroethylene, hexane, etc. have a significant association with PD based on both animal and human studies showing oxidative stress, mitochondria dysfunction, calcium homeostasis disruptions, and α-syn aggregation. Compounds associated with industrial manufacturing show a significant effect on PD risk by disrupting crucial processes in DAergic neurons.

Pesticides

Pesticide exposure is another major risk factor for idiopathic PD. Whereas, pesticides are known to contribute to PD, but are not considered a causative agent. Early research

focused on the pesticide paraquat because it shares structural similarities to MPP^+ and results in similar toxicological effects. More recently, other pesticides such as maneb, dithiocarbmates, pyrethroids, rotenone, and organochlorines have also been studied as possible agents contributing to PD (Moretto and Colosio 2011). One such compound is dieldrin, an organochlorine pesticide that is associated with an increased risk of PD in both epidemiological and mechanistic studies resulting in almost a 2-fold increased risk of PD (Fleming et al. 1994; Corrigan et al. 1998; Corrigan et al. 2000; Kanthasamy et al. 2005; Hatcher et al. 2007; Weisskopf et al. 2010; Moretto and Colosio 2011).

The genetic and environmental factors that contribute to PD converge at common mechanisms such as mitochondrial dysfunction, oxidative stress, and impaired protein degradation (Fleming 2017). Although monogenetic mutations only account for a small proportion of PD, the identified genetic risk variants from GWAS studies have identified hundreds of commonly occurring variants increasing the risk of PD (Nalls et al. 2019). Together genetic risk variants and environmental risk factors can interact synergistically to contribute to disease risk and progression (Steece-Collier et al. 2002; Bellou et al. 2016; Fleming 2017) (Figure 1.3).

Epigenetics

Epigenetics is defined as a set of mechanisms that regulate gene expression without modifying the DNA sequence itself and are meiotically and mitotically heritable in dividing cells (Berger et al. 2009; Dupont et al. 2009). Generally, epigenetics refers to a set of three major mechanisms: 1) histone modifications and the regulation of chromatin structure, 2) covalent modifications of DNA, and 3) non-coding RNA-mediated mechanisms that affect gene expression and/or the other epigenetic mechanisms

(Marques et al. 2011; Marques and Outeiro 2013). Epigenetic marks are sensitive to the environment and play a critical role in the regulation of gene expression; thus, they are thought to be a potential mediator of the relationship between genes, the environment, and disease (Bollati and Baccarelli 2010; Faulk and Dolinoy 2011; Allis and Jenuwein 2016; Cavalli and Heard 2019).

DNA modifications

One of the most well-studied epigenetic marks is the covalent modification of the fifth position of cytosine in DNA (5-methylcytosine, 5mC) by DNA methyltransferases (DNMTs) (Moore et al. 2013). More recently, further oxidation of 5mC to 5-hydroxymethylation (5hmC) by the ten-eleven translocase (Tet) family of enzymes was recognized as a critical epigenetic mark, particularly in stem cells and the brain

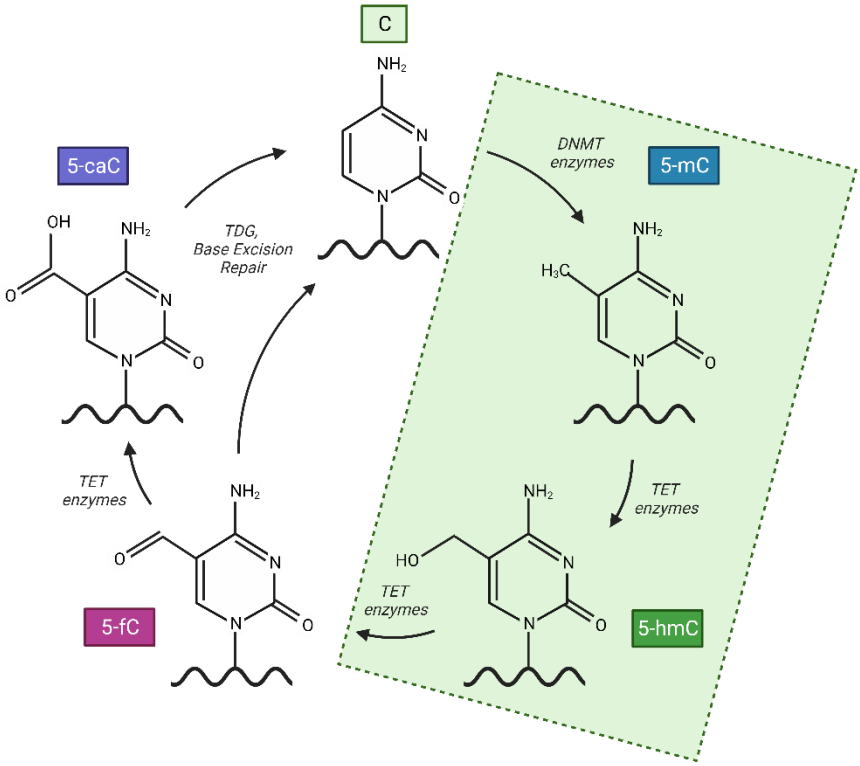

Figure 1.4. DNA methylation biochemical pathway. Made in BioRedner.

(Tahiliani et al. 2009; Kriaucionis and Tahiliani 2014; Cheng et al. 2015; Rasmussen and Helin 2016; Parker et al. 2019; Shekhawat et al. 2021); Tahiliani et al., 2009) (Figure 1.4). Each of these marks has a distinct set of “writers” and “readers” that catalyze their generation and recognize these marks such that they play a critical role in the regulation of gene expression (Cheng et al., 2015). 5hmC is thought to be particularly important in the central nervous system and the response to neurotoxicants, where 5hmC is highly enriched with levels 10-fold higher than levels in stem cells (Cheng et al., 2015; Globisch et al., 2010; Kochmanski & Bernstein, 2020). Mounting evidence indicates that exposure to environmental toxicants is associated with epigenetic changes and altered trajectories of age-related epigenetic changes, particularly for DNA modifications (Baccarelli and Bollati 2009; Bollati and Baccarelli 2010; Faulk and Dolinoy 2011; Lardenoije et al. 2015; Lardenoije et al. 2018; Cavalli and Heard 2019). Of relevance to this dissertation, previous work has found that developmental dieldrin exposure results in differential DNA methylation on several genes (Joesph Kochmanski et al. 2019). DNA modifications occur most often on cytosine nucleotides before guanines (a CpG dinucleotide) (Moore et al. 2013; Kriaucionis and Tahiliani 2014). Approximately 70% of gene promoters are located within CpG islands and methylation of CpG islands and within promoters can result in stable silencing of gene expression. In the gene body, DNA methylation contributes to cell-specific gene expression and regulation (Moore et al. 2013).

Th e role of 5hmC is yet unclear, however, there are high expression levels of 5hmC in the brain; there is a ten-fold increase in 5hmC expression in the brain as compared to embryonic stem cells (Kochmanski and Bernstein 2007; Cheng et al. 2015). In

transcriptionally active genes in neuronal tissue, 5mC expression is reduced, however, 5hmC is elevated in active genes.

Epigenetic reprogramming

DNA methylation is a vital part of mammalian development. Two rounds of epigenetic reprogramming during embryogenesis and gametogenesis occur to reset the zygote or germ cells for further differentiation (Bollati and Baccarelli 2010; Faulk and Dolinoy 2011; Smallwood and Kelsey 2012) (Figure 1.5). During gametogenesis, the genome-wide DNA methylation is erased in primordial germ cells. Following sex determination, there is an asymmetrical sex-specific re-methylation that occurs. After fertilization, a second wave of demethylation occurs. In the male embryo, methylation begins during meiosis and the epigenetic landscape is established before birth. However, in females, the primordial germ cells remain unmethylated and are later established with each estrous cycle. During this, global

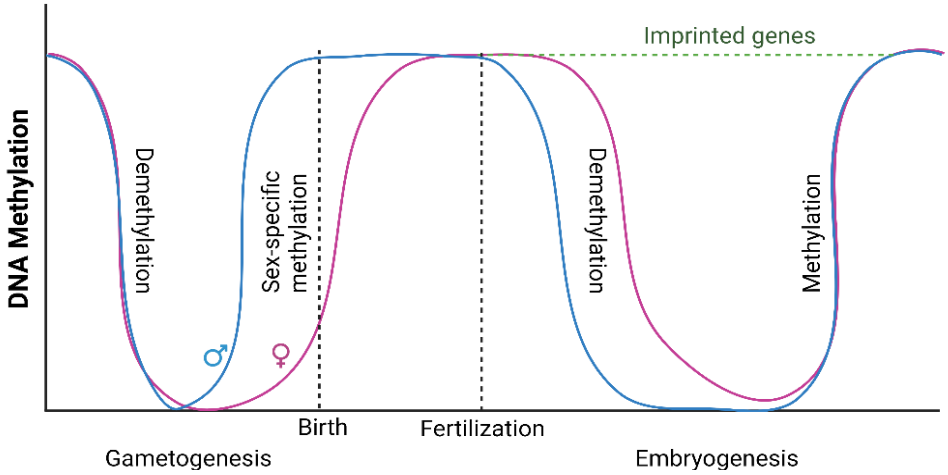

Figure 1.5. Epigenetic reprogramming in development. The y-axis shows relative DNA methylation levels. Th x-axis shows phases of development. The blue line represents the paternal genome. The pink represents maternal genome. The green dashed line represents imprinted genes. Early in gametogenesis, there is a universal demethylation followed by a sex-specific re-methylation. After fertilization ther is a symmetrical demethylation followed by re-methylation during embryogenesis. Made in BioRedner.

demethylation occurs across the genome except at imprinted genes (Kappil et al. 2015). Following fertilization, the paternal genome is demethylated rapidly, and the maternal genome is erased more slowly. With cell-lineage determination, DNA methylation is re-established in the developing embryo (Bollati and Baccarelli 2010; Faulk and Dolinoy 2011; Smallwood and Kelsey 2012). Due to the dynamics of the DNA methylation landscape, developmental periods are particularly sensitive to environmental influences and can mediate disease susceptibility.

Developmental Origins of Health and Disease Hypothesis

Exposure to various solvents, metals, and pesticides is involved in PD risk. Developmental exposure to these compounds may affect disease risk later in life (Heindel and Vandenberg 2015; Baird et al. 2017). The developmental origins of the health and disease hypothesis state that there are certain sensitive periods such as pre- and peri-natal periods that are exceptionally sensitive to environmental exposures that may impact disease trajectory later in life (Baird et al., 2017; Heindel & Vandenberg, 2015). Epigenetic mechanisms, such as DNA modifications contribute to this increased sensitivity to the environment during developmental phases.

Epigenetics in PD

It is thought that epigenetics play an important role in the development of PD since environmental factors are a major component of the etiology of PD, and epigenetic marks are sensitive to the environment (Tsalenchuk et al. 2023). Many studies have investigated the epigenetic effects of several environmental exposures in human PD. In summary, these studies have shown several differential DNA methylation marks in PD cases as a result of exposure to various factors including coffee consumption, vitamin

E, PD drugs, lead, organochlorine pesticides, organophosphate pesticides, heavy metals, manganese, and exercise (Reviewed Tsalenchuk et al. 2023).

Epigenetic studies have shown that the SNCA gene may have differential epigenetic regulation in PD. Specifically, some studies have shown a reduction in SNCA methylation in the SN of PD patients (Jowaed et al. 2010; Matsumoto et al. 2010). Histone modifications resulting in SNCA silencing and histone deacetylase inhibitors have proven efficacy in neuroprotection against PD in several *in vivo* models (Kontopoulos et al. 2006; Outeiro et al. 2007; Kidd and Schneider 2010; Monti et al. 2010; Song et al. 2010; Jin et al. 2011; Kidd and Schneider 2011; Chen et al. 2012). Other PD-associated genes such as LRRK2 and Parkin have also been shown to have differential- epigenetic regulation in PD cases, many of which are related to differential regulation of micro-RNAs (Asikainen et al. 2010; Gehrke et al. 2010). Others have noted differential modification and variant expression of genes that function in the cycle of methylation and demethylation including DNA methyltransferase 1 and Ten-eleven-translocase-1 in PD patients (Desplats et al. 2011; Shu et al. 2019). These epigenetic modifications associated with environmental factors and PD-related genes show that epigenetic mechanisms are a crucial link between environmental exposures, PD risk genes, and the development of disease.

Synaptic and vesicular integrity in Parkinson's Disease

The Dopaminergic synapse

As discussed above, nigrostriatal degeneration is a pathological hallmark of PD and is thought to underly the motor symptoms of the disease. The main synapse that is lost when this pathway degenerates is the synapse of nigral DA neurons onto medium spiny

neurons in the striatum. Within the presynaptic terminal of these synapses, the vesicular packaging of DA into vesicles by type II vesicular monoamine transporter (VMAT2) serves two key functions: to package DA into vesicles for release into the synaptic cleft and to protect the cell from cytosolic DA.

For DA release, the arrival of an action potential at the synaptic terminal triggers a calcium influx. Cytosolic calcium binds to synaptotagmin proteins on the vesicle to initiate vesicular fusion with the pre-synaptic membrane resulting in the release of vesicular contents into the synaptic space (Bellani et al. 2010). Synaptic DA can be cleared from the synapse via DAT on the presynaptic terminal and re-packaged into vesicles via VMAT2. Synaptic DA can bind to post-synaptic receptors including D1-like (D1R) and D2-like (D2R) or D2R located on the pre-synaptic membrane functioning as autoreceptors. The machinery of the DAergic synapse is essential for maintaining DA homeostasis, and disruption in the synaptic machinery can result in DAergic dysfunction.

Cytosolic DA levels are primarily regulated by the transport of DA by the DAT and VMAT2. Synaptic vesicles within DA neurons also prevent DA oxidation and enzymatic breakdown of toxic metabolites by sequestering cytosolic DA in the acidic environment of the vesicle (Alter et al., 2013). When not readily packaged into vesicles, cytosolic DA can undergo enzymatic deamination or autoxidation to form harmful, reactive metabolites including H_2O_2, 3,4-Dihydroxyphenylacetaldehyde (DOPAL), DA quinones,

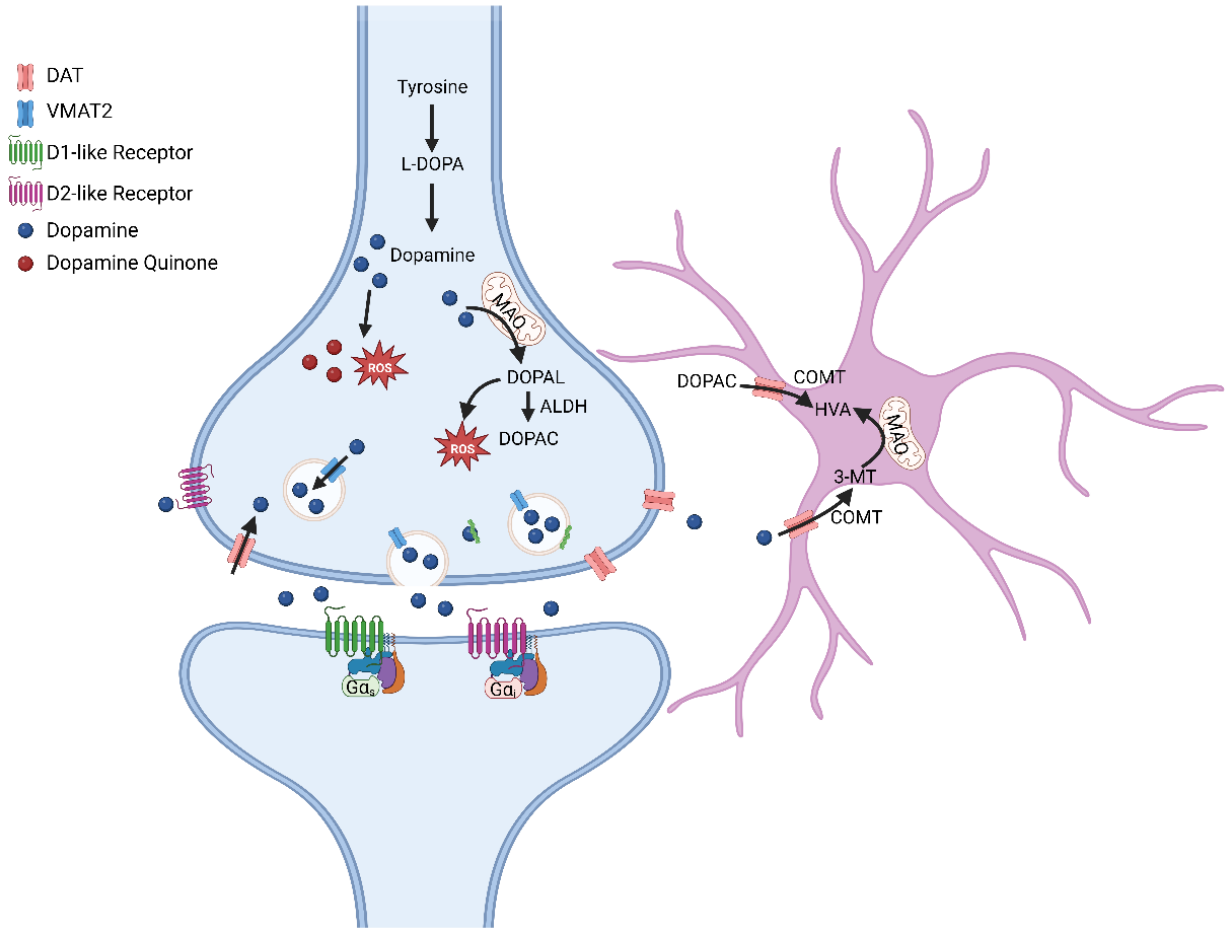

Figure 1.6. The dopaminergic synapse. Tyrosine is converted to L-DOPA by Tyrosine hydroxylase which is the rate limiting enzyme in DA syntehsis. L-DOPA is converted to DA by DOPA decarboxylase. DA is packaged into synpatic vesicles via VMAT2 which can bind to the membrane and release DA upon an action potential. Synaptic dopamine can bind to D1-like or D2-like receptors. D1 is a $G\alpha_s$ located on the post-synaptic terminal. D2 is a $G\alpha_i$ located on the post-synaptic terminal and the pre-synaptic terminal where it can function as an autoreceptors. Synaptic dopamine and the DA metabolite, DOPAC can be taken up by surrounding astrocytes and degraded to form the metabolites, HVA. Synpatic DA can also be taken up into the synaptic terminal via the DAT. Cytosolic dopamine can be autoxidized or enzymatically degraded into toxic metabolites. Synaptic dopamine and the DA metabolite, DOPAC can be taken up by surrounding astrocytes and degraded to form the metabolites, HVA. Made in BioRedner.

and superoxide (Alter et al. 2013). When the ratio of DAT to VMAT2 increases, more DA can enter the pre-synaptic terminal via DAT, but there is less VMAT2 available to sequester cytosolic DA resulting in increased cytosolic DA and increased DA-related

toxicity. On the other hand, a decreased DAT: VMAT2 ratio would decrease the susceptibility to DA-related toxicity as less DA can enter the terminal via DAT, but more DA is capable of being sequestered by VMAT2. A lowered ratio of DAT: VMAT2 ultimately reduces cytosolic DA concentrations and lowers the potential for DA autoxidation and toxic metabolite formation (Miller et al. 1999)(Figure 1.6).

The functions of α-synuclein at the synapse

α-syn is primarily found in neurons and plays a role in many neuronal functions including regulation of synaptic transmission, mitochondrial homeostasis, gene expression, protein phosphorylation, and fatty acid binding. Within neurons, α-syn is primarily localized in presynaptic terminals where it interacts with synaptic vesicles, suggesting that α-syn is involved in regulating DA neurotransmission and homeostasis (Perez et al. 2002; Yavich et al. 2004a). α-syn was found to interact with almost all the proteins involved in DA synthesis and handing. In multiple studies, a-syn was shown to inhibit tyrosine hydroxylase (TH) activity and DA synthesis, modulate DAT activity and localization, and increase the amount of VMAT2 on vesicles, (Bellani et al., 2010; Bridi & Hirth, 2018; Cheng et al., 2011; Roy, 2017; Venda et al., 2010). α-syn is a negative regulator of TH activity and DA synthesis. Overexpression of α-syn inhibits TH activity, whereas downregulation of α-syn enhances TH activity and DA synthesis (Cheng et al. 2011). It is suggested that α-syn may bind to the serine residues in the N-terminus of TH when TH is in a dephosphorylated state and preventing TH phosphorylation or activation, therefore reducing TH activity (Cheng et al. 2011).

In addition to regulating DA synthesis, α-syn is thought to facilitate synaptic transmission by maintaining and regulating vesicle trafficking and mediating vesicular docking and

exocytosis (Larsen et al. 2006; Mosharov et al. 2009; Scott and Roy 2012a; Choi et al. 2013; Vargas et al. 2014; Wang et al. 2014). Monomeric α-syn reversibly binds and interacts with vesicle membranes and maintains recycling pool vesicles in primary neuronal culture. Consistent with this, α-syn null mice show decreased DA stores (Davidson et al. 1998; Abeliovich et al. 2000; Scott and Roy 2012b). α-syn also mediates synaptic vesicle clustering in the presynaptic terminal in a Ca^{2+}-dependent manner. Treatment with DA exacerbates synaptic clustering of synaptic vesicles only (Burré et al. 2014) Overall, physiologically normal α-syn plays an important role in mediating synaptic vesicle clustering and inducing the SNARE complex formation resulting in the regulation of neurotransmitter release (Burré et al., 2014; Cabin et al., 2002; Choi et al., 2013; Diao et al., 2013; Fouke et al., 2021; V. Gao et al., 2023; Gaugler et al., 2012; Lautenschläger et al., 2018; D. Scott & Roy, 2012; Vargas et al., 2014; L. Wang et al., 2014; Yavich et al., 2004a).

α-syn regulates DA synthesis by interacting with the enzymes TH and L-aromatic amino acid decarboxylase (AADC) modulating the synthesis of DA (Peng et al. 2005; Tehranian et al. 2006; Rietdijk et al. 2017). Mice with α-syn null mutation (loss of function mutation) display an increased rate of vesicular refilling, but stable DA release upon repeated electrical stimulations compared to mice with normally expressed α-syn (Yavich et al. 2004). On the other hand, overexpression models of α-syn results in reduced DA release, reduced size and density of the recycling pool, and altered organization of synaptic vesicles (Larsen et al. 2006; Nemani et al. 2010).

Dopamine-α-synuclein cycle

DA and α-syn can both result in neurotoxic byproducts themselves, and they also feed into a circle of neurotoxicity where DA and α-syn interact and exacerbate the toxic effects of each other (Venda et al. 2010; Roy 2017). Cytosolic DA levels must be closely maintained in DAergic neurons. If not properly maintained, cytosolic DA can be autoxidized or enzymatically degrading forming toxic intermediates that can be detrimental to the integrity and function of DAergic neurons (Alter et al. 2013; Wong et al. 2019).

The function of α-syn is closely tied to maintaining synapses, specifically vesicular trafficking and neurotransmitter packaging, synthesis, and release (Venda et al. 2010; Roy 2017). Therefore, if α-syn expression or function is altered, this will result in effects on DA handling. Since DAergic synapses are tightly regulated, a change in α-syn could result in disrupted DA release, packaging, synthesis, and cytosolic DA concentrations (Alter et al. 2013; Wong et al. 2019). On the other hand, changes in DA signaling can also mediate effects on α-syn. DA mishandling resulting in increased DA autoxidation

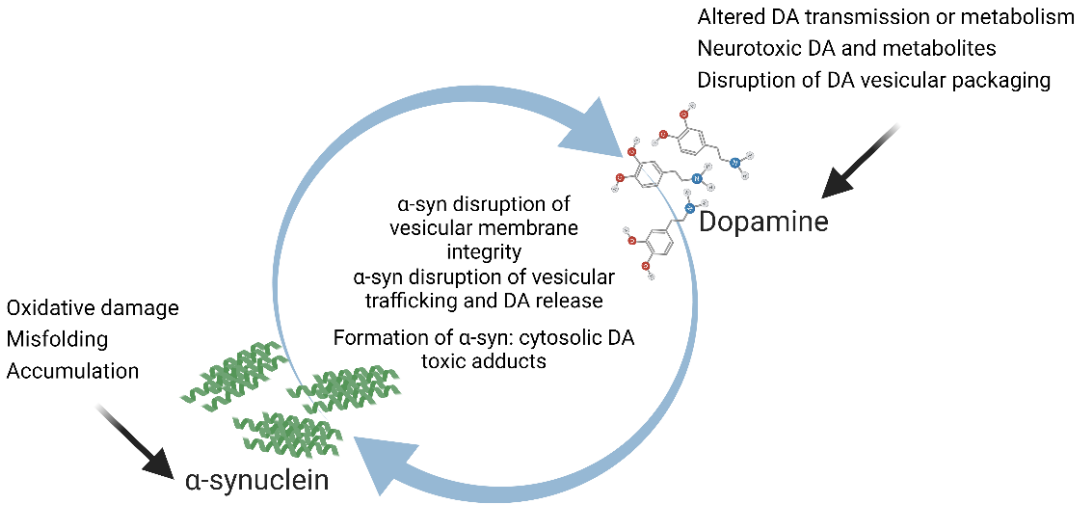

Figure 1.7. Interactions between dopamine and α-synuclein. Made in BioRedner.

and toxic intermediates can induce oxidative damage on α-syn which could affect α-syn folding and aggregation. Therefore, this interaction between DA and α-syn is crucial for maintaining properly functioning DA synapses (Roy 2017) (Figure 1.7).

Compensatory mechanisms in Parkinson's disease

Compensatory changes in the nigrostriatal system in DA are defined by increases in DAergic activity including DA release, DA turnover, DA uptake, and TH activity following PD-related degeneration (Hornykiewicz 1966; Bernheimer et al. 1973; Zigmond et al. 1984; Onn et al. 1986; Zhang et al. 1988; Snyder. GL et al. 1990; Liang and Jiang 1991; Zigmond et al. 1993; Moore and Zigmond 1994; Bezard and Gross 1997; Zigmond 1997; Zigmond et al. 1998; Bezard et al. 2001; Bustos et al. 2004; Blesa et al. 2017; Bezard and Gross). Early compensatory changes in the nigrostriatal system that precede neurotoxicity and degeneration are well-documented in human PD, multiple animal models of PD, and most recently reported in a norepinephrine deficit model (Zigmond et al. 1984; Onn et al. 1986; Zhang et al. 1988; Snyder. GL et al. 1990; Zigmond et al. 1993; Moore and Zigmond 1994; Zigmond 1994; Bezard and Gross 1997; Zigmond 1997; Zigmond et al. 1998; Molina-Mateo et al. 2017; Iannitelli et al. 2023). These compensatory mechanisms were observed in multiple neurotransmitter systems.

Early compensatory increases in DA precede DA dysfunction and degeneration

In both the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA), which are neurotoxicants that are extensively used to model DAergic loss and degeneration, there appears to be an early compensatory increase in many aspects of the nigrostriatal system that precedes DA loss and DA

neuron death (Onn et al. 1986; Zhang et al. 1988; Graham et al. 1990; Snyder. GL et al. 1990; Liang and Jiang 1991; Alexander et al. 1993; Zigmond et al. 1993; Rozas et al. 1998; Bezard, Dovero, Prunier, Ravenscroft, Chalon, Guilloteau, Alan R Crossman, et al. 2001). A model of DAergic depletion via MPTP in non-human primates showed an upregulation of TH immunoreactive neurons in the striatum by 3.5-fold compared to controls, which may show that nigrostriatal neurons are capable of compensation (Betarbet et al. 1997). In a rat striatal slice lesioned with 6-hydroydopamine, there are significant increases in stimulation-induced DA release compared to controls suggesting that remaining striatal terminals compensate for 6-OHDA-induced depletion of DA neurons (Snyder. GL et al. 1990). In rats lesioned with 6-OHDA, there is an increase in the presence of striatal DA terminal 4 months post-lesion compared to immediately after lesion as well as increases in levels of TH, DA, and 3,4-Dihydroxyphenylacetic acid (DOPAC) content compared to control rats (Onn et al. 1986). In 6-OHDA lesioned rats at 1-month post-lesion, there was only a 17.2% increase in DA content suggesting that the remaining striatal terminals likely undergo compensatory physiological changes to increase synthesis and release of DA (Onn et al. 1986) These findings are consistent with other monoaminergic models, including norepinephrine (NE), which shares similar synaptic machinery to DA neurons. In a model of NE degeneration in the locus coeruleus, there are significant increases in NE turnover and signaling as well as behavioral phenotypes despite the loss of NE neurons (Iannitelli et al. 2023).

This increase in DA activity that precedes DA loss and degeneration may be due to the regulation of DA receptors. In the MPTP model of DAergic degeneration, there is an early upregulation of the D2R binding in striatal neurons which occurs only at a time

point before the most severe motor deficits (Bezard et al. 2001). D2R density in the striatum was shown to increase in a variety of models of DA depletion (Graham et al. 1990; Alexander et al. 1993; Decamp et al. 1999; Aubert et al. 2005; Chefer et al. 2008; Sun et al. 2013; Blesa et al. 2017).

Serotonergic signaling

Some research suggests that an upregulation of serotonergic innervation in the striatum may play a role in these compensatory increases in DA signaling (Zhou et al. 1991; Gaspar et al. 1993; Rozas et al. 1998; Maeda et al. 2003; Mounayar et al. 2007; Gagnon et al. 2016; Blesa et al. 2017; Wile et al. 2017). In differentiated PC12 cells, dieldrin exposure increased tryptophan hydroxylase transcript expression, the rate-limiting step in serotonin (5-HT) synthesis, and a decrease in expression of genes related to 5-HT storage, degradation, and several 5-HT receptors (Slotkin and Seidler 2008). Most of the 5-HT-containing neurons are in the raphe nuclei which make projections throughout the basal ganglia, including the SNpc and the striatum (Di Matteo et al. 2008). Striatal increases in 5-HT concentrations were shown to facilitate DA release from nigrostriatal neurons in a variety of rat microdialysis studies (Blandina et al. 1989; Bonhomme et al. 1995; De Deurwaedere et al. 1996; Reed et al. 2013). Serotonergic projection in the striatum has also been implicated in PD, specifically in the context of L-DOPA treatment and facilitating L-DOPA-induced dyskinesias since 5-HT neurons are capable of storing and releasing DA (Carta et al. 2007; Reed et al. 2013). Models of DA depletion induced by 6-OHDA and MPTP in several animal models including neonatal and adult rats as well as adult non-human primates show sprouting of serotonergic neurons innervating the striatum post-lesioning (Zhou et al. 1991;

Gaspar et al. 1993; Karstaedt et al. 1994; Rozas et al. 1998; Maeda et al. 2003; Mounayar et al. 2007; Gagnon et al. 2016; Pagano et al. 2018; Jiménez-Sánchez et al. 2020).

Glutamatergic signaling

Evidence suggests glutamate signaling is likely involved since glutamatergic signaling regulates DA synthesis and release via N-methyl-D-aspartate (NMDA) receptors in the SN forming a negative feedback loop (Zigmond 1994; Bustos et al. 2004). In line with this, glutamatergic synapse remodeling and altered vesicular glutamate transporter were observed in animal models of PD and postmortem PD brains (Smith et al. 2009; Villalba et al. 2015; Villalba and Smith 2018). In differentiated PC12 cells, treated with dieldrin, there is a significant increase in transcript expression relating to glutamatergic signaling (Slotkin and Seidler 2009). These results in PC12 paralleled the effects of the organophosphate, chlorpyrifos, which is known to exert toxic effects by promoting excitotoxicity (Slotkin and Seidler 2009; Rush et al. 2010). Dieldrin-treated cells resulted in a significant increase in genes encoding for ionotropic receptors in response to dieldrin such as *gria1* (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) 1), *grin1* (NMDA 1), and *grina* (NMDA-associated protein) (Slotkin and Seidler 2009). Ionotropic receptors such as NMDA and AMPA are thought to contribute to excitotoxicity because they are ligand-gated ion channels permeable to Ca^{2+}, a driver of oxidative stress and cell death (Dong et al. 2009). It has also been proposed that enhanced sub-thalamic nucleus signaling observed in PD could result in exacerbated glutamatergic signaling in surviving DA neurons which results in excitotoxicity (Sharman et al. 2000;

Obeso et al. 2004; Mehler-Wex et al. 2006; Wu et al. 2012; Blandini et al.; Riederer et al.).

Excitotoxicity

In the basal ganglia, GABAergic neurons from the striatum innervate structures in the indirect pathway and ultimately serve as a negative regulator to glutamatergic neurons that play a role in initiating signaling to the motor cortex to control motor movements (Obeso et al. 2000; Wu et al. 2012; Maiti et al. 2017; Blandini et al.; Riederer et al.). The upregulation of glutamatergic signaling promotes excitotoxicity by activating the glutamatergic NMDA receptor, which can also promote Ca^{2+} overabundance (Caudle and Zhang 2009; Ambrosi et al. 2014; Blandini 2014; Wang et al. 2020). High levels of Ca^{2+} in the mitochondrial or cytoplasm can result in the formation of reactive oxygen species (ROS) and reactive nitrogen species which can induce mitochondrial dysfunction (Ambrosi et al., 2014; Post et al., 2018). Elevated levels of ROS impair ATP production by inhibiting the function of several steps in the mitochondrial electron transport chain and overall excitotoxicity. Ca^{2+} overabundance can also activate pro-apoptotic factors and open the mitochondrial permeability transition pore leading to the release of cytochrome C and neuronal death (Ambrosi et al. 2014).

Excitotoxicity was an observed factor contributing to PD-related pathogenesis. Degeneration of nigrostriatal DAergic neurons disinhibition to the STN which promotes hyperexcitation of glutamatergic neurons projecting from the STN to the GPe, GPi, and SNpc (Rodriguez-Farre and Suñol 1995; Bezard and Gross 1998; Caudle and Zhang 2009; Blandini 2014). Understanding DA-related compensatory mechanisms provides important insight into ways in which development exposures may induce lasting

changes in synaptic function in DAergic nigrostriatal neurons. A variety of PD models were instrumental in studying many of the potential mechanisms underlying PD-related compensatory DA responses.

Parkinson's disease models

Genetic models

Many genetic models of PD target genes linked to familial, monogenic forms of the disease including *SNCA, LRRK2, DJ-1, PINK1*, and *PRKN* (Shimohama et al. 2003; Blandini and Armentero 2012; Blesa et al. 2012; Jagmag et al. 2016). Due to the central role of α-syn in PD, many models were made targeting *SNCA* (Lill 2016). These include a range of α-syn overexpression models or models that express the mutant form of α-syn, including the disease-linked mutations A53T, A30P, and E46K. Nigrostriatal degeneration, Lewy-like inclusions, and motor impairments are inconsistent among these α-syn models and few show all three of these features (Shimohama et al. 2003; Blandini and Armentero 2012; Blesa et al. 2012; Jagmag et al. 2016). These models are variable in how closely they mimic important aspects of the disease, but few show nigrostriatal degeneration, α-syn pathology, and motor impairments. The A53T mutant mice produce inclusions resembling LBs, and motor impairment, but generally do not produce overt nigrostriatal loss. The A30P mouse model produces LB-like inclusions, does not produce nigrostriatal degeneration, and some studies have shown motor impairments, but this seems to be promoter-specific. Expressing an E56K α-syn transgene inserted into the ROSA26 locus did not produce α-syn inclusions, nigrostriatal degeneration, or motor impairments (Shimohama et al. 2003; Blandini and Armentero 2012; Blesa et al. 2012; Jagmag et al. 2016). In LRRK2 mouse models, it was reported

that the R1441G and R1441C variants produce motor impairments, but no effects on PD hallmarks (Nuytemans et al. 2010; Jagmag et al. 2016). However, The G2019S variant was shown to induce nigrostriatal loss and variable effects on motor phenotypes, but no formation of LB-like inclusions. However, exon 1, 29, and 30 of LRRK2 have resulted in inclusion formation (Nuytemans et al. 2010; Jagmag et al. 2016). Only a few Parkin exon deletion models have shown significant motor impairments. DJ-1 exon 2 deletion and exon 7 inactivation models show motor impairments, but no PD-like pathology (Jagmag et al. 2016). Only a few of these genetic models result in PD-associated pathology and motor impairments, and many of the results are variable between promoters used and between research groups.

A transgenic model that successfully recapitulates many of these key aspects of PD is the VMAT2LO mouse model that expresses ~5% of normal VMAT2 levels (Caudle et al. 2007; Miller et al. 2011; Alter et al. 2013; Lohr et al. 2016). VMAT2LO mice display age-associated nigrostriatal dopamine degeneration, increased accumulation of α-syn, progressive non-motor, and L-DOPA-responsive motor deficits, and extranigral monoaminergic dysfunction and degeneration (Caudle et al. 2007; Miller et al. 2011; Alter et al. 2013; Lohr et al. 2016).

Neurotoxicant models

In addition to genetic models, toxicant models that induce DAergic loss and degeneration were developed. Classic toxicant models include (6-OHDA), (MPTP), rotenone, and paraquat (Jagmag et al. 2016). 6-OHDA is a metabolite of DA that was used to model certain aspects of PD (Blesa et al. 2012). Due to the resemblance of DA, 6-OHDA is a substrate for several catecholaminergic transporters including the

norepinephrine transporter and DAT allowing its entry into DAergic and noradrenergic neurons (Martin et al. 1976; Blesa et al. 2012). Once in the neuron, cytosolic 6-OHDA accumulates and undergoes auto-oxidation inducing the formation of reactive oxygen species, catecholamine quinones, and hydrogen peroxide resulting in oxidative stress and degeneration of affected neurons (Simola et al. 2007; Jagmag et al. 2016). 6-OHDA must be stereotactically injected directly into the SNpc, medial forebrain bundle, or the striatum because it is hydrophilic and will not cross the blood-brain barrier (Simola et al. 2007; Jagmag et al. 2016) (Jagmag et al. 2016). Injection of 6-OHDA results in unilateral degeneration of DAergic and noradrenergic projections, depending on the injection site, resulting in motor impairments including dyskinetic limb movements, and asymmetrical rotation induced by DA-releasing agents like amphetamine. However, 6-OHDA models do not produce Lewy-like aggregates (Luthman et al. 1989; Simola et al. 2007; Blandini et al. 2008; Jagmag et al. 2016).

MPTP and its active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), were widely used as *in vivo* and *in vitro* models of PD, respectively. The MPTP model is often used to study the effects of DAergic depletion because of its robust ability to deplete striatal DA and lesion nigrostriatal neurons (Blesa et al. 2012; Blesa and Przedborski 2014; Jagmag et al. 2016). MPTP is not neurotoxic itself but can enter the brain where it is metabolized by MAO-B in astrocytes to MPP^+ (Jagmag et al. 2016). MPP+ is selectively toxic to DAergic neurons because of its high affinity for the DAT and significantly lower affinity to other monoaminergic transporters. In the cytosol, MPP^+ can be sequestered via VMAT2 into synaptic vesicles or it can concentrate in the mitochondria (Rarnsaysb and Singers 1986). MPP^+ is a mitochondrial complex 1 inhibitor and is thought to kill

DAergic cells by inhibiting ATP production and stimulating superoxide radical formation (Jagmag et al. 2016). While MPP^+ can affect all DAergic neurons throughout the brain, neurons in the SNpc are more susceptible than DA neurons in the ventral tegmental area, mimicking selective vulnerability seen in PD (Varastet et al. 1994; Blesa et al. 2011; Blesa et al. 2012). A wide range of animal models using MPTP were developed. In these models, MPTP is given by several different routes, including oral gavage, various types of injections, and direct stereotaxic injection into the brain. Compared to 6-OHDA, a benefit of the MPTP model is that can be administered systemically due to its lipophilicity. Dosing paradigms also vary by dose, duration, and frequency of administration. One of the most common, reliable, and reproducible lesions is caused by its systemic sub-cutaneous or intraperitoneal administration. In general, MPTP does not induce α-syn aggregation in rodents, and α-syn pathology has only been observed in some non-human primate MPTP models (Jackson-Lewis and Przedborski 2007; Blesa et al. 2012). Similarly, motor impairments are typically present in non-human primates MPTP models but less apparent in mice.

Due to the lack of astrocytes in many cell culture models, the active metabolite MPP^+ is used as an *in vitro* model of PD since MPTP *in vivo* is metabolized by MAO-B in astrocytes. In cells expressing DAT, MPP^+ induces robust degeneration by entering the cell via DAT and inhibiting complex 1 of the electron transport chain (Lopes et al. 2017). MPP^+ is an established model of DAergic degeneration in a variety of cell models including primary dopamine cultures from mice and rats, organotypic slice cultures, induced pluripotent stem cells (iPSCs), and cell lines including Lund University Human

Mesencephalic Cells (LUHMES), SH-SY5Y, MN9D, PC12, and N27 cells (Lopes et al. 2017).

N′-dimethyl-4,4′-bipyridinium dichloride (paraquat) is one of the most widely used herbicides worldwide and was developed into a PD model (Tanner et al. 2011; Jagmag et al. 2016). Due to paraquat's apparent structural similarity to MPTP and DA, it was thought that paraquat similarly induces degeneration via DAT-mediated uptake, but this mechanism was called into question (Richardson et al. 2005; Miller 2007). However, paraquat does cross the blood-brain barrier, enter DAergic neurons, induce reactive oxygen species, oxidative stress, and eventually neurodegeneration via a non-DAT-mediated mechanism (Richardson et al. 2005; Miller 2007). In mice, direct injection into the brain produces more consistent toxicity than systemic administration, which does not yield consistent results. While injection of a strong oxidant directly into nigral neurons produced degeneration of these cells, it remains controversial whether or not this model is toxicologically relevant to human PD (Miller 2007).

Rotenone is a naturally occurring compound, occurring in several species of plants, that is used as an insecticide (Betarbet et al. 2000; Tanner et al. 2011; Jagmag et al. 2016). Like MPP^+, rotenone is a mitochondrial complex 1 inhibitor that can easily enter the brain. Chronic systemic administration in rodents results in a slow and selective degeneration of nigrostriatal DAergic neurons, Lewy-like aggregates, and PD-like motor impairments. Because rotenone is lipophilic and can cross the blood-brain barrier, it can be administered systemically without a need for stereotaxic injection. However, rotenone exposure is associated with high mortality rates, making it difficult to replicate as a PD model (Tanner et al. 2011; Jagmag et al. 2016).

Most neurotoxicant models produce rapid degeneration failing to mimic the protracted course of the disease. There is also a lack of consistency and often a lack of formation of LB-like inclusions. In models where LB-like inclusions are formed, this is often due to supraphysiological levels of oxidative stress. Therefore, these neurotoxicant models can be used for studying DAergic degeneration and the effects of DA loss but are not ideal for investigating environmental exposures in PD as well as the pathogenesis of sporadic PD (Blesa et al. 2012).

Alpha-synuclein pre-formed fibril model of PD

Many PD therapeutics fail clinical trials partly because the PD models used during *in vivo* testing fail to model the key pathological hallmarks of the disease (Jagmag et al. 2016). Therefore, new work was aimed at improving PD models by reproducing both nigrostriatal degeneration and Lewy bodies. More recently, the α-syn pre-formed fibrils (PFFs) model of PD was used to mimic both synucleinopathy and DAergic degeneration (Luk et al. 2012; Paumier et al. 2015; Patterson et al. 2019). Unlike other models, such as 6-OHDA and MPTP, the PFF model recapitulates the slow progression of PD, the pathological hallmarks, and PD-like motor deficits (Luk et al. 2012; Paumier et al. 2015; Patterson et al. 2019).

In the PFF model, recombinant α-syn monomers are used to form α-syn PFFs through a 7-day fibrilization process. Before use, α-PFFs are sonicated to form ~50 nm fragments, which is the optimal length for seeding α-syn inclusions. When PFFs are injected into rodent brains or applied to cell cultures, they are taken up into synaptic terminals. Within the synaptic terminal, they seed the formation of soluble, endogenous α-syn into insoluble phosphorylated inclusions that resemble Lewy-bodies in that they are

proteinase-K resistant, contain amyloid structure, are ubiquitinated, detergent-insoluble, and contain α-syn phosphorylated at serine 129 (pSyn) (Volpicelli-Daley et al. 2011; Luk et al. 2012). Eventually, this leads to neuronal dysfunction and degeneration within the context of normal physiological levels of endogenous α-syn. SNCA knock-out animals are also not susceptible to PFF-induced pathology, indicating that PFFs mechanisms of action are through the recruitment of endogenous α-syn (Luk et al. 2012).

The mechanisms by which α-PFFs enter cells are not fully understood, but some evidence suggests they are internalized via endocytosis, micropinocytosis, and/or tunneling nanotubules (Valdinocci et al. 2017). Internalized α-syn PFFs were found to colocalize with markers of early endosomes like Rab5 suggesting PFF uptake via endocytosis potentially mediated by clathrin/dynamin-1 (Eisbach and Outeiro 2013; Bieri et al. 2018). Early studies suggested that lymphocyte-activation gene 3, which is a member of the immunoglobulin superfamily of receptors with known functions as an inhibitory receptor on T-cells may mediate PFF uptake via receptor-mediated endocytosis, but more recent studies found that lymphocyte-activation gene 3 is not expressed in human or murine neurons (Emmenegger et al. 2021).

Another potential mechanism for PFF uptake is through micropinocytosis, an actin-dependent mechanism that facilitates membrane ruffling mediated by the cell surface marker, heparan sulfate proteoglycan (HSPG) (Valdinocci et al. 2017; Bieri et al. 2018; Rodriguez et al. 2018). HSPGs are a cell surface and extracellular matrix protein thought to bind PFFs and promote uptake via late endosomes or lysosomes (Valdinocci et al. 2017; Bieri et al. 2018; Rodriguez et al. 2018). Transmembrane 9 superfamily member 2 is another endosomal marker that is one of the highest expressed genes in

the brain, and its highest activity is in the substantia nigra and may be involved in α-syn PFF uptake (Schimmöller et al. 1998; Wadman 2016; Valdinocci et al. 2017). FACS-based genome-wide CRISPR/Cas9 knockout screening identified Transmembrane 9 superfamily member 2 because of its role in regulating the expression of HSPG biosynthesis (Vanderperre et al. 2023). Other studies have investigated the role of neurexins, a presynaptic cell adhesion protein, and sodium and potassium ATPase as another mechanism involved in PFF uptake (Bieri et al. 2018; Rodriguez et al. 2018). This model was initially developed in mice and was later replicated in rats (Luk et al. 2012; Patterson et al. 2019). In mice, intrastriatal injection of PFFs results in pale cytoplasmic diffuse and neuritic inclusions 1-month in the substantia nigra, cortex, and olfactory bulbs, regions connecting to the striatum (Luk et al. 2012). Nigral inclusions peak at 1-month and then decline as nigrostriatal neurons begin to degenerate (Luk et

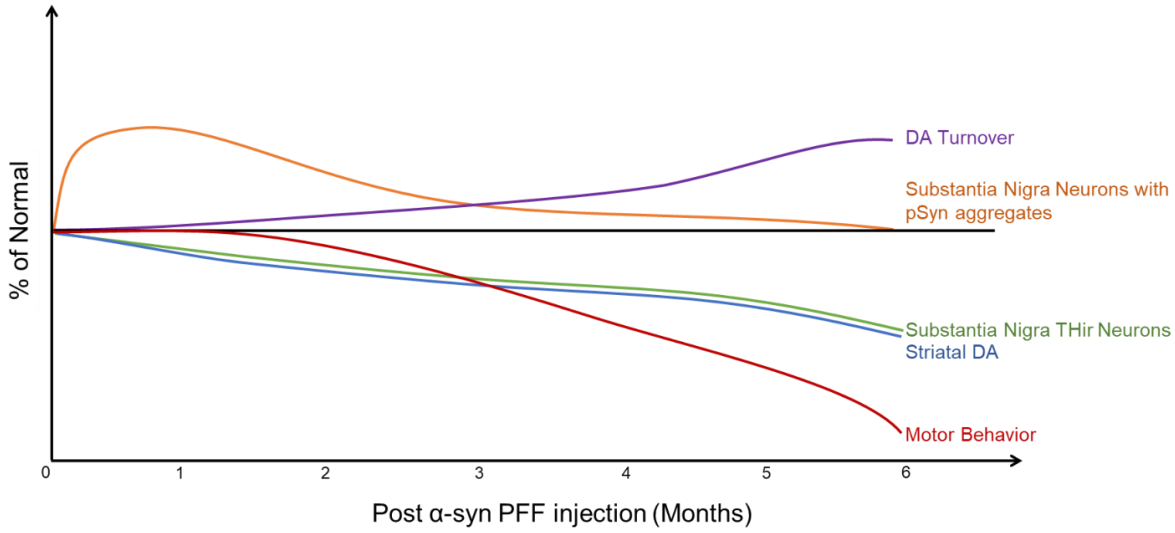

Figure 1.8. Schematic showing the timecourse of the α-synuclein PFF model in mice. The x axis shows the months post-PFF injection. Y axis shows the percent of control. The purple line represents the relative changes in DA turnover. The orange line shows SN neurons containing pSyn aggregates. The green shows relative changes in SN TH immunoreactive neurons. The blue represents changes in striatal DA levels. The red shows changes in motor behavior. Made in BioRedner.

al. 2012). At 2 months post-injection, there is a significant decrease in striatal levels of DA and the DA metabolites DOPAC and homovanillic acid (HVA) (Gezer et al. 2020). Also, at 2 months post-injection there is an increase in DA turnover indicated by the ratio of HVA: DA and a female-specific increase in the ratio of DOPAC: DA (Gezer et al. 2020)(Figure1.8).

By 3 months post-injection, the inclusions become dense and perinuclear and are found in the thalamus, amygdala, and contralateral cortex. At 3 months post-injection, there are significant reductions in striatal DA concentration and a significantly reduced latency to fall on the wire hang test (Luk et al. 2012).

At 6 months post-injection, striatal TH and DAT expression is significantly decreased (Luk et al. 2012). There is also a more significant decrease in TH immunoreactive neuron loss in male mice compared to female mice at 6 months post-injection (35% compared to 20% loss respectively) (Gezer et al. 2020). Striatal DA, DOPAC, and HVA levels are significantly reduced in both male and female mice. Additionally, there is a significant increase in DA turnover indicated by increased ratios of DOPAC: DA and HVA: DA in both sexes at 6 months post-injection (Gezer et al. 2020). Motor behavioral impairments are also observed at 6 months post-PFF injection. There is a significant decrease in the rotarod and wire hang test (Luk et al. 2012). 6-months post-PFF injections, the number of steps was significantly reduced and the number of errors per step was significantly increased in male mice. However, there were no significant differences in female mice injected with PFFs on the challenging beam (Gezer et al. 2020). This combination of effects shows a DA deficit and a Parkinsonian-like phenotype, showing a sex-specific difference in the PFF model (Figure 1.8).

PFF-induced pathology from intrastriatal injections seems to be selective to the nigrostriatal pathway and brain regions with structural connectivity to the injection site. However, other DAergic populations, such as the ventral tegmental area are not affected by intrastriatal PFF injections because of the location of PFF injection and because nigrostriatal neurons are specifically vulnerable to toxicity (Luk et al. 2012). This model has also been replicated in rats. The overall sequence of events is similar, but the aggregation and degeneration may be more pronounced in rats. In addition, rats show some effects contralateral to the injection site with degeneration of nigral DAergic neurons and striatal TH terminals in the striatum at 6 months post-injection (Patterson et al. 2019). Contralateral degeneration has not been observed in mice, but it may appear at unmeasured later time points. Neuroinflammation has also been studied in the rat PFF model. Reactive microglia and major histocompatibility complex-II expression levels form near psyn-positive inclusions in the nigra and follow a similar temporal pattern as the appearance and disappearance of inclusions (Duffy et al. 2018).

The α-syn PFF model has also been successfully recapitulated in iPSCs and primary rodent neuronal cultures (Luk et al. 2009; Volpicelli-Daley et al. 2011; Volpicelli-Daley et al. 2014; Gao et al. 2019a; Ross et al. 2020a). Many aspects of the in vivo model can be recapitulated in these cells, including the formation of phosphorylated inclusions and compact puncta, mitochondrial dysfunction, oxidative stress, deficits in neuronal excitability, increased autophagy, loss of synaptic markers, and eventual degeneration of neurons (Luk et al. 2009; Volpicelli-Daley et al. 2011; Volpicelli-Daley et al. 2014; Ross et al. 2020a). Specifically, initial work was done in primary hippocampal neurons because of their high levels of α-syn. In this system, phosphorylated inclusions are

present 4 days after PFF application and compact puncta form by 7 days. At 14 days post-treatment, 20-30% of treated primary hippocampal neurons degeneration, showing mitochondrial oxidant stress, deficits in neuronal excitability, increased autophagy, and loss of synaptic markers (Volpicelli-Daley et al. 2011; Volpicelli-Daley et al. 2014). Additional work has applied the PFF model to SH-SY5Y cells to study aggregate clearance pathways and PFF-induced mitochondrial dysfunction (Perfeito et al. 2014; Choi et al. 2018; Gao et al. 2019b; Ross et al. 2020b; Pantazopoulou et al. 2021; Feng et al. 2022; Lin et al. 2022).

Multiple-hit Hypotheses in PD

The majority of PD cases are sporadic and are thought to be caused by a combination of common genetic risk variants and environmental exposures which suggests that a combination of insults is required for disease development (Sulzer 2007). Therefore, multiple hit models can be used to study the effects of factors that may not cause PD on their own, but instead prime the system for increased susceptibility to another insult or hit. This multiple-hit model is often used to study the effects of environmental factors on disease risk (Sulzer 2007). Previous work has established a two-hit dieldrin/MPTP model, and this has recently been extended to include a dieldrin/PFF model to study the effects of developmental dieldrin exposure on PD risk (Richardson et al. 2006a; Gezer et al. 2020).

Dieldrin

Dieldrin Overview

Dieldrin is an organochlorine pesticide that was associated with increased sporadic PD risk in epidemiological, post-mortem, and mechanistic studies (Tanner and Langston

1990; Semchuk et al. 1992; Fleming et al. 1994; Le Couteur et al. 1999; Priyadarshi et al. 2000; Ritz and Yu 2000; Tanner and Aston 2000; Priyadarshi et al. 2001; Kanthasamy et al. 2005; Ascherio et al. 2006; Brown et al. 2006; Richardson et al. 2006a; Steenland et al. 2006; Hatcher et al. 2007; Elbaz et al. 2009; Weisskopf et al. 2010; Moretto and Colosio 2011; Tanner et al. 2011; Wirdefeldt et al. 2011; Caudle et al. 2012; Freire and Koifman 2012; Gezer et al. 2020). Since dieldrin was phased out in the 1970s and 1980s, the potential for new, acute exposure to dieldrin is low. However, the health effects of past exposures will continue for decades as the population currently diagnosed with PD and those that will develop PD in the next 20-30 years were likely exposed to dieldrin before its phase-out (de Jong et al. 1997; Jorgenson 2001; Meijer et al. 2001; Kanthasamy et al. 2005).

Dieldrin in vitro studies

In vitro approaches were used to test the direct effects of dieldrin on DAergic toxicity using a variety of cell lines. Collectively, these studies show that at high doses, there are dieldrin-induced effects on DAergic neurons resulting in increased toxicity and degeneration, and some effects were observed on α-syn (Figure 1.9).

Relevant to synucleinopathy, rat mesencephalic N27 cells overexpressing α-syn resulted in proteasomal dysfunction and eventually apoptosis, and this effect was exacerbated by dieldrin exposure (Sun et al. 2005). Dieldrin exposure (30 μM) affects the ubiquitin-proteasome system by decreasing proteasomal activity in a dose-dependent manner and this effect is more pronounced in cells overexpressing α-syn. This suggests that dieldrin may exacerbate α-syn related proteasomal and apoptotic responses playing a role in PD pathology (Sun et al. 2005).

At lower concentrations of dieldrin (2.5 μM and 25 μM), there is a significant reduction in energy metabolism via Seahorse XFe24 analysis. It was further found that dieldrin-induced mitochondria impairments were likely related to endoplasmic reticulum (ER) stress as the transcripts for ER-related apoptotic genes were significantly increased (Schmidt et al. 2017). Similarly, dieldrin treatment has promoted caspase-3-dependent activity resulting in DNA fragmentation and apoptosis in PC12 cells, a rat pheochromocytoma cell line (Kitazawa et al. 2003). Dieldrin treatment also generates reactive oxygen species and increases lipid peroxidation in PC12 cells (Kitazawa et al. 2001).

Dieldrin has higher selectivity for DAergic PC12 cells compared to pancreatic α-endocrine and human cortical GABAergic neuron cells when comparing cell viability in cells treated with dieldrin. Dieldrin exposure displaces DA and results in a concentration-dependent increase in DA and the DOPAC release in DAergic, PC12 cells (Kitazawa et al. 2001). In a model of primary mesencephalic neuronal cultures from rats

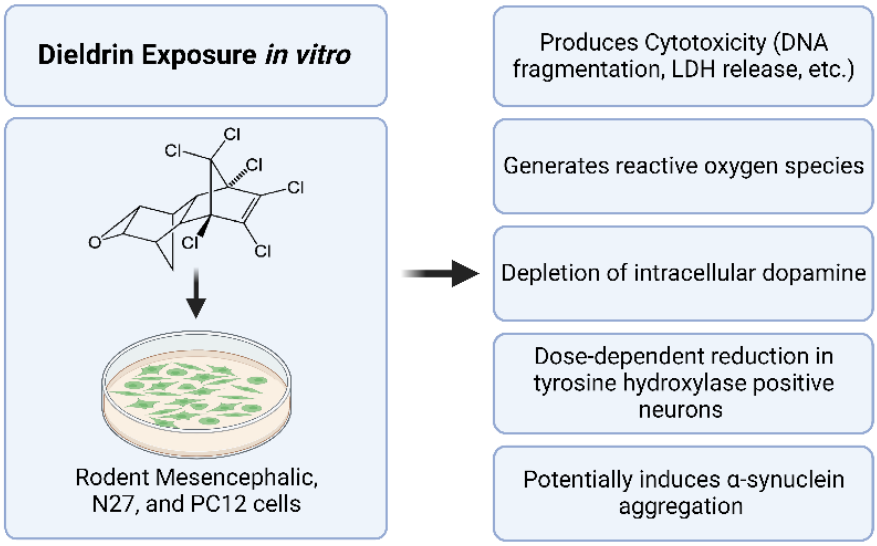

Figure 1.9. Summary of dieldrin *in vitro* studies. Made in BioRedner.

and mice, dieldrin exposure (0.01 -100 μM) resulted in a concentration-dependent decrease in DAergic and GABAergic neurons. However, GABAergic neurons were less sensitive to dieldrin than DAergic neurons. Supporting this, plasma membrane uptake of tritium-labeled DA was significantly reduced by dieldrin compared to tritium-labeled GABA uptake (Sanchez-Ramos et al. 1998). Overall, this data indicates that dieldrin is selectively toxic to DAergic neurons, although the concentrations of dieldrin used in these *in vitro* studies may not be relevant to concentrations of dieldrin in human exposure.

Dieldrin in vivo studies

Chronic dieldrin exposure results in reduced levels of DA, norepinephrine, and serotonin through multiple brain structures and models. Specifically, in an oral dieldrin exposure paradigm in rats (50 ppm for 8 weeks), NE concentrations were reduced in several brain areas including the hippocampus, striatum, oblongata, and pons between 2- and 4 weeks post-treatment. At 8 weeks, NE levels returned to normal or above normal throughout the brain regions (Wagner and Greene 1978). In-ring doves exposed to dieldrin (200ppm) reduced levels of DA levels as well as NE levels in ring dove brains (Heinz et al. 1980). Similarly, chronic dietary exposure (30ppm of dieldrin) to mallard ducks resulted in 5-HT, NE, and DA concentrations in brains (Sharma et al. 1977). Further evidence confirms that dieldrin exposure results in alterations in the nigrostriatal pathway and DA handling (Hatcher et al. 2007). In a mouse model of chronic dieldrin (up to 3mg/kg) administration, there is an observed reduction in striatal DA metabolites including DOPAC and HVA concentrations. Additionally, there were significant decreases in striatal DAT expression and uptake. In addition to DA mishandling, dieldrin

also affected oxidative stress by inducing increases in striatal levels of cysteinyl-catechol, protein carbonyls, α-syn, and redox potential of glutathione (Hatcher et al. 2007). Overall, this shows that dieldrin exposure can induce changes in the DAergic systems, and as mentioned above DA handling is finely tuned and any perturbation to the system can be detrimental to the neuron.

Dieldrin mechanisms of action of adult dieldrin exposure

Dieldrin is thought to exert its toxic effects by targeting and inhibiting $GABA_A$ receptors causing an inhibition of Cl^- influx (Pomps et al. 1993; Rodriguez-Farre and Suñol 1995; Ikeda et al. 1998; le Corronc et al. 2002; Le Corronc et al. 2002; Vale et al. 2003; Zhao et al. 2003; Heusinkveld and Westerink 2012). Prenatal exposure to dieldrin reduces expression of $GABA_A$ receptor subunit mRNA expression as well as reduced binding capacity of $GABA_A$ receptors in the rat brainstem (Narahashi et al. 1995; Liu et al. 1997; Brannen et al. 1998; Liu et al. 1998). Dieldrin exposure can also alter Ca^{2+} signaling and activity. Specifically, dieldrin exposure can modulate calmodulin-dependent and independent Ca^{2+} ATPase activity which can impair Ca^{2+} metabolism and signaling in rat synaptosomes and heart sarcoplasmic reticulum (Mehrotra B.D. et al. 1988; Mehrotra B.D. et al. 1989).

Dieldrin also induces oxidative stress, and this is particularly relevant in the nigrostriatal system. Supporting this, dieldrin exposure was shown to increase cysteinyl-DOPAC and cysteinyl-DA, increase protein carbonyls, and reduce the redox potential for glutathione, specifically in the striatum (Hatcher et al. 2007). Synthesis and degradation of DA can result in hydrogen peroxide, reactive oxygen species, autoxidation, or the formation of toxic DA intermediates like DA-quinones. DA-quinones can react with sulfhydryl groups

resulting in exacerbated oxidative damage resulting in the formation of cysteinyl-adducts which were shown to be increased by dieldrin exposure (Hatcher et al. 2007). Dieldrin exposure in PC12 cells has also been shown to produce a dose-dependent decrease in mitochondrial membrane potential, an increase in lactic acid dehydrogenase activity, and an increase in ROS generation indicating worsened oxidative stress (Kitazawa et al. 2001). DAergic neurons are specifically sensitive to oxidative stress and have a particularly low antioxidant capacity, dieldrin exposure exacerbates this oxidative stress which can impair the function of DA neurons and result in degradation (Hatcher et al. 2007).

Developmental dieldrin exposure model

In this dissertation, we utilize a previously established developmental dieldrin exposure model that was designed to mimic human exposures at a critical developmental period (Richardson 2006, Kochmanski 2019, Gezer 2020). In this model, male mice developmentally exposed to dieldrin show an increased susceptibility later in life to the DAergic toxicant, MPTP, and synucleinopathy induced by α-syn PFFs. In this model, female mice are fed dieldrin before mating and throughout mating, gestation, and

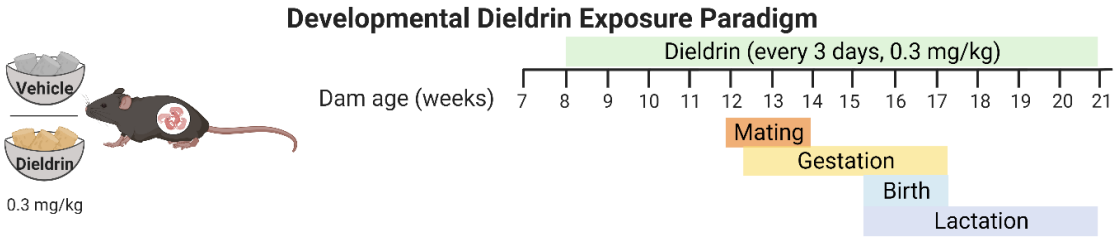

Figure 1.10. Developmental dieldrin exposure paradigm. Female mice are fed dieldrin (0.3mg/kg) or vehicle prior to mating and throughout lactation. Offspring are developmentally exposed through the placenta and breast milk. Made in BioRedner.

lactation at doses of 0.3 mg/kg of dieldrin (Richardson et al. 2006) (Figure 1.10). This exposure paradigm is based on the developmental origins of the health and disease hypothesis described above, which states that environmental exposures during critical periods of development affect disease risk and trajectory later in life (Heindel and Vandenberg 2015; Baird et al. 2017). PD is a disease of the aged, but given the long prodromal phase, the neurodegenerative process likely begins decades before clinical diagnosis, therefore, exposures early in life may contribute to sporadic PD. It is unknown if the mechanisms of action for developmental dieldrin exposure are the same as adult exposure to dieldrin. However, these early developmental changes may produce a poised state of silent neurotoxicity, where the effects of early life exposures are unmasked by challenges later in life, the cumulative effects of exposures over the lifespan, or the effects of aging (Cory-Slechta et al. 2005; Kraft et al. 2016). Likewise, dieldrin exposure does not cause overt toxicity by traditional measures on its own but increases the vulnerability to future insults.

In previous studies using this model, male offspring at 12 weeks of age showed increased vulnerability in both MPTP and the α-syn PFF model (Richardson 2006; Gezer 2020). At 12 weeks after birth, the developmentally exposed offspring males showed a significant increase in DAT and VMAT2 protein in dieldrin-exposed (0.3 mg/kg) animals before MPTP administration. This increase in DAT and VMAT2 was exacerbated in male offspring. There was also an increase in nuclear receptor related-1 (Nurr1) and paired-like homeodomain transcript factor 3 mRNA which are two nuclear transcription factors that regulate DAT and VMAT2 expression, but only at higher concentrations of dieldrin (1 and 3 mg/kg) and more so in female mice. Striatal DA

turnover was also significantly increased by developmental dieldrin exposure. Specifically, the DOPAC/DA ratio was increased at all doses ranging from 0.3 to 3 mg/kg only in male mice. Overall, it shows that developmental dieldrin exposure increased DA dysfunction and increased DAT, VMAT2, and Nurr1 levels in male offspring at 12 weeks of age. Although there was an increase in DAT, VMAT2, and NURR1 levels at a lesser extent in female mice developmentally exposed to dieldrin, DA turnover was not affected at any dose in female mice (Richardson et al. 2006). This indicates that there is a male-specific exacerbation to developmental dieldrin exposure resulting in increased DA mishandling and turnover.

To determine the risk of developmental dieldrin exposure to PD, Richardson et al. developed a two-hit developmental dieldrin/MPTP model. In this model, female mice are exposed to dieldrin, and the developmentally exposed offspring are administered MPTP (2 injections of 10mg/kg MPTP subcutaneously). Dieldrin exposure exacerbated the MPTP-induced loss of striatal DA in a dose-dependent manner in mice, and this effect was greater in male mice (Richardson et al. 2006). The ratio of DAT: VMAT2 was significantly increased at 0.3 mg/kg only in male mice, indicating increased DAergic vulnerability to toxicity (Richardson et al. 2006).

To recapitulate these findings in a model of synucleinopathy, Gezer et al. used a developmental dieldrin/α-syn PFF model where PFFs were intrastriatally injected in developmentally exposed mice at 3 months of age. There was no significant dieldrin-induced effect on PFF-induced nigral phosphorylated-synuclein at 1 and 2 months post-PFF injection (Gezer et al. 2020). At 6 months post-PFF injection, when PFF induced significant DAergic degeneration, there was no effect of dieldrin on nigral neuronal

counts or TH-immunoreactive neurons. At 2 and 6 months post-PFF injection, striatal DA and DA metabolite levels were measured using HPLC to assess DA turnover. At 6 months post-PFF injection, but not 2 months, there was a dieldrin-induced increase in the ratio of HVA: DA in male mice indicative of increased DA turnover. The challenging beam assessment was used as a more sensitive measure of motor coordination. In this test, mice are placed on a beam covered in grates and must walk across the beam toward their home cage. Closer to the home cage, the beam becomes narrower and the time to traverse, the number of steps, and the number of errors are recorded (Figure 1.11A). A combination of all three factors indicates the severity of DAergic deficit. Dieldrin on its own does not lead to deficits on the challenging beam similar to vehicle/saline injected animals. The vehicle-exposed animals injected with PFFs showed fewer steps and more errors per step indicating a worsened DA deficit compared to control. Male mice developmentally exposed to dieldrin and injected with PFFs showed a significant increase in the time to traverse the beam, more steps, but fewer errors/step since they had more time to compensate for errors. This combination of effects shows that developmental dieldrin exposure exacerbates PFF-induced motor impairments and deficits in DA handling in male mice (Gezer et al. 2020)(Figure 1.11B). This shows that developmental dieldrin exposure may prime the nigrostriatal system for PD-related toxicity induced by either MPTP or PFFs and that male mice may have heightened susceptibility. In conclusion, this data aligned with previous MPTP data showing a dieldrin-induced male-specific exacerbation to Parkinsonian toxicity resulting in a worsened motor deficit and increases in DA mishandling.

The levels of glial fibrillary protein (GFAP), a mark of astrological proliferation, and α-syn levels were also exacerbated by MPTP in dieldrin-exposed animals in a dose-dependent manner (Richardson et al. 2006). In line with this, developmental dieldrin exposure induced effects on neuroinflammatory genes and pathways in a sex-specific manner (Gezer et al. 2020). In conclusion, these data aligned with previous MPTP data showing a dieldrin-induced male-specific exacerbation to parkinsonian toxicity resulting

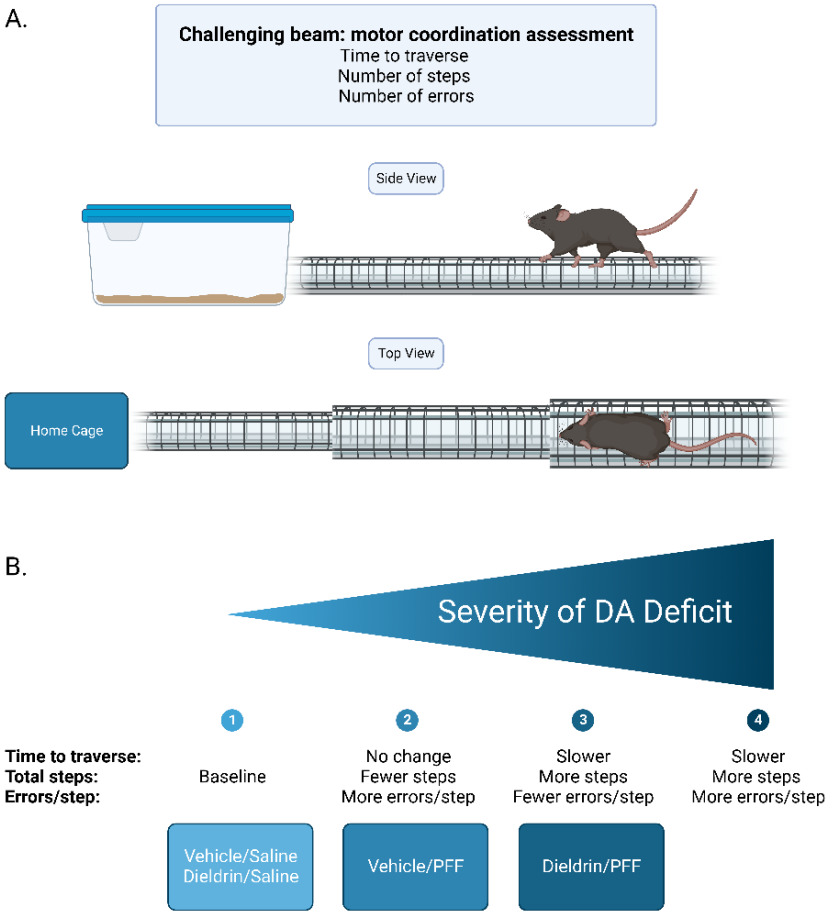

Figure 1.11. Motor behavioral deficits identified in the dieldrin/PFF two-hit model. A) Schematic describing the challenging beam motor coordination assessment. B) Overview of the identified motor deficits (time to traverse, total steps, and errors/step) and severity of DA deficits dieldrin/PFF animals and controls. Made in BioRedner.

in a worsened motor deficit and increases in DA mishandling. Mechanisms underlying this dieldrin-associated increase in susceptibility remain incompletely defined and are the main focus of this dissertation.

The epigenome is a potential mediator of the relationship between developmental dieldrin exposure, increased neuronal vulnerability, and adult disease. We previously characterized changes in DNA modifications in developmentally exposed offspring (Kochmanski 2019). We found that dieldrin exposure established a sex-specific poised epigenetic state early in life and hypothesize that this may mediate susceptibility to PD-related neurotoxicity in adulthood (Kochmanski et al., 2019). Specifically, using the same developmental dieldrin exposure paradigm, we performed reduced representation bisulfite sequencing on DNA isolated from the midbrain and identified 115 differentially methylated CpGs in males and 478 in female 3-month-old, developmentally exposed C57BL/6 mice. There was also many sex-specific differential expression in protein-coding transcripts associated with DAergic development (Kochmanski et al., 2019). This suggests that developmental dieldrin-induced differential modification of genes associated with DAergic development may contribute to and underly the dieldrin-induced exacerbations of MPTP and PFFs. Genes identified in this study are the source of candidate genes explored in Chapter 4 of this dissertation.

New approach methodologies

While animal models traditionally provide the most physiologically relevant system to model disease, in vivo experiments have several limitations. They can be time-consuming, expensive, and resource-intensive, require large numbers of animals, have high variability, and lack translation from animal models to humans (Bal-Price 2018).

New approach methodologies (NAMs) are methods designed to replace animal testing in assessing chemical or drug toxicity and offer advantages for neurotoxicity testing, disease modeling, and drug screening (Hogberg et al. 2013; Anderson et al. 2021). Multiple NAMs were developed to screen chemicals for developmental neurotoxicity (DNT) and additional methods in this area are needed as an efficient and translatable approach to assess DNT (Bal-Price 2018).

The ability to use cells of human origin is a major advantage of NAMs and may be more translatable in modeling human disease and toxicity than in vivo rodent models. More than 90% of compounds in clinical trials for drug development fail because of effects that were not observed in vivo testing, partly due to species differences (Hogberg et al. 2013). Human-derived cells can be used for in vitro NAMs at increasing levels of complexity, ranging from adherent cells to organoids.

As a result, recent work was aimed at developing and advancing *in vitro* new approach methodologies (NAMs) to screen chemicals for their toxicological effects, especially for DNT testing (Carstens et al. 2022). Developing NAMs for DNTs requires a battery of assays to investigate several neurodevelopmental processes. For example, a battery of assays that measure neurite outgrowth, ATP assays, proliferation, apoptosis, neural network formation, and synaptogenesis was developed by the U.S. EPA to evaluate chemicals for potential DNT (Carstens et al. 2022). Human-derived cells can be used for *in vitro* NAMs at varying levels of complexity, ranging from adherent cells to organoids. While human stem cells derived from embryonic, umbilical, bone marrow, or central nervous tissues can be differentiated into a diverse set of neuronal types, there are ethical issues and issues of availability that limit their utility (Hogberg et al. 2013). As an

alternative, iPSCs from fibroblasts or adult somatic cells can be reprogrammed into an embryonic stem cell-like state via the induction of pluripotent genes (Ye et al 2013). iPSCs can be further differentiated into to form different types of neuronal populations including glial populations (Hogberg et al. 2013) However, generating iPSCs is difficult and only a small fraction of cells is reprogrammed after pluripotent induction. In addition, differentiating mature glial cells and neurons has proven to have low reproducibility and can be both time-consuming and expensive (Hogberg et al. 2013). Therefore, multiple groups have developed NAMs using differentiated immortalized cell lines that have technical advantages. In addition, differentiating these cells into 3D neurospheres can improve survival, model cell-to-cell interactions and synapse formation, and recapitulate neuronal activity such as network activity and activity seen in *in vivo* models (Hogberg et al. 2013; Anderson et al. 2021).

Recent advances show that three-dimensional (3D) neuronal models are better at recapitulating the complexity of the central nervous system than 2D adherent cell models. One advantage is that the synaptic interactions and cell morphology are more physiologically relevant in 3D cultures compared to traditional monolayer cultures (Hogberg and Smirnova 2022). 3D models include spheroids, organoids, and organ-on-a-chip models. Spheroids are the simplest of these models and are clusters of one specific cell type that be used to investigate the role of a specific population of cells (Hogberg and Smirnova 2022). For example, spheroids generated from LUHMES cells can be differentiated into DAergic-like neurons to study PD (L. Smirnova et al. 2016; Harris et al. 2017a; Harris et al. 2018; Leite et al. 2019; Ko et al. 2020). Organoids contain a complex mixture of cells with a long differentiation process that more closely

recapitulates aspects of human neuronal development and maturation and better represents the architecture and connection in human brains (Hogberg and Smirnova 2022). Recent advances have incorporated vascularization, immune cells including microglia, cerebral spinal fluid secretion, and the blood-brain barrier into organoid models (Hogberg and Smirnova 2022). However, organoids are generally derived from induced pluripotent stem cells, which are highly variable in their reprogramming and differentiation resulting in low reproducibility and are expensive and labor-intensive (Hogberg et al. 2013).

The most complex 3D models include microfluidic devices and brain-on-chip models. Microfluidic models use microchannels to provide gradients of different growth factors, chemokines, or other substances to 3D neurospheres or organoids to model controlled microenvironments (Hogberg et al. 2013; Alépée 2014; Anderson et al. 2021; Hogberg and Smirnova 2022). Brain-on-a-chip models are similar to organoids but may include compartmentalized neuronal chambers, microfluidic devices, and organized cellular layers to model different neuronal interactions (Amirifar et al. 2022). The blood-brain barrier has commonly been modeled using brain-on-a-chip technology to capture the neurovascular, neurons, glia, and synaptic connections (Amirifar et al. 2022; Hogberg and Smirnova 2022). Brain-on-a-chip models are superior at modeling *in vivo* complexity and cellular interactions to screen toxicants or pharmaceutical compounds (Amirifar et al. 2022; Hogberg and Smirnova 2022) However, there are some limitations with complex models, including low reproducibility, difficulty with scaling the appropriate proportions of cell populations, complications with co-culture of different cell types, and the associated high cost and labor (Amirifar et al. 2022). In Chapters 3 and 4 of this

dissertation, we utilized 3D neurospheres derived from Lund Human Mesencephalic (LUHMES) cells and SH-SY5Y cells because of the well-documented protocols for differentiation into DAergic-like cells.

LUHMES cells are a human mesencephalon-derived cell line that can be differentiated into morphologically and biochemically mature DA-like neurons and are increasingly used for in vitro research (Lotharius et al. 2002; Lotharius et al. 2005; Scholz et al. 2011a; Pöltl et al. 2012; Schildknecht et al. 2013; Krug et al. 2014; X.M. Zhang et al. 2014a; Efremova et al. 2015; Hirsch et al. 2015; Oliveira et al. 2015; Tong et al. 2017) These cells are derived from 8-week-old female human embryonic mesencephalic tissue (Smirnova et al., 2016) 3D LUHMES spheres were developed as a high-throughput toxicity screening platform to take advantage of the fact that 3D cell models show better differentiation and survival (L Smirnova et al. 2016; Harris et al. 2017b; Tong et al. 2017) After differentiation into 3D neurospheres, these cells express TH, DAT, VMAT2 and α-syn (Scholz et al. 2011b; L. Smirnova et al. 2016; Harris et al. 2017a; Lauter et al. 2020; Tüshaus et al. 2020). This cell model is well established and demonstrates robust performance in high-throughput neurotoxicity studies, including studies of rotenone, another neurotoxicant relevant to PD (Scholz et al. 2011c; Scholz et al. 2011b; X. Zhang et al. 2014; X.M. Zhang et al. 2014b; L. Smirnova et al. 2016; Harris et al. 2017a; Harris et al. 2018; Leite et al. 2019; Ko et al. 2020; Tong et al. 2020; Tüshaus et al. 2020; Yamaguchi et al. 2020; Leah et al. 2021; Nicolai et al. 2022; Tong et al. 2022; Ali et al. 2023; Capinha et al. 2023).

SH-SY5Y is a sub-cell line of SK-N-SH cells, which are derived from a neuroblastoma from a 4-year-old female (Xicoy et al., 2017). Early characterization of SH-SY5Y cells

showed that the cells may have a catecholaminergic phenotype displaying enzymatic activity for both DA and norepinephrine such as TH, dopamine-β-hydroxylase, acetylcholinesterase, and choline acetyltransferase (Xicoy et al. 2017) Shipley et al. has developed methods to induce differentiation in SH-SY5Y cells with retinoic acid and specific growth factors to form a DAergic-like phenotype (Shipley et al. 2016).

Goals of the current research

The overall working hypothesis of this project is that developmental exposure to the organochlorine pesticide dieldrin alters PD risk by establishing a poised epigenetic state early in life that mediates susceptibility to Parkinsonian neurotoxicity in adulthood (Kochmanski et al. 2019) (Figure 1.12). These dieldrin-induced changes exacerbate PD-associated dysfunction in the nigrostriatal pathway and behavioral dysfunction (Richardson et al. 2006; Gezer et al. 2020). The overall goal of this project is to link the observed epigenetic modifications to functional differences in neuronal phenotype and neuronal susceptibility to synucleinopathy.

The goal of this project is three-fold.

1) Analyze VMAT2-mediated DA uptake and neurotransmission in striatal terminals in the two-hit dieldrin/α-syn PFF model *in vivo*. (Chapter 2)
2) Adapt the α-syn PFF model for use in 3D LUHMES and SH-SY5Y neurospheres. (Chapter 3)
3) Test if differentially modified candidate genes mediate α-syn PFF- or MPP^+-induced toxicity in 3D LUHMES neurospheres (Chapter 4)

This project has characterized the separate and combined effects of dieldrin and synucleinopathy on functional measures of DA uptake, release, and vesicular packaging

in the striatum of mice and provides additional insight into the synaptic mechanisms underlying dieldrin-induced exacerbation of synucleinopathy-induced deficits. Through this project, we have also assessed the role of developmental dieldrin-induced differentially modified genes, Nuclear receptor subfamily 4 group A member 2 (*NR4A2)* and *Ephrin Type-B receptor 2 (EPHB2)* in DAergic differentiation and MPP^+-induced toxicity in the 3D LUHMES cell model. This has addressed a gap in knowledge between epigenetic regulation and functional outcomes that alter neuronal vulnerability and disease susceptibility. Results from this project have generated important data regarding the effect of regulation of expression of these genes and provide new data to build on in future studies that explore the roles of those specific epigenetic changes. Combined with previous epigenetic data, these results can guide future studies that use epigenome editing to target genes that play a role in PD-related toxicity.

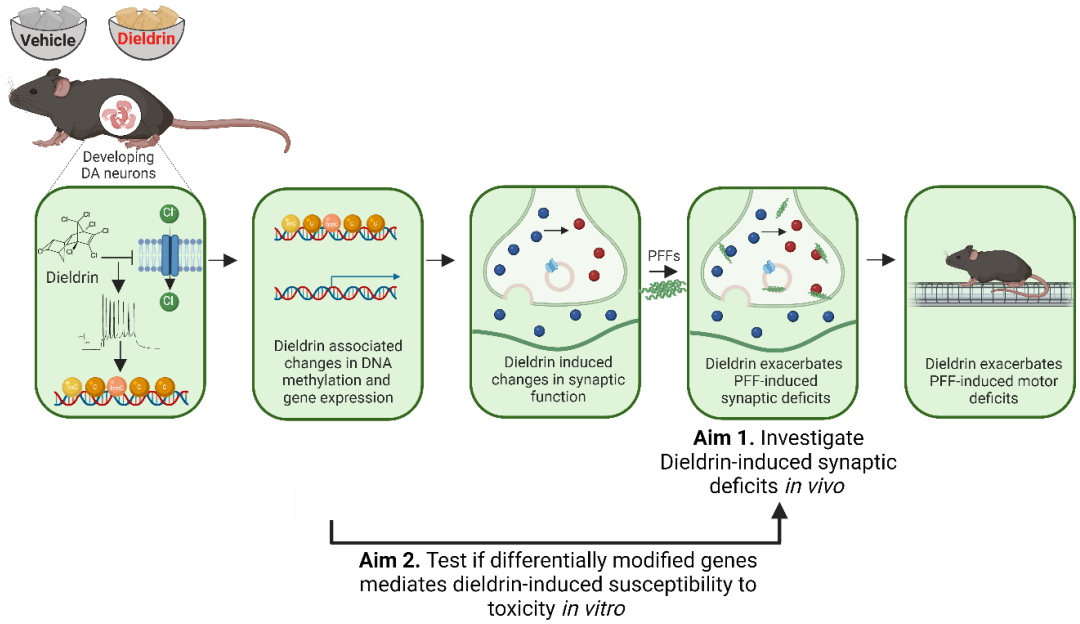

Figure 1.12. Working hypothesis for developmental dieldrin-induced exacerbation of parkinsonian toxicity. Developmental dieldrin exposure induces functional changes in the developing dopaminergic neurons in the offspring by inhibiting the chloride influx via $GABA_A$ receptors and increasing neuronal excitation. Dieldrin can induce changes in DNA methylation and gene expression which results in alterations n synaptic function. The addition of a second hit (the α-syn PFFs) results in dieldrin-induced exacerbation of PFF-induced synaptic deficits resulting in an exacerbated Parkinsonian phenotype specifically in male mice. Aim 1 of this dissertation assess the dieldrin-induced changes in synaptic and vesicular integrity using the dieldrin/PFF two-hit model *in vivo*. Aim 2 links differential gene expression to neuronal function and susceptibility *in vitro*. Made in BioRedner.

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Chapter 2: Developmental exposure to the Parkinson's disease-associated organochlorine pesticide dieldrin alters dopamine neurotransmission in α-synuclein pre-formed fibril (PFF)-injected mice

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Running head: Dieldrin alters dopamine transmission in the α-syn PFF model

Abstract

Parkinson's disease (PD) is the fastest-growing neurological disease worldwide, with increases outpacing aging and occurring most rapidly in recently industrialized areas, suggesting a role of environmental factors. Epidemiological, post-mortem and mechanistic studies suggest that persistent organic pollutants, including the organochlorine pesticide dieldrin, increase PD risk. In mice, developmental dieldrin exposure causes male-specific exacerbation of neuronal susceptibility to MPTP and synucleinopathy. Specifically, in the α-synuclein (α-syn) pre-formed fibril (PFF) model, exposure leads to increased deficits in striatal dopamine (DA) turnover and motor deficits on the challenging beam. Here, we hypothesized that alterations in DA handling contribute to the observed changes and assessed vesicular monoamine transporter 2 (VMAT2) function and DA release in this dieldrin/PFF two-hit model. Female C57BL/6 mice were exposed to 0.3 mg/kg dieldrin or vehicle every 3 days by feeding, starting at 8 weeks of age, and continuing throughout breeding, gestation, and lactation. Male offspring from independent litters underwent unilateral, intrastriatal injections of α-syn PFFs at 12 weeks of age and vesicular ^{3}H-DA uptake assays and fast-scan cyclic voltammetry (FSCV) were performed 4 months post-PFF injection. Dieldrin induced an increase in DA release in striatal slices in PFF-injected animals, but no change in VMAT2 activity. These results suggest that developmental dieldrin exposure increases a compensatory response to synucleinopathy-triggered striatal DA loss. These findings are consistent with silent neurotoxicity, where developmental exposure to dieldrin primes the nigrostriatal striatal system to have an exacerbated response to

synucleinopathy in the absence of observable changes in typical markers of nigrostriatal dysfunction and degeneration.

Introduction

Parkinson's disease (PD) is a multi-system disorder pathologically defined by the degeneration of dopaminergic neurons in the nigrostriatal pathway and the formation of α-synuclein (α-syn)-containing Lewy bodies. PD is the most common movement disorder, the second most common neurogenerative disease, and one of the fastest-growing neurological diseases (de Lau and Breteler 2006). From 1990 to 2016, the prevalence of PD has more than doubled globally (Ray Dorsey et al. 2018). In addition, a recent study suggests that PD incidence in the US is 50% higher than previously estimated, with 90,000 diagnoses per year (Willis et al. 2022). Of relevance to this work, the authors reported PD incidence rates higher in certain geographic areas including the "Rust Belt," a region with a history of heavy industrial manufacturing. This is consistent with epidemiological research that shows an association between increased risk of PD and environmental factors associated with industrialization, including heavy metals, solvents, and pesticide exposures (Semchuk et al. 1992; Tanner and Aston 2000; Ascherio et al. 2006; Brown et al. 2006; de Lau and Breteler 2006; Steenland et al. 2006; Hatcher et al. 2008; Cicchetti et al. 2009; Elbaz et al. 2009; Moretto and Colosio 2011; Tanner et al. 2011; Wirdefeldt et al. 2011; Freire and Koifman 2012; Fleming 2017; Ray Dorsey et al. 2018; De Miranda et al. 2022). Multiple epidemiological studies have found elevated levels of organochlorines in general in the serum and brain of PD subjects (Corrigan et al. 1998; Corrigan et al. 2000; Steenland et al. 2006; Elbaz et al. 2009; Freire and Koifman 2012). Of relevance here, one study reported a specific association between dieldrin levels and PD risk with an odds ratio of 1.95 per

interquartile range in non-smokers, while other organochlorines did not show an association (Weisskopf et al. 2010). In addition, when combined with post-mortem analysis and mechanistic studies, a potential role for dieldrin in PD emerges (Tanner and Langston 1990; Semchuk et al. 1991; Semchuk et al. 1992; Fleming et al. 1994; Corrigan et al. 1998; Le Couteur et al. 1999; Corrigan et al. 2000; Priyadarshi et al. 2000; Ritz and Yu 2000; Tanner and Aston 2000; Priyadarshi et al. 2001; Kanthasamy et al. 2005; Ascherio et al. 2006; Brown et al. 2006; Steenland et al. 2006; Hatcher et al. 2007; Elbaz et al. 2009; Weisskopf et al. 2010; Moretto and Colosio 2011; Tanner et al. 2011; Wirdefeldt et al. 2011; Caudle et al. 2012; Freire and Koifman 2012).

Because dieldrin was phased out in the 1970s and 1980s, the potential for new, acute exposure to dieldrin is low. However, the health effects of past exposures will continue for decades as the population currently diagnosed with PD and those that will develop PD in the next 20-30 years were likely exposed to dieldrin before its phase-out during critical neurodevelopmental periods (de Jong et al. 1997; Jorgenson 2001; Meijer et al. 2001; Kanthasamy et al. 2005). Furthermore, well-established models of dieldrin exposure have demonstrated that dieldrin induces oxidative stress, is selectively toxic to dopaminergic cells, disrupts striatal dopamine (DA) activity, and may promote α-syn aggregation (Sanchez-Ramos et al. 1998; Chun et al. 2001; Kitazawa et al. 2001; Kitazawa et al. 2003; Kanthasamy et al. 2005; Richardson et al. 2006; Hatcher et al. 2007; Moretto and Colosio 2011). Thus, dieldrin serves as an important representative PD-related toxicant that has well-characterized animal exposure paradigms and provides a roadmap for understanding how environmental exposures confer PD risk (Kochmanski et al. 2019; Gezer et al. 2020)

Here, we utilize a mouse developmental dieldrin exposure model where exposure induces sex-specific stable alterations in the DA system that increase susceptibility to subsequent exposure to both α-synuclein (α-syn) pre-formed fibril (PFF)-induced synucleinopathy and MPTP in male, but not female, offspring, suggesting that this model is broadly applicable to investigating how this exposure affects PD risk and neuronal susceptibility (Richardson et al. 2006; Luk et al. 2012a; Luk et al. 2012b; Kochmanski et al. 2019; Gezer et al. 2020) In this model, dams are fed dieldrin (0.3 mg/kg, every 3 days) throughout mating, gestation, and lactation and F1 pups are assessed for toxicity in PD models at 12 weeks of age (Richardson et al. 2006; Kochmanski et al. 2019; Gezer et al. 2020). This dose was chosen based on a previous dose-response study and our results in the two-hit dieldrin/PFF model (Richardson et al. 2006; Kochmanski et al. 2019; Gezer et al. 2020). Mice were exposed through oral ingestion by the dam because the most likely route of exposure to dieldrin in humans is through ingestion of contaminated foods (ATSDR 2022). In this two-hit model, we previously reported a male-specific dieldrin-associated exacerbation of synucleinopathy-induced increases in DA turnover at 6 months, but not at 2 months, as well as an exacerbation of motor deficits on challenging beam at 6 months (Gezer et al. 2020). We also reported no dieldrin effect on the number of α-syn aggregates 1 and 2 months after PFF injection or the reductions in total striatal dopamine by HPLC at 2 and 6 months post-PFF injection. We also demonstrated that synucleinopathy-induced loss of DA neurons by TH and NeuN counts in the SN at 6 months is not exacerbated by dieldrin exposure (Figure 6). While we are unaware of specific epidemiological evidence of sex differences for the dieldrin-related increase in PD risk, this sex specificity of our

observed phenotype is consistent with known sex differences in dopaminergic vulnerability to parkinsonian toxicants and our previously reported sex-specific epigenetic effects of developmental dieldrin exposure (Baldereschi et al. 2000; Elbaz et al. 2002; van den Eeden et al. 2003; Wooten et al. 2004; Haaxma et al. 2007; Taylor et al. 2007; Alves et al. 2009; Weisskopf et al. 2010; Gillies et al. 2014; Georgiev et al. 2017; Kochmanski et al. 2019; De Miranda et al. 2019; Adamson et al. 2022).

Based on the observed exacerbation in PFF-induced increases in striatal DA turnover by dieldrin, we hypothesized here that dieldrin-induced alterations in DA packaging and synaptic vesicle function contribute to the exacerbated toxicity in PFF-injected animals. Proper packaging of DA into synaptic vesicles is critical for DA neurotransmission and neuronal health. (Alter et al. 2013). Because cytosolic DA is metabolized to DOPAC and HVA and broken down into toxic products, disruption of DA handling and packaging can increase cytosolic DA and lead to oxidative stress and acceleration of the toxic interplay between dysregulated α-syn and DA (Graham et al. 1978; Zigmond et al. 1984; Onn et al. 1986; Zhang et al. 1988; Snyder. GL et al. 1990; Zigmond et al. 1993; Ben-Scachar et al. 1995; Hastings et al. 1996; Bezard and Gross 1997; Zigmond 1997; Uhl 1998; Zigmond et al. 1998; Gainetdinov et al. 1999; Caudle et al. 2007; Guillot et al. 2008; Taylor et al. 2009; Miller et al. 2011; Alter et al. 2013; Meiser et al. 2013; Lohr et al. 2014; Molina-Mateo et al. 2017; Mor et al. 2017; Iannitelli et al. 2023). To test this, we assessed VMAT2 function by vesicular uptake assay and DA release and uptake by fast-scan cyclic voltammetry (FSCV) in the dieldrin/PFF two-hit model 4 months post-PFF injection in male F1 offspring developmentally exposed to dieldrin. Testing at 4 months allowed us to capture changes in the striatal synapse prior to significant

nigrostriatal degeneration. Assessing these endpoints at 6 months when degeneration of striatal terminals and nigral cell bodies is already pronounced would test mainly the effects of degeneration, rather than the functional changes that precede it.

Methods

Animals

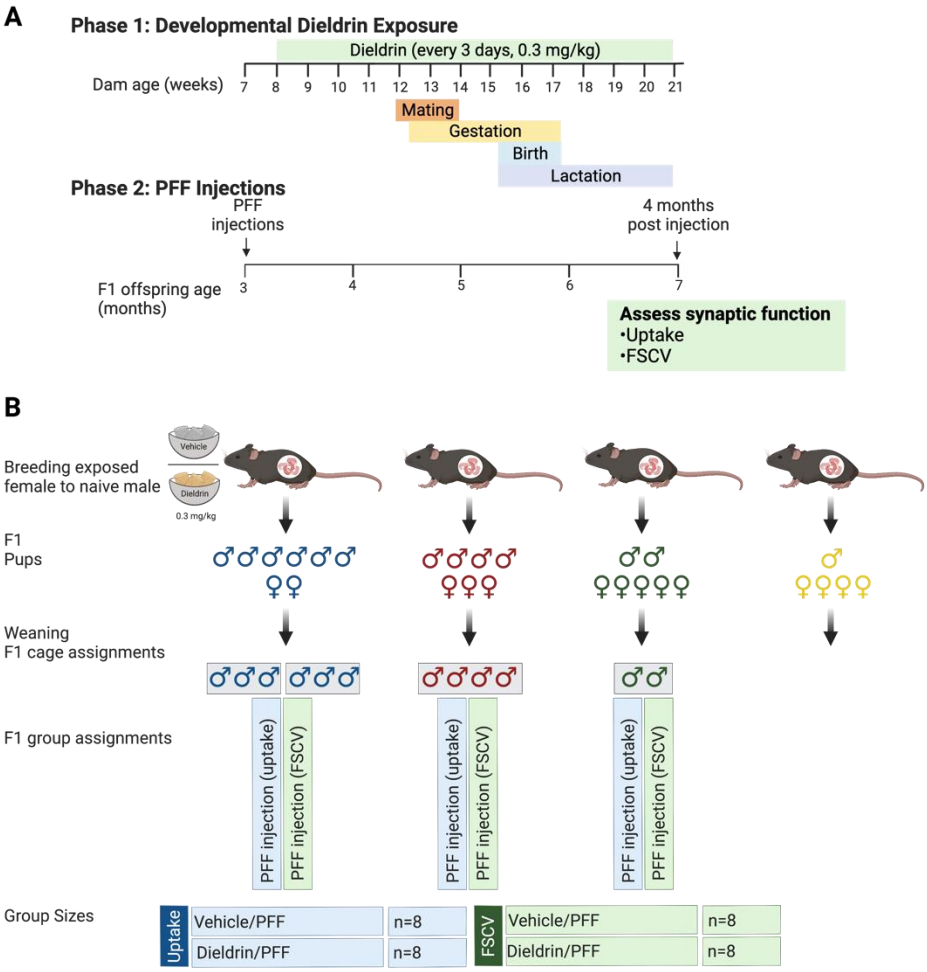

Figure 2.1. Experimental design including dosing schedule, weaning strategy, cage and group assignments. A) Timeline of dieldrin-PFF two-hit model: At 8 weeks of age, female C57BL/6 mice dieldrin exposure began via oral administration of 0.3mg/kg dissolved in corn oil and injected into peanut butter pellets. At 12 weeks of age, mating began, and exposure continued through weaning of pups. F1 pups were weaned 3 weeks after birth and separated by litter and sex (2-4 animals per cage). At 3 months of age, male pups underwent intrastriatal injections of PFFs and were individually housed after surgery. B) Cage, group assignments, and group numbers: Male F1 offspring (F1) that underwent intrastriatal PFF-injections were assigned to endpoints such that every animal for each endpoint came from an independent litter. The Fourth F1 litter is an example of a litter excluded from endpoint assignments due to individual housing. Made in BioRender.

Male (11 weeks old) and female (7 weeks old) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Animal husbandry and colony maintenance was completed as previously described (Kochmanski et al. 2019; Gezer et al. 2020). All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Michigan State University.

Dieldrin exposure paradigm

Dosing was carried out as previously described (Kochmanski et al. 2019; Gezer et al. 2020). Adult C57BL/6 (8-week-old) female animals were treated throughout breeding, gestation, and lactation (Figure 2.1A). Mice were administered 0.3 mg/kg dieldrin (ChemService, CAS# 60-57-1) dissolved in corn oil vehicle and mixed with peanut butter pellets every 3 days (Gonzales et al. 2014; Kochmanski et al. 2019; Gezer et al. 2020). This dose was chosen based on a previous dose-response study and our results in the two-hit dieldrin/PFF model (Richardson et al. 2006; Kochmanski et al. 2019; Gezer et al. 2020). Mice were exposed through oral ingestion by the dam because the most likely route of exposure to dieldrin in humans was through ingestion of contaminated foods and ingestion of the resulting contaminated breast milk (ATSDR) 2022). Control mice received an equivalent amount of corn oil vehicle in peanut butter. Four weeks into female exposure, unexposed C57BL/6 males (8–12 weeks old) were introduced for breeding. Offspring were weaned at 3 weeks of age and separated by litter and by sex, with 2-4 animals per cage (Figure 2.1B). At 12 weeks of age, male offspring from independent litters were selected for PFF or monomer injection. This time point was chosen based on previous results demonstrating increased neuronal

susceptibility to this age (Richardson et al. 2006; Gezer et al. 2020). This developmental dieldrin dosing paradigm has been previously used in our lab to study the role of epigenetics and its effects on synucleinopathy-induced toxicity (Kochmanski et al. 2019; Gezer et al. 2020).

Preparation of α-synuclein PFFs and fibril size verification

Recombinant mouse α-syn monomers and PFFs were provided by the Luk lab, stored at -80 °C, and prepared as previously described (Luk et al. 2012a; Patterson et al. 2019). Over 500 fibrils were measured to determine the average fibril length of 45.06nm +/- 14.7nm (Figure 2.2). Fibril length was assessed before and after surgeries to ensure

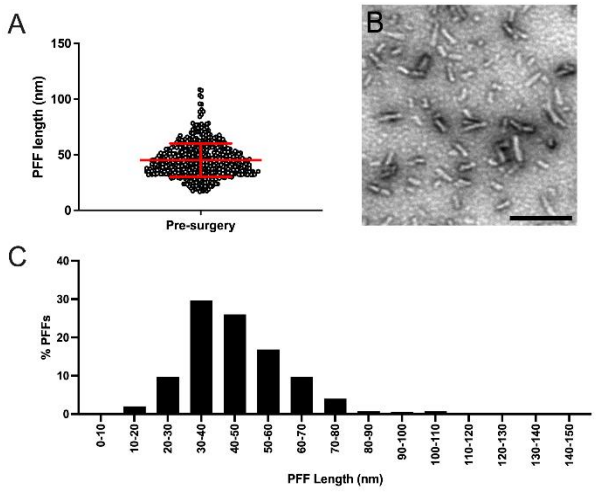

Figure 2.2. Verification of α-synuclein PFF size. A) PFF length distribution determined via TEM. Each point represents a measured fibril length, the error bars denote standard deviation. B) Representative TEM image of sonicated fibrils. C) Frequency distribution of PFF lengths post-sonication.

that fibrils did not re-aggregate over the duration of the surgeries. All measurements were performed with ImageJ (Schneider et al. 2012).

Intrastriatal injection of α-syn PFFs

At 12 weeks of age, animals received unilateral intrastriatal PFF injections according to their cage and group assignment (Figure 2.1B). Surgeries were performed as previously

described (Luk et al. 2012a; Gezer et al. 2020). Mice received a total of 5 µg of PFFs (2.5 µL injection of 2 µg/µL PFFs) and received a single intrastriatal injection (anterior-posterior (AP) +0.2, medial-lateral (ML) +2.0, dorsal-ventral (DV) -2.6) with a flow rate of 0.5 µl/ml. post-surgery, mice received 1mg/kg of sustained-release buprenorphine by subcutaneous injection and were monitored closely until they recovered from anesthesia. In the three days following recovery, animals were monitored daily for adverse outcomes. A small subset of animals (n=2 for FSCV and n=4 for uptake) received α-syn monomer injections as a negative control to ensure that there were no effects of surgery itself. Animals were singly housed following surgeries for the duration of the experiment, consistent with our previous study (Gezer et al. 2020).

Vesicular 3H-dopamine uptake

Animals were killed by cervical dislocation and hemisected. Half of the brain from each group was homogenized for each statistical n, and vesicular DA uptake was performed as previously described (Staal et al. 2000; Caudle et al. 2007; Bernstein et al. 2012; Lohr et al. 2014). Data was normalized to protein level determined by BCA assay and expressed as pmol DA/mg protein/minute.

Fast-scan cyclic voltammetry

Animals were killed by cervical dislocation and brains were sectioned in oxygenated, 4°C artificial cerebrospinal fluid (aCSF) at 300 µm thick using a vibratome (Campden Instruments 5100mz-Plus) (Ferris et al. 2014). FSCV was carried out in the lateral, dorsal striatal sections as previously described (Yorgason et al. 2011; Ferris et al. 2014; Lohr et al. 2014; Ramsson et al. 2015; Ramsson 2016; Everett et al. 2022).

Carbon fiber glass microelectrodes were constructed using a vacuum to pull carbon fiber through a glass capillary tube, pulled using a horizontal electrode puller, broken, and sealed with paraffin (Ramsson et al. 2015). Microelectrodes were cycled for at least 15 minutes before recording at a frequency of 60Hz, then cycled at 10Hz until stable (Takmakov et al. 2010; Ramsson et al. 2015; Ramsson 2016; Davis et al. 2020). Carbon fiber microelectrodes were calibrated using a pipette-based calibration system by adding a dilute stock DA solution to a buffer and measuring the oxidation and/or reduction (Ramsson 2016). All cycling and recordings occurred with a triangle waveform (-0.4 to 1.3V to -0.4V; 400V/s 10Hz) (Lohr et al. 2014; Ramsson et al. 2015; Kang et al. 2021; Everett et al. 2022). Dopamine release was elicited with a bipolar twisted electrode (PlasticsOne) and a 350 μA, 4 ms monophasic optically isolated stimulus pulse (Neurolog NL800). Data was collected and analyzed using Demon Voltammetry and Analysis Software (Wake Forest Innovations) (Yorgason et al. 2011). A five-recording survey of two different dorsal striatal release sites per hemisphere in 2 different slices was taken for each animal with a 5-min rest interval between each stimulation (Lohr et al. 2014; Everett et al. 2022). Peak Dopamine, upward velocity (DA release), downward velocity (uptake; a V_{max} estimate for DAT uptake), and tau (uptake; a K_m estimate for DAT uptake) were calculated for each recording (Figure 5A) (Everett et al. 2022). Ipsilateral values were normalized to contralateral values to account for animal-to-animal variability.

Immunohistochemistry

The rostral remainder of the brains used for FSCV were immersion fixed in 4% paraformaldehyde for 24 hours and placed into 30% sucrose in PBS at 4°C for

immunohistochemistry. Fixed brains were frozen on a sliding microtome and sliced at 40 µm coronally. Free-floating sections were stored in cryoprotectant (30% sucrose, 30% ethylene glycol, 0.05 M PBS) at −20 °C. A 1:6 series of the entire rostral portion of the brain was used for staining and two nigral sections per animal were selected for imaging and quantification. Nonspecific staining was blocked with 10% normal goat serum, and sections were then incubated overnight in appropriate primary antibodies in TBS with 1% NGS/0.25% Triton X-100 followed by appropriate secondary antibodies for 2 hours (Table 2.1). Slides were cover-slipped with VECTASHIELD Vibrance Antifade Mounting Medium (VectaLabs) with DAPI and imaged on a Zeiss AxioScan 7 Digital Slide Scanning Microscope. Analysis of pSyn counts for immunohistochemistry was completed using the object colocalization module in the HALO Image Analysis Platform (Indica Labs). The SNpc was manually traced as the region of interest based on TH staining and pSyn-positive objects within this region were identified on two sections per animal from the same level.

Table 2.1. Antibodies Used for Immunohistochemistry

Antibody	Host	Supplier	Dilution	RRID
Tyrosine Hydroxylase	Rabbit	Millipore AB152	1:4,000	AB_390204
α-synuclein (Phospho S129)/81A	Mouse	Abcam Ab184674	1:10,000	AB_2819037
Goat-anti-Mouse IgG2A Cross-Absorbed Alexa Fluor 555	Goat	Invitrogen A21137	1:500	AB_2535776
Goat-anti-Rabbit IgG (H+L) Cross-Absorbed Alexa Fluor 647	Goat	Invitrogen A21244	1:500	AB_2535812

Data Analysis and Statistics

Statistical analysis and graphing were performed using GraphPad Prism 9. Vehicle-exposed animals injected with PFFs (Vehicle/PFF) and dieldrin-exposed animals

injected with PFFs (Dieldrin/PFF) were compared with two-tailed, unpaired t-tests. All data are shown as mean +/- SD and the cutoff for statistical significance was $p<0.05$. Monomer-injected animals (Vehicle/Monomer, Dieldrin/Monomer) were used as controls to ensure there were no effects of surgery on its own, but these were not included in the statistical analysis, consistent with our preregistration and power analysis (Bernstein and Boyd 2022).

Results

Confirmation of PFF-induced seeding of pSyn-positive aggregates

To confirm PFF-induced seeding, nigral slices were stained for TH and phosphorylated-synuclein (pSyn) from the remaining tissue of brains used for FSCV. We were unable to confirm seeding in animals used for uptake since the entire brain was used for that assay. We confirmed seeding in all animals used for FSCV and counted the number of pSyn-positive objects. At 4 months post-injection, as expected, we observed pSyn positive inclusions ipsilateral, but not contralateral, SN, in both dieldrin and vehicle/PFF groups (Figure 2.3A-B). Consistent with previous results, dieldrin did not affect the number of pSyn-positive inclusions (Figure 2.3C)(Gezer et al. 2020).

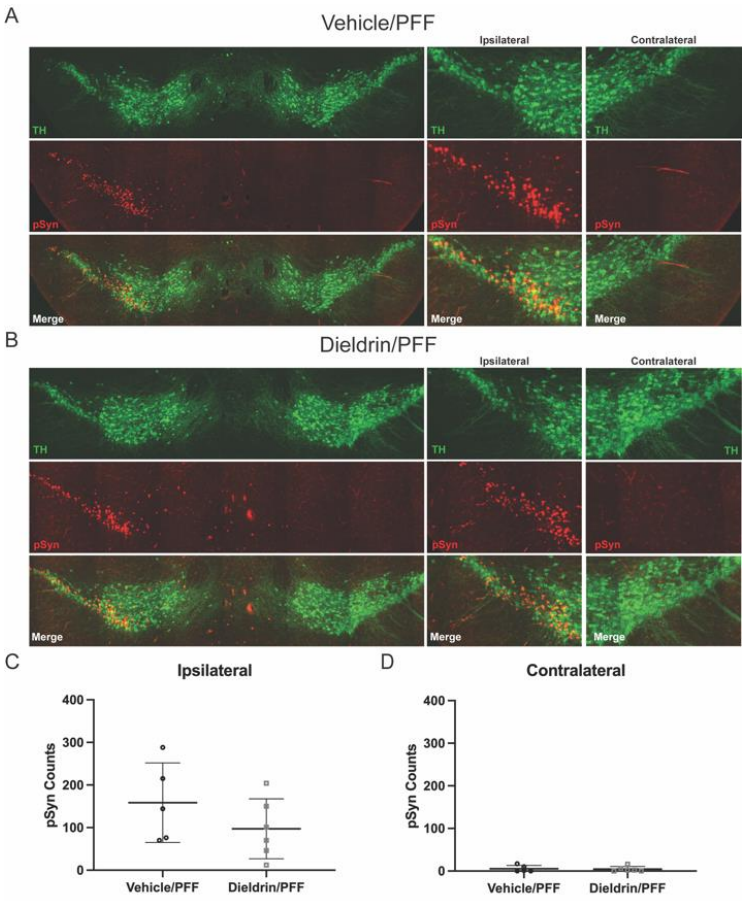

Figure 2.3. Confirmation of PFF-induced seeding in FSCV animals. A) Representative images from nigral tissue sections stained with TH (green) and pSyn (red) from a vehicle/PFF (A) and dieldrin/ PFF (B) animals 4 months post-PFF injection. C) pSyn counts in the SNpc show no effect of dieldrin on pSyn-positive objects in the SNpc ipsilateral to the PFF injection (*p=0.2441*). D) As expected, there were no pSyn-positive objects contralateral to the injection in either group of animals. All data are shown as mean +/- SD.

Developmental dieldrin exposure increases DA release in PFF-injected animals

FSCV was performed in striatal slices to determine if developmental dieldrin exposure affects evoked DA release or uptake in PFF-injected animals 4 months after PFF injection (Figure 2.5A-D). There was a significant increase in both peak DA concentration and upward velocity, a measure of DA release, in the dieldrin/PFF group compared to the vehicle/PFF group (p=0.0394 and p=0.0434, respectively) (Figure 2.5E, F). However, there was no significant difference in DAT uptake as measured by

tau or downward velocity, which are measures of DAT K_m and V_{max} (p=0.6435 and 0.5303 respectively) (Figure 2.5G, H). Calculated values are shown in Table 2. We verified that there was no difference in any metric on the contralateral side to confirm that dieldrin alone did not affect DA release or uptake (Supplemental Figure 2A-D). We also compared ipsilateral to contralateral metrics in the vehicle/PFF group and observed no significant effect of PFFs alone (Supplemental Figure 2E-H). Monomer-injected animals in both the vehicle and dieldrin-exposed groups showed similar outcomes on all FSCV metrics.

Table 2.2. FSCV values (ipsilateral/contralateral) for vehicle/PFF and dieldrin/PFF groups (mean ± standard deviation)

Treatment	Peak Dopamine (µM)	Upward Velocity (µM/s)	Downward Velocity (µM/s)	Tau (s)
Vehicle/PFF	1.102 ± 0.4470	1.097 ± 0.4483	0.2439 ± 1.736	1.038 ± 0.1970
Dieldrin/PFF	2.028 ± 0.9815	1.886 ± 0.8306	0.8454 ± 1.858	1.098 ± 0.2817

Developmental dieldrin exposure does not alter VMAT2 uptake velocity in PFF-injected animals

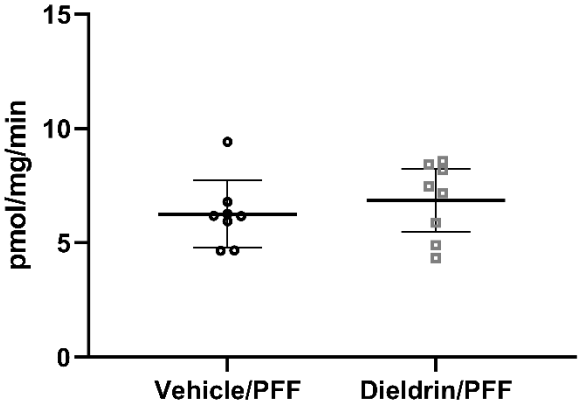

Figure 2.4. Dieldrin does not affect VMAT2 uptake velocity in PFF-injected male F1 offspring. There was no difference in uptake velocity ipsilateral to injection site 4 months post-PFF injection (p= 0.4524). All data shown as mean +/- SD.

To determine if dieldrin exposure alters VMAT2 function in PFF-injected animals, uptake assays were performed at 4 months post-PFF injection. Somewhat surprisingly, there was no difference in VMAT2-mediated uptake velocity between the vehicle/PFF and the dieldrin/PFF groups ipsilateral to the injection site (Figure 2.4). As expected, there was no difference in uptake contralateral to the injection site, showing that dieldrin alone did not affect uptake velocity (Supplemental Figure 1A). In addition, uptake was equivalent between the ipsilateral and contralateral sides within the vehicle/PFF group, demonstrating no significant effect of PFFs alone (Supplemental Figure 1B). Observed uptake velocity was consistent with previously published values for VMAT2 uptake velocity in WT C57BL/6 mice (Lohr et al. 2014). Vehicle and dieldrin-exposed animals injected with monomer showed similar VMAT2 uptake velocity in the hemisphere ipsilateral to the injection (vehicle/monomer: 7.020 ± 2.224 pmol/mg/min, n = 4; dieldrin/monomer: 5.460 ± 1.678 pmol/mg/min, n = 4).

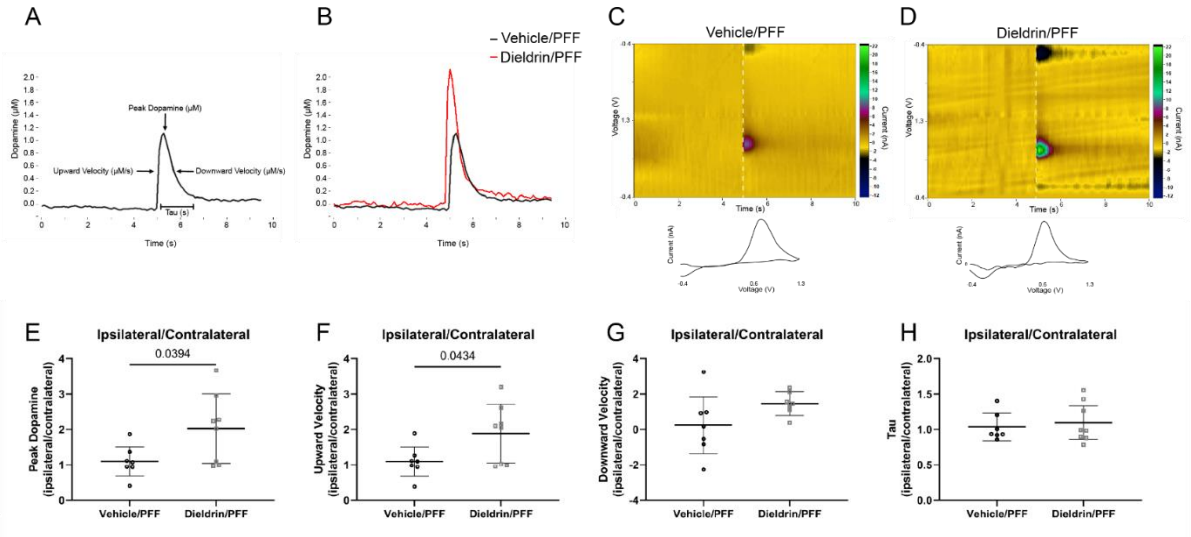

Figure 2.5. Dieldrin/PFFs increase peak dopamine and upward velocity in striatal tissues measured using FSCV. 4-months post-PFF injection, animals were killed and FSCV was performed in dorsal striatum. A) Example dopamine versus time graph showing each quantified metric. B) Representative dopamine versus time graph for the groups vehicle/PFF (black) and dieldrin/PFF (red). C,D) Representative dopamine concentration vs time plot for (C) vehicle/PFF and (D) dieldrin/PFF following stimulation at t=5 secs. E-H) FSCV metrics represented as ipsilateral values normalized to contralateral values. E) Quantification of peak dopamine showed a significant dieldrin-related increase (p= 0.0394). F) Quantification of upward velocity showed a significant dieldrin-related increase (p= 0.0434). G) Quantification of downward velocity showed no significant effect of dieldrin (p= *0.5303*). H) Quantification of tau showed no significant effect of dieldrin (p= *0.6435*). Each individual data point represents a sum of 20 recordings per animal. All data shown as mean +/- SD.

Discussion

A model of environmental risk and silent neurotoxicity in PD

Based on the results reported here, we expand our model for how developmental dieldrin exposure leads to increased susceptibility to synucleinopathy-induced deficits in motor behavior (Richardson et al. 2006; Kochmanski et al. 2019; Gezer et al. 2020). In this model, exposure to dieldrin occurs during prenatal and postnatal development. The half-life of dieldrin in mouse brain is less than a week, so no detectable dieldrin remains in the brain of F1 offspring a few weeks after weaning (World Health Organization. et al. 1989; Richardson et al. 2006; Hatcher et al. 2007). When dieldrin is present in the

developing brain, it is thought to act on developing DA neurons by inhibiting $GABA_A$ receptor-mediated chloride flux, resulting in increased neuronal activity (Narahashi et al. 1995; Narahashi 1996; Liu et al. 1997; Lauder et al. 1998; Paladini and Tepper 1999; Okada et al. 2004). Based on previous results, we propose that this net increase in neuronal activity modifies the dopamine system through persistent sex-specific changes in epigenetic mechanisms, leading to the dysregulation of genes important for dopamine neuron development and maintenance in the substantia nigra and for the neuroinflammatory system in the striatum (Kochmanski et al. 2019; Gezer et al. 2020). These changes alter the response of the nigrostriatal system to future insults via persistent alterations in striatal dopamine synapses that manifest as an early increase in compensatory mechanisms triggered by synucleinopathy-induced striatal DA loss in adult male mice (Figure 4) (Gezer et al. 2020).

Our results are also consistent with the idea of silent neurotoxicity, where the effects of early life exposures are unmasked by challenges later in life, the cumulative effects of exposures over the lifespan, or the effects of aging (Cory-Slechta et al. 2005; Kraft et al. 2016). In such a paradigm, developmental exposure to dieldrin primes the nigrostriatal striatal system in male offspring to have an exacerbated response to synucleinopathy induced by α-syn PFFs in the absence of observable changes in typical markers of nigrostriatal dysfunction and degeneration. In support of this, our previous studies identified persistent epigenetic and transcriptomic changes in genes related to DAergic differentiation and maintenance in the midbrain and altered expression of neuroinflammatory genes in the striatum at 12 weeks of age following developmental dieldrin exposure (Kochmanski et al. 2019; Gezer et al. 2020). In a parallel study, we

are also tracking the longitudinal patterns of dieldrin-induced epigenetic changes across the timeline of this entire two-hit model from birth to 9 months of age to determine if dieldrin alters the trajectory of epigenetic changes across the lifespan.

Taken together, these results suggest that exploring dieldrin-induced changes that produce this high susceptibility state is critical to advancing our understanding of how exposures contribute to increased risk of PD and underscores the need to study PD-related exposures across the lifespan, particularly during sensitive periods of neurodevelopment.

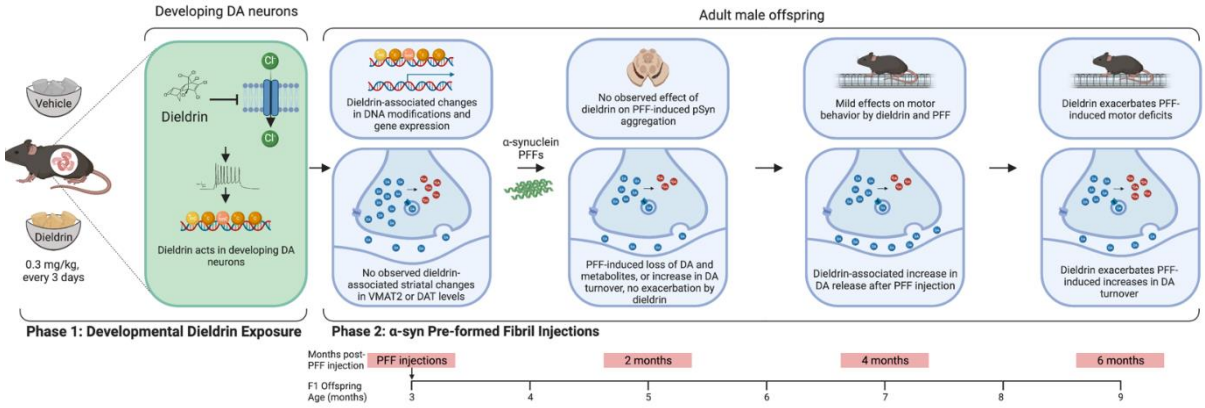

Figure 2.6: Overview of developmental dieldrin/PFF two-hit model. Made in BioRender.

Developmental dieldrin exposure alters the dopaminergic response to synucleinopathy-triggered dopamine deficits

Here, we demonstrate that developmental dieldrin exposure alters response to synucleinopathy and enhances DA release in PFF-injected male animals 4 months after PFF-injection (Figure 2.5,Figure 2.4). These results are consistent with our previous observation of an exacerbated increase in DA turnover at 6 months, summarized in Figure 2.7 (Gezer et al. 2020). If more DA is released at this 4 month, but DAT and VMAT2 uptake velocities are unchanged, this could lead to the increased DA turnover

observed at 6-month post-PFF injection (Alter et al. 2013). Importantly, our current data was collected 4 months post PFF injection while our previous data showed increase striatal DA turnover at 6 months, suggesting that this enhanced DA release precedes effects on DA turnover and motor behavior (Gezer et al. 2020). Of note, in our previous study, we reported that pSyn aggregation at 1 and 2 months, the loss of total striatal DA and its metabolites, DOPAC and HVA, at 2 and 6 months post-PFF injection and loss of nigral DA neurons at 6 months were not affected by dieldrin exposure (Gezer et al. 2020) (Figure 6). Taken together, despite similar levels of synucleinopathy-induced pathology and total tissue DA levels in dieldrin and vehicle exposed animals, dieldrin exposed animals display an increase in evoked DA release at 4 months post-PFF injection.

While FSCV has been utilized in α-syn knockout and α-syn overexpressing models, to our knowledge, this is the first study to perform FSCV in either the dieldrin or α-syn PFF mouse model with intrastriatal injection (Yavich et al. 2004; Threlfell et al. 2021; Somayaji et al.). A previous paper performed FSCV in the mouse α-syn PFF model via intranigral injections at 2- and 5 months of age and reported decreased DA release in older animals only at 1- and 2 months post-injection (Sun et al. 2021). These and other studies in different α-syn models indicate a critical role for a-syn in DA release, synaptic vesicle fusion, vesicle trafficking, and regulation of synaptic vesicle pool size (Abeliovich et al. 2000; Murphy et al. 2000; Volles et al. 2001; Bellani et al. 2010; Cheng et al. 2011; Xilouri et al. 2013; Ingelsson 2016; Dagra et al. 2021). Given that we did not observe any effect of dieldrin or PFF alone on DA release, dieldrin exposure appears to cause changes in the synaptic terminal that prime the nigrostriatal system for an exacerbated

response to synucleinopathy and striatal DA loss, resulting in early enhanced DA release and an eventual increase DA turnover (Supplementary Figure 2)(Kochmanski et al. 2019; Gezer et al. 2020). Early nigrostriatal compensatory changes are well-documented in human PD, multiple animal models of DA deficits, and more recently in a model of other monoaminergic (norepinephrine) loss (Zigmond et al. 1984; Onn et al. 1986; Zhang et al. 1988; Snyder. GL et al. 1990; Zigmond et al. 1993; Zigmond 1994; Bezard and Gross 1997; Zigmond 1997; Zigmond et al. 1998; Molina-Mateo et al. 2017; Iannitelli et al. 2023). Together, this suggests that dieldrin induces changes in the synaptic terminal that increase the compensatory response to early synucleinopathy-induced striatal DA loss that contributes to greater long-term increases in DA turnover due to increases in cytosolic DA, the resulting oxidative stress, and acceleration of the toxic interplay between dysregulated α-syn and DA (Zigmond et al. 1984; Onn et al. 1986; Zhang et al. 1988; Snyder. GL et al. 1990; Zigmond et al. 1993; Bezard and Gross 1997; Zigmond 1997; Zigmond et al. 1998; Molina-Mateo et al. 2017; Iannitelli et al. 2023). Such a relationship between DA and α-syn is well-established and interfering with either can lead to a cycle of neurotoxicity where DA and α-syn interact and exacerbate the toxic effects of one another (Perez et al. 2002; Yavich et al. 2004; Peng et al. 2005; Tehranian et al. 2006; Nemani et al. 2010; Venda et al. 2010; Roy 2017).

Developmental dieldrin exposure does not affect DAT- or VMAT2-mediated uptake in PFF-injected animals

We expected to see a relative increase in DAT function compared to VMAT2 function 4 months after PFF injection that was greater in animals developmentally exposed to dieldrin would lead to increased cytosolic DA and DA turnover and explain the dieldrin-induced exacerbation of synucleinopathy-induced changes in DA turnover (Richardson et al. 2006; Gezer et al. 2020). However, we did not observe any dieldrin related effect on VMAT2 uptake in PFF-injected animals (Figure 5). It is possible that there is an effect on VMAT2 uptake velocity in the intact system that was not observed here due to methodology. Specifically, isolating synaptic vesicles for this assay removes them from their biological context and measures persistent changes in function (Caudle et al. 2007; Lohr et al. 2014; Lohr et al. 2015; Lohr et al. 2016). In addition, this assay

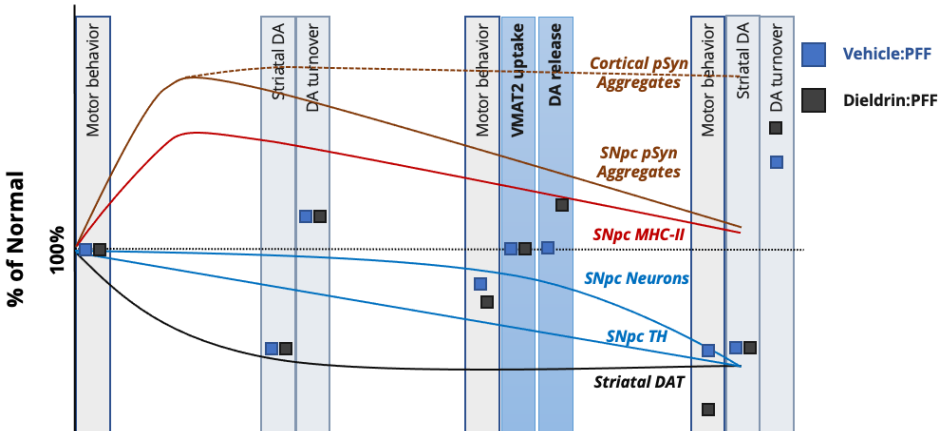

Figure 2.7: Summary of observed changes in the dieldrin PFF two hit model. Timelines show representative changes synuclein pathology, microglial activation, striatal loss, and nigral degeneration in the PFF model based on published literature, shown as the percent change in these markers compared to a saline/monomer injected mouse. Grey boxes indicate previous results from our lab in the dieldrin PFF two hit model. Blue boxes indicate FSCV and uptake results reported here at 4 months post-PFF injection. Blue and grey squares represent results from vehicle:PFF and dieldrin:PFF animals, respectively.

involves homogenizing the entire hemisphere of the brain, so effects may be diluted by DAergic vesicles from areas of the brain not affected in our PFF model, including unaffected terminals within the striatum. Thus, this assay may not have the sensitivity to detect a small change in this small subpopulation of terminals. Unfortunately, technical limitations preclude us from performing this assay on unilateral striata from the mouse brain.

It is also possible to increase the relative levels of DAT to the VMAT2 function by affecting DAT function without alerting VMAT2 function. Multiple prior studies show that both dieldrin and PFFs can impact DAT expression and function (Richardson et al. 2006; Hatcher et al. 2007; Luk et al. 2012a; Sossi et al. 2022). Specifically, developmental dieldrin exposure was previously reported to lead to increases in striatal DAT levels at 12 weeks of age and changes in the DAT/VMAT2 ratio, but we did not observe the same effect in our previous study (Richardson et al. 2006; Gezer et al. 2020). In the mouse PFF model, striatal DAT protein expression at 6 months post-PFF injection in C57BL/6 mice was observed, but not at 1- and 3 months (Luk et al. 2012a). Thus, it is possible that dieldrin-induced increases in striatal DAT function lead to a less severe loss of DAT following PFF-induced synucleinopathy and a relative increase of DAT to VMAT2 function. However, we observed no change in DAT uptake in this study as measured by Tau and downward velocity, measures of DAT K_m and V_{max}, from FSCV data (Figure 2.5). Thus, it is possible that if more DA is released, but neither DAT- nor VMAT2-mediated uptake velocity is changed, the observed increase in turnover is due to both intracellular breakdown and extracellular metabolism of DA. Despite these caveats regarding VMAT2 and DAT uptake, this new data is consistent with our previous

results in this two-hit model showing no increase in α-syn pathology but an enhanced response to synucleinopathy in dieldrin-exposed F1 male offspring (Gezer et al. 2020).

Synucleinopathy alone does not affect DA release 4 months after PFF injection

Contrary to our hypothesis, we did not observe a synucleinopathy-induced decrease in DA release despite ~45% loss of total striatal DA levels 2 months post-PFF (Luk et al. 2012a; Gezer et al. 2020). This discrepancy is likely due to methodological differences and the underlying biology of DA neurons. HPLC measures total tissue DA levels from tissue punches, while FSCV measures extracellular DA only from the area immediately surrounding the electrode. Biologically, while there is a significant loss of total tissue DA at 2 months post-PFF injection, reductions in DA release at these synapses may be delayed relative to this loss. Most striatal DA synapses are silent, and the majority of synaptic vesicles are located within the reserve pool rather than the readily releasable pool (Goldstein 2012; Goldstein 2013; Trudeau et al. 2014; Sulzer et al. 2016; Goldstein 2021). Additionally, within the striatum of PFF-injected animals, we expect only a third to a half of terminals to be affected. Together, this leaves a pool of both surviving neurons and vesicles within affected neurons to maintain DA release. Consistent with this, we previously observed only mild PFF-associated effects on motor behavior at 4 months post-PFF injection, suggesting that DA release is maintained even with a 45% loss of total striatal DA at 2 months post-PFF (Gezer et al. 2020). In line with this, it is generally accepted that DA-related symptoms in human PD do not present until at least 30% of dopaminergic neurons in the nigrostriatal pathway are lost, suggesting that remaining neurons release sufficient quantities of DA despite the loss of total DA (Cheng et al. 2010).

Data Availability

This study was preregistered with Open Science Framework at https://doi.org/10.17605/osf.io/qv4ya (Bernstein and Boyd 2022).

All data acquired and analyzed for this study are available in the Dryad Data Repository https://doi.org/10.5061/dryad.qz612jmmq (Bernstein and Boyd 2023).

CRediT Author Statement

Sierra L. Boyd: Investigation, Software, Formal Analysis, Writing – Original Draft, Writing – Review & Editing, Visualization, Project Administration; **Nathan C. Kuhn:** Methodology, Investigation, Project Administration; **Joseph R. Patterson:** Investigation, Validation, Writing – Review & Editing; **Anna C. Stoll:** Investigation; **Sydney A. Zimmerman:** Investigation; **Mason R. Kolanowski:** Investigation; **Joseph J. Neubecker:** Investigation; **Kelvin C. Luk:** Resources; **Eric S. Ramsson:** Conceptualization, Investigation, Supervision, Writing – Review & Editing; **Caryl E. Sortwell:** Conceptualization, Investigation, Supervision, Writing – Review & Editing; **Alison I. Bernstein:** Conceptualization, Supervision, Data Curation, Writing – Review & Editing, Funding Acquisition

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Conflicts of Interest Statement: All authors declare that they have no conflicts of interest.

TABLES AND FIGURES

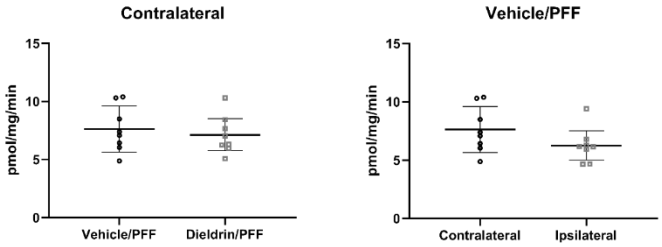

Figure S2.1. Developmental dieldrin exposure or PFF injection alone do not affect VMAT2-mediated vesicular uptake. A) There was no effect of dieldrin on VMAT2-mediated uptake on the non-injected contralateral side (*p=0.5972*). B) There was no effect of PFF on uptake from comparing ipsilateral to contralateral in vehicle/PFF animals (*p=0.0871*). Each data point represents an individual animal. All data shown as mean +/- SD.

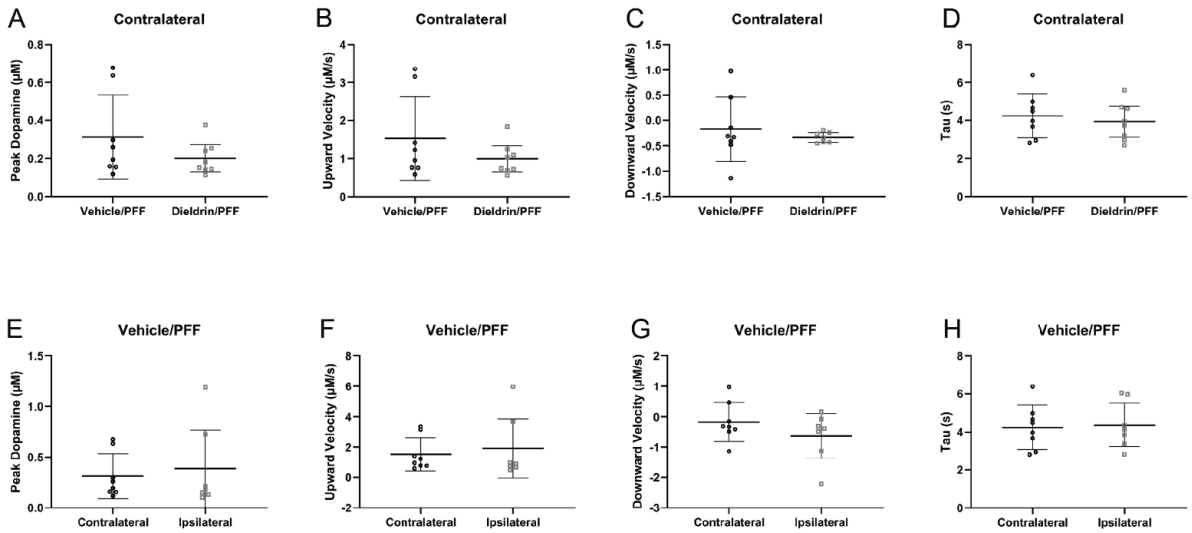

Figure S2.2. Developmental dieldrin exposure or PFF injection alone do not affect FSCV metrics. A-D) There was no dieldrin-related effect on the contralateral side on peak dopamine, upward velocity, downward velocity, or tau between vehicle/PFF versus dieldrin/PFF, indicating that dieldrin alone has no effect on FSCV metrics at this time point (*p=0.2033, 0.2189, 0.5133*, and *0.5736*, respectively). E-H) There was no PFF-related effect on peak dopamine, upward velocity, downward velocity, or tau between the contralateral versus ipsilateral striata, indicating that PFF injection alone has no effect on FSCV metrics at this point (*p=0.6708, 0.6562, 0.2395*, and *0.8440* respectively). Each individual data point represents a sum of 20 recordings per animal. All data shown as mean +/- SD.

Table S2.1. FSCV values (ipsilateral/contralateral) for each individual animal in vehicle/monomer and dieldrin/monomer groups

Treatment	Peak Dopamine (μM)	Upward Velocity (μM/s)	Downward Velocity (μM/s)	Tau (s)
Vehicle/Monomer	0.624602447	0.640540391	0.91393031	1.1802483
Dieldrin/Monomer	1.239490439 0.884641345	1.192634403 0.900459075	1.095703498 -1.283024995	1.29314412 0.945159564

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Chapter 3: α-synuclein preformed fibrils do not seed aggregation or induce toxicity in LUHMES or SH-SY5Y 3D neurospheres

Abstract

Recently, 3D neurosphere models have emerged as an important new approach methodology (NAM) for neurotoxicity testing, disease modeling, and drug screening because these show improved differentiation and survival compared to many 2D adherent systems. Here, we aimed to use α-synuclein (α-syn) preformed fibrils (PFFs) in Lund Human Mesencephalic (LUHMES) and SH-SY5Y 3D neurospheres to model synucleinopathy-induced toxicity *in vitro* and assess if neurotoxicants or specific target genes alter this toxicity. Previously, α-syn PFFs were used to induce synucleinopathy *in vivo* in mice and rats, as well as in *in vitro* models including rodent primary neurons and adherent human SH-SY5Y cells, as Parkinson's disease models. However, this model has not yet been adapted for use in 3D *in vitro* models. We confirmed that after differentiation, both SH-SY5Y and LUHMES neurospheres express the DAergic and neuronal markers tyrosine hydroxylase (TH), vesicular monoamine transporter 2 (VMAT2), and α-syn, but only LUHMES neurospheres express dopamine transporter (DAT) at measurable levels. Consistent with this, LUHMES, but not SH-SY5Y, neurospheres were susceptible to (MPP^+) toxicity assessed by ATP and neurite outgrowth assays, as DAT is required for methyl-4-phenylpyridinium (MPP^+) uptake into cells. While treatment of both LUHMES and SH-SY5Y neurospheres with human α-syn PFFs at concentrations ranging from 0.5 µg/ml to 2 µg/ml led to a concentration-dependent increase in detergent-insoluble α-syn, no toxicity was observed. Uptake of PFFs into cells requires specific proteins at the synaptic terminals, thus, we suspect that these neurospheres do not have well-developed synapses and lack the machinery to take up PFFs and seed aggregation of endogenous α-syn and/or that levels of α-syn in

neurites is too low to allow efficient seeding. Our observation of concentration-dependent increases in detergent-insoluble α-syn may be due to the accumulation of PFF aggregates on the cell surface. Together, our data shows that α-syn PFFs do not seed aggregation of endogenous α-syn or induce toxicity in LUHMES and SH-SY5Y neurospheres.

Introduction

While animal models traditionally provide the most physiologically relevant system to model disease, *in vivo* experiments have several limitations. They can be time-consuming, expensive, and resource-intensive, require large numbers of animals, have high variability, and lack translation from animal models to humans (Bal-Price 2018). New approach methodologies (NAMs) are methods designed to replace animal testing in assessing chemical or drug toxicity and offer advantages for neurotoxicity testing, disease modeling, and drug screening (Hogberg et al. 2013; Anderson et al. 2021). Multiple NAMs were developed to screen chemicals for developmental neurotoxicity (DNT) and additional methods in this area are needed as an efficient and translatable approach to assess DNT (Bal-Price 2018).

The ability to use cells of human origin is a major advantage of NAMs and may be more translatable in modeling human disease and toxicity than *in vivo* rodent models. More than 90% of compounds in clinical trials for drug development fail because of effects that were not observed during *in vivo* testing, partly due to species differences (Hogberg et al. 2013). Human-derived cells can be used for *in vitro* NAMs at increasing levels of complexity, ranging from adherent cells to organoids. Here, we utilized 3D neurospheres derived from Lund Human Mesencephalic (LUHMES) cells and SH-SY5Y cells because of the well-documented protocols for differentiation into DAergic-like cells.

LUHMES cells are derived from 8-week-old female human embryonic mesencephalic, can be differentiated into morphologically and biochemically mature dopamine-like neurons, and are increasingly used for *in vitro* research (Lotharius et al. 2002; Lotharius et al. 2005; Scholz et al. 2011a; Pöltl et al. 2012; Schildknecht et al. 2013; Krug et al. 2014; X.M. Zhang et al. 2014a; Efremova et al. 2015; Hirsch et al. 2015; Oliveira et al. 2015; L. Smirnova et al. 2016; Tong et al. 2017a). 3D LUHMES neurospheres were developed as a high-throughput toxicity screening platform to take advantage of the fact that 3D cell models show better differentiation and survival than 2D LUHMES cultures (L Smirnova et al. 2016; Harris et al. 2017a; Tong et al. 2017a) After differentiation into 3D neurospheres, these cells express TH, DAT, VMAT2 and α-syn (Scholz et al. 2011b; L. Smirnova et al. 2016; Harris et al. 2017b; Lauter et al. 2020; Tüshaus et al. 2020). This cell model is well established and demonstrates robust performance in high-throughput neurotoxicity studies, including studies of rotenone, another neurotoxicant relevant to PD (Scholz et al. 2011c; Scholz et al. 2011b; X. Zhang et al. 2014; X.M. Zhang et al. 2014b; L. Smirnova et al. 2016; Harris et al. 2017b; Harris et al. 2018; Leite et al. 2019; Ko et al. 2020; Tong et al. 2020; Tüshaus et al. 2020; Yamaguchi et al. 2020; Leah et al. 2021; Nicolai et al. 2022; Tong et al. 2022; Ali et al. 2023; Capinha et al. 2023).
SH-SY5Y cells are a sub-cell line of SK-N-SH cells, which are derived from a neuroblastoma from a 4-year-old female (Xicoy et al., 2017). Early characterization of SH-SY5Y cells showed that the cells may have a catecholaminergic phenotype displaying enzymatic activity for both dopamine (DA) and norepinephrine such as tyrosine hydroxylase (TH), dopamine-β-hydroxylase, acetylcholinesterase, and choline acetyltransferase (Xicoy et al. 2017) Shipley et al. has developed methods to induce

differentiation in SH-SY5Y cells with retinoic acid and specific growth factors to form a DAergic-like phenotype (Shipley et al. 2016). Previous work has reported growing SH-SY5Y on protein scaffolds and for shorter durations of time, which likely alter the differentiation trajectory of the neurospheres (Song et al. 2012; Innala et al. 2014; Wang et al. 2014; Ito et al. 2017; Marrazzo et al. 2019; Bastiaens et al. 2020; Limboonreung et al. 2020; Lin et al. 2020; Fiore et al. 2022; Welch and Tsai 2022). However, to our knowledge 3D SH-SY5Y neurospheres have not been previously formed in suspension like the LUHMES cell protocol utilized here. Therefore, we have adapted existing differentiation methods to culturing SH-SY5Y neurospheres in suspension and characterized the DAergic-like phenotype for our model of SH-SY5Y neurospheres.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a canonical DAergic toxicant used for *in vivo* PD research due to its ability to induce robust and specific DAergic degeneration (Crossman et al. 1987; Janson et al. 1992; Bezard et al. 1997; Freyaldenhoven et al. 1997; Grünblatt et al. 2000; Mandavilli et al. 2000; Speciale 2002; Meissner et al. 2003; Schildknecht et al. 2017; Huang et al. 2018). *In vivo*, MPTP is taken up into astrocytes and metabolized by MAO to form the active metabolite, 1-methyl-4-phenylpyridinium (MPP^+), which then enters DAergic neurons via DAT. Inside neurons, MPP^+ is a potent inhibitor of complex 1 of the electron transport chain. For *in vitro* models, the active metabolite, MPP^+ is often used because neuronal cultures lacking astrocytes are unable to convert MPTP to MPP^+ (Richardson et al. 2007; Jagmag et al. 2016).

The MPP^+ model of DAergic degeneration was well established and utilized in a wide range of cell lines and primary neuronal cultures from mice and rats (Song et al. 2012;

Geng et al. 2017; Ito et al. 2017; Ko et al. 2020; Limboonreung et al. 2020; Beliakov et al. 2023a; Beliakov et al. 2023b). Treating 2D adherent or 3D LUHMES neurospheres with MPP^+ results in a concentration-dependent decrease in cell viability and mitochondrial membrane potential (L. Smirnova et al. 2016; Tong et al. 2017b; Ko et al. 2020; Beliakov et al. 2023a). A large number of papers report using the MPP^+ model in SH-SY5Y cells grown in a variety of conditions, with varying levels of toxicity dependent on growth and differentiation conditions (Nicotra and Parvez 2002; Song et al. 2012; Wang et al. 2014; Ito et al. 2017; Ko et al. 2020; Limboonreung et al. 2020; Ioghen et al. 2023). The MPTP/MPP+ model is well-established and frequently used in PD research. While it does not replicate all aspects of the disease, it remains a useful tool for studying the susceptibility of DAergic neurons. Despite this, it remains a useful tool for studying the susceptibility of DAergic neurons.

To model Lewy-like aggregation and the protracted course of DAergic degeneration, the α-syn PFF model was established *in vivo* and successfully recapitulated in iPSCs and primary rodent neurons (Luk et al. 2009; Volpicelli-Daley et al. 2011; Volpicelli-Daley et al. 2014; Gao et al. 2019a; Ross et al. 2020a). In this model, PFFs produced from recombinant α-syn template endogenous α-syn form protease-resistant, detergent-insoluble, ubiquitinated, and hyperphosphorylated inclusions and eventually lead to cell death (Luk et al., 2009; Volpicelli-Daley et al., 2011, 2014). Many aspects of the *in vivo* model can be recapitulated *in vitro*, including the formation of phosphorylated inclusions, mitochondrial dysfunction, oxidative stress, deficits in neuronal excitability, increased autophagy, loss of synaptic markers, and eventual degeneration of neurons (Luk et al. 2009; Volpicelli-Daley et al. 2011; Volpicelli-Daley et al. 2014; Ross et al.

2020a). Initial work in primary hippocampal neurons, chosen because of their high levels of α-syn, showed that phosphorylated inclusions are present 4 days after application of PFFs and compact puncta form by 7 days. By 14 days post-treatment, 20-30% of treated primary hippocampal neurons degenerate, showing mitochondrial oxidant stress, deficits in neuronal excitability, increased autophagy, and loss of synaptic markers (Volpicelli-Daley et al. 2011; Volpicelli-Daley et al. 2014). Some work has extended the PFF model in 2D SH-SY5Y cells to study aggregate clearance pathways and mitochondrial dysfunction associated with PFF-induced aggregation (Perfeito et al. 2014; Choi et al. 2018; Gao et al. 2019b; Ross et al. 2020b; Pantazopoulou et al. 2021; Feng et al. 2022; Lin et al. 2022).

While the α-syn PFF model has successfully been used in multiple *in vitro* systems, it has not yet been used in LUHMES cells or any 3D neurosphere model. Therefore, in this study, we aimed to adapt the PFF model to 3D LUHMES and SH-SY5Y neurospheres to take advantage of advances in the development of NAMs. Here, we demonstrate that LUHMES, but not SH-SY5Y neurospheres are susceptible to MPP^+-induced cell loss as measured by ATP and neurite outgrowth assays, consistent with the observed lack of DAT expression in SH-SY5Y neurospheres. We also show a concentration-dependent PFF-induced increase in detergent-insoluble α-syn in both LUHMES and SH-SY5Y neurosphere cultures, but no effect on cell viability. Based on these findings, we suspect that the synapses in these neurospheres may not form the proper synaptic connections and/or do not have enough endogenous levels of α-syn to template α-syn PFFs to form aggregates. Overall, our data show that neither LUHMES

nor SH-SY5Y neurospheres are not susceptible to α-syn PFF-induced aggregation or toxicity.

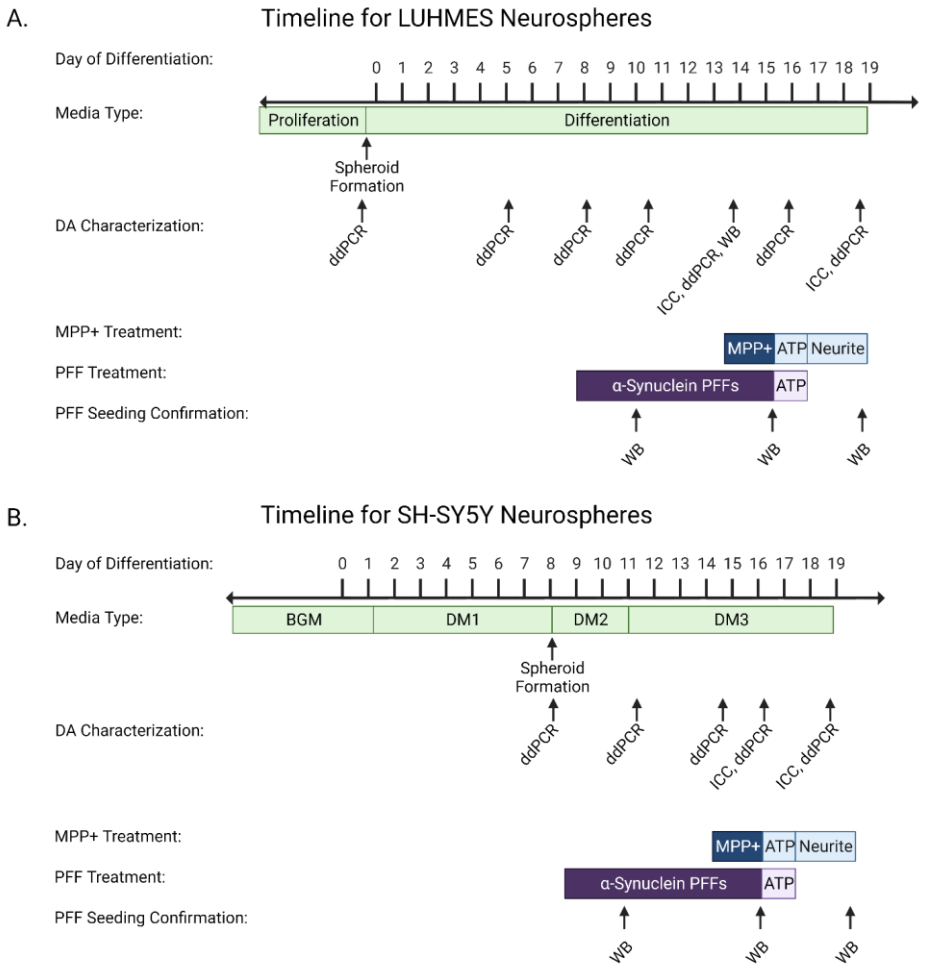

Figure 3.1. Differentiation timeline for LUHMES and SH-SY5Y cells. A) Timeline for LUHMES experiments. On day 0, spheroid formation and media are replaced from proliferation to differentiation media. MPP^+ treatment is added on day 14, ATP assay on day 16, and neurite outgrowth assays on day 17. α-syn PFFs are added on Day 8, ATP assays on day 16, and samples to confirm PFF seeding via western blots are collected on days 10, 16, and 18. B) Timeline for SH-SY5Y cells. On day 0, basic growth media is replaced for differentiation media 1. On Day 8, differentiation media 1 is exchanged for differentiation media 2 when spheroid formation begins. On day 11, differentiation media 2 is replaced with differentiation media 3. α-syn PFFs are added on Day 9, ATP assays on day 17, and samples to confirm PFF seeding via western blots are collected on days 11, 17, and 19. For both LUHMES and SH-SY5Y, samples were collected throughout the differentiation time course for DAergic characterization using ddPCR, immunocytochemistry, and western blots. Made in BioRender.

Methods

LUHMES Cell Culture

LUHMES cells (ATCC CRL-2927, RRID: CVCL_B056) were grown as previously described with modifications to the differentiation media supplements. (L. Smirnova et al. 2016; Harris et al. 2017b; Tong et al. 2017b; Harris et al. 2018; Leite et al. 2019) For proliferation, cells were grown in flasks coated with 50 µg/ml Poly-L-ornithine and 1 µg/ml Fibronectin. Proliferation Media was completely exchanged every other day, and cells were passaged every 3-4 days (Table 1).

For differentiation, cells were trypsinized with TrypLE Express (Gibco) and seeded at 2.25 x 10^6 cells/ml in cell-repellent 6-well plates (Corning) in 2 ml/well Differentiation Media and this was designated Differentiation Day 0 (Harischandra et al., 2020) (Table 2). The 6-well plates were placed on an orbital shaker at 90 rpm in an incubator (37°, 5% CO_2) for the remainder of the experiment. Two days after seeding the 6-well plates (Differentiation Day 2), 1 ml of media was exchanged for 1 ml of Differentiation Media with 20 nM Taxol (Sigma-Aldrich) to inhibit proliferation. Two days after Taxol treatment (Differentiation Day 4), 1.8 ml of media was removed from each well and replaced with 2 ml of differentiation media to wash out Taxol. After the Taxol washout, half media changes were completed every other day for the remainder of the experiment as previously described (L. Smirnova et al. 2016; Harris et al. 2017b; Tong et al. 2017b; Harris et al. 2018; Leite et al. 2019)

Table 3.1. LUHMES Proliferation Media

Reagent	Vendor	Catalog Number	Final Concentration
DMEM/F12 Base Media	Invitrogen	12-634-028	-
L-Glutamine	Gibco	A2916801	2 mM
N2 Supplement	Gibco	17502048	1 X
Recombinant Fibroblast Growth Factor	R&D Systems	5114-TC	40 ng/mL

Table 3.2. LUHMES Differentiation Media

Reagent	Vendor	Catalog Number	Final Concentration
DMEM/F12 Base Media	Invitrogen	12-634-028	-
L-Glutamine	Gibco	A2916801	2 mM
N2 Supplement	Gibco	17502048	1 X
N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt	Sigma-Aldrich	D0627	100 mM
Tetracycline	Sigma-Aldrich	T7660	1 µg/mL
Recombinant Glial Cell Line Derived Neurotrophic Factor	R&D Systems	212-GD	20 ng/ml
Brain Derived Neurotrophic Factor	R&D Systems	11166-BD-050	10 ng/ml
Human TGF-Beta3 Recombinant Protein	Invitrogen	RP-8600	20 ng/ml
Recombinant Human Leukemia Inhibitory Factor	Gibco	PHC9484	10 ng/ml

SH-SY5Y Cell Culture

Table 3.3. SH-SY5Y Basic Growth Media

Reagent	Vendor	Catalog Number	Final Concentration
MEM Base Media	Gibco	11095098	-
Heat Inactivated Fetal Bovine Serum	Cytiva	SH3008803HI	15 %
L-Glutamine	Gibco	A2916801	2 mM
Penicillin-Streptomycin	Gibco	15-140-122	1 X

Table 3.4. SH-SY5Y Differentiation Media 1

Reagent	Vendor	Catalog Number	Final Concentration
MEM Base Media	Gibco	11095098	-
Heat Inactivated Fetal Bovine Serum	Cytiva	SH3008803HI	2.5 %
L-Glutamine	Gibco	A2916801	2 mM
Penicillin-Streptomycin	Gibco	15-140-122	1 X
All-Trans Retinoic Acid	Biogems	3027949	10 µM

Table 3.5. SH-SY5Y Differentiation Media 2

Reagent	Vendor	Catalog Number	Final Concentration
MEM Base Media	Gibco	11095098	-
Heat Inactivated Fetal Bovine Serum	Cytiva	SH3008803HI	1 %
L-Glutamine	Gibco	A2916801	2 mM
Penicillin-Streptomycin	Gibco	15-140-122	1 X
All-Trans Retinoic Acid	Biogems	3027949	10 µM

Table 3.6. SH-SY5Y Differentiation Media 3

Reagent	Vendor	Catalog Number	Final Concentration
Neurobasal Base Media	Gibco	21103049	-
L-Glutamine	Gibco	A2916801	2 mM
Penicillin-Streptomycin	Gibco	15-140-122	1 X
All-Trans Retinoic Acid	Biogems	3027949	10 µM
B27 Plus Supplement	Gibco	A3582801	1 X
N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt	Sigma-Aldrich	D0627	2 mM
Potassium Chloride	Sigma-Aldrich	P3911	20 mM
Brain Derived Neurotrophic Factor	R&D Systems	11166-BD-050	50 ng/mL

SH-SY5Y cells (ATCC CRL-2266, RRID: CVCL_0019) were grown in uncoated flasks while proliferating in Basic Growth Media as previously described (Shipley et al. 2016) (Table 3). Once the cells were 80% confluent, the media was completely exchanged to Differentiation Media 1 Differentiation Day 0 (Table 4). On Differentiation Day 8, the cells were trypsinized using Trypsin-EDTA 0.05% (Gibco) and plated in 6-well at 2.50 $x10^6$ cells/ml in cell-repellent 6-well plates (Corning) at 2 ml/well in Differentiation Media 1 (Table 4). The 6-well plates were placed on an orbital shaker at 90 rpm in an incubator (37°, 5% CO_2) for the remainder of the experiment. One day after seeding, the 6-well plates (Differentiation Day 9), and 2 ml of Differentiation Media 2 were added to the wells (Table 5). On Differentiation Day 10, 3 ml of media was removed and replaced with 1 ml of Differentiation Media 2 (Table 2). On Differentiation Day 14, 1 ml of media was exchanged for 1 ml of Differentiation Media 3 (Table 5). Half of the media was exchanged with Differentiation Media 3 every two days for the remainder of the experiment.

MPP+ Treatment

MPP^+ was resuspended in PBS at a stock concentration of 10 mM in PBS. On the day of treatment, MPP^+ was diluted in media to make 2x the final concentration, and 1 ml of media was removed from each well of the 6-well plate and replaced with 1 ml of 2x media with MPP^+. Each well was treated with concentrations ranging from 0.001 - 200 µM of MPP^+. At 48 hours post-treatment, 8 spheroids from each 6-well were transferred to Matrigel-coated plates (1 spheroid per well) for neurite outgrowth assays and the remaining spheroids in each MPP^+ treated well were used for ATP assays.

Preparation of α-synuclein PFFs and fibril size verification

Recombinant human α-syn PFFs were provided by the Luk lab, stored at -80°C, and prepared via a Q500 Sonicator with a cup-horn assembly and chiller (Qsonica) as previously described (Luk et al. 2012; Volpicelli-Daley et al. 2014; Patterson et al. 2019). To determine the average fibril length, transmission electron microscopy (TEM) was completed as previously described (Patterson et al. 2019). To briefly describe this process, PFF samples were prepared on Formvar/carbon-coated copper grids (EMSDIASUM, FCF300-Cu). Grids were imaged with a JEOL JEM-1400+ TEM. TEM images were analyzed for fibril length in ImageJ (Schneider et al. 2012). 500 fibrils were measured to determine the average fibril length of 38.94 nm +/- 11.65 nm.

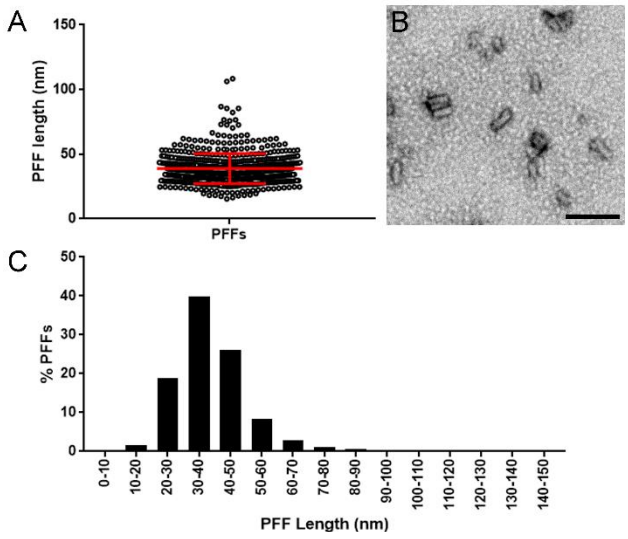

Figure 3.2. Verification of α-synuclein PFF size. A) PFF length distribution determined via TEM. Each point represents a measured fibril length, the error bars denote standard deviation. B) Representative TEM image of sonicated fibrils. C) Frequency distribution of PFF lengths post-sonication.

PFF Treatment

Human α-syn PFFs (0.5 µg/ml to 2.0 µg/ml) diluted in PBS were added to 1 ml of media and added to neurosphere cultures on Differentiation Day 8 for LUHMES and SH-SY5Y. 48-hour post-treatment, 1 ml of media was removed and replaced with fresh media. Half media changes were conducted every 48 hours.

RNA Isolation, Reverse Transcription, and droplet digital PCR

All spheroids from one well of a 6-well plate were collected using a wide bore pipette tip at indicated time points, spun at 500xg for 5 minutes, the media was aspirated. To rinse, cell pellets were resuspended in PBS and spun again at 500xg for 5 minutes. The PBS was aspirated, and the rinsed cells were frozen as pellets stored at -80°C until RNA extraction (Figure 1). RNA was isolated from frozen cell pellets resuspended in QIAzol (Qiagen) using a Quick-RNA MiniPrep Kit as directed (Zymo Research). RNA integrity and concentration were measured via NanoDrop UV-Vis spectrophotometer (ThermoScientific). For quality control, 260/280 absorbance ratios for all samples were in the 1.8-2.2 range.

Purified RNA was loaded in equal amounts of 1,000 ng and reverse transcribed via iScript MasterMix (BioRad) to form cDNA. cDNA was diluted 1:10 for a final RNA amount of 100ng and analyzed via droplet digital PCR to assess expression of TH (Hs00165941_m1), SLC6A3 (Hs00997374_m1), SLC18A2 (Hs00996835_m1), SNCAIP (H200914722_m1), and MK167 (HS04260396_m1) using ddPCR Mastermix (BioRad) and TaqMan Gene Expression Assays (ThermoFisher). Samples were partitioned with a BioRad QX200 droplet generator and amplified and analyzed using a BioRad QX200

droplet reader. All TaqMan assays were completed with at least 3 biological replicates from independent experiments.

Sequential protein expression for insoluble α-synuclein western blotting

To confirm insoluble α-synuclein in PFF-treated neurospheres, spheroids from each well of the 6-well plate were collected, processed, and frozen as stated for RNA extraction at 2, 5, and 10 days post-treatment. Cell pellets were thawed on ice and resuspended with 1% Triton in Tris Buffered Saline (TBS) and protease inhibitors, then sonicated for 2 pulses, 30% amplitude with a probe sonicator. Lysates were spun at 100,000xg at 4°C for 30 minutes. The supernatant was collected as the soluble fraction. The pellet was sonicated in 2% sodium dodecyl sulfate (SDS) in TBS and collected as the insoluble fraction. Protein levels for the soluble and insoluble fractions were quantified by BCA protein assay (Volpicelli-Daley et al. 2014).

Immunocytochemistry

Neurospheres were transferred to Netwell inserts and fixed with 4% PFA for 30 minutes. Non-specific staining was blocked with 5% normal goat serum (NGS), 1% bovine serum albumin, and 0.1% Triton for 1 hour. Fixed neurospheres were incubated overnight in the appropriate primary antibodies in TBS with 1% NGS/0.1% Triton X-100 followed by appropriate secondary antibodies for 2 hours. Spheroids were stained with DAPI for 10 minutes following secondary incubation. Slides were cover-slipped with VECTASHIELD Vibrance Antifade Mounting Medium (VectaLabs). Stained neurospheres were imaged on a Lionheart Fx Automated Microscope (BioTek).

Table 3.7. Antibodies used for Immunohistochemistry

Antibody	Host	Supplier	Dilution	RRID
Tyrosine Hydroxylase	Rabbit	Millipore AB152	1:1,000	AB_390204
α-synuclein	Rabbit	Abcam Ab212184	1:500	AB_2941889
DAPI		Invitrogen D1306	1:10,000	AB_2629482
Goat-anti-Rabbit IgG (H+L) Cross-Absorbed Alexa Fluor 488	Goat	Invitrogen A1108	1:500	AB_621843

Collection of cell lysates and western blotting

Cell pellets were resuspended in RIPA buffer with protease inhibitors and homogenized with a pestle**.** Lysates were spun at 1,000 xg for 5 minutes, and the supernatant was collected as the lysate for western blotting.

18 µg of protein for soluble and insoluble α-syn western blots were loaded onto AnyKD Criterion midi gel (BioRad) gels. 20 µg of protein for lysates for TH, DAT, and VMAT2 were loaded onto Novex 10% Bis-Tris gels (Invitrogen).

Proteins were electrophoretically transferred to nitrocellulose membranes (BioRad). For α-syn blots only, membranes were fixed in 0.4% paraformaldehyde for 30 minutes immediately after transfer. Membranes were stained with Revert 700 Total-Protein Stain (LI-COR) for 5 minutes and imaged with a LI-COR Odyssey CLx. Membranes were blocked with Odyssey blocking Buffer (LI-COR), and incubated in either α-syn (Invitrogen), Tyrosine hydroxylase (Millipore), DAT (Sigma), or VMAT2 primary antibodies overnight at 4°C. After washing, membranes were incubated in goat anti-Rb 800CW (LI-COR) for 2 hours and imaged with a LI-COR Odyssey CLx.

Table 3.8. Antibodies used for Western Blotting

Antibody	Host	Supplier	Dilution	RRID
Tyrosine Hydroxylase	Rabbit	Millipore AB152	1:1,000	AB_390204
VMAT2	Rabbit	Miller Lab	1:10,000	
DAT	Rabbit	Sigma D6944	1:1,000	AB_1840807
IRDye 800CW anti-Rabbit IgG	Goat	LI-COR Biosciences 926-32211	1:10,000	AB_621843

ATP Assays

For ATP assays, 1 ml of media from each well of the 6-well neurosphere plate was removed and replaced with 1 ml of Cell Titer-Glo 3D reagent (Promega). Neurospheres were lysed by shaking the plates at 700 rpm for 20 minutes at room temperature in a Thermomixer (Eppendorf). To avoid cellular debris, the supernatant was transferred to a white 96-well assay plate in triplicate (Corning). ATP standards were diluted in media and pipetted in triplicate in the white 96-well assay plate. The plate was incubated for 10 minutes in the dark and luminescence was read using a Synergy H1 plate reader at an integration time of 1.5 seconds and a reading height of 7.00 mm (BioTek). Blanks were subtracted and samples were calibrated to the ATP standard to calculate ATP concentration (μM). All ATP cytotoxicity assays were completed with at least 3 biological replicates from independent experiments, with 4 technical replicates per experiment.

Neurite Outgrowth Assays

48 hours before neurite analysis, black 96-well optical bottom plates (Corning) were coated with growth factor reduced Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning) diluted 1:24 in DMEM/F12 and placed in an incubator to polymerize overnight. Following polymerization, Matrigel was aspirated and replaced

with either LUHMES Differentiation Media made with phenol-free DMEM/F12 (Gibco) or SH-SY5Y Differentiation Media 3 made with phenol-free Neurobasal (Gibco). Individual spheroids were transferred to single wells in the Matrigel-coated plate. 24 hours after plating, neurites were stained with a neurite outgrowth staining kit (ThermoFisher) for 30 minutes as directed. At the end of incubation, half of the solution was replaced with a 3x Background Suppression Dye. Neurospheres were imaged with a Lionheart Fx Automated Microscope at 4x using a GFP filter cube (BioTek).

Images were then analyzed in Gen5 software. Briefly, a primary mask around the spheroid body is generated by thresholding and a secondary mask is generated by thresholding without a ring around the spheroid body (Figure 3). The ratio of the areas of the secondary mask to the primary mask provides a measure of neurite outgrowth. Each well was manually checked for the following exclusion criteria: wells containing multiple spheroids, spheroids touching the edge of the well, or spheroid or neurites no longer attached to the plate. For each treatment condition, 8 neurospheres were individually transferred to a 96-well plate to ensure at least 4 technical replicates per concentration were included after the exclusion, and 3 biological replicates were used from independent experiments for all neurite outgrowth assays.

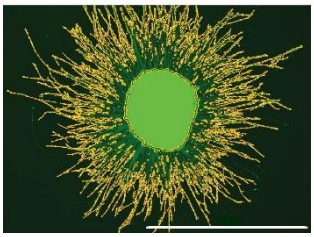

Figure 3.3. Representative neurite outgrowth analysis. Scale bar represents 1000 µM.

Statistical analysis

Independent, biological replicates are defined as cells from separate independent thaws and differentiation of LUHMES cells. At least three biological replicates were used for every endpoint. Technical replicates were defined as follows for each outcome measure. After lysis for the ATP assay, the lysate is pipetted in triplicate into the white 96-well assay plate for 3 technical replicates for every biological replicate. For the neurite outgrowth assay, 8 neurospheres were transferred from each well (concentration of MPP^+) of the 6-well plate to a Matrigel-coated 96-well plate. After the exclusion mentioned in the above section, at least 4 neurospheres per concentration were used as technical replicates for every biological replicate. For both assays, technical replicates were averaged and normalized to untreated control. Normalized data for all independent biological replicates was then averaged. Nonlinear regression curve fitting was used in GraphPad Prism.

Results

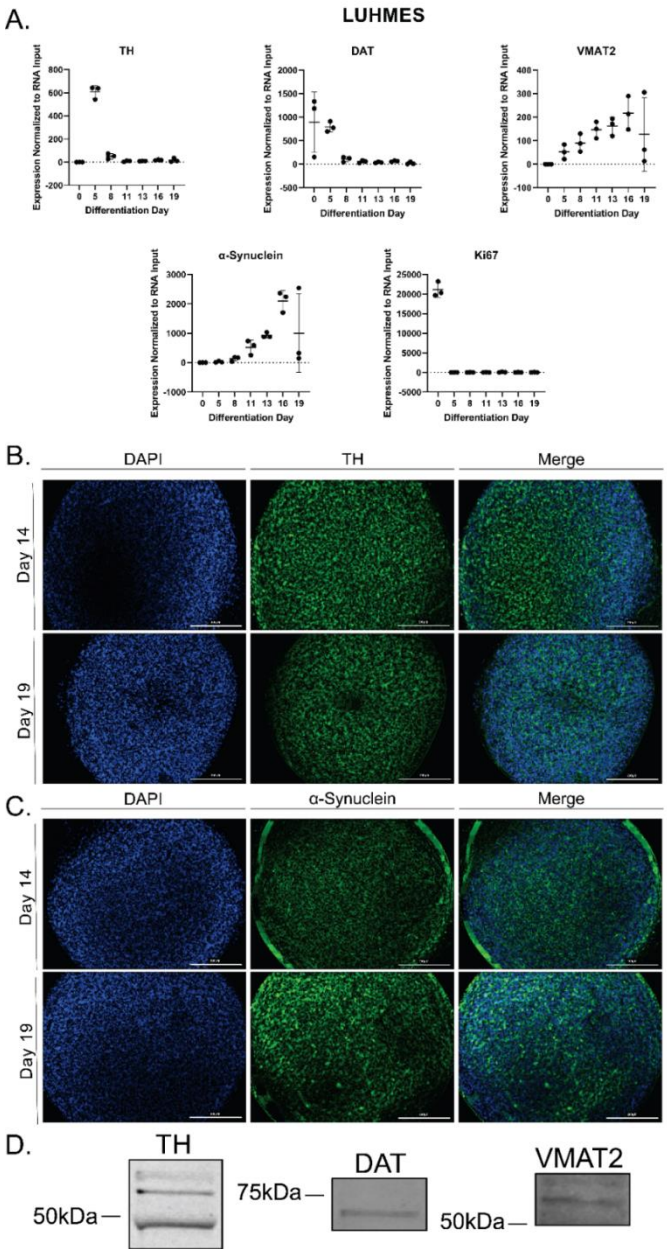

Figure 3.4. LUHMES neurospheres express DAergic markers after differentiation. A) mRNA expression for TH, DAT, VMAT2, α-synuclein, and Ki67 at days Differentiation Days 0, 5, 8, 13, 16, and 19 B) Representative Immunocytochemistry for DAPI (blue), TH (green) and merge images of neurospheres at differentiation days 14 (top) and 19 (bottom) C) Representative Immunocytochemistry for DAPI (blue), α-synuclein (green), and merge images at days 14 (top) and 19 (bottom). D) Representative western blots for TH, DAT, and VMAT2 at Differentiation days 0, 10, 15, and 18. C) Representative western blots for TH, DAT and VMAT2 at Differentiation Day12. Graphs show mean ± SD for 3 independent experiments.

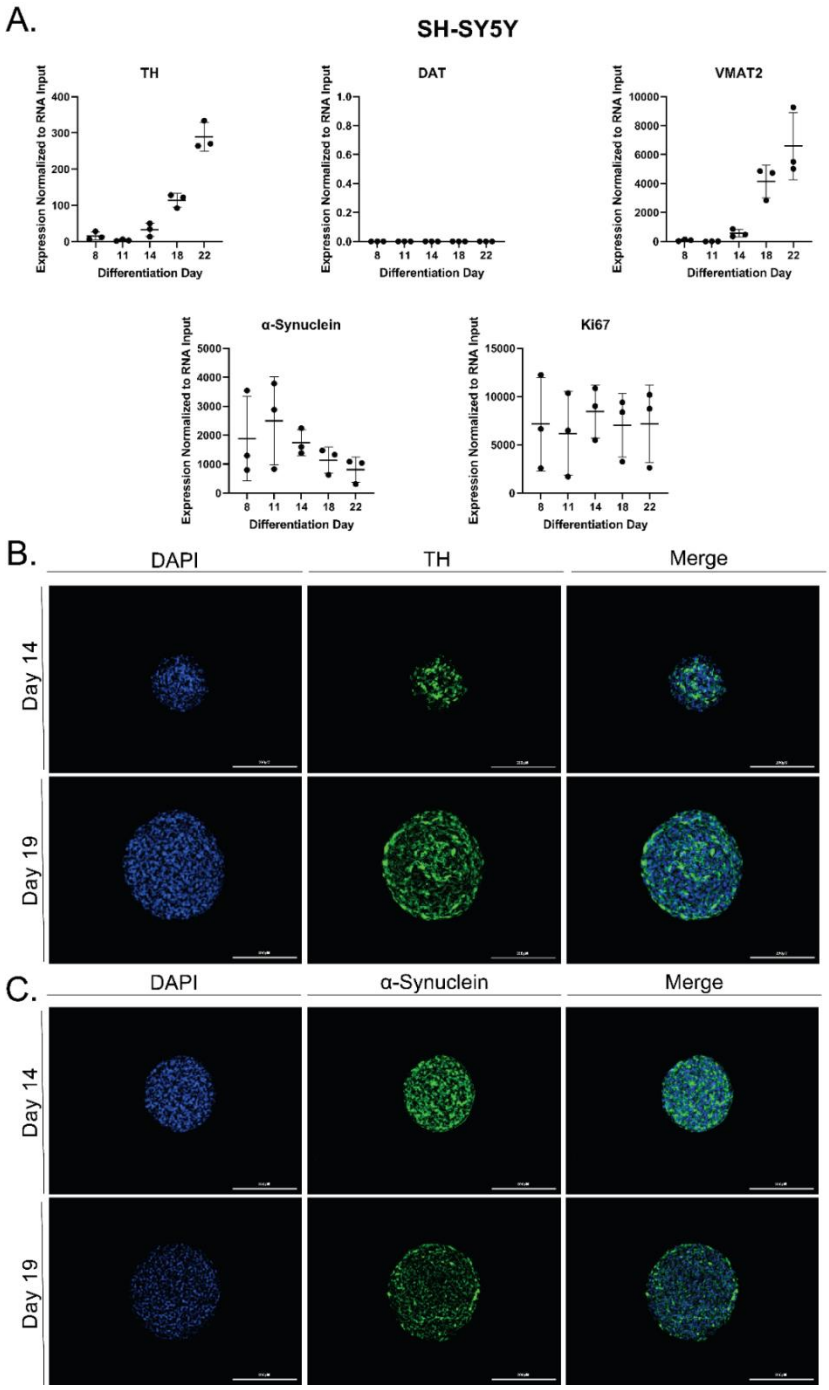

Figure 3.5. SH-SY5Y neurospheres express some DAergic markers after differentiation. A) mRNA expression for TH, DAT, VMAT2, α-synuclein, and Ki67 at days Differentiation Day 0, 5, 8, 13, 16, and 19 B) Representative Immunocytochemistry for DAPI (blue), TH (green) and merge images of neurospheres at Differentiation Day 14 and 19 (top) C) Representative Immunocytochemistry for DAPI (blue), α-synuclein (green), and merge images at days 14 (top) and 19 (bottom). Graphs show mean ± SD for 3 independent experiments.

Confirmation of DAergic differentiation in LUHMES neurospheres

To confirm that differentiated LUHMES neurospheres develop a DAergic-like phenotype, we assessed mRNA and protein levels of DAergic markers (TH, DAT, and VMAT2), α-syn, and the proliferation marker Ki67. Neurospheres were collected throughout the differentiation time course for ddPCR analysis of mRNA levels. LUHMES neurospheres had detectable levels of TH, DAT, and VMAT2, during differentiation (Figure 4A). Following Taxol treatment, Ki67 mRNA levels were non-detectable in LUHMES neurospheres (Figure 4A). Raw values for ddPCR are shown in table 9. To confirm protein expression of DAergic markers, LUHMES neurospheres were stained for α-syn and TH at Differentiation Day 14 and 19, and expression of both proteins was confirmed (Figure 4B). Expression of TH, DAT, and VMAT2 protein was confirmed at Differentiation Day 12 by western blot (Figure 4C).

Table 3.9. ddPCR values for LUHMES Cells (Average Copies per 20µl)

Differentiation Day	TH	DAT	VMAT2	α-Syn	Ki67
0	0.467	891.333	0.000	2.4	21080.000
5	607.333	794.000	52.667	26.667	32.400
8	50.667	108.000	90.667	129.333	27.200
11	8.533	56.000	146.667	526.667	23.800
13	9.067	38.667	162.000	931.333	88.000
16	18.200	64.000	217.333	2100.00	35.200
19	16.800	23.467	127.067	1004.00	28.933

Confirmation of DAergic Differentiation in SH-SY5Y Neurospheres

To confirm that differentiated SH-SY5Y neurospheres develop a DAergic-like phenotype, we also assessed these cells for mRNA and protein expression of the same markers. SH-SY5Y neurospheres showed detectable mRNA expression for TH, VMAT2, and α-syn throughout differentiation (Figure 5A). However, there were no detectable levels of DAT at any timepoint in SH-SY5Y cells (Figure 5A). SH-SY5Y neurospheres express the proliferation marker of Ki67 throughout differentiation (Figure 5A). Raw values for ddPCR is shown in table 10. Protein expression for TH and α-syn was verified with immunocytochemistry at Differentiation Days 14 and 19 (Figure 5B).

Table 3.10. ddPCR Values for SH-SY5Y Cells (Average Copies per 20µl)

Differentiation Day	TH	DAT	VMAT2	α-Syn	Ki67
8	21.364	0.000	84.467	1877.333	7153.333
11	1.867	0.000	6.667	2496.667	6184.000
14	59.547	0.000	560.667	1739.333	8446.667
18	148.104	0.000	4146.667	1142.667	7013.333
22	328.741	0.000	6593.333	810.667	7180.000

MPP+ induces toxicity in LUHMES, but not SH-SY5Y, neurospheres

LUHMES neurospheres were treated with various concentrations of MPP^+, the active metabolite of MPTP at Differentiation Day 13, a timepoint where DAT is expressed. Toxicity was measured with ATP assays 48 hours post MPP^+ treatment, showing a concentration-dependent decrease in ATP content in LUHMES neurospheres (IC_{50}=17.08µM) (Figure 6A). MPP^+-induced toxicity was also measured in neurite

outgrowth assays, which showed a concentration-dependent decrease in normalized neurite outgrowth (IC_{50}=54.90μM) (Figure 6B-C). Consistent with a lack of DAT expression, SH-SY5Y neurospheres were not susceptible to MPP^+ treatment across the same concentrations applied to LUHMES cells or up to 1 mM MPP^+ (data not shown).

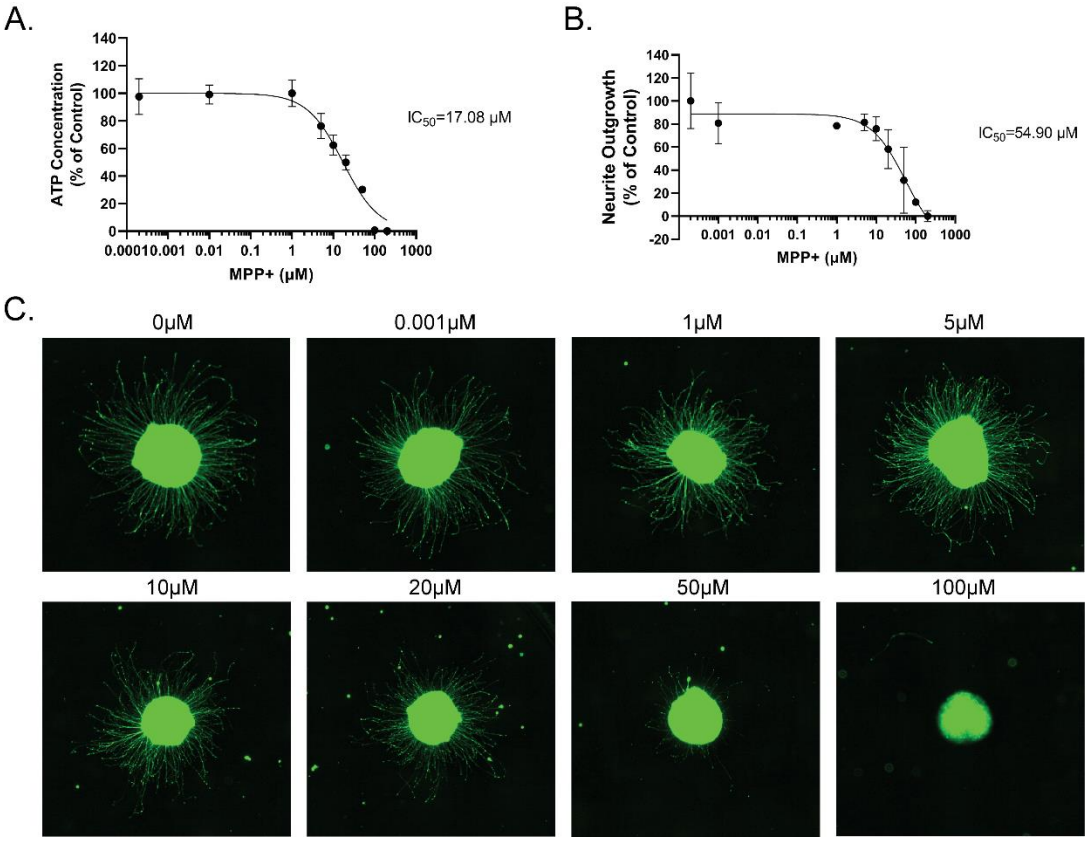

Figure 3.6. MPP^+ concentration-response curves generated from ATP and neurite outgrowth assays in LUHMES neurospheres. (A) and neurite outgrowth assays (B-C) confirm expected susceptibility of 3D LUHMES neurospheres to MPP^+. A) ATP assay. B) Neurite outgrowth assay C) Representative neurite outgrowth images in MPP^+ treated LUHMES neurospheres. Graphs show mean ± SD for 3 independent experiments.

Increase in detergent-insoluble α-syn after application of α-syn PFFs

Next, α-syn PFFs were added to neurospheres at Differentiation Day 9. This time point was selected because the cells express α-syn and expression of endogenous α-syn protein is required for PFF-induced seeding. Samples were collected at three-time

points post-PFF treatment and analyzed for Triton-soluble and insoluble α-syn. One of the features of α-syn aggregates is that they are insoluble in Triton X-100 detergent. As expected, there was a concentration-dependent increase in Triton-insoluble α-syn protein in both LUHMES and SH-SY5Y neurospheres at each timepoint post-treatment (2, 5, and 10 days) (Figure 7). However, it appears that there is a decrease in Triton-soluble α-syn was also detected in all samples.

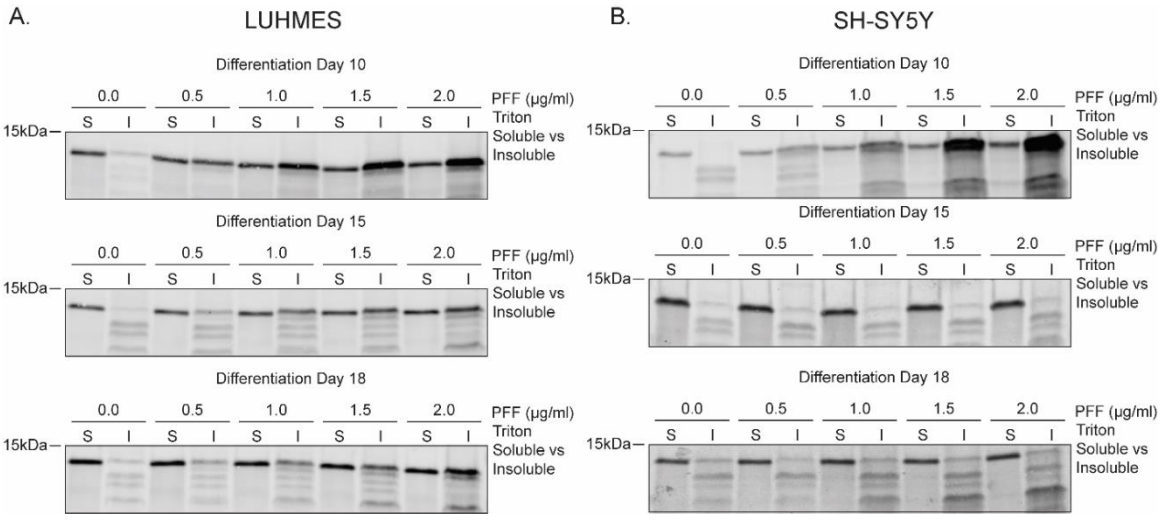

Figure 3.7. α-synuclein PFF treatment induces a concentration-dependent increase in Triton-insoluble α-synuclein in both LUHMES and SH-SY5Y neurospheres. Representative western blots for Triton soluble and insoluble α-synuclein Differentiation Days 10, 15, and 18 (2,7, and 10 days post-PFF treatment respectively) in A) LUHMES and B) SH-SY5Y neurospheres.

Assessment of α-Synuclein PFF-induced toxicity

To determine if α-syn PFF treatment is toxic in 3D LUHMES and SH-SY5Y neurospheres, we collected samples for ATP assays at Differentiation Day 18 (20 days after treatment). However, there were no significant effects of α-syn PFFs on ATP concentrations in either cell line at concentrations that induced Triton-insoluble α-syn formation in either LUHMES or SH-SY5Y neurospheres (Figure 8).

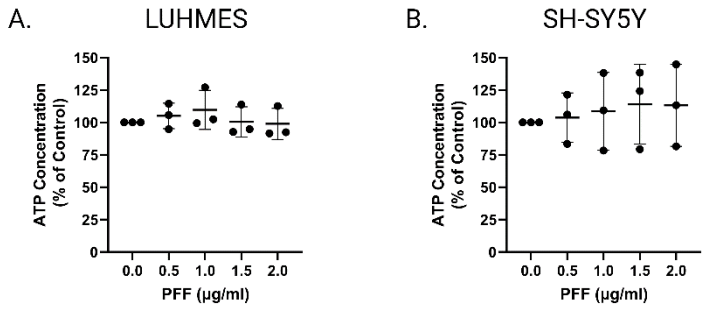

Figure 3.8. α-synuclein PFF treatment does not induce toxicity in LUHMES or SH-SY5Y neurospheres. ATP concentration at Differentiation Day 18 (10 days post-PFF treatment) in A) LUHMES and B) SH-SY5Y neurospheres. Data is shown as percent of control. Graphs show mean ± SD for 3 independent experiments.

Discussion

α-synuclein-PFFs may increase detergent insoluble α-synuclein but do not induce toxicity

The goal of this project was to adapt the α-syn PFF model to 3D DAergic neurospheres. However, while we observed an initial increase in detergent-insoluble α-syn, seeding does not appear to have occurred and there is no toxicity associated with PFF treatment in either LUHMES and SH-SY5Y neurospheres (Figures 7 and 8). In this model, we did not observe any effects of PFFs on ATP concentration in either cell line at concentrations where we saw insoluble α-syn (Figure 7). We suspect that this is because α-syn PFFs did not seed the formation of aggregates of endogenous α-syn intracellularly for multiple possible reasons.

First, we may be detecting insoluble α-syn binding to the membrane of cells rather than internalized aggregates. Second, PFFs may be internalized, but levels of endogenous α-syn in neurites are too low to allow efficient seeding; the expression level of endogenous α-syn is known to be a critical factor for successful seeding. To model synucleinopathy in these cells, future studies could potentially utilize cells

overexpressing α-syn. However, the supraphysiological α-syn in overexpression levels has the potential to result in non-PD related pathophysiological effects, potentially reducing the translatability of this model (Duffy et al. 2018).

Third, because PFF uptake into cells requires specific proteins at the synaptic terminals, these neurospheres may not have well-developed synapses and lack the machinery to take up PFFs to seed aggregation of endogenous α-syn. Unfortunately, while it is possible to assess if the PFFs were properly internalized in the neurospheres using fluorescently tagged PFFs to visualize and confirm PFF uptake, this is technically beyond the scope of this project (Karpowicz et al. 2017; Valdinocci et al. 2017). To maintain host compatibility, another critical component of successful seeding by PFFs, we used human PFFs in human cells, which also makes it difficult to differentiate endogenous from exogenous α-syn (Luk et al. 2016). The use of PFFs generated from another species could enable the differentiation of endogenous from exogenous α-syn, but seeding efficiency would have likely been greatly reduced. Together, our data suggests that adapting the α-syn PFF model to 3D LUHMES or SH-SY5Y neurospheres faces significant technical challenges and may require additional manipulations that would also reduce its translational potential.

3D LUHMES neurospheres as a model for assessing DAergic toxicity

Here, we used protocols adapted from previous work to enhance the differentiation of LUHMES neurospheres. We replicated and extended the characterization of the DAergic-like phenotype in 3D LUHMES neurospheres by measuring RNA and protein expression of key DAergic markers (TH, DAT, and VMAT2) up to Differentiation Day 19 (Shipley et al. 2016; L. Smirnova et al. 2016; Harris et al. 2017b; Harischandra et al.

2020)(Figure 4). Previous work did not characterize the DAergic phenotype of 3D LUHMES in longer-term cultures such as day 19, which is essential when using long-term models such as the PFF model. In LUHMES neurospheres, TH and DAT RNA levels peak early in differentiation and decrease over time. However, western blot analysis shows that TH and DAT protein levels are still detectable at Differentiation Day 12 and ICC shows TH expression at Differentiation Day 19 (Figure 4C). In addition, the susceptibility of these cells to MPP^+ at Differentiation Day 13 confirms that DAT protein is expressed at this time point (Figure 6). This discrepancy between RNA and protein levels is consistent with the established feedback loops between DA levels and expression of TH and DAT through mechanisms including D2 autoreceptors activation and feedback inhibition by DA itself (Sulzer et al. 2010; Daubner et al. 2011; Chen et al. 2020). As expected, Ki67 RNA levels were undetectable after Taxol treatment indicating that these neurospheres are no longer proliferating, consistent with a mature neuronal phenotype. Expanding on previous characterizations, we demonstrated that these cells also express α-syn RNA and protein, further supporting their utility in studying mechanisms relevant to PD (Figure 4). Together, this data adds to the growing body of evidence supporting the use of 3D LUHMES neurospheres for studies of DAergic toxicity.

The relevance of SH-SY5Y cells is highly dependent on growth and differentiation conditions

In contrast to the LUHMES cells, 3D SH-SY5Y neurospheres differentiated in suspension do not express DAT and are not susceptible to the DAergic toxicant MPP^+ (Figure 5A). They are also not post-mitotic and continue to divide, as demonstrated by

sustained Ki67 expression and increasing size from Differentiation Day 14 to 19 (Figure 5A). Other groups have reported inconsistent expression of DAT in SH-SY5Y cells (Presgraves et al. 2004; Song et al. 2012; Wang et al. 2014; Geng et al. 2017; Ito et al. 2017; Ko et al. 2020; Limboonreung et al. 2020) (Figure 5). Together, this suggests that 3D SH-SY5Y neurospheres differentiated with this protocol may not be a useful model of DA neurons.

Consistent with DAT expression in LUHMES cells but not SH-SY5Y cells, we showed that LUHMES, but not SH-SY5Y neurospheres, are susceptible to MPP^+-induced toxicity (Figure 5). Because DAT is required for MPP^+ entry, and our SH-SY5Y neurospheres do not express DAT mRNA at any time point with this differentiation protocol, it is likely that MPP^+ is not taken up into these cells (Figure 5). Other groups have demonstrated MPP^+ toxicity in SH-SY5Y cells using different differentiation and growth conditions with MPP^+ concentrations ranging from 2mM to 5mM. Adherent SH-SY5Y cells grown in 2D are susceptible to MPP^+ using assays for mitochondrial function, apoptosis, and ATP concentration (Presgraves et al. 2004; Song et al. 2012; Wang et al. 2014; Geng et al. 2017; Limboonreung et al. 2020). 3D SH-SY5Y neurospheres grown in Matrigel rather than in suspension have also been shown to be susceptible to high concentrations of MPP^+, although the media and differentiation conditions used were different than the conditions used here (Ko et al. 2020). In addition, DAT expression appears to be variable between differentiation protocols, 2D vs 3D models, and research groups which likely contributes to the inconsistencies in susceptibility to MPP^+ (Presgraves et al. 2004; Song et al. 2012; Wang et al. 2014; Geng et al. 2017; Ito et al. 2017; Ko et al. 2020; Limboonreung et al. 2020)

Here, we used a stepwise decrease in FBS, and additional media supplements intended to improve the differentiation of SH-SY5Y cells (Neurobasal growth media, B27 supplement, and cAMP). Lastly, the differentiation protocol reported here for SH-SY5Y neurospheres includes an 18-day retinoic acid differentiation protocol rather than a 9-day retinoic acid protocol used in the Ko et al. paper. Overall, our methods for SH-SY5Y neurospheres involve a more complex differentiation and spheroid formation protocol. Using this SH-SY5Y protocol there is an increase in TH expression, which is a crucial marker for a DAergic phenotype. Previous work was unable to detect any TH using their model of differentiation, suggesting that the cell population was not DAergic (Ko et al. 2020). However, while we did detect TH in these cells, we did not detect another key DAergic marker, DAT, at any time point (Figure 5). In addition, these cells continue to proliferate, as indicated by sustained levels of Ki67 at all time points. Together, this indicates that using this protocol, differentiated SH-SY5Y cells are not mature post-mitotic, DAergic-like neurons.

Therefore, the differentiation and spheroid formation protocol reported here only incrementally enhanced the DAergic-like differentiation of SH-SY5Y cells and allowed us to form neurospheres grown in suspension. We were unable to demonstrate MPP^+-induced toxicity in our model of SH-SY5Y neurospheres, although we did not test extremely high concentrations of MPP^+. Many groups use SH-SY5Y cells to study Parkinson's disease and related neurodegeneration, however, the LUHMES neurosphere model is better at recapitulating a DAergic phenotype and is also susceptible to the DAergic-toxicant, MPP^+ at physiologically relevant concentrations. Therefore, the SH-SY5Y neurosphere model is not suitable for modeling PD-like

degeneration or related toxicity due to the lack of DAergic phenotype (lacking DAT expression and high Ki67 proliferation markers) and because of its resistance to MPP^+. We also demonstrate here, that neither LUHMES nor SH-SY5Y neurospheres are susceptible to PFF-induced toxicity.

TABLES AND FIGURES

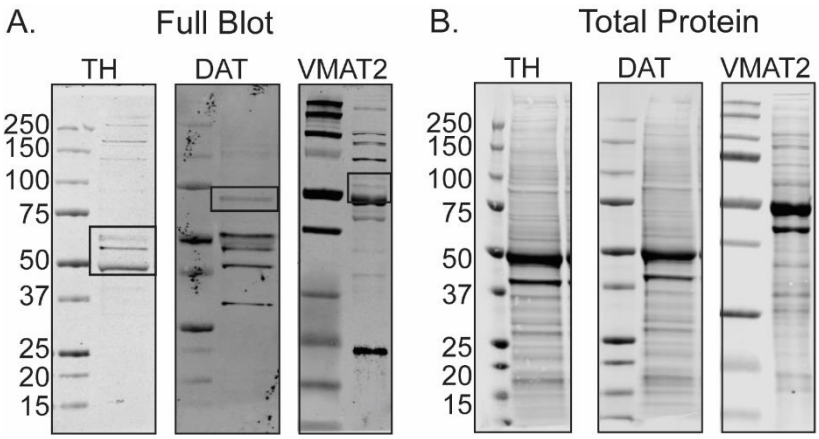

Figure S3.1. Full western blots and total protein staining for Figure 4. A) Full representative blots for and B) Representative Revert stain.

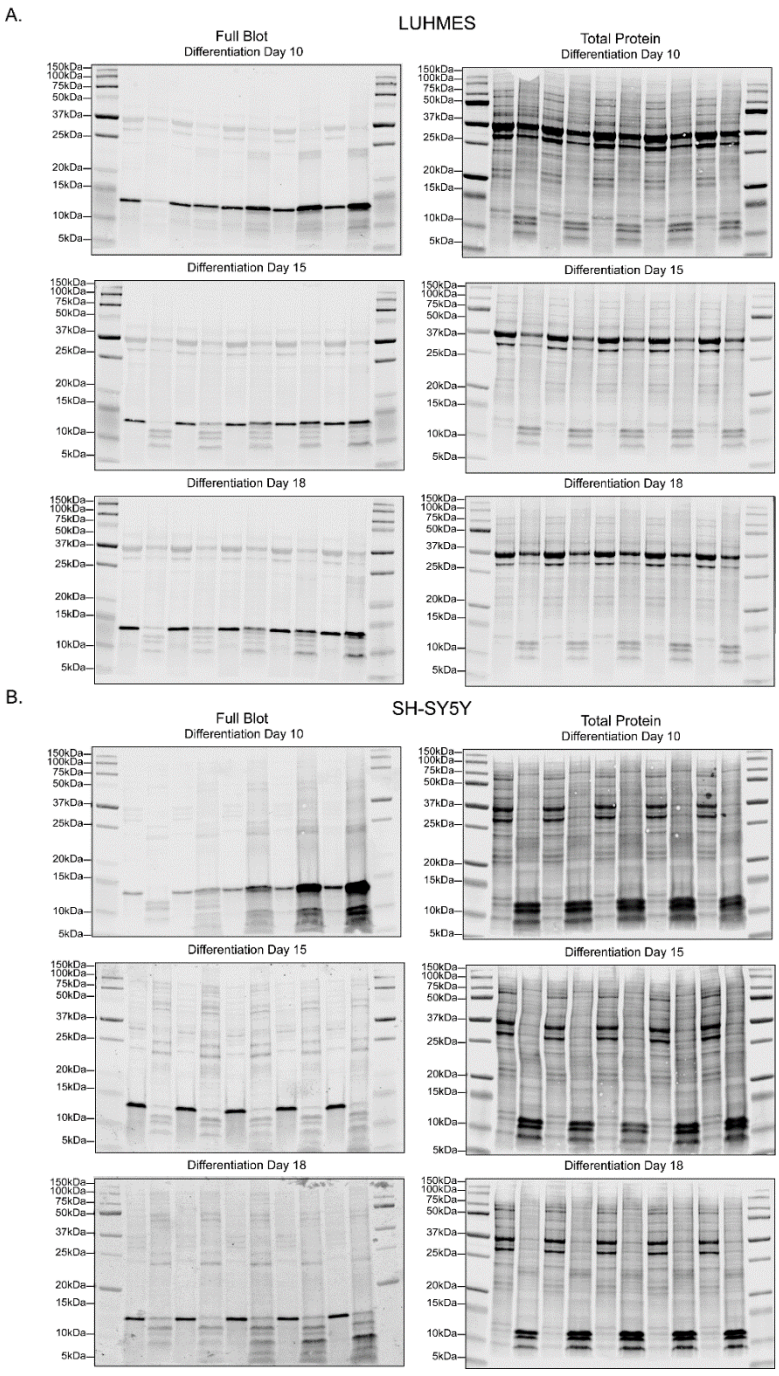

Figure S3.2. Full western blots and total protein staining for Figure 7. Full representative blots for A) LUHMES and B) SH-SY5Y Representative Revert stain for A) LUHMES neurospheres treated with PFFs and B) SH-SY5Y neurospheres treated with PFFs.

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Chapter 4: EPHB2 and NR4A2 regulate dopaminergic differentiation and markers of dopaminergic vulnerability in neurospheres, but not MPP^+-induced toxicity

Abstract

Multiple lines of evidence show an association between exposure to persistent organic pollutants (POPs) and an increased risk of Parkinson's disease (PD). Dieldrin, an organochlorine pesticide, is a specific POP that is associated with an increased risk of PD in both epidemiological and mechanistic studies. In a series of previous studies, we and others demonstrated that developmental exposure to dieldrin in mice leads to a male-specific increase in susceptibility in both the MPTP and α-synuclein pre-formed fibril models. Because epigenetic marks are sensitive to the environment, established during cellular differentiation, and regulate gene expression throughout the lifespan, we characterized DNA modifications induced by developmental dieldrin exposure. We identified sex-specific changes in DNA modifications in genes related to dopaminergic (DAergic) neuron development, suggesting that this exposure establishes a poised epigenetic state early in life that may mediate the observed changes in susceptibility later in life. To model this *in vitro*, we utilized a 3D Lund human mesencephalic (LUHMES) neurosphere model, where we overexpress or knockdown differentially modified target genes prior to differentiation and assess susceptibility to 1-methyl-4-phenylpyridinium (MPP^+), a DAergic toxicant. Here, we focus on two candidate genes that are differentially modified by developmental dieldrin exposure: nuclear receptor subfamily group A member 2 (*Nr4a2),* a transcription factor critical for DAergic development, and ephrin receptor B2 (*Ephb2), a* receptor tyrosine kinase involved in axonal guidance and survival. We show that altered *NR4A2 and EPHB2* expression disrupts the DAergic phenotype of 3D LUHMES neurospheres and alters the ratio of the dopamine transporter (DAT) to the vesicular monoamine transporter 2 (VMAT2), a

marker of DAergic susceptibility to toxicity. However, there were no observed effects of these candidate genes on MPP^+-induced toxicity. Therefore, we have modeled developmental dieldrin-induced differentially modified candidate genes and showed that modified expression of *NR4A2* and *EPHB2* affects DAergic differentiation. Although there were no overt effects on MPP^+-induced toxicity, these subtle changes may contribute to DAergic dysfunction and the many factors involved in parkinsonian toxicity.

Introduction

Epidemiological studies show an association between exposure to persistent organic pollutants and an increased risk of Parkinson's disease (PD) (Ascherio et al., 2006; Brown et al., 2006; Caudle et al., 2012; Elbaz et al., 2009; Freire & Koifman, 2012; Le Couteur et al., 1999; Priyadarshi et al., 2000; Ritz & Yu, 2000; Semchuk et al., 1992; Steenland et al., 2006; Tanner & Aston, 2000; Tanner & Langston, 1990; Wirdefeldt et al., 2011). When combined with postmortem analysis and mechanistic studies, a role for specific compounds in PD emerges. One such compound is dieldrin, an organochlorine (OC) pesticide that is associated with an increased risk of PD in both epidemiological and mechanistic studies (Corrigan et al., 1998, 2000; Fleming et al., 1994; Hatcher et al., 2007; Kanthasamy et al., 2005; Moretto & Colosio, 2011; Weisskopf et al., 2010). Because dieldrin was phased out in the 1970s and 1980s, the potential for new, acute exposure to dieldrin is low. However, the health effects of past exposures will continue for decades as the population currently diagnosed with PD and those that will develop PD in the next 20-30 years were likely exposed to dieldrin before its phase-out (de Jong et al., 1997; Jorgenson, 2001; Kanthasamy et al., 2005; Meijer et al., 2001). Furthermore, well-established models of dieldrin exposure have demonstrated that dieldrin induces oxidative stress, is selectively toxic to dopaminergic (DAergic) cells,

disrupts striatal dopamine (DA) activity, and may promote α-syn aggregation (Chun et al., 2001; Hatcher et al., 2007; Kanthasamy et al., 2005; Kitazawa et al., 2001, 2003; Moretto & Colosio, 2011; Richardson et al., 2006; Sanchez-Ramos et al., 1998).

The epigenome is a potential mediator of the relationship between exposures, genes, and disease. Multiple studies suggest that exposure to OCs and dieldrin can induce epigenetic changes. Organochlorines are associated with hypomethylation of repetitive DNA elements in blood from Greenlandic Inuit (Rusiecki et al., 2008). In mice, dieldrin induces histone hyperacetylation in the striatum and substanita nigra (SN) of mice treated for 30 days (Song et al., 2010). We also recently found that developmental dieldrin exposure establishes a sex-specific poised epigenetic state early in life that may mediate observed changes in susceptibility to neurotoxicity in the parkinsonian models, MPTP and α-synuclein (α-syn) pre-formed fibrils (PFF), in adult animals (Gezer et al., 2020; Kochmanski et al., 2019; Richardson et al., 2006) Thus, dynamic environmental exposures to these compounds may induce fixed changes in the epigenome, creating a poised epigenetic state in which developmental exposures program a modified response to later-life challenges. As PD prevalence grows, it is critical to define how environmental exposures affect the epigenetic mechanisms involved in disease susceptibility and etiology (Dorsey et al., 2007).

The mechanism by which these epigenetic changes may alter PD risk remains unknown. This is a common knowledge gap in neuroepigenetic studies; as a field, we can identify epigenetic changes associated with disease but linking these changes with functional outcomes and altered susceptibility remains a challenge. Here, we modified the expression of two candidate genes in proliferating Lund human mesencephalic

(LUHMES) cells before induction of differentiation to DAergic-like neurons. After differentiation, a second hit with 1-methyl-4-phenylpyridinium (MPP^+), a DAergic toxicant and the active metabolite of MPTP, was used to parallel the *in vivo* two-hit models to assess whether each candidate gene mediates neuronal susceptibility.

LUHMES cells can be differentiated into morphologically and biochemically mature dopamine-like neurons and are increasingly used for *in vitro* research (Efremova et al., 2015; Krug et al., 2014; Lotharius et al., 2002, 2005; Noelker et al., 2015; Oliveira et al., 2015; Pöltl et al., 2012; Schildknecht et al., 2013; Scholz et al., 2011; Tong et al., 2017; X. M. Zhang et al., 2014). 3D LUHMES neurospheres were developed as a high-throughput toxicity screening platform to take advantage of the fact that 3D cell models show better differentiation and survival (Harris et al., 2017; Smirnova et al., 2016; Tong et al., 2017). In addition, LUHMES cells have well-established use and display robust performance in high-throughput neurotoxicity studies, including studies of rotenone, another PD-related neurotoxicant (Beliakov et al., 2023; Harris et al., 2018; Hogberg & Smirnova, 2022; Nicolai et al., 2022; Tong et al., 2017).

To select candidate genes, we filtered the 288 genes with female-specific differential modifications and 83 genes with male-specific differential modifications for genes with confirmed expression in 1) neurons (Brain RNA-Seq database) to match our neuronal cell model, 2) midbrain based on our previous RNA-Seq data, and 3) undifferentiated 3D LUHMES cells cultures to ensure we can modify these genes in our model, 180 genes remained (Kochmanski et al., 2019; Pierce et al., 2018; Y. Zhang et al., 2014). We then considered a priori knowledge of the genes, whether genes showed differential expression in our previous RNA-Seq data, network analysis of these 180 genes in

StringDB, and the function of the genes in those networks (Szklarczyk et al., 2015, 2017). Based on these additional criteria, we selected a set of candidate genes. Here, we report results for nuclear receptor subfamily 4 group A member 2 (*NR4A2*) and Ephrin Receptor B2 (*EPHB2).*

NR4A2 encodes the nuclear receptor related-1 (Nurr1) protein, a transcription factor critical for DAergic neuron maintenance and development that may contribute to the pathogenesis of PD (Decressac et al., 2013; Dong et al., 2016; Luo, 2012; Smits et al., 2003). Both clinical and experimental data demonstrate that dysregulation of Nurr1 function leads to DA neuron dysfunction (Decressac et al., 2013). In addition, compounds that activate Nurr1 protein and *Nr4a2* gene therapy can enhance DA neurotransmission and protect DA neurons from toxicant and microglia-mediated neuroinflammation (Dong et al., 2016). Data in rat midbrain, human midbrain, and the human neuroblastoma cell line, SK-N-AS, demonstrate that there are multiple Nurr1 splice variants expressed in these cells and these isoforms have variable ability to activate transcription of target genes including DAT and TH, suggesting that regulation of isoform-specific expression could be a critical regulatory mechanism in DA neurons (Michelhaugh et al., 2005). In our developmental dieldrin exposure study, we identified a female specific-hypermethylated site within an intron *Nr4a2*, making epigenetic regulation of *Nr4a2* isoform by environmental toxicants, a novel potential mechanism by which developmental exposure to dieldrin may alter PD susceptibility (Kochmanski et al., 2019).

EPHB2 encodes Ephrin type-B receptor 2 (Ephb2), a receptor tyrosine kinase that binds the receptor-binding domain of ephrin-B ligands; these proteins communicate across

extracellular space allowing for cell-cell bidirectional signaling (Martínez & Soriano, 2005). Ephrins and the Eph receptors show dynamic expression patterns in the developing central nervous system (CNS) and are expressed in most adult CNS cell types (Yang et al., 2018). The *EPHB2* signaling pathway is involved in many developmental processes in the CNS, including migration of neural progenitors to the dentate gyrus, regulation of axon guidance, dendritic spine formation, glutamatergic synaptogenesis, and long-term potentiation (Catchpole & Henkemeyer, 2011; Flanagan & Vanderhaeghen, 1998; Henderson et al., 2001; Kayser et al., 2006; Takasu et al., 2002). We observed female-specific intronic DMC in *Ephb2* and increased expression of a protein-coding transcript (Kochmanski et al., 2019). In our String network analysis, *Ephb2* appears in a highly connected Rho GTPase network, with specific genes showing differential methylation in our developmental dieldrin exposure model. While previous results demonstrate an association between *EPHB2* and Alzheimer's disease, little work has investigated whether *EPHB2* plays a role in PD (Cissé et al., 2011; Simón et al., 2009).

In these experiments, we overexpressed and knocked down each candidate gene and assessed effects on the expression of key DAergic markers in differentiated LUHMES cells including tyrosine hydroxylase (TH), the dopamine transporter (DAT), and the vesicular monoamine transporter 2 (VMAT2). The effects of candidate gene modification on DAergic toxicity to MPP^+ were also assessed. Here, we showed that *NR4A2* and *EPHB2* gene modifications during proliferation affected DA markers after differentiation, and *EPHB2* knockdown significantly altered the ratio of DAT:VMAT2, which is a key

indicator of DAergic susceptibility to toxicity. However, there was no effect of *NR4A2* or *EPHB2* gene modification on MPP⁺-induced toxicity in a 3D LUHMES model.

Methods

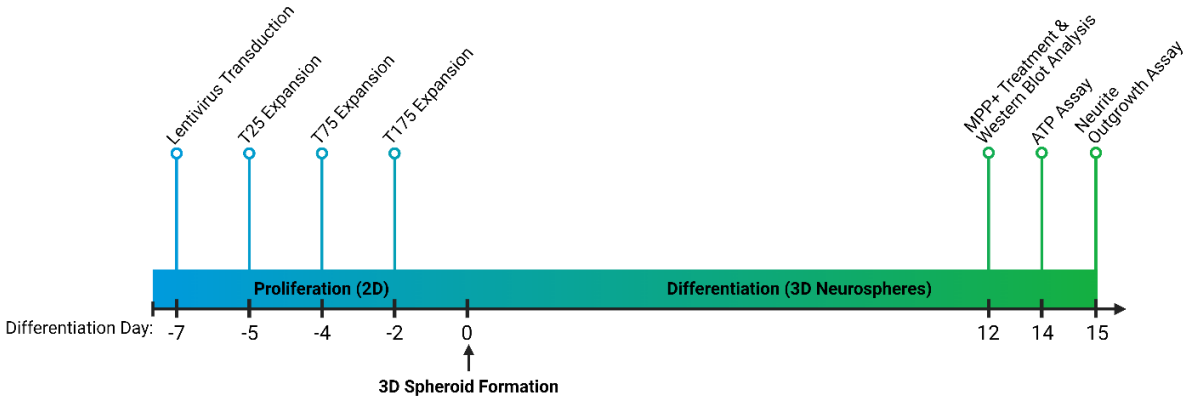

Figure 4.1. Experimental design for the current study. B) Timeline of lentivirus-mediated gene modification and MPP⁺ two-hit experiments. During proliferation (D-7), LUHMES cells are transduced with a lentivirus to knockdown or overexpress target genes. Transduced cells are expanded in 2D for one week. At D0, cells are trypsinized and seeded into 6-well plates to begin spheroid formation and dopaminergic differentiation. At D0, samples are collected to verify knockdown or overexpression. At Differentiation Day 12, each well of neurospheres are treated with a different concentration of MPP+ and samples are also collected to determine the dopaminergic differentiation of the neurospheres. 48-hours post-MPP⁺ treatment, 8 neurospheres for each concentration of MPP⁺ are transferred to a Matrigel-coated plate and ATP assays are completed the remaining spheroids in the MPP⁺-treated 6-well plate. On Differentiation Day 15, spheroids on growing the Matrigel-coated plate are live-stained and imaged. Made in BioRender.

Animals

Male (11 weeks old) and female (7 weeks old) C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Animal husbandry and colony maintenance was completed as previously described (Kochmanski et al. 2019; Gezer et al. 2020). All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Michigan State University. Adult female mice (8 weeks old) were

bred, and offspring were euthanized at 3 months of age. Animals were euthanized by pentobarbital overdose and intracardially perfused with 0.9% cold saline followed by cold 4% paraformaldehyde perfusions for 30 minutes. Brains were extracted, post-fixed in 4% paraformaldehyde for 48 hours, and placed in 30% sucrose.

RNAscope with co-immunofluorescence

Mouse nigral sections were cut at 40 µm on a freezing stage sliding knife microtome and stored in cryoprotectant solution at -20°C. Sections were washed in TBS-Triton-X (TBS-Tx) several times, incubated in 0.3% hydrogen peroxide for 1 hour mounted on a Histobond+ adhesion slide (VWR), and dried on a slide warmer. Slides were washed in TBS-X several times and incubated at 60°C overnight. Slides were incubated in antigen retrieval buffer (ACD Biosciences) for 10 minutes, rinsed, and incubated in protease III solution (ACD BioSciences) for 30 minutes and washed with water. Slides were then incubated with an RNA scope probe (Mm-NR4A2-C1: ACD BioSciences) diluted 1:50 for 2 hours at 40°C. Two washes were performed using wash buffer (ACD BioSciences) and then incubated with amplification solution 1 for 30 minutes, washed, incubated in amplification solution 2 for 15 minutes, washed, incubated in amplification solution 3 for 30 minutes, washed, and incubated in amplification solution 4 Alt B-Fluorophore Atto 550 (ACD BioSciences) for 15 minutes. For co-immunofluorescence, slides were rinsed, blocked in 10% normal goat serum in TBS-Tx for 1 hour, and incubated in primary antibodies (TH and NeuN) diluted in 1% NGS in TBS-Tx overnight at room temperature (Table 1). Slides were washed and incubated in secondary antibodies diluted in 1% TBS-Tx for 2 hours, washed, and coverslip with VectaShield Vibrance (VectaLabs). Slides were imaged on an Axioscan 7 (Zeiss).

Table 4.1. Assays and Antibodies used for Fluorescent RNAscope

Antibody	Host	Supplier	Dilution	RRID
Mm-Nr4A2-C1		ACD BioSciences 423351	1:50	SCR_012481
Tyrosine Hydroxylase	Rabbit	Millipore AB152	1:400	AB_390204
Neuronal Nuclei	Chicken	Sigma ABN91	1:200	AB_11205760
Goat-anti-Rabbit IgG (H+L) Cross-Absorbed Alexa Fluor 488	Goat	Invitrogen A11008	1:250	AB_143165
Goat-anti Chicken IgG (H+L) Cross-Absorbed Alexa Fluor 647	Goat	Invitrogen A21469	1:250	AB_2535872

LUHMES Cell Culture

LUHMES cells (ATCC CRL-2927, RRID: CVCL_B056) were grown as previously described with modifications to the differentiation media supplements. (Harris et al., 2017, 2018; Leite et al., 2019; Smirnova et al., 2016; Tong et al., 2017) For proliferation, cells were grown in 6-well plates or flasks coated with 50 µg/ml poly-L-ornithine and 1 µg/ml fibronectin. Proliferation Media was completely exchanged every other day, and cells were passaged every 3-4 days (Table 1).

For differentiation, cells were trypsinized with TrypLE Express (Gibco) and seeded at 2.25 x 10^6 cells/ml in cell-repellent 6-well plates (Corning) in 2 ml/well Differentiation Media and this was designated Differentiation Day 0 (Harischandra et al., 2020) (Table 2). The 6-well plates were placed on an orbital shaker at 90 rpm in an incubator (37°, 5% CO_2) for the remainder of the experiment. Two days after seeding the 6-well plates (Differentiation Day 2), 1 ml of media was exchanged for 1 ml of Differentiation Media with 20 nM paclitaxel (Sigma-Aldrich) to inhibit proliferation. Two days after paclitaxel treatment (Differentiation Day 4), 1.8 ml of media was removed from each well and

replaced with 2 ml of differentiation media to wash out paclitaxel. After the paclitaxel washout, half media changes were completed every other day for the remainder of the experiment as previously described (Harris et al., 2017, 2018; Leite et al., 2019; Smirnova et al., 2016; Tong et al., 2017).

Table 4.2. LUHMES Proliferation Media

Reagent	Vendor	Catalog Number	Final Concentration
DMEM/F12 Base Media	Invitrogen	12-634-028	-
L-Glutamine	Gibco	A2916801	2 mM
N2 Supplement	Gibco	17502048	1 X
Recombinant Fibroblast Growth Factor	R&D Systems	5114-TC	40 ng/mL

Table 4.3. LUHMES Differentiation Media

Reagent	Vendor	Catalog Number	Final Concentration
DMEM/F12 Base Media	Invitrogen	12-634-028	-
L-Glutamine	Gibco	A2916801	2 mM
N2 Supplement	Gibco	17502048	1 X
N6,2′-O-Dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt	Sigma-Aldrich	D0627	100 mM
Tetracycline	Sigma-Aldrich	T7660	1 µg/mL
Recombinant Glial Cell Line Derived Neurotrophic Factor	R&D Systems	212-GD	20 ng/ml
Brain Derived Neurotrophic Factor	R&D Systems	11166-BD-050	10 ng/ml
Human TGF-Beta3 Recombinant Protein	Invitrogen	RP-8600	20 ng/ml
Recombinant Human Leukemia Inhibitory Factor	Gibco	PHC9484	10 ng/ml

Lentivirus Production

siRNAs targeting EPHB2 (GCCCAAGTTCGGCCAAATTGT) and NR4A2 (CTCCAGAGTTTGTCAAGTTTA) were designed using algorithms as previously described (Benskey et al., 2015; Toro Cabrera & Mueller, 2016). Scrambled control siRNAs containing the same nucleotide composition as target siRNAs in random order were also produced, scrambled EPHB2 (ATTAGTCGCTAAGTCAGGACC) and scrambled NR4A2 (GATATTACCGATATTGTCGCT). siRNAs oligonucleotides were cloned into an shRNA backbone downstream of the H1 promoter and the knockdown

efficiency of each respective shRNA was tested using a dual luciferase assay (Promega Dual-Luciferase Reporter Assay System) (Benskey et al., 2015). Following screening, the shRNAs that produced the highest degree of knockdown, and corresponding scrambled control shRNAs, were subcloned into a lentiviral genome that also contained a blue florescent protein (BFP) reporter gene. For overexpression studies, human EPHB2 and NR4A2 cDNA were cloned into a lentiviral genome under the control of the control of chicken β-actin (CBA) promoter. Overexpression vectors contained an NE tag on the N-terminus (Shu Leong Ho Labs). The control condition for the overexpression was untreated cells.

Lentiviruses were packaged through the co-transfection of human embryonic kidney 293T (HEK293T) cells with a pHEF VSV-G pseudotyping envelop vector (Addgene #22501), a pNHP packaging vector (Addgene #22500), and the lentiviral genome containing the respective shRNAs, or cDNAs (Coleman et al., 2003). Briefly, HEK293T cells were grown to 70% confluency in 3x150mm culture dishes in 293T (ATCC CRL-3216) medium containing 5% FBS, penicillin/streptomycin in DMEM base media. Polyethyleneimine (PEI) was used to transfect HEK293T cells with the above plasmids and cells were at 37°C and 5% CO2. 48 hours post-transduction, all media was aspirated and replaced with 26ml of viral media containing 2% FBS, Penicillin/streptomycin using DMEM base media. 24 hours after the media change, producer cell media was harvested and an additional 26ml of viral media was added to the 293T cells. Harvested media was concentrated by first removing cellular debris via a 675 xg 5-minute centrifugation spin and filtering the supernatant via a 0.45 µm filter to clarify the media. Media was added to 3,35ml ultracentrifugation tubes and underlaid

with 2ml of a 20% sucrose solution and centrifuged at 82,700 x g for 2 hr at 4°C. The pellets were collected in 500µl of PBS. 24 hours later, producer media was again collected, harvested, clarified, and concentrated as stated above, and the pellet was resuspended and combined with the same 500µl lentivirus containing PBS solution from the previous day. Lentiviruses were mixed well, aliquoted, and stored at -80°C (Coleman et al., 2003; Combs et al., 2021). Lenti X P24 Rapid Titration Elisa Kit (TaKaRa bio) was used according to the manufacturer's protocol to determine lentiviral physical titers.

Lentivirus Transduction in LUHMES Cells

LUHMES cells were seeded in poly-L-ornithine and fibronectin-coated 6-well plates at a density of 300,000 cells/well in 2 ml of proliferation media. 24 hours after seeding, media was removed from wells and replaced with 1.5ml of proliferation media. After, lentivirus was diluted in PBS and added to each well at a concentration of 1.0 pg of P24 (determined by Lenti X P24 Rapid Titration Elisa) was added to the cells by evenly distributing throughout the well. 24 hours later, an additional 0.5ml of media was added to each well. 24 hours later the cells were trypsinzed and passaged to a poly-L-ornithine and fibronectin T25 flask and expanded.

MPP+ Treatment

MPP^+ was resuspended in PBS at a stock concentration of 10 mM. On the day of treatment, MPP^+ was diluted in media to make 2x the final concentration, and 1 ml of media was removed from each well of the 6-well plate and replaced with 1 ml of 2x media with MPP^+. Each well was treated with concentrations of 20 µM (ATP viability assays) or 50 µM (neurite outgrowth assays), equivalent to the previously calculated

IC_{50} for these assays in these cells (Chapter 3). At 48 hours post-treatment, 8 spheroids from each 6-well were transferred to Matrigel-coated 96-well plates for neurite outgrowth assays and the remaining spheroids in each MPP^+ treated well were used for ATP assays.

Collection of cell lysates and western blotting

To confirm DA marker protein expression (TH, DAT, VMAT2) and confirm candidate protein expression (NR4A2 or EPHB2), spheroids from 1-well of the 6-well plate were pooled, collected, processed, and frozen as stated above at Differentiation Day 12. Cell pellets were resuspended in RIPA buffer with protease inhibitors, lysates were spun at 1,000 xg for 5 minutes, and the supernatant was collected as the lysate for western blotting.

For TH, DAT, VMAT2, EPHB2, and NR4A2 (overexpression blots only) western blots, 20 µg of protein was loaded onto Novex 10% Bis-Tris gels (Invitrogen) and co-blotted with a standard curve of protein ranging from 5 µg-30 µg. For NR4A2 blots confirming NR4A2 knockdown only, 35 µg of protein was loaded onto Novex 10% Bis-Tris gels (Invitrogen) and co-blotted with a standard curve of protein ranging from 7.5µg-45µg. For TH, the bands quantified are ~50-62kDa, DAT is ~70kDa, VMAT2 is ~56kDa, NR4A2 is ~72kDa, and EPHB2 is ~130kDa.

Proteins were electrophoretically transferred to nitrocellulose membranes (BioRad). Membranes were stained with Revert 700 Total-Protein Stain (LI-COR) for 5 minutes and imaged with a LI-COR Odyssey CLx. Membranes were blocked with Odyssey blocking Buffer (LI-COR), and incubated in either NR4A2 (Invitrogen), EPHB2 (Invitrogen) TH (Millipore), DAT (Sigma), or VMAT2 primary antibodies overnight at 4°C.

After washing, membranes were incubated in goat anti-Rabbit 800CW (LI-COR) or goat-anti-Mouse IgG_1 (for EPHB2 only) for 1 hour and imaged with a LI-COR Odyssey CLx. Bands of interest were normalized to total protein revert stain and the standard curve was included in each blot.

Table 4.4. Antibodies used for Western Blotting

Antibody	Host	Supplier	Dilution	RRID
NR4A2	Rabbit	Invitrogen PA5-13416	1:1,000	AB_2153896
EPHB2	Mouse IgG_1	Invitrogen 37-1700	1:500	AB_2533302
Tyrosine Hydroxylase	Rabbit	Millipore AB152	1:1,000	AB_390204
VMAT2	Rabbit	Miller Lab	1:10,000	
DAT	Rabbit	Sigma D6944	1:1,000	AB_1840807
IRDye 800CW anti-Rabbit IgG	Goat	LI-COR Biosciences 926-32211	1:10,000	AB_621843
IRDye 800CW anti-Mouse IgG_1	Goat	LI-COR Biosciences 926-32350	1:10,000	AB_2782997

ATP Assays

The baseline effects of overexpressing and knocking down expression of *NR4A2* or *EPHB2* on cell viability (ATP assay) of LUHMES cells was assessed in the absence of MPP^+, while the impact of these gene changes on viability was assessed after exposure to 20 µM MPP^+. The 20 µM MPP^+ dose was based on previous data in our lab (Chapter 3, Figure 6) where we identified that the IC_{50} value for MPP^+ exposure in spheroids for ATP assays was near 20 µM MPP^+ (i.e 17.08 µM MPP^+).

For ATP assays, 1 ml of media from each well of the 6-well neurosphere plate was removed and replaced with 1 ml of Cell Titer-Glo 3D reagent (Promega). Neurospheres were lysed by shaking the plates at 700 rpm for 20 minutes at room temperature in a Thermomixer (Eppendorf). To avoid cellular debris, the supernatant was transferred to a white 96-well assay plate in triplicate (Corning). ATP standards were diluted in media and pipetted in triplicate in the white 96-well assay plate. The plate was incubated for 10 minutes in the dark and luminescence was read using a Synergy H1 plate reader at an integration time of 1.5 seconds and a reading height of 7.00 mm (BioTek). Blanks were subtracted and samples were calibrated to the ATP standard to calculate ATP concentration (μM). All ATP cytotoxicity assays were completed with at least 3 biological replicates from independent experiments, with 4 technical replicates per experiment.

Neurite Outgrowth Assays

The baseline effects of overexpressing and knocking down expression of *NR4A2* or *EPHB2* on neurite outgrowth of LUHMES cells were assessed in the absence of MPP^+, while the impact of these gene changes on viability was assessed after exposure to 50 μM MPP^+. The 50 μM MPP^+ dose was based on previous data in our lab where we

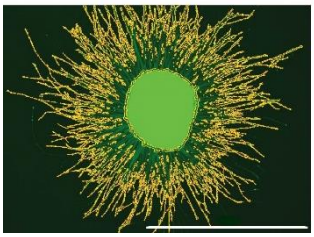

Figure 4.2. Representative neurite outgrowth analysis. Scale bar represents 1000 μM.

identified that the IC_{50} value for MPP^+ exposure in spheroids for neurite outgrowth assays was near 50 μM MPP^+ (i.e 54.90 μM MPP^+).

48 hours before neurite analysis, black 96-well optical bottom plates (Corning) were coated with growth factor reduced Matrigel® Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning) diluted 1:24 in DMEM/F12 and placed in an incubator to polymerize overnight. Following polymerization, Matrigel was aspirated and replaced with LUHMES Differentiation Media made with phenol-free DMEM/F12 (Gibco). Individual spheroids were transferred to single wells in the Matrigel-coated plate. 24 hours after plating, neurites were stained with a Neurite Outgrowth Staining Kit (ThermoFisher) for 30 minutes as directed. At the end of incubation, half of the solution was replaced with a 3x Background Suppression Dye. Neurospheres were imaged with a Lionheart Fx Automated Microscope at 4x using a GFP filter cube (BioTek). Images were then analyzed in Gen5 software. Briefly, a primary mask around the spheroid body is generated by thresholding and a secondary mask is generated by thresholding within a ring around the spheroid body. The ratio of the areas of the secondary mask to the primary mask provides a measure of neurite outgrowth (Figure 2). Each well was manually checked for the following exclusion criteria: wells containing multiple spheroids, spheroids touching the edge of the well, or spheroid or neurites no longer attached to the plate. Images were exported from Gen5 and figures were generated in Adobe Illustrator.

Statistical analysis

Independent experiments are cells from separate differentiations. After lysis for the ATP assay, the lysate is pipetted in triplicate in the white 96-well assay plate which

comprises 3 technical replicates for every independent replicate. For the Neurite outgrowth assay, 8 neurospheres were transferred from each well (concentration of MPP^+) of the 6-well plate to a Matrigel-coated 96-well plate. For western blot analysis, at least 3 biological replicates were used for every analysis. For all statistics reported here, a paired two-tailed T-test was used with a p-value cutoff of 0.05 using GraphPad Prism 9.

Results

Confirmation of NR4A2 expression in dopaminergic nigrostriatal neurons

To confirm that our candidate genes are expressed in DAergic nigrostriatal neurons, RNAscope for *NR4A2* was performed with co-immunofluorescence for tyrosine hydroxylase (TH) and neuronal nuclei (NeuN) in 3-month-old mouse nigral tissue of male mice. Here, we show that *NR4A2* mRNA is expressed in TH-immunoreactive, NeuN-immunoreactive neurons of the substantia nigra (SN). However, we determined that EPHB2 transcripts-per-million from RNA sequencing in the midbrain was 3.0 TPM is

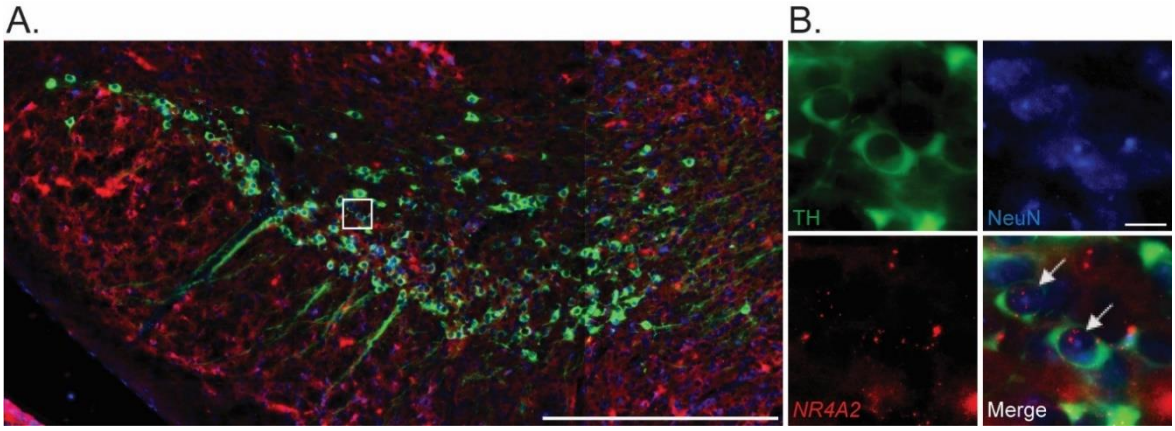

Figure 4.3. Confirmation of *NR4A2* RNA expression in dopaminergic nigrostriatal neurons. A) Substantia nigra indicating location of images in panel B. Scale bar represents 550μm. B) Higher magnification showing TH in green, NeuN in blue, *NR4A2* RNA scope in Red, and merged images. Scale bar represents 10μm.

below the limit of detection for RNAscope (Allen Brain Atlas). Therefore, *EPHB2* mRNA via RNAscope was undetectable.

Confirmation of NR4A2 overexpression

To confirm *NR4A2* overexpression, lysates were collected at Differentiation Day 12 and western blots were run and probed for Nr4a2 protein. Here, we show an approximately 3-fold increase in Nr4a2 protein in transduced LUHMES neurospheres after differentiation (*p=0.0173*) (Figure 4).

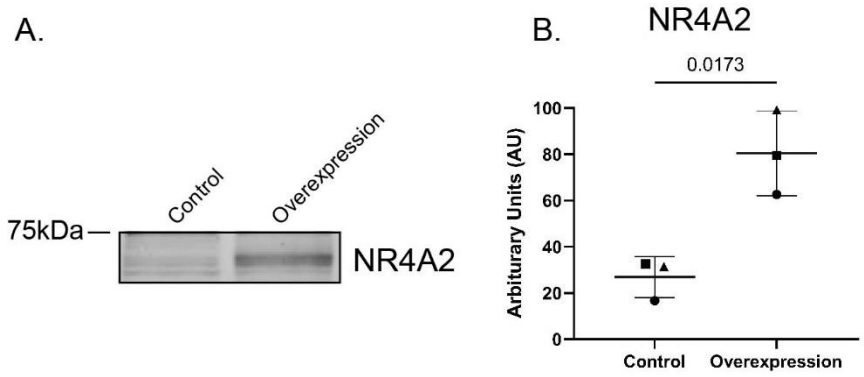

Figure 4.4. Confirmation of *NR4A2* Overexpression. A) Representative western blot for Nr4a2. B) Quantification of 3 biological replicates show a signficant decrease in protein (*p=0.0173*) (n=3 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

NR4A2 overexpression during proliferation decreases the expression of dopaminergic phenotype markers in differentiated 3D LUHMES neurospheres

On Differentiation Day 12, lysates were collected and western blots were run and probed for DAergic phenotype markers including TH, DAT, and VMAT2. Western bot analysis shows that *NR4A2* overexpression during proliferation modifies the expression of DAergic markers in differentiated neurospheres. Here, we show an approximate 40% decrease in TH expression (*p=0.0240*) (Figure 5A, B). DAT expression was not significantly affected (*p=0.2137*), while VMAT2 protein was decreased by approximately

27% compared to control (*p=0.0247*) (Figure 5A, C-D). The ratio of DAT:VMAT2 was not significantly affected by *NR4A2* overexpression (*p=0.5847*) (Figure 5E).

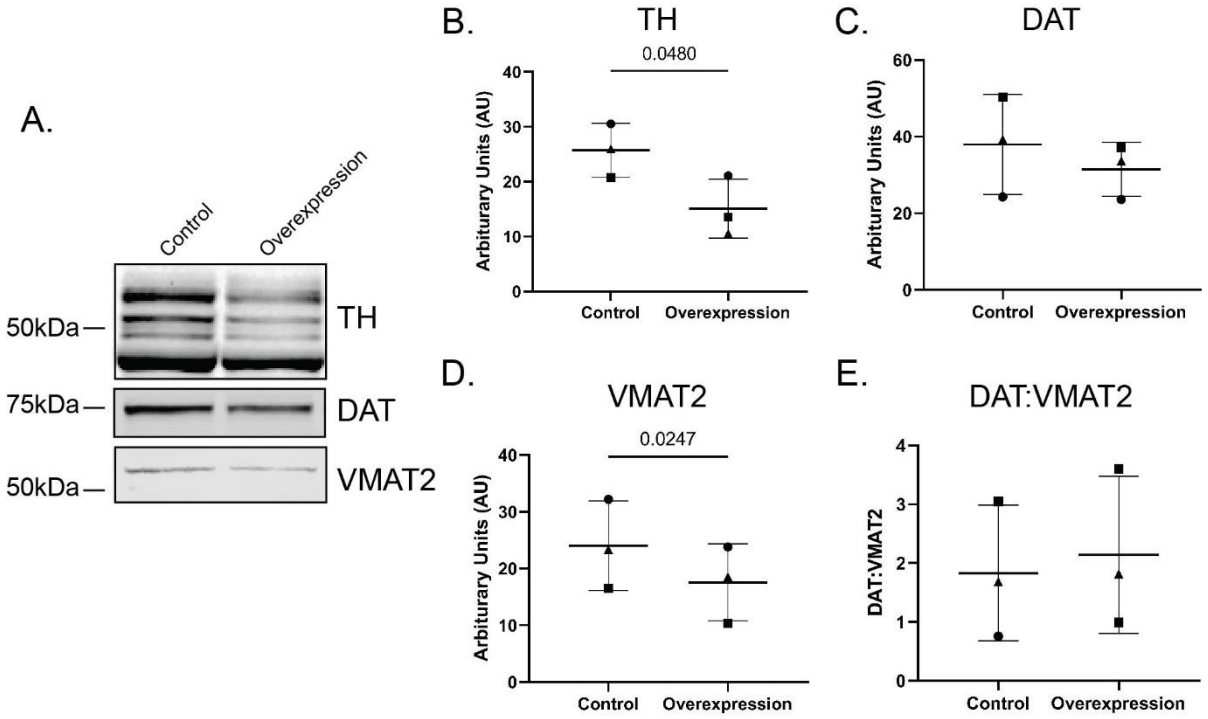

Figure 4.5. *NR4A2* overexpression affects the dopaminergic differentiaon of LUHMES neurospheres. A) Representative blots for TH, DAT, VMAT2 protein. B) TH protein is signficantly decreased in *NR4A2* overexpressing neurospheres (*p= 0.0240*). C) DAT expression is not affected by *NR4A2* overexpression (*p= 0.2137*). D) VMAT2 expression is significantly decreased (*p=0.0247*). The ratio of DAT:VMAT2 is not significantly affected (*p= 0.5847*) (n=3 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

NR4A2 overexpression does not affect MPP+-induced toxicity

To determine if *NR4A2* overexpression affects the health of the cells at baseline, ATP and neurite outgrowth assays were completed on control and overexpressing neurospheres. At baseline, ATP concentration was not affected by *NR4A2* overexpression (*p= 0.2951*). Neurite outgrowth was also not affected by *NR4A2*

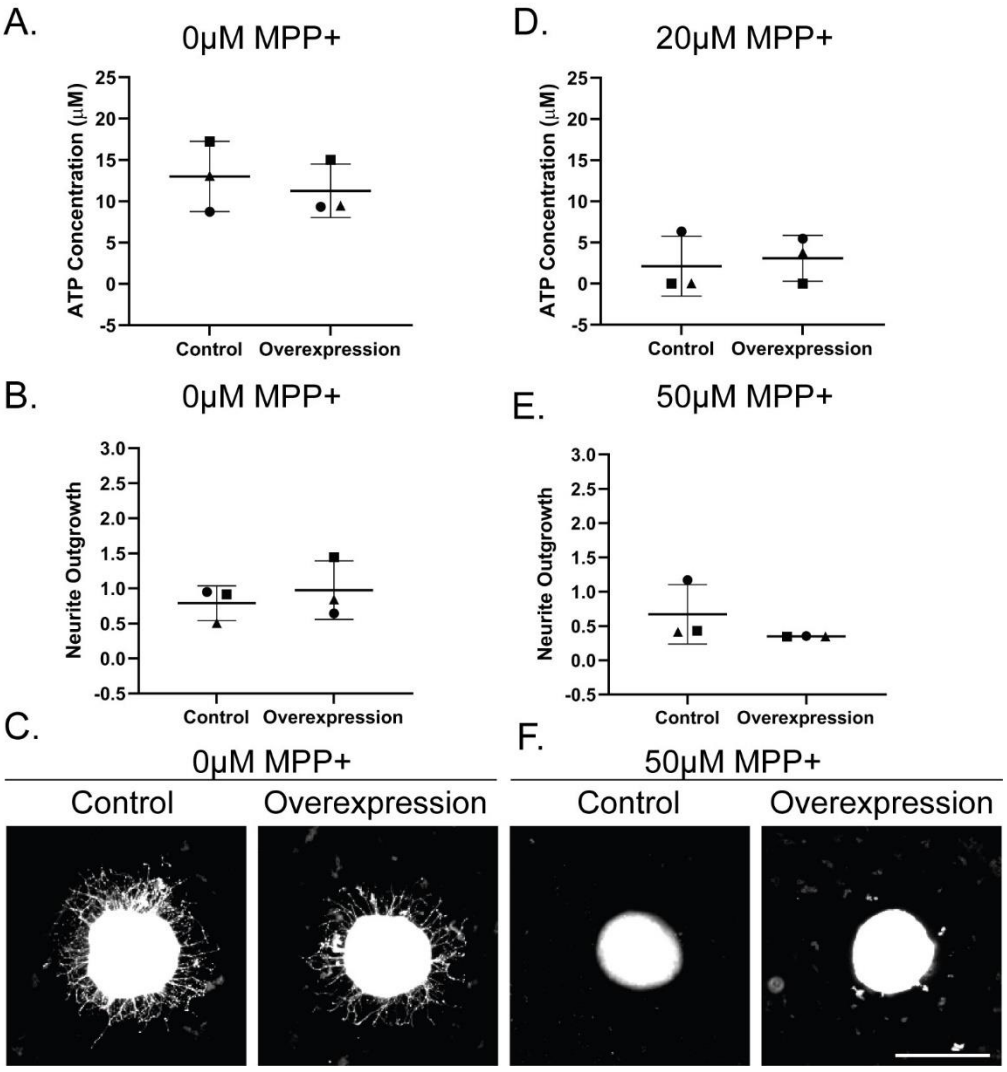

Figure 4.6. *NR4A2* overexpression does not affect baseline ATP and neurite outgrowth assays nor MPP$^+$-induced toxicity. A) ATP concentration (μM) is not affected in NR4A2 overexpressing neurospheres (*p= 0.2951*). B) Relative neurite outgrowth is not affected in *NR4A2* overexpressing neurospheres compared to control neurospheres (*p= 0.2964*). C) Representative neurite outgrowth image of control (left) and *NR4A2* overexpressing neurospheres (right) at baseline D) ATP concentration (μM) is not affected in *NR4A2* overexpressing neurospheres treated with 20 μM MPP$^+$ (*p= 0.5963*). E) Relative neurite outgrowth is not affected in *NR4A2* overexpressing neurospheres (*p= 0.3216*). F) Representative neurite outgrowth image of control (left) and *NR4A2* overexpressing (right) neurospheres treated with 50 μM. (n=3 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD. Scale bar represents 1000μm.

overexpression (*p= 0.2964*) (Figure 6A-C). As a second hit, MPP^+ concentrations of either 20 µM or 50 µM were added to neurospheres based on previously determined IC_{50} values for ATP and neurite outgrowth assays, respectively. *NR4A2* overexpression did not affect MPP^+ (20 µM)-induced decreases in ATP concentration (*p=0.5963)* (Figure 6D). *NR4A2* overexpression did not affect MPP^+ (50 µM)-induced decreases in neurite outgrowth (Figure 6E-F).

Confirmation of NR4A2 knockdown

On Differentiation Day 12, lysates were collected, and western blots were run and probed for Nr4A2 protein. Western blot analysis showed an approximately 45% decrease in Nr4a2 protein in *NR4A2* knockdown neurospheres (*p= 0.0165*) (Figure 7).

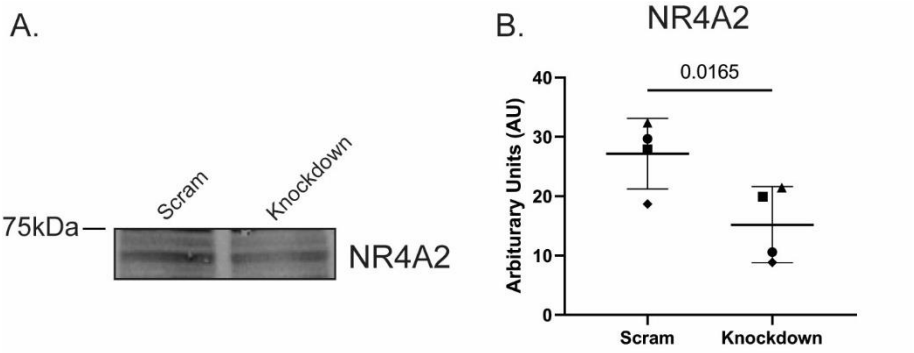

Figure 4.7. Confirmation of NR4A2 Knockdown. A) Representative western blot for Nr4a2. B) Quantification of 4 biological replicates show a significant decrease in Nr4a2 (*p=0.0165*). (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

NR4A2 knockdown during proliferation increases the expression of dopaminergic phenotype markers in differentiated 3D LUHMES neurospheres

Lysates were collected at Differentiation Day 12 and run on western blots probing for DAergic phenotype markers (TH, DAT, and VMAT2) to determine if knockdown affects DAergic differentiation. *NR4A2* knockdown neurospheres showed a 36% increase in TH expression following differentiation (*p= 0.0304*). DAT expression was significantly

decreased by 9% compared to control (*p= 0.0032*), and there was an approximate 40% increase in VMAT2 protein expression (*p= 0.0225*). The ratio of DAT: VMAT2 was not significantly affected in *NR4A2* knockdown neurospheres (*p= 0.1815*) (Figure 8).

NR4A2 knockdown does not affect MPP+-induced toxicity

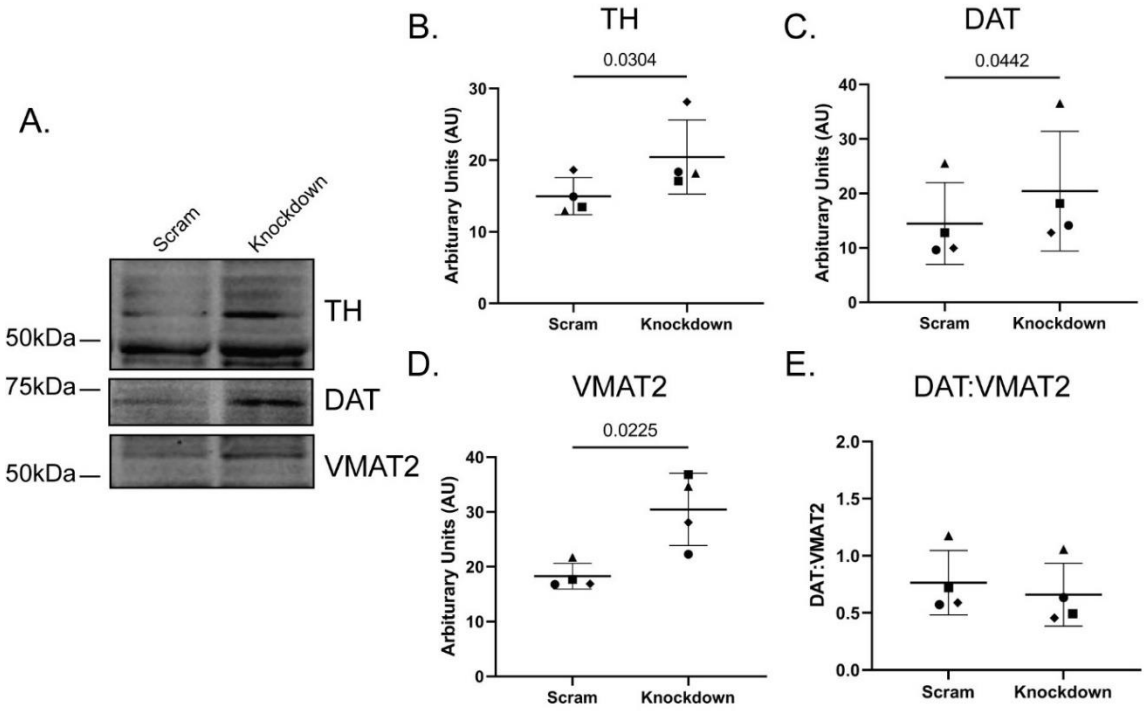

Figure 4.8. *NR4A2* Knockdown affects the dopaminergic differentiation of LUHMES neurospheres. A) Representative blots for TH, DAT, VMAT2 protein. B) TH protein is signficantly increased in *NR4A2* knockdown compared to scrambled neurospheres (*p=0.0304*). C) DAT expression is signficantly decreased (*p=0.0032*). D) VMAT2 expression is significantly increased compared to scrambled (*p=0.0225*). The ratio of DAT:VMAT2 is not significantly affected by *NR4A2* knockdown neurospheres (*p= 0.1815*). (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

To test if NR4A2 knockdown affects baseline cell health, ATP and neurite outgrowth assays were completed on scramble and knockdown neurospheres. ATP concentration (µM) was significantly decreased in *NR4A2* knockdown neurospheres compared to scrambled shRNA controls (*p= 0.0320*) (Figure 9A). However, relative neurite outgrowth is not affected by *NR4A2* knockdown at baseline (*p= 0.5771*) (Figure 9B-C). As a

second hit, MPP^+ concentrations of either 20 μM or 50 μM were added to neurospheres based on previous IC_{50} values for ATP and neurite outgrowth assays respectively. *NR4A2* knockdown did not affect MPP^+ (20μM)-induced decreases in ATP concentration (*p=0.2301*) (Figure 9D). *NR4A2* knockdown also did not affect MPP^+(50μM)-induced decreases in relative neurite outgrowth in neurospheres (*p= 0.0898* respectively) (Figure 9E-F).

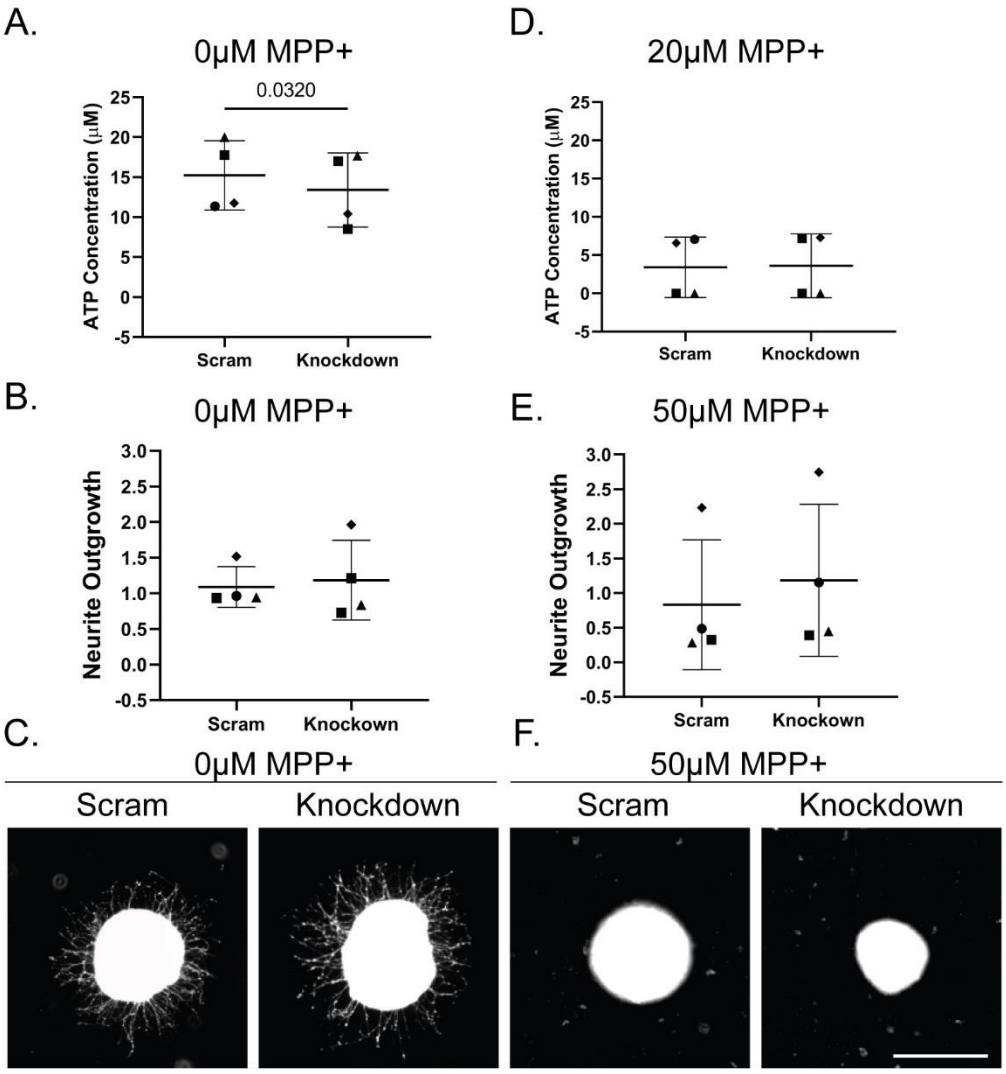

Figure 4.9. *NR4A2* knockdown reduces baseline ATP, but not relative neurite outgrowth or MPP$^+$-induced toxicity. A) ATP concentration (μM) is signficantly decreased in NR4A2 knockdown neurospheres compared to sham treated (*p= 0.0320*). B) Relative neurite outgrowth is not affected in *NR4A2* knockdown neurospheres compared to scrambled neurospheres (*p= 0.5771*). C) Representative neurite outgrowth image of scrambled (left) and *NR4A2* knockdown neurospheres (right) at baseline D) ATP concentration (μM) is not affected in *NR4A2* knockdown neurospheres treated with 20 μM MPP$^+$ (*p= 0.2301*). E) Relative neurite outgrowth is not affected in *NR4A2* knockdown neurospheres (*p= 0.0898*). F) Representative neurite outgrowth image of scrambled (left) and *NR4A2* knockdown (right) neurospheres treated with 50 μM. (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD. Scale bar represents 1000μm.

Confirmation of EPHB2 overexpression

To determine the relative knockdown in Ephb2 protein, western blots were run and probed for Ephb2 using lysates collected at Differentiation Day 12. Western blot quantification shows that differentiated neurospheres transduced with *EPHB2* lentivirus results in ~128% increase in Ephb2 protein (*p =0.0053*) (Figure 10).

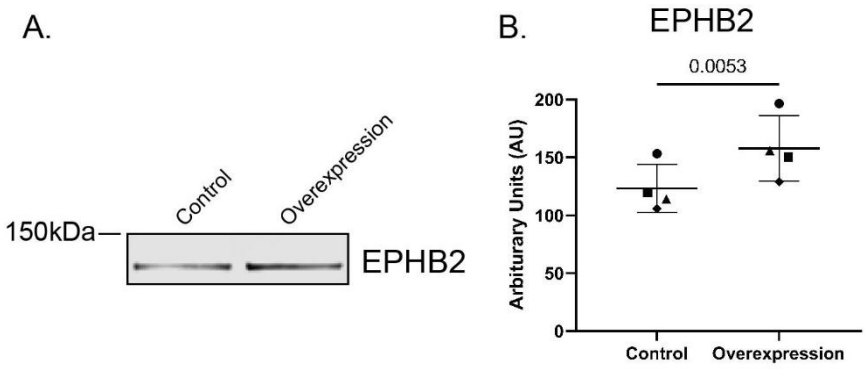

Figure 4.10. Confirmation of *EPHB2* Overexpression. A) Representative western blot for *Ephb2*. B) Quantification of 4 biological replicates show a significant increase in Ephb2 (*p= 0.0053*). (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

EPHB2 overexpression during proliferation modifies the expression of dopaminergic phenotype markers in differentiated 3D LUHMES neurospheres

On Differentiation Day 12, lysates were collected and western blots were run and probed for DAergic phenotype markers including TH, DAT, and VMAT2. *EPHB2* overexpression results in a significant decrease in TH protein by ~7% (*p= 0.0043*). Similarly, DAT expression was not significantly increased compared to control (*p= 0.0578*). VMAT2 protein was increased by 25% (*p= 0.0135*). The ratio of DAT:VMAT2 was not significantly affected in *EPHB2* overexpressing neurospheres (*p= 0.0604*) (Figure 11).

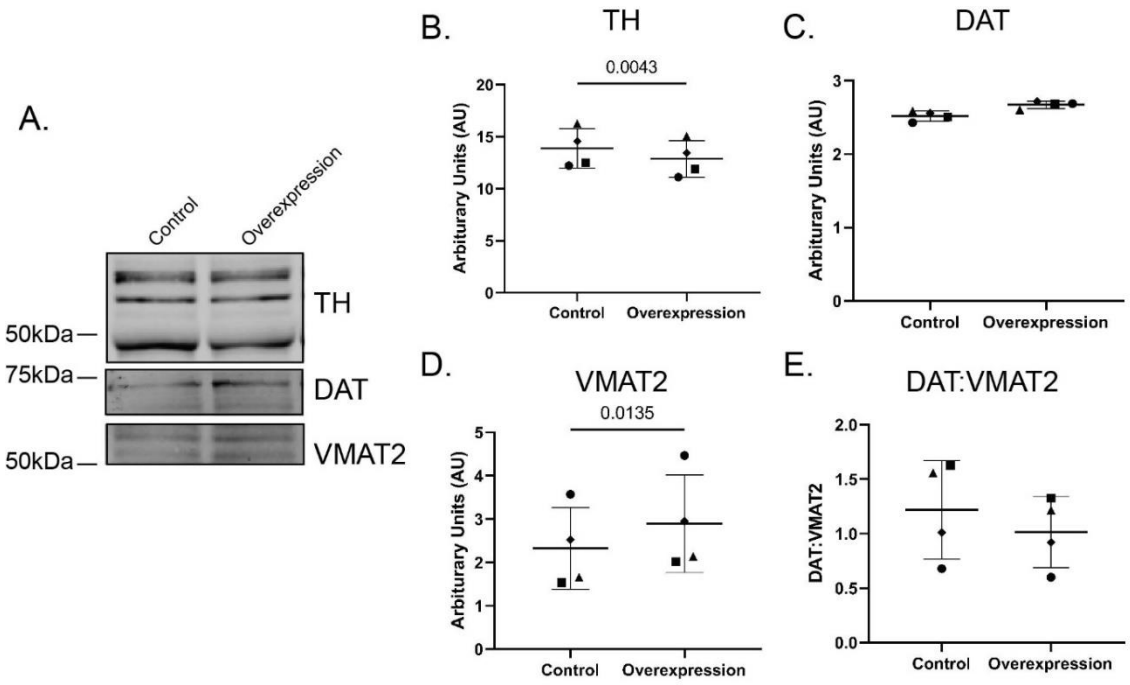

Figure 4.11. *EPHB2* Overexpression affects the dopaminergic differentiaon of LUHMES neurospheres. A) Representative blots for TH, DAT, VMAT2 protein. B) TH protein is signficantly decreased in *EPHB2* overexpressing neurospheres compared to control (*p= 0. 0043*). C) DAT expression was not significantly affected (*p= 0.0578*). D) VMAT2 expression is significantly increased compared to scrambled (*p= 0.0135*). The ratio of DAT: VMAT2 is signficantly reduced in *EPHB2* overexpressing neurospheres (*p= 0.0604*). (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

EPHB2 overexpression does not affect MPP+-induced toxicity

ATP and neurite outgrowth assays were completed on control and *EPHB2* overexpressed neurospheres to determine the effect of EPHB2 on cell health. At baseline, ATP concentration (μM) and relative neurite outgrowth are not affected by *EPHB2* overexpression (*p= 0.8367* and *p= 0.4476*, respectively) (Figure 12A-C). To determine if *EPHB2* overexpression modifies the response to MPP^+-induced toxicity, cells were treated with 20μM of MPP^+ for ATP assays or 50μM MPP+ for neurite outgrowth assays. *EPHB2* overexpression did not affect MPP^+-induced decreases in ATP concentration (*p= 0.3910*) (Figure 12D). *EPHB2* overexpression also did not affect

MPP$^+$-induced decreases in relative neurite outgrowth in neurospheres (*p= 0.5146*) (Figure 12E-F).

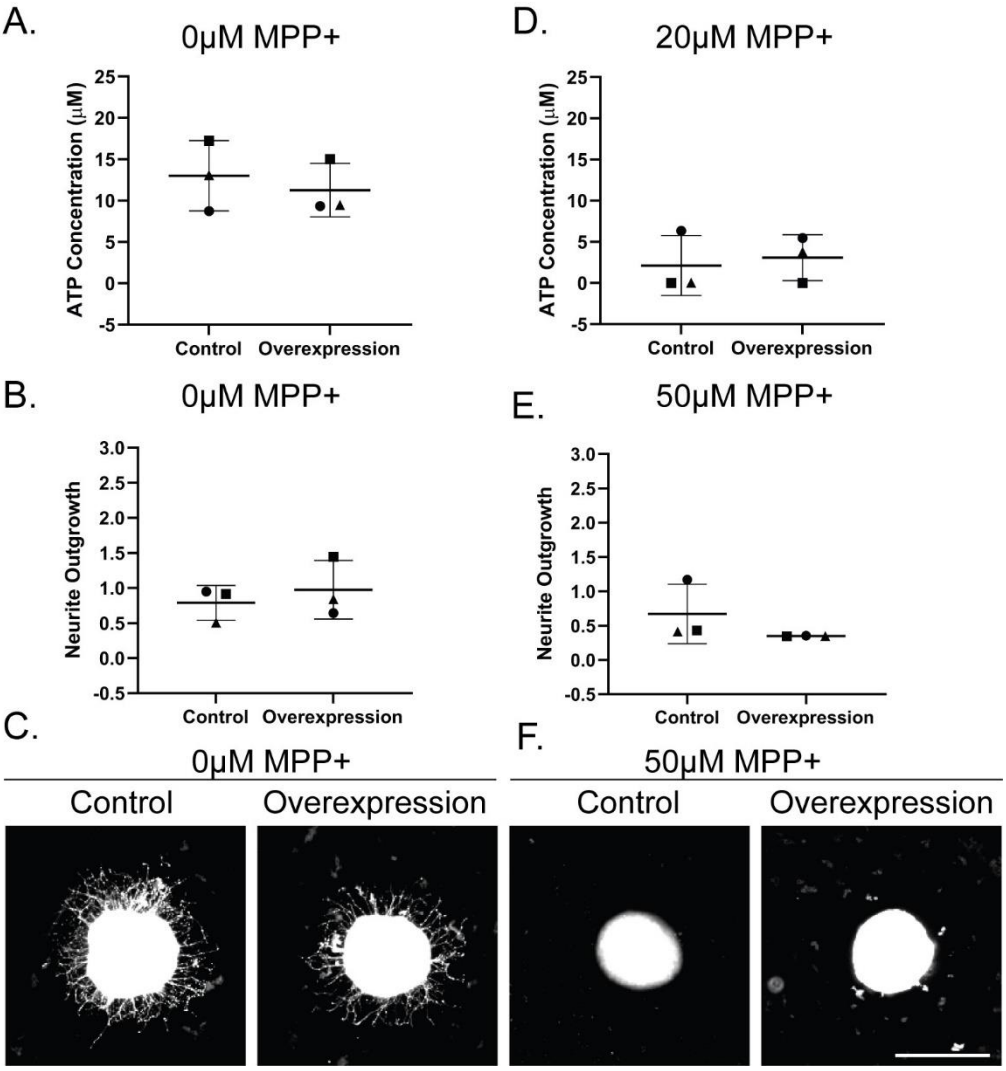

Figure 4.12. *EPHB2* overexpression does not affect baseline ATP and relative neurite outgrowth nor MPP$^+$-induced toxicity. A) ATP concentration (μM) is not affected by *EPHB2* overexpression compared to control neurospheres (*p=0.8367*). B) Relative neurite outgrowth is not affected in *EPHB2* overexpression neurospheres compared to control (*p= 0.4476*). C) Representative neurite outgrowth image of control (left) and *EPHB2* overexpression neurospheres (right) at baseline D) ATP concentration (μM) is not affected in *EPHB2* overexpression neurospheres treated with 20 μM MPP$^+$ (*p= 0.3910*). E) Relative neurite outgrowth is not affected in *EPHB2* overexpression neurospheres (*p= 0.5146*). F) Representative neurite outgrowth image of control (left) and *EPHB2* overexpression (right) neurospheres treated with 50 μM. (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD. Scale bar represents 1000μm.

Confirmation of EPHB2 knockdown

Lysates were collected, run on western blots, and probed for Ephb2 protein on Differentiation Day 12 to confirm *EPHB2* knockdown. Western blot quantification shows that differentiated neurospheres transduced with an *EPHB2* shRNA lentivirus result in an approximate 56% decrease in *EPHB2* protein (*p= 0.0164*) (Figure 13).

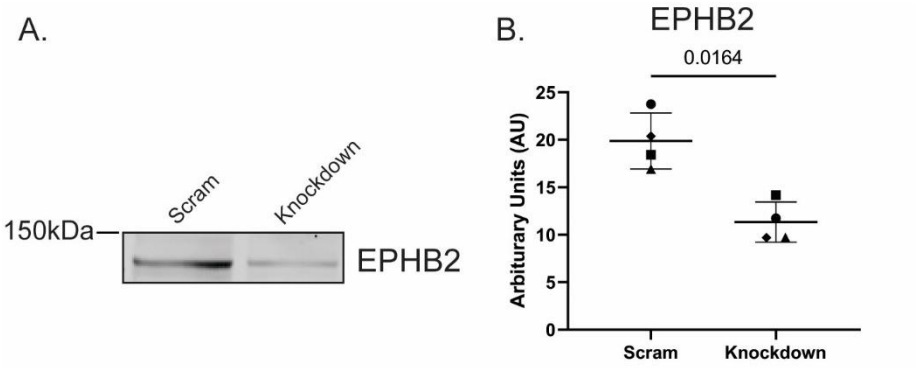

Figure 4.13. Cofirmation of *EPHB2* Knockdown. A) Representative western blot for *EPHB2*. B) Quantification of 4 biological replicates show a significant increase in *EPHB2* (*p= 0.0164*) (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

EPHB2 knockdown during proliferation modifies the expression of dopaminergic phenotype markers in differentiated 3D LUHMES neurospheres

Western blots were run and probed for the DAergic phenotype markers (TH, DAT, VMAT2) using lysates collected at Differentiation Day 12 to determine if *EPHB2* knockdown affects DAergic differentiation. *EPHB2* knockdown resulted in a ~34% increase in TH protein in differentiated neurospheres compared to scramble transduced (*p= 0.0099*). There was also a 33% decrease in DAT protein (*p=0.0199*). VMAT2 protein expression was significantly reduced by 48% (*p= 0.0061*). The ratio of DAT:VMAT2 was significantly increased compared to scramble-transduced neurospheres (*p=0.0152*) (Figure 14).

EPHB2 knockdown does not affect MPP+-induced toxicity

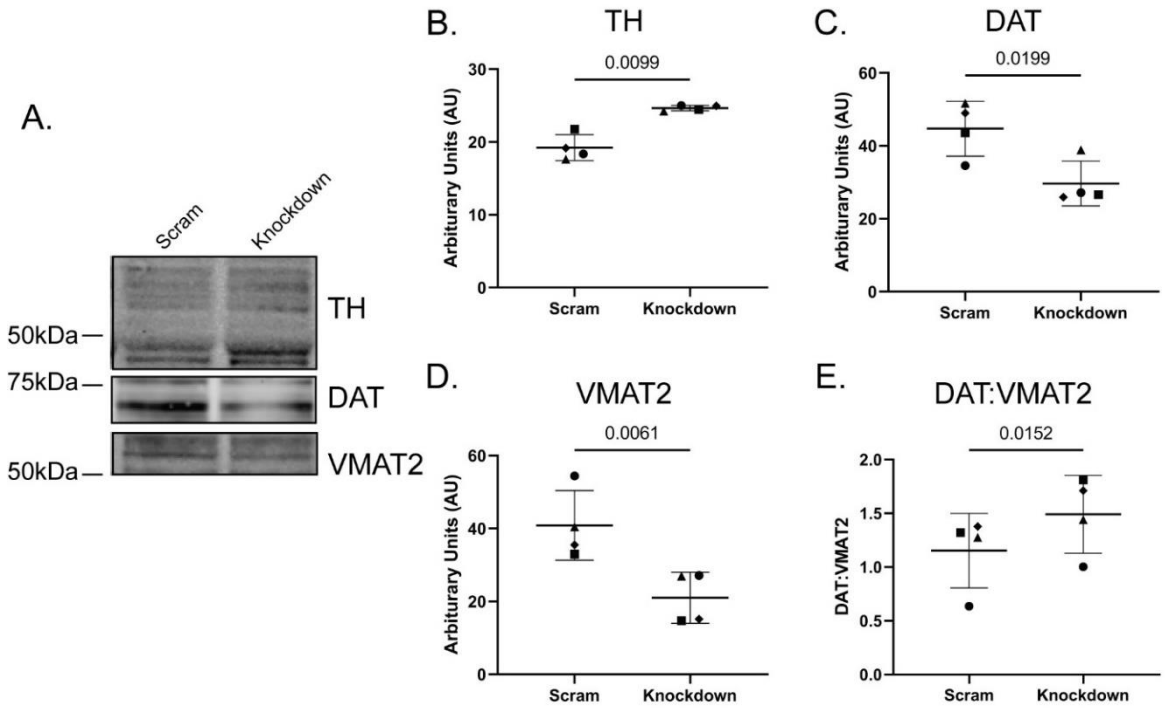

Figure 4.14. *EPHB2* knockdown affects the dopaminergic differentiaon of LUHMES neurospheres. A) Representative blots for TH, DAT, VMAT2 protein. B) TH protein is signficantly increased in *EPHB2* knockdown compared to scrambled (*p= 0.0099*). C) DAT expression is signficantly decreased (*p= 0.0199*). D) VMAT2 expression is significantly decreased compared to scrambled (*p= 0.0061*). The ratio of DAT:VMAT2 is signficantly increased (*p= 0.0152*). (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD.

To determine if *EPHB2* knockdown affects the baseline health of the cells, ATP and neurite outgrowth assays were completed on scrambled control and knockdown neurospheres. At baseline, ATP concentration (μM) and relative neurite outgrowth are not affected by *EPHB2* knockdown (*p= 0.4586* and *p=0.9556*, respectively) (Figure 15A-C). To determine if *EPHB2* knockdown affects toxicity induced by MPP^+, neurospheres were treated with 20μM MPP^+ for ATP assays or 50μM MPP^+ for neurite outgrowth assays. *EPHB2* knockdown did not affect MPP^+-induced decreases in ATP concentration (*p= 0.7575*) (Figure 15D). MPP^+-induced decreases in relative neurite

outgrowth were not affected by *EPHB2* knockdown in neurospheres (*p= 0.9002)*) (Figure 15E-F).

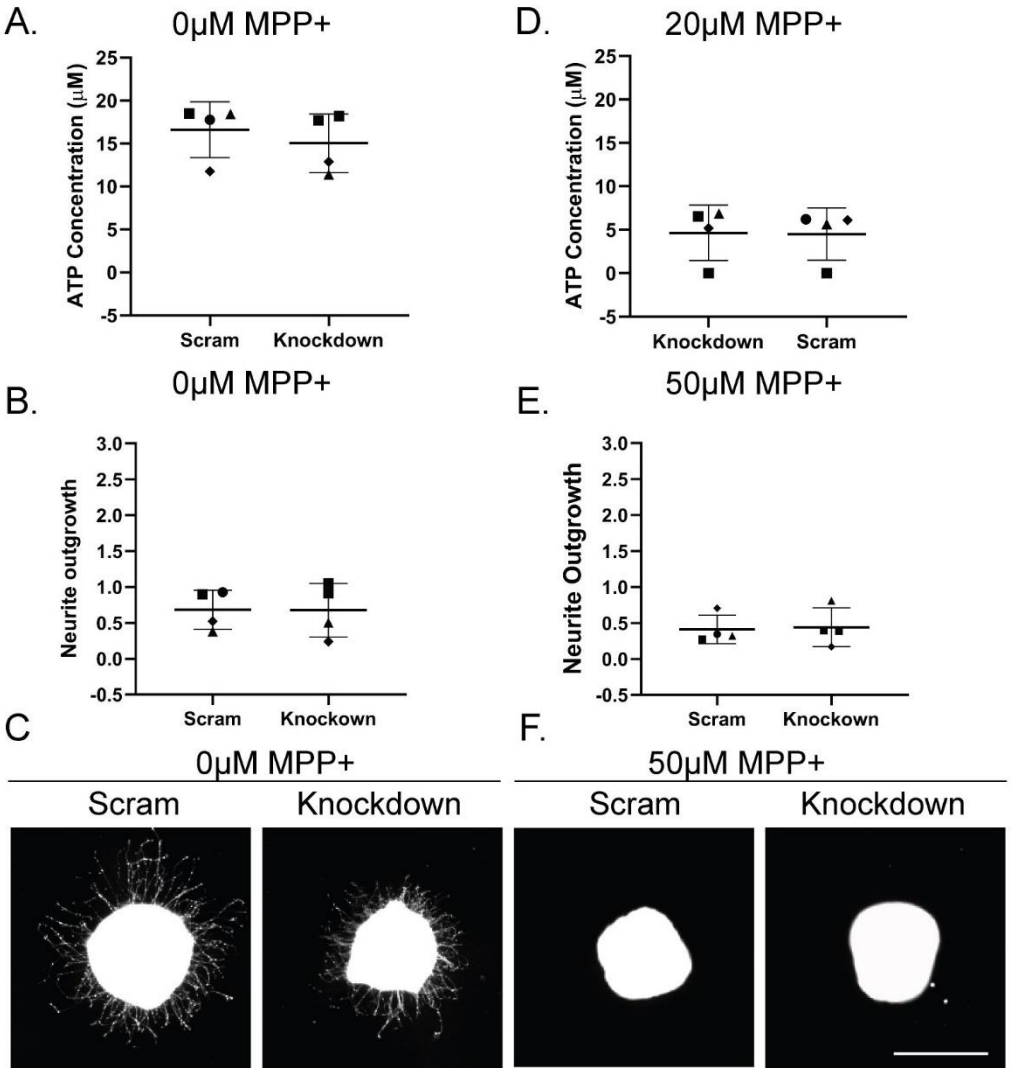

Figure 4.15. *EPHB2* knockdown does not affect baseline ATP or relative neurite outgrowth nor MPP^+-induced toxicity. A) ATP concentration (μM) is not affected by *EPHB2* knockdown compared to scrambled neurospheres (*p= 0.4586*). B) Relative neurite outgrowth is not affected in *EPHB2* knockdown neurospheres compared to scrambled (*p= 0.9556*). C) Representative neurite outgrowth image of scrambled (left) and *EPHB2* knockdown neurospheres (right) at baseline D) ATP concentration (μM) is not affected in *EPHB2* knockdown neurospheres treated with 20 μM MPP^+ (*p= 0.7575*). E) Relative neurite outgrowth is not affected in *EPHB2* knockdown neurospheres (*p= 0.9002*). F) Representative neurite outgrowth image of scrambled (left) and *EPHB2* knockdown (right) neurospheres treated with 50 μM. (n=4 and each replicate are indicated by a different symbol; paired two-tailed t-test). Data shown as mean +/- SD. Scale bar represents 1000μm.

Table 4.5. Summary of Modified *NR4A2* Expression on Differentiated LUHMES Neurospheres

	DA Phenotype			DA Vulnerability	Baseline		MPP+-Induced Toxicity	
	TH	**DAT**	**VMAT2**	**DAT: VMAT2**	**ATP**	**Neurite Outgrowth**	**ATP**	**Neurite Outgrowth**
NR4A2 Overexpression	↓	No Change	↓	No Change	No Change	No Change	No Change	No Change
NR4A2 Knockdown	↑	↑	↑	No Change	No Change	No Change	No Change	No Change

Table 4.6. Summary of Modified *EPHB2* Expression on Differentiated LUHMES Neurospheres

	DA Phenotype			DA Vulnerability	Baseline		MPP+-Induced Toxicity	
	TH	**DAT**	**VMAT2**	**DAT: VMAT2**	**ATP**	**Neurite Outgrowth**	**ATP**	**Neurite Outgrowth**
EPHB2 Overexpression	↓	No Change	↑	No Change	No Change	No Change	No Change	No Change
EPHB2 Knockdown	↑	↓	↑	↑	No Change	No Change	No Change	No Change

Discussion

NR4A2 modification during a developmental phase modifies the expression of dopaminergic markers following differentiation

Here, we show that *NR4A2* modifies the expression of DAergic proteins TH, DAT, and VMAT2 (Figures 5 and 8). The role of *NR4A2* (Nurr1 protein) in DAergic regulation and development has been well-studied. In adult rat-derived neuronal precursor cells, Nurr1 overexpression increases TH but does not affect other DAergic proteins including the D2-like receptors (D2R), VMAT2, and aromatic l-amino acid decarboxylase (AADC) (Sakurada et al., 1999). In human DAergic, MN9D cells, Nurr1 overexpression

increases DA content, AADC, and VMAT2 levels 24 hours after induced overexpression (Hermanson, 2003). In line with this, TH expression is significantly upregulated immediately after Nurr1 transduction in adult rat hippocampal progenitor cells (Sakurada et al., 1999). During early stages of neurodevelopment in Nurr1 knockout mice VMAT2, AADC, and TH expression is significantly downregulated at Embryonic day 13.5. Since Nurr1 knockout is lethal soon after birth, levels during adulthood have not been investigated in this model, but Nurr1 deficient heterozygous mice at birth show a decrease in DA in the midbrain and striatum, but no changes in the number of midbrain DA neurons (Zetterström et al., 1997). In contrast, Le et al. did not observe any differences in striatal DA in adult Nurr1 heterozygous mice (Le et al., 1999, 2003). These models show that *NR4A2* regulates DAergic protein expression, but Nurr1 activity is highly dependent on timing and phase of development in DAergic neurons. It is important to note that lasting changes in gene expression can induce compensatory mechanisms to tune the effects of DAergic protein expression during neurodevelopment. A feedback loop involving DA activation of DA presynaptic autoreceptors which are known to reduce phosphorylation of TH leading to decreased DA synthesis, packaging, and release (Chen et al., 2020; Daubner et al., 2011; Ford, 2014; Sulzer et al., 2016).

Nurr1-mediated effects on DAergic markers and DA levels seem to be variable over time and these differences may be explained by compensatory mechanisms (Moore & Zigmond, 1994; Zigmond et al., 1998). Nurr1 protein was shown to bind the NGFI-B response element site on the TH promoter directly regulating TH activity (Kim et al., 2003). However, a more recent study shows that Nurr1 represses TH promoter activity

in human neural stem cells (hNSC), but transactivates the TH promoter in differentiated SH-SY5Y cells (Kim et al., 2013). This suggests that potentially Nurr1 undergoes a functional switch from a transcriptional repressor to an activator during DAergic development (Kim et al., 2013).

Here, we show that sustained *NR4A2* overexpression during the differentiation phase decreases TH and VMAT2 expression once cells are fully differentiated. In contrast, *NR4A2* knockdown results in increased TH, DAT, and VMAT2 protein. Our *NR4A2*-mediated effects on these DAergic proteins are in line with previous work showing that Nurr1 protein targets TH, DAT, VMAT2, and AADC, crucial DAergic genes (Bannon et al., 2002; Decressac et al., 2013; Hermanson, 2003; Jankovic et al., 2005; Jin et al., 2006; Kim et al., 2003; Kim et al., 2006; Kim et al., 2013; Luo, 2012; Michelhaugh et al., 2005; Rodríguez-Traver et al., 2016; Sakurada et al., 1999; Smits et al., 2003). It is likely that *NR4A2* modification before the differentiation phases causes persistent changes in the DAergic development of neurospheres and may involve compensatory or negative feedback regulation of these markers. For example, *NR4A2* overexpression may induce an early upregulation of TH resulting in increases in DA that trigger the negative feedback loop, leading to a later reduction in TH. As mentioned above Nurr1 can undergo a functional switch to either activate or repress DAergic gene expression during neurodevelopment to finely control differentiation. It is also possible that in our model, we have mirrored the NR4A2-mediated repression of TH expression that others noted (Kim et al., 2013). In our previous in vivo study, developmental dieldrin exposure resulted in a female-specific hypermethylation of a CpG in *Nr4a2,* and we expected that overexpression of this gene would be protective against toxicity by maintaining

dopaminergic phenotype and integrity (Kochmanski et al., 2019). Here, we show that *NR4A2* overexpression decreases the DAergic phenotype of these cells after differentiation, while *NR4A2* knockdown increases the DAergic phenotype in fully differentiated LUHMES cells.

EPHB2 expression modifies dopaminergic differentiation during neurodevelopmental phases

We show *EPHB2* modifies the expression of DAergic proteins, TH, DAT, and VMAT2 (Figures 9 and 12). The role of *EPHB2* in the development of DAergic neurons has not yet been investigated. However, some work shows that the Ephrin (Eph) family receptors play an important role in orchestrating the topographic connections of DAergic neurons of the nigrostriatal and mesolimbic pathways during development (Xiao et al., 2006; Yue et al., 1999). The Eph family receptors are located throughout the CNS and are thought to play a role in axonal guidance, neurite outgrowth, and synaptic plasticity (Blits-Huizinga et al., 2004). *Ephb1* is primarily found in the midbrain, with the highest concentrations found in the SN and the ventral tegmental area (VTA), whereas *Ephb2* is expressed greatest in the striatum (Passante et al., 2008; Xiao et al., 2006; Yue et al., 1999). On embryonic day 18, *Ephb2* is expressed in the nucleus accumbens (NAc) and the striatum in mice. After birth (postnatal day 1-7), *Ephb2* expression increases in the NAc and striatum. In adulthood, *Ephb2* expression decreases in the NAc and the striatum indicating a potential role of Ephb2 in DAergic development in controlling SN innervation (Passante et al., 2008; Xiao et al., 2006; Yue et al., 1999). Given that the highest expression of *Ephb2* occurs during embryonic development and immediately after birth, it is not surprising that we were unable to detect *Ephb2* mRNA via RNAscope

in 3-month-old mouse nigral tissue even though it was previously detected by more sensitive measures in midbrain dissections via RNAseq in 3-month mice (Kochmanski et al., 2019).

To test the effect of ephrin-B2 on nigral DAergic neurons, Yue et al. co-cultured embryonic day 18 VTA or nigral neurons on a monolayer of control NIH-3T3 cells or a monolayer of cells from a stable ephrin-B2-expressing NIH-3T3 cell line (Yue et al., 1999). In nigral DAergic and ephrin-B2 expressing co-cultures, there was an approximate 50% decrease in TH-immunoreactive neurons and a reduction in average neuritic length. However, there was not a loss of TH-immunoreactive neurons or average neuritic length in VTA DAergic and ephrin-B2 expressing co-cultures. These effects on TH immunoreactive neuron loss and reductions in neurite outgrowth were moderately rescued by treatment with an *EPHB2* antagonist. This suggests that ephrin-B2 signaling may function to selectively inhibit nigral DAergic neuron growth to guide DAergic development in the SN (Yue et al., 1999).

Since *EPHB2* is highly expressed in DAergic neurons during development and plays an essential role in guiding DAergic innervation, it is not surprising that altering the expression of *EPHB2* during the LUHMES proliferation phase affects the DAergic differentiation of LUHMES neurospheres. Specifically, we observed a reduction in TH, but an increase in VMAT2 and no change in DAT when *EPHB2* is overexpressed (Figure 11). On the other hand, we observed an increase in TH, but a reduction in DAT and VMAT2 in *EPHB2* knockdown neurospheres (Figure 14). Our observed changes in TH expression align with previous research indicating that *EPHB2* during development

selectively inhibits nigral DAergic neuron growth to maintain proper SN development and interactions.

NR4A2 and EPHB2 regulate markers of DAergic susceptibility to toxicity but do not affect effect overt MPP+-induced degeneration

We hypothesized that *NR4A2* overexpression would be protective against MPP^+-induced toxicity. Although there were no significant changes in the expression of the *Nr4a2* protein-coding transcript in female animals, there was a slight increase in expression and observed female-specific hypermethylation. Since the phenotype associated with dieldrin-induced exacerbation of PFF is specific to male mice, and because of the known functions of *Nr4a2* in DAergic neuron development we expected *NR4A2* to protect against DAergic toxicity. Contrary to this hypothesis, we show here that *NR4A2* knockdown upregulates DAergic markers and maintains a lower DAT:VMAT2 ratio (Kochmanski et al., 2019). The ratio of DAT:VMAT2 is an indicator of DAergic susceptibility to toxicity. With a lower ratio of DAT:VMAT2 less DA is taken up via DAT and more is sequestered by VMAT2 resulting in less overall cytosolic DA, which is prone to autoxidation and the forming toxic intermediates (Miller et al., 1999). *NR4A2* knockdown did not affect the ratio of DAT:VMAT2, and there were no effects of modified *NR4A2* expression on MPP^+-induced toxicity were observed here (Figures 6 and 9).

It was expected that *EPHB2* overexpression would serve as a protective gene mitigating the effects of MPP^+ because of its hypermethylation and increased expression in developmental dieldrin-exposed female mice and its functions in axonal guidance and neurite outgrowth. The ratio of DAT:VMAT2 is decreased with *EPHB2* overexpression, while *EPHB2* knockdown increases the ratio of DAT:VMAT2 (Figures 11 and 14). The

ratio of DAT:VMAT2 was previously used as an indicator of increased susceptibility to DAergic toxicity (Miller et al., 1999). However, we did not observe any effects of *EPHB2* on MPP^+-induced toxicity (Figures 12 and 15).

Alterations of the developmental dieldrin-induced differentially modified candidate genes during proliferation, *NR4A2*, and *EPHB2* affect the DAergic phenotype of neurospheres following differentiation. This work is based on the hypothesis that these changes in DAergic gene expression modify susceptibility later in life. However, we were unable to recapitulate toxicity to the second hit of MPP+ in this *in vitro* system. Since *NR4A2* and *EPHB2* are two of hundreds of genes differentially modified by dieldrin exposure, likely, these genes are not the sole mediators of exacerbated parkinsonian toxicity and may have only small effects on toxicity on their own that are not apparent in this simplified model (Kochmanski et al., 2019). Modifying one candidate gene may have small effects on susceptibility to toxicity, which may require more sensitive endpoint measures to detect. For example, other groups working towards improving new approach methodologies for neurotoxicity screening show that the most sensitive endpoint for measuring neurotoxicity is electrophysiological endpoints including measures of network connectivity (Carstens et al., 2022).

Although there were no observed changes in MPP^+-induced toxicity in LUHMES neurospheres, it is still possible that altered expression of *EPHB2* and *NR4A2* could affect disease susceptibility *in vivo*. The 3D LUHMES neurosphere model was used as opposed to more complex models because we were specifically interested in the role of these candidate genes in DAergic neurons. However, due to the simplicity of the neurosphere model, we might not observe the same effects that we would *in vivo*.

Additionally, concentrations of MPP^+ were selected based on previous IC_{50} values for ATP and neurite outgrowth assays. However, the levels of toxicity at these doses were higher here than previously observed, with greater effects observed at these doses, particularly for the neurite outgrowth assays. In previous work (Chapter 3, Figure 6) the average relative neurite outgrowth value was near 2.0 in untreated control cells. However, in this set of experiments, untreated control cells showed an average relative neurite outgrowth value between 0.5 and 1.0 (Figures 6B, 9B,12B, and 15B). In previous experiments reported in Chapter 3, values in this range were consistent with neurite outgrowth observed at 20 μM MPP^+. Here, cells treated with 20 μM MPP^+ had almost no neurites, which is more consistent with our highest MPP^+ concentrations used in Chapter 3. Therefore, untreated and unmodified cells in these experiments started with far less developed neurites compared to previous work, limiting our ability to detect differences (Chapter 3, Figure 6). This is likely due to the additional passaging and manipulation steps required for viral transduction and cell expansion during the proliferation phase of these cells, indicating that the passage number and handling of LUHMES cells are extremely important and can cause variation in the cellular response. To perform the ATP and neurite outgrowth assays in the future, individual neurospheres should be treated with MPP^+ rather than an entire well for a 6-well plate to reduce the amount of passaging needed to expand the transduced cells. Another approach would be to complete these experiments in a 2D format instead.

Here, we show that developmental dieldrin-induced differentially modified genes *NR4A2* and *EPHB2* regulate the expression of DAergic markers in a 3D LUHMES neurosphere model. These changes in the DAergic phenotype of cells result in an altered

DAT:VMAT2 ratio, an indicator of susceptibility to DA degeneration, but did affect MPP^+ toxicity in this model. Since environmental factors such as dieldrin exposure play a significant role in PD, and developmental periods are specifically vulnerable to such factors, we must continue to study and identify the link between developmental exposures and disease development later in life.

TABLES AND FIGURES

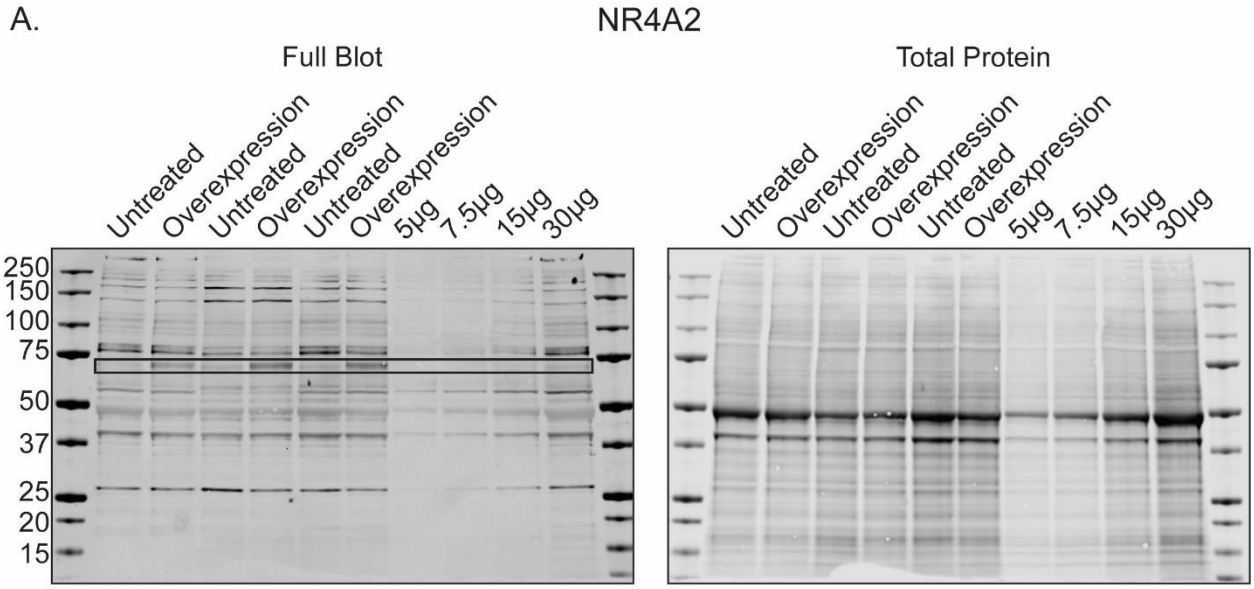

Figure S4.1. Confirmation of *NR4A2* overexpression full blot and total protein stain.

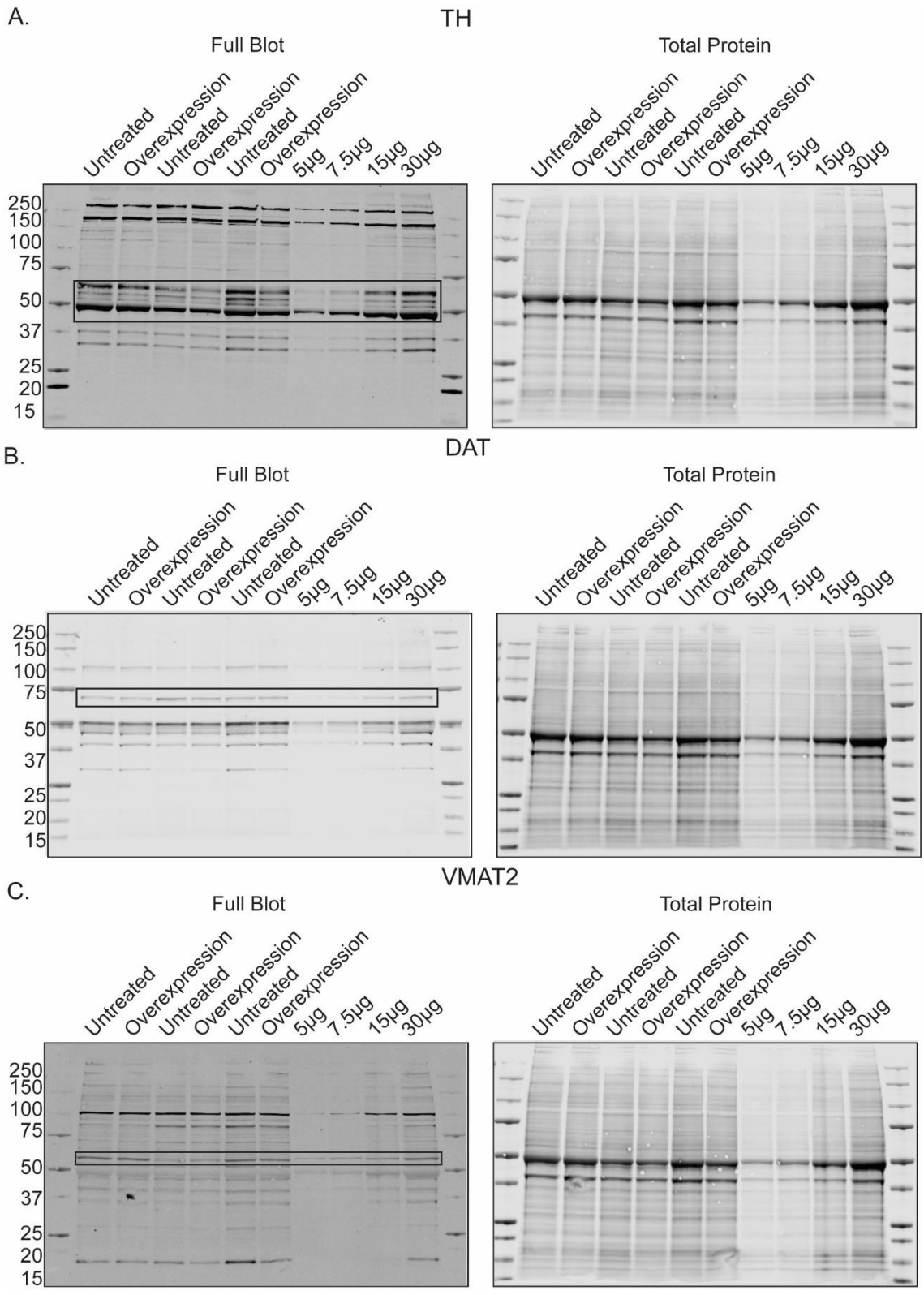

Figure S4.2. Full blots and total protein stains for *NR4A2* overexpression experiments. A) TH B) DAT C) VMAT2.

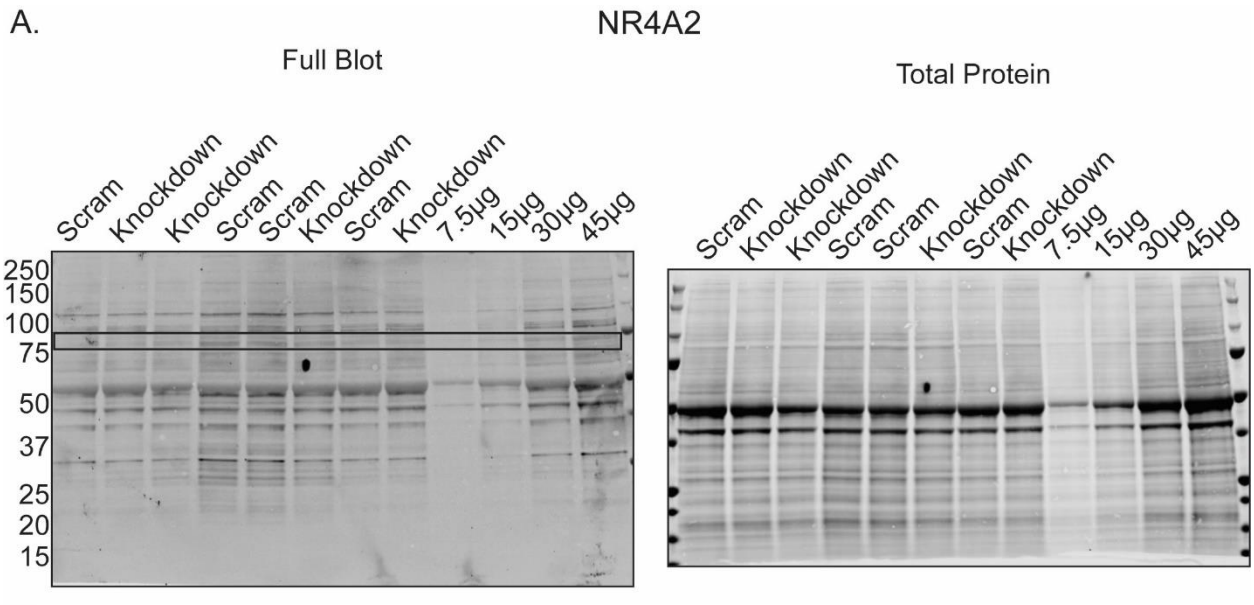

Figure S4.3. Confirmation of *NR4A2* knockdown full blot and total protein stain.

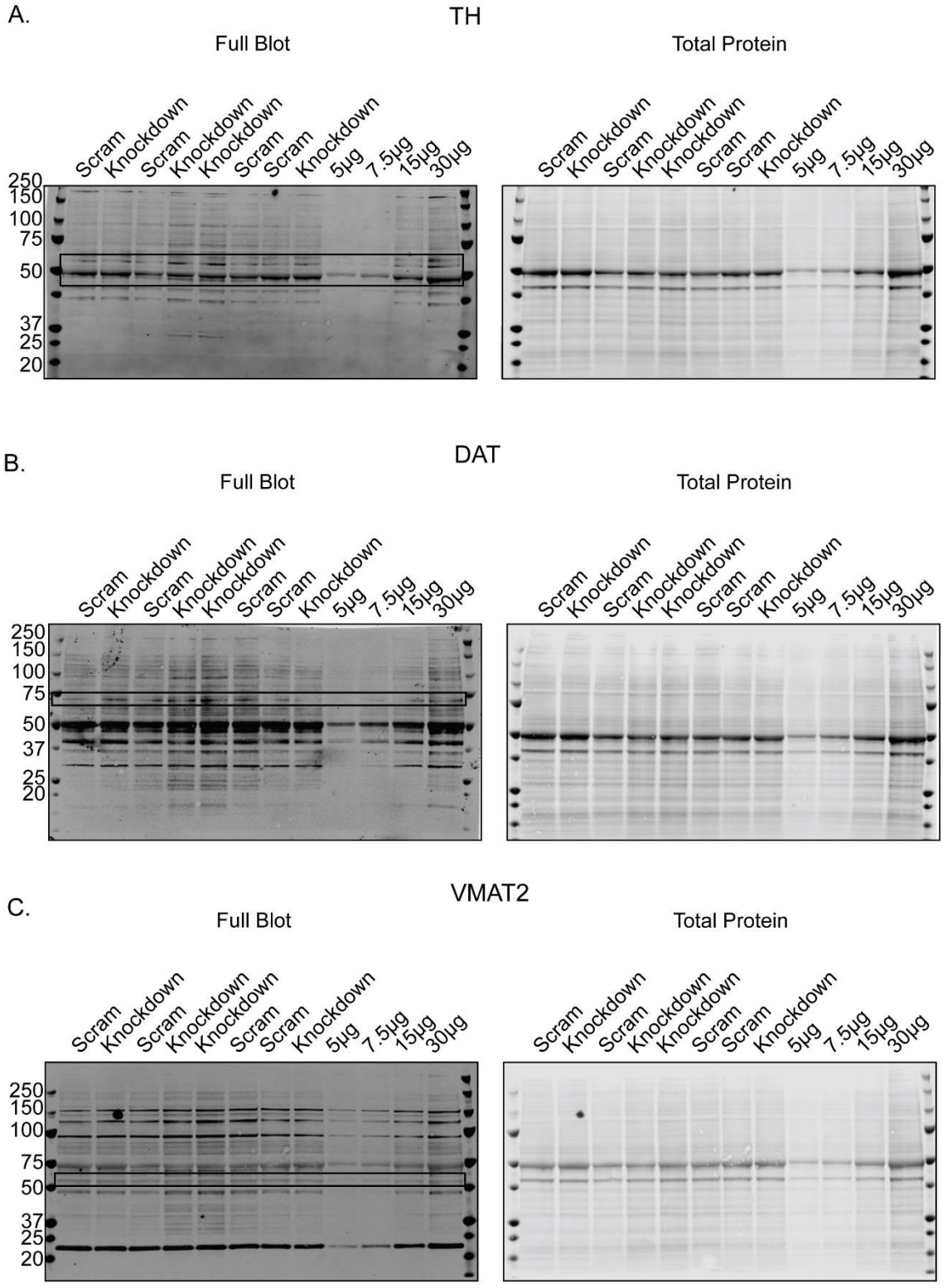

Figure S4.4. Full blots and total protein stains for *NR4A2* knockdown experiments. A) TH B) DAT C) VMAT2.

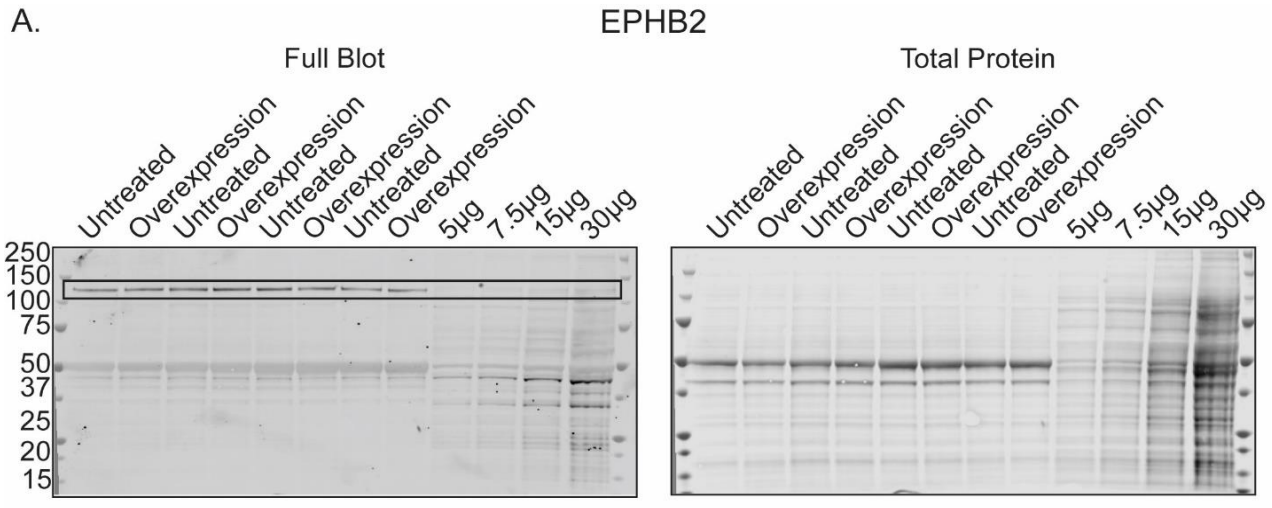

Figure S4.5. Confirmation of *EPHB2* overexpression full blot and total protein stain.

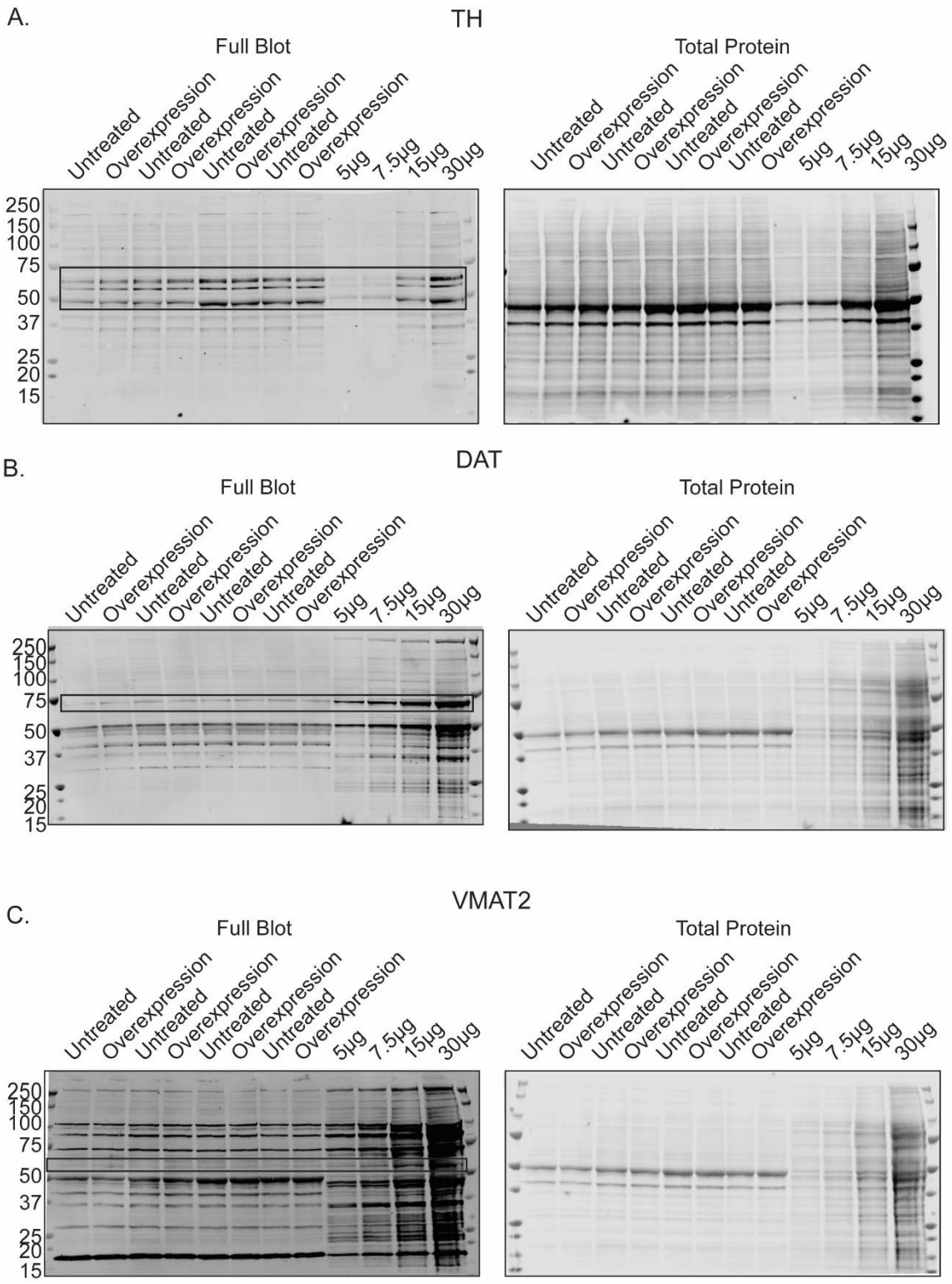

Figure S4.6. Full blots and total protein stains for *EPHB2* overexpression experiments. A) TH B) DAT C) VMAT2.

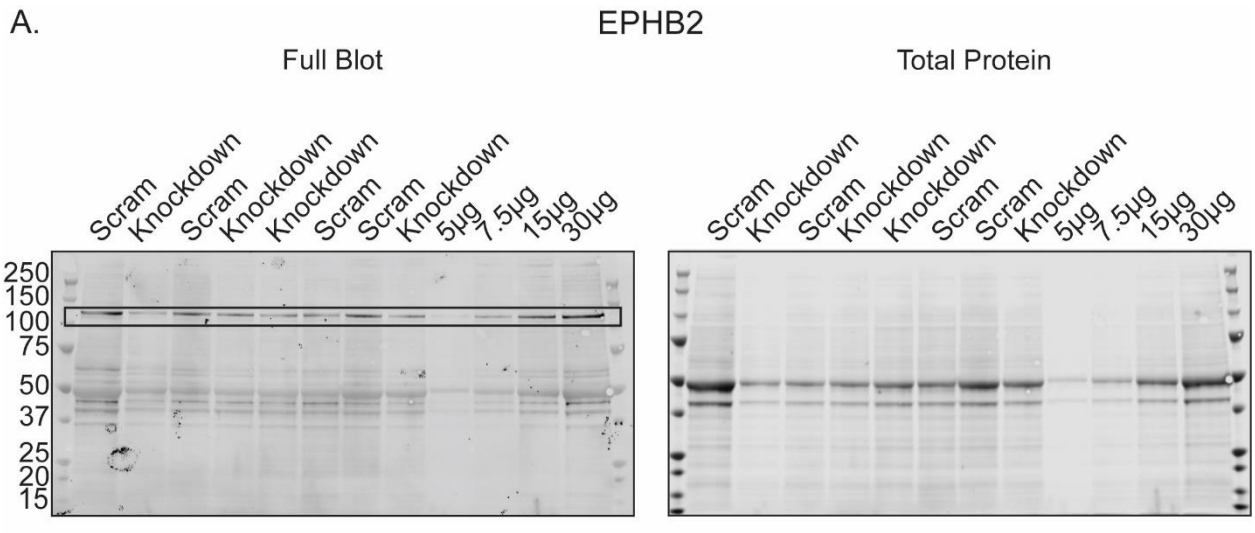

Figure S4.7. Confirmation of *EPHB2* knockdown full blot and total protein stain.

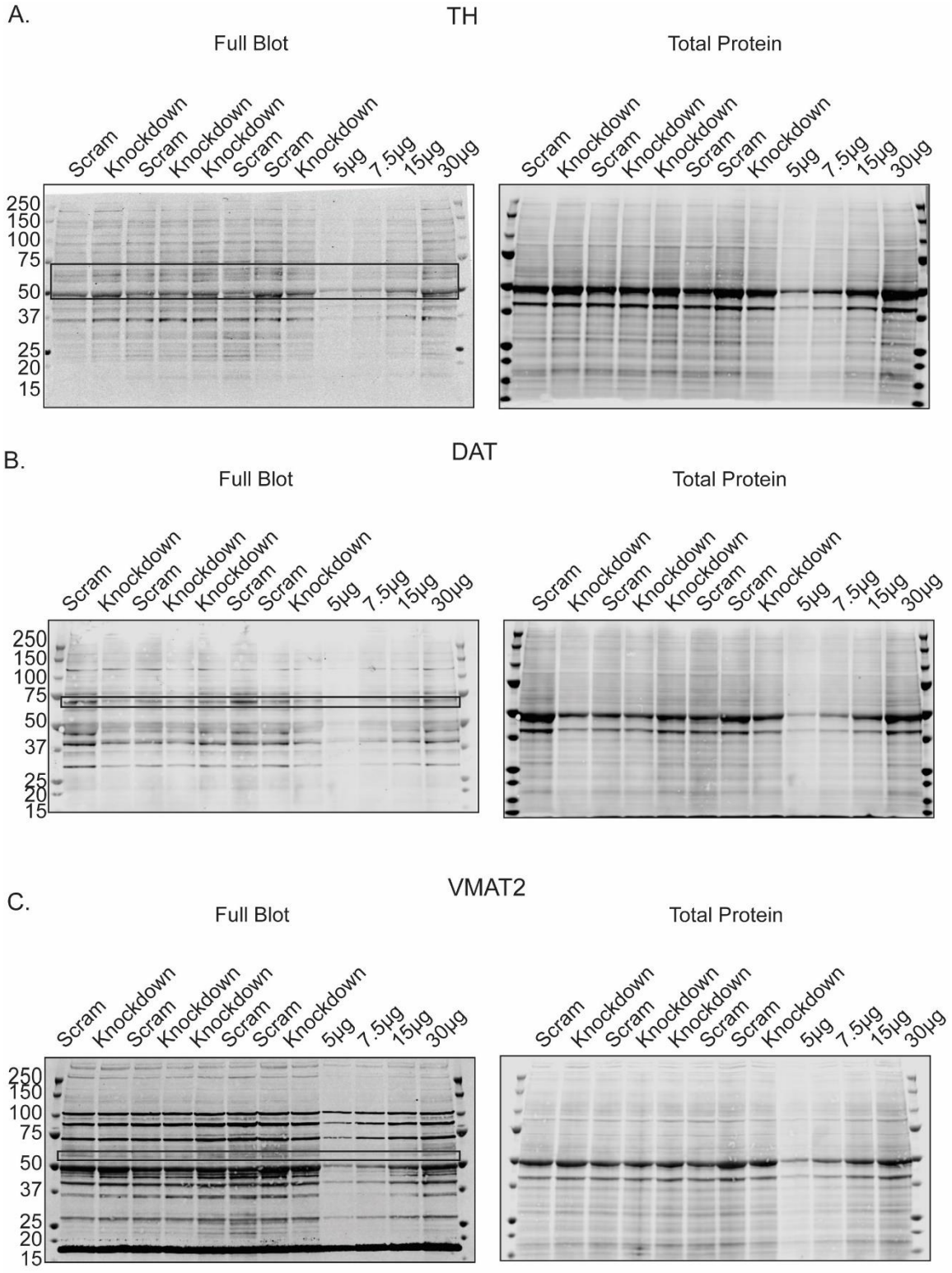

Figure S4.8. Full blots and total protein stains for *EPHB2* knockdown experiments. A) TH B) DAT C) VMAT2.

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Chapter 5: Conclusions

Overview

Epidemiological studies have consistently shown an association between increased risk of Parkinson's disease (PD) and exposure to environmental factors, such as heavy metals, solvents, and pesticide exposures, including the organochlorine pesticide dieldrin (Brown et al., 2006; Cicchetti et al., 2009; Corrigan et al., 1998, 2000; de Lau & Breteler, 2006; De Miranda et al., 2022; Dorsey et al., 2018; Elbaz et al., 2009; S. M. Fleming, 2017; Freire & Koifman, 2012; Hatcher et al., 2008; Moretto & Colosio, 2011; Semchuk et al., 1992a; Steenland et al., 2006; Tanner et al., 2011; Tanner & Aston, 2000). Additional research in post-mortem, animal models, and *in vitro* systems shows a link between dieldrin exposure and increased susceptibility of DAergic (DAergic) neurons (Ascherio et al., 2006; Brown et al., 2006; Caudle et al., 2012; Elbaz et al., 2009; L. Fleming et al., 1994; Freire & Koifman, 2012; Gezer et al., 2020; Hatcher et al., 2007; Kanthasamy et al., 2005; Le Couteur et al., 1999; Moretto & Colosio, 2011; Priyadarshi et al., 2000, 2001; Richardson et al., 2006; Ritz & Yu, 2000; Semchuk et al., 1992b, 1992a; Steenland et al., 2006; Tanner et al., 2011; Tanner & Aston, 2000; Tanner & Langston, 1990; Weisskopf et al., 2010; Wirdefeldt et al., 2011). Despite this body of work, specific mechanisms underlying this association are not well understood. Previous work in mice has established two-hit models demonstrating that developmental exposure to dieldrin induces alterations in developing DAergic neurons and a male-specific increase in susceptibility in PD models, including the DAergic toxicant, MPTP, and the α-synuclein (α-syn) pre-formed fibril (PFF) model (Gezer et al., 2020; Richardson et al., 2006). We have demonstrated that this exposure causes persistent sex-specific changes in epigenetic mechanisms, and dysregulation of genes

important for dopamine neuron development and maintenance in the substantia nigra and for the neuroinflammatory system in the striatum (Kochmanski et al. 2019; Gezer et al. 2020). These early changes may prime the synapse for heightened sensitivity later in life. This idea can be described by the term silent neurotoxicity, where the effects of early life exposures are unmasked by challenges later in life, the cumulative effects of exposures over the lifespan, or the effects of aging (Cory-Slechta et al. 2005; Kraft et al. 2016).

In this study, we expand on existing data using our previously established *in vivo* developmental dieldrin/PFF two-hit exposure model (Chapter 2) and an *in vitro* 3D neurosphere model (Chapters 3 and 4) to elucidate mechanisms by which developmental dieldrin exposure results in exacerbated toxicity later in life.

Developmental dieldrin exposure primes the nigrostriatal system for an exacerbated response to synucleinopathy

The results reported in Chapter 2 show that there are functional differences in dopamine neurotransmission at nigrostriatal synapses in male mice developmentally exposed to dieldrin after PFF injection. Specifically, our results demonstrate that there is increased striatal dopamine (DA) release in response to PFF-induced striatal DA loss in dieldrin-exposed mice 4 months after PFF injection (Figure 2.6). In previous work, there was a dieldrin-associated exacerbation of PFF-induced deficits in motor behavior and an increase in striatal DA turnover at 6 months post-injection, but no change in the degree of nigral α-syn pathology (1 and 2 months), degeneration of nigral DA neurons (6 months), or loss of striatal DA (2 and 6 months) (Gezer et al., 2020; Luk et al., 2012). In this study, we did not observe any effect of dieldrin or PFF alone on DA release (Figure

2.4). Taken together, dieldrin exposure appears to cause changes in the striatal synaptic terminals that prime the nigrostriatal system for an exacerbated response to synucleinopathy. This effect is associated with striatal DA loss, early enhanced DA release, and greater long-term increases in DA turnover and cytosolic DA. These lead to increased oxidative stress, and acceleration of the toxic interplay between dysregulated α-syn and DA (Gezer et al., 2020; Luk et al., 2012). Although there is a reduction in total DA content by 2 months post-PFF injection, here we showed dieldrin-induced increases in striatal evoked DA release upon stimulation using FSCV in PFF-injected mice at 4 months post-PFF injection (Chapter 2). This increase in evoked DA release, despite loss in total striatal DA connect, is consistent with an extensive body of literature on compensatory mechanisms that occur early in human PD, multiple animal models of DA deficits, and most recently in a model of depletion of a related monoamine, norepinephrine (Zigmond et al. 1984; Onn et al. 1986; Zhang et al. 1988; Snyder. GL et al. 1990; Zigmond et al. 1993; Zigmond 1994; Bezard and Gross 1997; Zigmond 1997; Zigmond et al. 1998; Molina-Mateo et al. 2017; Iannitelli et al. 2023).This new work provides further support for the hypothesis that developmental dieldrin exposure induces changes in the nigrostriatal striatal DAergic system that primes it to have an exacerbated response to synucleinopathy induced by α-syn PFFs in male offspring, despite the absence of observable changes in typical markers of nigrostriatal dysfunction and degeneration (Figure 5.1). Taken together, these results highlight the importance of exploring dieldrin-induced changes that produce this high susceptibility state in advancing our understanding of how exposures contribute to increased risk of PD.

Based on our results, future studies should explore the following questions:

- What specific compensatory mechanisms underlie the increase in dopamine in developmental dieldrin-exposed PFF-injected animals?

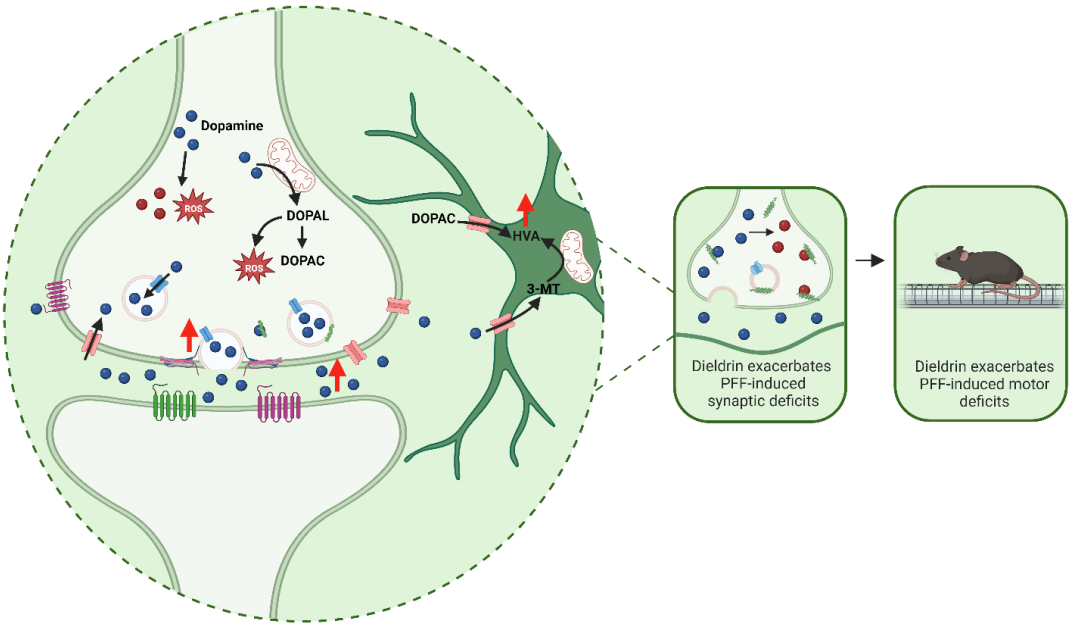

Figure 5.1. Developmental dieldrin exposure induces early synaptic changes later exacerbating PFF-induced toxicity and motor deficits. Made in BioRender.

  - Are glutamatergic and serotonergic signaling altered in dieldrin-exposed animals? Studies of DA degeneration show that glutamatergic and serotonergic innervation on striatal neurons is upregulated (Slotkin & Seidler, 2009). Glutamatergic receptors including NMDA receptors negatively regulate DA synthesis and release in the SN (Bustos et al., 2004; Moore & Zigmond, 1994; Zigmond, 1994). Dieldrin exposure *in vitro* was shown to increase NMDA and AMPA subunit expression. Since NMDA and AMPA receptors mediate glutamatergic signaling and are involved in the process of excitotoxicity, it may be important to determine if these

glutamatergic signaling pathways underly the exacerbation of dieldrin-induced increases in DA release in PFF injected animals (Dong et al., 2009).

- Is calcium homeostasis affected by dieldrin? Since calcium homeostasis is an important regulator in neurotransmitter release (Ambrosi et al., 2014; Dong et al., 2009; Post et al., 2018). Dieldrin exposure can alter Ca^{2+} signaling by regulating expression of Ca^{2+} ATPase activity resulting in impaired Ca^{2+} metabolism and signaling (Mehrotra et al., 1988, 1989). Ca^{2+} regulation is important in preventing excitotoxicity and downstream effects including activation of apoptotic factors, cytochrome C production, reactive oxygen species formation, and effects on the mitochondria (Ambrosi et al., 2014; Post et al., 2018). In PD, voltage-gated L-type Ca^{2+} ^{cha}nnels (Ca_v) are affected. Specifically, Ca_v1 channel is reduced and $Ca_v1.3$ is increased in surviving neurons. This increase in $Ca_v1.3$ expression is thought to play an important role in facilitating excitotoxicity and related degeneration in PD (Post et al., 2018).

- Are there alterations in synaptic vesicle pools and trafficking in developmental dieldrin-exposed PFF-injected animals? Studies in different α-syn models indicate a critical role for a-syn in DA release, synaptic vesicle fusion, vesicle trafficking, and regulation of synaptic vesicle pool size (Abeliovich et al., 2000; Bellani et al., 2010; Cheng et al., 2011; Dagra et al., 2021; Ingelsson, 2016; Murphy et al., 2000; Volles et al., 2001; Xilouri et al., 2013). It is also known that most striatal DA synapses are silent, and the majority of synaptic vesicles are

located within the reserve pool rather than the readily releasable pool as discussed in Chapter 2 (Goldstein, 2012, 2013, 2021; Sulzer et al., 2016; Trudeau et al., 2014). Additionally, within the striatum of PFF-injected animals, we expect that only one-third to one-half of DA terminals are affected. Together, this leaves a pool of both surviving neurons and vesicles within affected neurons to maintain DA release. Therefore, there may be decreases in the reserve pool of synaptic vesicles or changes in vesicular trafficking dynamics that we were unable to capture in previous experiments.

Failure to recapitulate the dieldrin/PFF two-hit model using in vitro 3D neurospheres model

Methods for studying developmental neurotoxicity (DNT) *in vitro* remain limited making it difficult to screen potential toxicants or investigate mechanisms of action involved in DNT. Therefore, the overall goal of the work reported in Chapters 3 and 4 of this dissertation was to develop an *in vitro* two-hit system to parallel our *in vivo* model. The purpose of Chapter 3 was to adapt the α-syn-PFF model to 3D neurospheres. Although we were able to replicate previous work and induce a DAergic-like phenotype in neurospheres formed using the human cell lines, LUHMES, the enhanced differentiation and spheroid formation protocol implemented for SH-SY5Y neurospheres did induce a DAergic-like phenotype. While this protocol increased TH expression in these cells, differentiated SH-SY5Y cells were not post-mitotic and did not express detectable levels of DAT, limiting their utility for our studies. We also confirmed that LUHMES neurospheres are susceptible to MPP^+, but SH-SH5Y neurospheres differentiated with this method do not express DAT and are not susceptible to MPP^+. Finally, we were

unable to adapt the α-syn PFF model in either LUHMES or SH-SY5Y neurospheres. We observed a concentration-dependent increase in detergent-insoluble α-synuclein after PFF application, but no toxicity was observed (Figure 3.6). Based on these results, in Chapter 4, we aimed to recapitulate the two-hit system *in vitro* system using the MPP^+ model in LUHMES neurospheres to study the role of specific genes of interest in PD-related DAergic toxicity.

Linking the role of developmental dieldrin-induced differentially modified candidate genes and the exacerbation of Parkinsonian toxicity

Developmental dieldrin exposure induces a poised epigenetic state early in life that mediates susceptibility to Parkinsonian toxicity in adulthood. Previous work has identified that developmental dieldrin exposure induces sex-specific differential methylation and protein-coding transcript expression in the mouse midbrain (Kochmanski et al., 2019). Specifically, 288 genes with female-specific differential methylation and 83 genes with male-specific differential methylation were identified. The list of genes was filtered based on confirmation of expression in neurons from the Brain RNAseq database, midbrain expression based on midbrain RNAseq data from Kochmanski et al., and expression in undifferentiated LUHMES cells from Pierce et al. (Kochmanski et al., 2019; Pierce et al., 2018; Zhang et al., 2016). Further selection was based on differential expression in our previous developmental dieldrin exposure study, network analysis via StringDB, and prior knowledge of gene function (Kochmanski et al., 2019). Based on these criteria, the nuclear receptor-related -1 (*NR4A2*) and the ephrin receptor B2 (*EPHB2*) were selected as candidate genes of interest for follow-up studies.

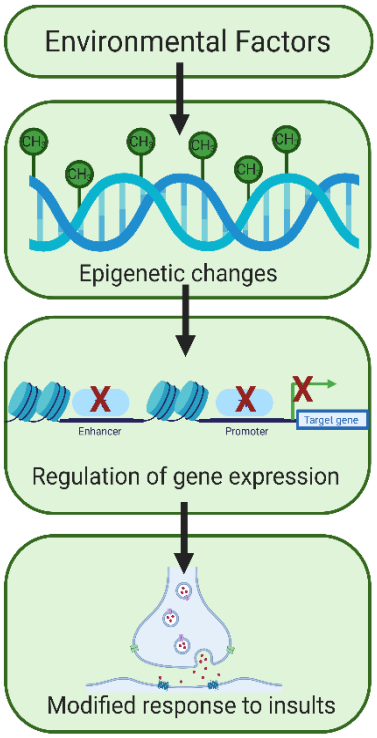

Figure 5.2 Environmental factors modify the epigenome, leading to changes in the regulation of gene expression and altered susceptibility to future insults. Made in BioRender.

In this project, the differential expression of these candidate genes during development was modeled by modifying the expression of candidate genes in proliferating LUHMES cells before induction of DAergic differentiation. After differentiation, a second hit with 1-methyl-4-phenylpyridinium (MPP+), a DAergic toxicant and the active metabolite of MPTP, was used to parallel the *in vivo* two-hit models to assess whether each candidate gene mediates neuronal susceptibility to PD-related toxicity (Figure 5.2).

NR4A2

NR4A2 encodes the nuclear receptor related-1 (NURR1) protein, a transcription factor critical for DAergic neuron maintenance and development (Decressac et al., 2013; J. Dong et al., 2016; Luo, 2012; Smits et al., 2003). Both clinical and experimental data demonstrate that dysregulation of Nurr1 function leads to DA neuron dysfunction and

that may contribute to the pathogenesis of PD (Decressac et al., 2013). In addition, compounds that activate Nurr1 protein and *Nr4a2* gene therapy in preclinical rodent models can enhance DA neurotransmission and protect DA neurons from toxicant and microglia-mediated neuroinflammation (J. Dong et al., 2016). Data in rat midbrain, human midbrain, and the human neuroblastoma cell line, SK-N-AS, demonstrate that there are multiple Nurr1 splice variants expressed in these cells. These isoforms have variable ability to activate transcription of target genes including DAT and TH, suggesting that regulation of isoform-specific expression could be a critical regulatory mechanism in DA neurons (Michelhaugh et al., 2005). In our developmental dieldrin exposure study, we identified a female specific-hypermethylated site within an intron *Nr4a2*. This result makes epigenetic regulation of *Nr4a2* by environmental toxicants potentially through differential isoform expression, a novel potential mechanism by which developmental exposure to dieldrin may alter PD susceptibility (Kochmanski et al., 2019). Since *Nr4a2* there was a slight increase in expression of the protein-coding transcript in female animals, and the phenotype associated with dieldrin-induced exacerbation of PFF is specific to male mice, we expected that *NR4A2* would be protective against toxicity. While we did not detect changes in expression in these changes, our data suggests there may be small transcript level changes that we were unable to detect and did not model here. Nevertheless, if there is an actual increase in expression in females, we would expect that knockdown of *NR4A2* would increase susceptibility and overexpression would result in no change or a decrease in susceptibility to PD-related toxicity.

Modifying the expression of the candidate gene, *NR4A2* during proliferation, affects the expression of DA markers after differentiation of neurospheres. It appears that *NR4A2* knockdown upregulates DAergic markers and maintains a lower DAT: VMAT2 ratio (Kochmanski et al., 2019). Previous work has shown that this ratio of DAT:VMAT2 is an indicator of a neuron's sensitivity to DAergic toxicity. A higher ratio would indicate an upregulation of DAT which would allow more DA uptake, but a lower expression of VMAT2 would result in less DA sequestering into synaptic vesicles resulting in increased cytosolic DA. Cytoslic DA is prone to autoxidation and enzymatic degradation forming toxic metabolites which can result in dopaminergic degeneration (Miller et al., 1999). *NR4A2* knockdown maintained a reduced ratio of DAT:VMAT2 suggesting that we would see decreased susceptibility to DAergic toxicity. The DAT:VMAT2 ratio in *NR4A2* overexpressing neurospheres was not significantly affected. However, neither modification of *NR4A2* expression affected MPP^+-induced toxicity. Therefore, altered *NR4A2* expression during a differentiation modeled *in vitro*, alters the differentiation of DAergic neurons, and knocking down *NR4A2* resulting in a decrease in a marker of susceptibility to toxicity as indicated by the ratio of DAT:VMAT2. Limitations and future directions are discussed below in conjunction with *EPHB2* findings.

EPHB2

EPHB2 encodes Ephrin type-B receptor 2 (EPHB2), a receptor tyrosine kinase that binds the receptor-binding domain of ephrin-B ligands; these proteins communicate across extracellular space allowing for cell-cell bidirectional signaling (Martínez & Soriano, 2005). Ephrins and the Eph receptors show dynamic expression patterns in the developing central nervous system (CNS) and are expressed in most adult CNS cell

types (Yang et al., 2018). The *EPHB2* signaling pathway is involved in many developmental processes in the CNS, including migration of neural progenitors to the dentate gyrus, regulation of axon guidance, dendritic spine formation, glutamatergic synaptogenesis, and long-term potentiation (Catchpole & Henkemeyer, 2011; Flanagan & Vanderhaeghen, 1998; Henderson et al., 2001; Kayser et al., 2006; Takasu et al., 2002). We observed female-specific hypermethylation of *Ephb2* and increased expression of a protein-coding transcript (Kochmanski et al., 2019). The phenotype and toxicity associated with dieldrin-induced exacerbation of PFFs was specific to male mice only, and because of the known reduced risk of PD in females, we expected that *EPHB2* expression would serve as a protective gene against PD-related toxicity. Alterations of *EPHB2* also modify the expression of DA markers and the indicator of DAergic vulnerability, the ratio of DAT: VMAT2 in neurospheres. *EPHB2* overexpression significantly reduced TH and DAT expression, but increased VMAT2 resulting in a decreased ratio of DAT: VMAT2 which is thought to be a protective marker against DAergic toxicity (Miller et al., 1999). On the other hand, *EPHB2* knockdown increased TH, but a reduction in DAT, VMAT2 levels, and an overall increase in DAT:VMAT2. Therefore, *EPHB2* expression plays an important role in regulating DAergic differentiation. The previously observed-developmental-dieldrin-induced hypermethylation and increased expression of *Ephb2* in female mice may serve as a protective factor by regulating DAergic development and maintaining a lower DAT: VMAT2 ratio. However, there were no observed alterations in MPP+-induced toxicity.

Alterations of the developmental dieldrin-induced differentially modified candidate genes during proliferation, NR4A2, and EPHB2 affect the DAergic phenotype and the ratio of DAT:VMAT2 of neurospheres following differentiation.

This work is based on the hypothesis that these changes in DAergic gene expression modify susceptibility later in life. Although we showed observed changes in an established marker of susceptibility to DAergic toxicity (DAT:VMAT2), we were unable to recapitulate toxicity to the second hit of MPP^+ in this *in vitro* system. Since *NR4A2* and *EPHB2* are two of hundreds of genes differentially modified by dieldrin exposure, likely, these genes are not the sole mediators of exacerbated Parkinsonian toxicity and may have only small effects on toxicity on their own that are not apparent in this simplified model (Kochmanski et al., 2019). Since MPP^+ produces robust and rapid DAergic degeneration, it is possible that this model is too potent to observe the effects of these candidate genes on mitigating or exacerbating toxicity to MPP^+. The gene modifications that were observed *in vivo* were sex-specific, and only male mice were susceptible to developmental dieldrin-induced exacerbations to PFFs while female mice did not show any changes in susceptibility indicating a potential role of sex-specific mechanisms (Gezer et al., 2020; Kochmanski et al., 2019). Specifically, the previous study showed that developmental dieldrin-induced sex-specific modifications of the expression of protein-coding transcripts for *Ephb2* were transcript-specific, and there were no significant changes in *Nr4a2* protein-coding transcript expression (Kochmanski et al., 2019). Since LUHMES cells are genetically female, we may not have been able to model this effect, depending on the sex-specific mechanism. However, there is not a comparable cell system derived from genetically males. In the previous study, there

were observed differential methylation patterns on these candidate genes *in vivo*. However, here we modified the expression of the full length of these genes as a proxy for the changes observed *in vivo*.

Based on these findings, future projects should address the following:

- What are the effects of modifying these genes *in vivo*? The ratio of DAT to VMAT2 is an indicator of susceptibility to DAergic toxicity. This ratio was used as a predictor of susceptibility in *in vivo* models using MPTP (Miller et al., 1999). However, the implications of this ratio have not yet been extended to *in vitro* models. Although we observed significant changes in the ratio of DAT: VMAT2 with *NR4A2* and *EPHB2* expression modification, there was no observed effect on MPP^+-induced toxicity. Therefore, this ratio may only serve as a predictor of vulnerability in more complex models that include other cell populations. Therefore, these modifications to candidate genes should be extended to more complex models to recapitulate the effects observed *in vivo*.
    - In addition to the DAT: VMAT2 ratio, these gene modifications altered the DAergic phenotype in differentiated LUHMES cells. The effects of these gene modifications on DA release and DA mishandling should be followed up on *in vivo*.
- Are more functional measures like network activity, firing rate, and measures of synchrony more sensitive to measuring the effects of differential gene modification on disease susceptibility? In new approach methodologies, a battery of assays is often used to screen for toxicity. In a high-throughput screen of compounds on neurodevelopmental toxicity, it was found that network activity

was the most sensitive assay in 2D adherent *in vitro* models in comparison to assays that we have used here like neurite outgrowth and ATP assays (Carstens et al., 2022). Therefore, more sensitive measures in neuronal functioning should be used to address other ways in which these genes may mediate toxic responses rather than hypothesizing that these genes affect overt toxicity to MPP^+.

- Does modifying *NR4A2* and *EPHB2* alter other functions involving the mitochondria? DAergic neurons are particularly sensitive to mitochondrial damage, and oxidative stress, and have low antioxidant capacity which can result in DAergic dysfunction (Haddad & Nakamura, 2015). Oxidative stress and DAergic dysfunction precede DAergic degeneration. Therefore, it is possible that these developmental dieldrin-induced differentially modified genes play a role in these early mitochondria alterations and DAergic dysfunction, but do not overtly affect ATP and neurite loss.

Concluding Remarks

Overall, this project has expanded our understanding of the link between developmental dieldrin exposure and PD risk. Consistent with previous literature, this work has demonstrated that developmental dieldrin exposure results in alterations in the dopamine system which contributes to an exacerbated susceptibility to PD. Furthermore, work was completed to optimize and develop methods to screen candidate genes or other toxicants in PD-related toxicity using a DAergic-like neurosphere model. Using this model, two candidate genes with developmental dieldrin exposure-induced modifications were screened for their effects on PD-like toxicity.

Using this model, it was demonstrated that altering these genes during differentiation alters the DAergic phenotype and markers of DAergic mishandling (Figure 5.3). In conclusion, this work has investigated the hypothesis that developmental exposures lead to persistent epigenetic changes in the regulation of gene expression that can result in alterations in DAergic function and increased disease susceptibility later in life. Since environmental factors play a significant role in PD, and developmental periods are specifically vulnerable to such factors, we must continue to study and identify the link between developmental exposures and disease development later in life.

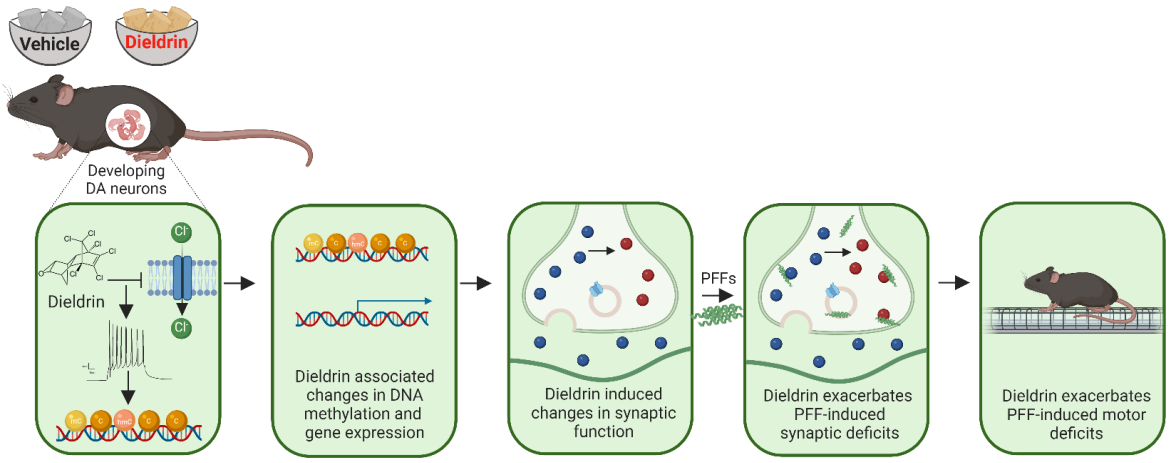

Figure 5.3. A model of developmental dieldrin exposure and exacerbated vulnerability to Parkinson's disease. Developmental exposures to dieldrin can alter developing dopaminergic neurons which can result in associated changes in gene modifications which induces alteration in striatal synapse function. With a second hit such as PFFs, this dopaminergic dysfunction is further exacerbated and unmasked resulting in detectable motor deficits. Made in BioRender.

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