# VESICULAR SEQUESTRATION OF DOPAMINE MODULATES NEURONAL HEALTH IN PARKINSON'S DISEASE MODELS

by

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Parkinson's disease (PD) is the second most commonly occurring neurodegenerative disease. It is characterized by motor dysfunctions resulting from a deficiency of dopamine in the striatum caused by degeneration of dopaminergic (DAergic) neurons in the nigrostriatal pathway. While there is no cure for PD, L-DOPA is commonly prescribed to alleviate the motor symptoms by restoring dopamine in the striatum. However, L-DOPA may jeopardize the health of the remaining neurons based on evidence that dopamine handling is dysregulated in PD, which is likely the result of deficient vesicular sequestration by the vesicular monoamine transporter 2 (VMAT2). Accordingly, VMAT2 mRNA, protein, and activity are decreased in PD patients. Deficits in vesicular sequestration result in cytosolic dopamine, which is detrimental to neuronal health due to two processes – metabolism and oxidation – which generate reactive metabolites and cause oxidative stress. It is vital to investigate the consequence of impaired dopamine sequestration to understand the pathogenesis of PD and to understand how to approach therapeutic intervention in PD.

This dissertation research studied the effect of viral-mediated decreased VMAT2 expression on neuronal health. A decrease in VMAT2 resulted in increased dopamine metabolism and oxidation, deficits in dopamine-mediated behaviors, generation of aberrant  $\alpha$ -synuclein, activation of PD-associated kinase LRRK2, and a loss of DAergic neurons in the SNpc with a corresponding degeneration of DAergic terminals in the striatum. The loss of DAergic neurons following decreased endogenous VMAT2 was rescued by reintroducing exogenous VMAT2.

These data suggest that decreasing VMAT2 expression is sufficient to induce PD associated pathogenic mechanisms of neurodegeneration, further implicating the role of dysfunctional dopamine packaging in the pathogenesis of PD. Given the toxic potential of cytosolic dopamine observed, the hypothesis that increased sequestration of dopamine would be protective against nigrostriatal degeneration in a PD model was tested. While we were able to successfully demonstrate viral-mediated overexpression of VMAT2, we were unable to detect VMAT2 overexpression-mediated protection. This data is in disagreement with previous *in vivo* studies and is likely due to limitations of our model.

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# **COMMON ABBREVIATIONS IN THIS DOCUMENT**

Aromatic amino acid decarboxylase (AADC)

Adeno-associated virus (AAV)

Catechol-O-methyl transferase (COMT)

Dopaminergic (DAergic)

Dopamine transporter (DAT)

3,4-Dihydroxyphenylacetic acid (DOPAC)

3,4-Dihydroxyphenylacetaldehyde (DOPAL)

1-3,4-dihydroxyphenylalanine (L-DOPA)

Monoamine oxidase (MAO)

1-methyl-4-phenylpyridinium (MPP+)

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

Parkinson's disease (PD)

Ribonucleic acid (RNA)

Small-hairpin ribonucleic acid (shRNA)

Substantia nigra (SN)

Substantia nigra pars compacta (SNpc)

Tyrosine hydroxylase (TH)

Vesicular monoamine transporter (VMAT)

Ventral tegmental area (VTA)

#### PREFACE

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#### **1.0 INTRODUCTION**

The goal of this work was to develop novel *in vivo* models to study the effect of vesicular sequestration of dopamine (3,4-dihydroxyphenethylamine) on neuronal health with implications for Parkinson's disease (PD). Although there is evidence that dopamine is involved in the pathogenesis of PD, previous *in vitro* and *in vivo* models of dopamine sequestration have been limited. The novel models presented here will allow for research into the role that dopamine plays in pathogenic mechanisms of neurodegeneration in PD. This dissertation project was divided into two aims: (1) characterize the impact of decreased vesicular sequestration of dopamine on dopaminergic (DAergic) neuronal health and investigate the mechanisms by which this impairment causes nigrostriatal DAergic neurons to become vulnerable to degeneration and (2) investigate the potential of targeting dopamine handling as a therapeutic intervention in PD models.

## **1.1 DOPAMINE BIOCHEMISTRY**

The catecholamine neurotransmitter dopamine regulates a number of physiological functions and behaviors including motor output and motivation. As such, dysregulation of dopamine transmission underlies many disease states including PD and psychiatric disorders. Dopamine has the potential to act as an endogenous neurotoxin when its vesicular sequestration is dysregulated. The two processes by which dopamine acts as a neurotoxin – oxidation and enzymatic degradation – occur when dopamine packaging is dysregulated and dopamine

accumulates in the cytosol (T. G. Hastings, Lewis, D.A., Zigmond, M.J., 1996). In order to limit these processes, and therefore the generation of reactive metabolites and reactive oxygen species, dopamine's synthesis, packaging, and degradation are tightly regulated.

## **1.1.1 DOPAMINE SYNTHESIS**

Dopamine is synthesized in the cytosol through an initial rate-limiting conversion of tyrosine to l-3,4-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH) (Nagatsu et al., 1964). L-DOPA is then converted to dopamine by aromatic amino acid decarboxylase (AADC) (Lovenberg et al., 1962). Following its synthesis, dopamine is rapidly sequestered into synaptic vesicles by the vesicular monoamine transporter 2 (VMAT2). After vesicular release into the synapse, dopamine is taken back up pre-synaptically by the dopamine transporter (DAT), where it can be metabolized in the cytosol or re-sequestered into synaptic vesicles. The synthesis, packaging, and reuptake machinery are physically associated in order to facilitate the rapid sequestration of dopamine following its synthesis and reuptake, thereby limiting the amount of dopamine in the cytosol (Egana et al., 2009).

## **1.1.2 DOPAMINE PACKAGING**

On the synaptic vesicle, there is a vacuolar-type ATPase, which is responsible for acidifying the interior of synaptic vesicles by pumping hydrogen ions into synaptic vesicles against a concentration gradient. This results in a highly acidic interior to the synaptic vesicle, which stabilizes vesicular dopamine and acts as the proton-motive force required by VMAT2 to load dopamine into the vesicle (Knoth et al., 1981). In this way, VMAT2 maintains neuronal health by

sequestering dopamine within vesicles where it is stable and isolated. There are two isoforms of VMAT - VMAT1 and VMAT2. VMAT2 is encoded by the gene SLC18a2 and found predominantly in the central nervous system, whereas VMAT1, encoded by SLC18a1, is primarily found in the periphery (Erickson et al., 1996). VMAT2 is responsible for the vesicular sequestration of all monoamines including serotonin and histamine. It is also necessary for adrenergic and noradrenergic transmission, as dopamine is the precursor to norepinephrine and the synthesis of norepinephrine occurs within synaptic vesicles following VMAT2-mediated sequestration of dopamine. VMAT2 has 12 transmembrane domains, with many potential sites of post-translational modification (Krantz et al., 1997) that may influence its localization (Fei et al., 2008; Waites et al., 2001; Yao et al., 2004; Yao & Hersh, 2007) and activity (Brunk et al., 2006; Torres & Ruoho, 2014). In particular, VMAT2's expression and activity has been shown to be modulated by oxidative modifications including nitrosylation (Eyerman & Yamamoto, 2007), and its activity is decreased following methamphetamine (Brown et al., 2002; Chu et al., 2010). The complexity of VMAT2's structure, including the many sites of post-translational modification, may influence its expression and cellular localization, which therefore would have downstream consequences on the amount of cytosolic dopamine present within a neuron.

In addition, a number of drugs have been identified that influence the activity of VMAT2. VMAT2's activity has been shown to be enhanced following buproprion (Rau et al., 2005), cocaine (Brown et al., 2001), dopamine receptor agonists (J. G. Truong, Newman, et al., 2004; Jannine G. Truong et al., 2003), apomorphine (J. G. Truong, Hanson, et al., 2004), and methylphenidate (Sandoval et al., 2002). In addition, the brain-gut peptide PACAP38 increases VMAT2 expression and protects against methamphetamine-induced toxicity (Guillot et al., 2008). Additionally, drug screens have identified a number of drugs that modulate VMAT2 activity, including dacarbazine,

which is used as a chemotherapeutic (Lawal et al., 2014). Alternatively, VMAT2 inhibitors developed to treat dyskinesia include tetrabenazine and reserpine (Frank, 2010). The specificity of these inhibitors has led to the use of [11C]dihydrotetrabenazine to visualize VMAT2 binding in PD patients by positron emission tomography (Bohnen et al., 2006).

## **1.1.3 DOPAMINE METABOLISM**

Dopamine is an organic base, and at physiologic pH in the cytosol of neurons, the amine group is protonated. Cytosolic dopamine is susceptible to oxidation due to its catechol ring. Oxidation occurs either through autooxidation (Herlinger et al., 1995) or enzymatic oxidation (T. G. Hastings, 1995) generating the highly reactive dopamine quinone and reactive oxygen species as a byproduct (Stokes et al., 1999). Dopamine oxidation occurs normally within DAergic neurons, which is evidenced by the presence of neuromelanin, a black substance visual to the eye (Sulzer et al., 2000) that accumulates within DAergic neurons over time (Xing et al., 2018). The substantia nigra, a population of DAergic neurons in the midbrain, is named after "black substance" from the presence of neuromelanin. These neurons degenerate in PD, which leads to a loss of the neuromelanin-containing cell bodies and therefore a loss of "black substance" in post-mortem tissue from PD patients.

The product of dopamine oxidation – the dopamine quinone – will attack free and proteinbound cysteine. Cysteine can be found in the cytosol in the form of glutathione – an endogenous antioxidant defense. If the dopamine quinone depletes the pool of glutathione, the neuron will have decreased antioxidant defense against additional oxidative insults, including further dopamine metabolism. Of note, there is evidence of a deficiency in endogenous antioxidants in PD as evidenced by depleted glutathione levels in the brains of post-mortem PD patients (Sian et al., 1994). However, *in vivo* experiments have demonstrated that by increasing antioxidant defenses, such as an increase in glutathione, dopamine-induced neurotoxicity can be attenuated (T. G. Hastings, Lewis, D.A., Zigmond, M.J., 1996).

Protein-bound cysteine is often found at the active site of proteins and the covalent modification of the quinone to protein-bound cysteine renders proteins inactive and signals for their degradation. Additionally, studies have shown that dopamine will react with the selenocysteine amino acid residue found in selenoproteins (Hauser et al., 2013). In comparison to cysteine, selenocysteine has a selenol group in place of the thiol group, which results in increased reactivity and increased participation in antioxidant reactions by proteins containing selenocysteine (Stadtman, 1996). Animal studies have demonstrated increased susceptibility of DAergic neurons to degeneration following selenium deficiency (Kim et al., 2000), and decreased susceptibility following selenium supplementation (Khan, 2010), suggesting that increased selenoprotein expression may be a potential therapeutic intervention in PD. Analysis from postmortem tissue from PD patients has revealed a deficiency in expression of selenoprotein P, but whether this is deficiency is causal in the pathogenesis of the disease or a consequence of disease pathogenesis is unclear (Bellinger et al., 2012).

If dopamine does not undergo oxidation, it is susceptible to enzymatic metabolism. The enzyme catechol-*O*-methyl transferase (COMT) (Axelrod & Tomchick, 1958) *O*-methylates dopamine (Männistö & Kaakkola, 1999), and monoamine oxidase (MAO) (Edmondson et al., 2004), which is located on the mitochondrial membrane (Schnaitman et al., 1967), converts dopamine into the metabolite 3,4-dihydroxyphenylacetaldehyde (DOPAL), while generating hydrogen peroxide as a byproduct (Rees et al., 2009). If allowed to accumulate, DOPAL will react with proteins and generate additional reactive oxygen species as a byproduct (Goldstein et al.,

2014). DOPAL normally will readily undergo further enzymatic metabolism by aldehyde dehydrogenase (ALDH) to form (3,4-dihydroxyphenylacetic acid) DOPAC. Similar to dopamine oxidation, the enzymatic degradation of dopamine generates reactive oxygen species as well as reactive metabolites and for this reason it is essential to minimize the cytosolic pool of dopamine in order to maintain neuronal health.

In addition to causing neuronal dysfunction by generating reactive oxygen species, oxidized dopamine has been shown to impair mitochondrial functioning (Berman, 1999; Brenner-Lavie et al., 2008; Burbulla et al., 2017; Hauser et al., 2013) and autophagy (Burbulla et al., 2017; Martinez-Vicente et al., 2008). Furthermore, dopamine and its metabolic products have been shown to interact with PD-associated factors including DJ-1 (Girotto et al., 2012; Van Laar et al., 2009) and  $\alpha$ -synuclein (Bisaglia, Tosatto, et al., 2010; Jinsmaa et al., 2016; Mor et al., 2017; Outeiro et al., 2009). The interaction between dopamine and its metabolites and  $\alpha$ -synuclein can cause downstream deficits in chaperone mediated autophagy (Martinez-Vicente et al., 2008), decreased glucocerebrosidase activity (Burbulla et al., 2017), damage to synaptic vesicles (Plotegher et al., 2017), and further mitochondrial dysfunction (Burbulla et al., 2017; Di Maio et al., 2016).

The metabolism of dopamine is thought to result in a higher baseline level of oxidative stress within DAergic neurons, which may explain the enhanced vulnerability to degeneration in DAergic neurons in PD. In addition, as summarized above, dopamine and its metabolites are highly reactive and can cause neuronal dysfunction. Importantly, the direct interaction of dopamine and its metabolites with PD-associated proteins associated suggests a contributing role of dopamine in the pathogenesis of PD.

#### **1.2 PARKINSON'S DISEASE**

PD is the second most commonly occurring neurodegenerative disease and the second most commonly occurring movement disorder. It impacts one in every 100 people over the age of 60, and is characterized by progressive and debilitating motor symptoms including resting tremor, rigidity, and postural instability resulting from degeneration of motor-controlling DAergic neurons in the nigrostriatal pathway (Dickson, 2012). This population of neurons has its cell bodies located in the midbrain in a nucleus called the substantia nigra pars compacta (SNpc), and the terminals in the caudate and putamen, collectively referred to as the striatum. The loss of these neurons results in a deficiency in DAergic transmission in the striatum, thereby causing motor deficits (Ehringer & Hornykiewicz, 1998). While other neuronal populations also undergo neurodegeneration in PD, the motor deficits resulting from nigrostriatal degeneration are the cardinal symptoms of PD and lead patients to seek medical attention. By the time that motor symptoms present, there is already significant loss of DAergic neurons in the nigrostriatal pathway (Giguere et al., 2018).

It is believed that the majority of cases of PD result from complex interactions between genetic predisposition and a lifetime accumulation of environmental exposures, paired with normal aging processes. This complexity has limited the development of therapeutic interventions because there is no singular therapeutic target for all cases of PD. Despite a variety of divergent causes, convergent pathogenic mechanisms of degeneration have been identified that appear to be common across all cases of PD, and novel therapeutic interventions currently being investigated target these processes. While therapeutic interventions that alleviate the motor deficits of PD exist, there are no disease-modifying interventions to slow or halt the progression of the disease. Additional research is necessary to better understand the pathogenic factors involved in degeneration and to test novel therapeutic interventions.

#### **1.2.1 PARKINSON'S DISEASE PATHOLOGY**

The pathology of PD has been well characterized and staged by Braak *et al.* 2003 demonstrating the progression of pathology and symptomology with disease progression (Braak et al., 2003). In post-mortem tissue from patients with PD, there is an apparent loss of the pigmented neuromelanin containing DAergic neuron cell bodies in the SN (Dickson, 2012). In the caudate and putamen, there is a progressive loss of DAergic terminals corresponding with disease progression. Within the remaining neurons in the SNpc that have not undergone degeneration, there is evidence of neuronal dysfunction. The pathologic hallmark of PD is the presence of protein inclusions called Lewy bodies that consist of insoluble proteins – the primary component of which is  $\alpha$ -synuclein (Spillantini et al., 1997).

The DAergic neurons in the SNpc are most commonly associated with PD because it is the degeneration of these neurons that results in the development of motor symptoms and leads to the eventual diagnosis of PD. There are prodromal symptoms of PD and common early signs include sleep disturbances, the loss of sense of smell, and constipation (Mahlknecht et al., 2015). It is known that other neuronal populations undergo degeneration in PD including noradrenergic neurons in the locus coeruleus that regulate sleep and attention, serotonergic neurons in the raphe nucleus involved in circadian rhythm regulation and alertness, and peripheral DAergic and noradrenergic neurons innervating the skin and heart (Dickson, 2012). Of interest, the DAergic neurons in the ventral tegmental area (VTA), which neighbor the SN, show resistance to degeneration, which suggests that not all DAergic neurons are the same or possess the same factors that regulate vulnerability to degeneration between the SNpc and VTA DAergic neurons is an active area of research, and it is known that VTA DAergic neurons show differences in gene

expression compared to the SNpc (Greene et al., 2005) including increased VMAT2 expression (Nirenberg et al., 1996), and decreased  $\alpha$ -synuclein expression (Solano et al., 2000) that may confer protection.

#### **1.2.2 PARKINSON'S DISEASE PATHOGENESIS**

Although the pathology of PD is well characterized, there is still a lot of work to be done to understand what causes PD. There is no singular genetic cause of PD, and both genetic and sporadic cases of PD exist. Inherited forms of PD are rare and only account for roughly 5-10% of all cases. Similarly, PD cases resulting strictly from environmental exposures are rare. The heritability of PD is estimated to be 27%, which suggests non-genetic factors play a critical role in the development of PD (D. Chang et al., 2017). As such, the majority of cases of PD are believed to result from interactions between predisposition genes and a lifetime accumulation of environmental factors paired with deficits resulting from the normal aging process (Cannon & Greenamyre, 2013; Horowitz & Greenamyre, 2010; Kalia & Lang, 2015; Pang et al., 2019; Polito et al., 2016; Priyadarshi et al., 2001).

## **1.2.3 GENETIC FACTORS IN PARKINSON'S DISEASE**

The first gene identified as causative in PD was  $\alpha$ -synuclein. Mutations in  $\alpha$ -synuclein are associated with inherited PD (Krüger et al., 1998; Polymeropoulos et al., 1997), as are gene duplications (Chartier-Harlin et al., 2004) and triplications (Singleton et al., 2003). Additional polymorphisms have been identified in genetic PD including gain-of-function mutations in the kinase LRRK2, which are the most prevalent mutations seen in genetic PD (Zimprich et al., 2004).

The protein DJ-1 has been implicated in PD, however, due to the multitude of cellular processes in which DJ-1 is involved, it is difficult to determine which critical function is responsible for increased risk of PD (Bonifati et al., 2002). Mutations in the mitochondrial protein PINK1 (Hatano et al., 2004; Rogaeva et al., 2004; E.M. Valente et al., 2003; E. M. Valente et al., 2004) and Parkin (Hattori et al., 1998; Kitada et al., 1998; Poorkaj et al., 2004; Shimura et al., 2000) have been identified, as well as mutations in GBA, which is involved in autophagy (Aharon-Peretz et al., 2004; Sidransky et al., 2009). Recent genome-wide association studies have identified additional risk loci in genes associated with increased risk of developing PD (D. Chang et al., 2017). The number and diversity of genes implicated in developing PD suggests that despite these divergent causes, there may be convergent mechanisms of degeneration common to all forms of the disease.

# **1.2.4 ENVIRONMENTAL FACTORS IN PARKINSON'S DISEASE**

Non-genetic components that contribute to the risk of developing PD encompass factors including age, sex, exposure to environmental pollutants or toxicants, diet and exercise, lifestyle choices, and occupation (Hancock et al., 2008). Certain occupations have been linked to developing PD, thought to be a consequence of occupation exposure to compounds such as pesticides, organic solvents, or metals (Tanner et al., 2009). In particular, manganese-induced parkinsonism has been well characterized (Lee, 2000; Montgomery Jr., 1995; Perl & Olanow, 2007). In addition, diet and exercise have been identified as modulators of risk of developing PD (Seidl et al., 2014). The non-genetic factors involved in PD risk are diverse and have been well reviewed (Ascherio & Schwarzschild, 2016).

The most well-known example of an environmental exposure resulting in rapid-onset of parkinsonian motor symptoms occurred in a group of people self-administering an opioid that

contained 1-methyl-4-pheynl-1,2,3,5-tetrahydropyridine (MPTP) contamination (Langston et al., 1983). MPTP is converted to 1-methyl-4-phenylpyridinium (MPP+) by MAO within astrocytes in the brain (Kopin, 1987). MPP+ is a specific DAergic neurotoxin (Javitch et al., 1984). It is believed its toxicity to DAergic neurons is in part due to MPP+ accumulating in DAergic neurons following transport by the plasma membrane dopamine transporter. MPP+ has also been shown to be a substrate for VMAT (Y. Liu et al., 1992), and MPP+ results in an increase in dopamine metabolism (S. J. Choi et al., 2015). The toxic effects of MPP+ are a result of its action as an inhibitor of complex I of the electron transport chain in mitochondria, which results in mitochondrial dysfunction (Cassarino et al., 1999; Murphy et al., 1995; Niklas et al., 1985). The discovery of acute-onset parkinsonism resulting from mitochondrial complex I inhibition was instrumental in understanding the role of mitochondrial dysfunction in the vulnerability of DAergic neurons to degeneration in PD.

Exposure to other mitochondrial complex I inhibitors have been identified as risk factors for developing PD. These include the organic pesticide rotenone, which has been linked to developing PD in migrant workers with increased exposure to the pesticide (Elbaz et al., 2009). Paraquat, another pesticide linked to developing PD (Tanner et al., 2011), acts as a redox cycler and generates free radicals (Bonneh-Barkay et al., 2005). The organic solvent trichloroethylene and its metabolites inhibit complex I and are associated with increased risk of PD (Gash et al., 2008). A study showing that risk of PD following pesticide exposure was mediated by genetic variants in DAT provides evidence supporting the interaction between genes and environment in the development of PD (Ritz et al., 2009).

## **1.2.5 CONVERGENT PATHOGENIC MECHANISMS IN PARKINSON'S DISEASE**

The diverse genetic and environmental factors involved in the development of PD converge on common pathogenic mechanisms including protein misfolding, intracellular trafficking deficits, oxidative stress, and mitochondrial dysfunction (Greenamyre & Hastings, 2004). These common pathogenic mechanisms have been the focus of much research into novel therapeutics with the hope that an intervention targeting a downstream pathogenic mechanism would result in an effective therapeutic intervention for all cases of PD. For example, recent work has identified activation of leucine-rich repeat kinase 2 (LRRK2) as a common pathogenic mechanism in genetic and idiopathic PD, suggesting the viability of LRRK2 inhibitors for PD treatment (Di Maio et al., 2018). Because both genetic and environmental factors are ubiquitously experienced by not only all neurons in the brain but all cells throughout the body, it is important to understand why only specific neuronal populations are vulnerable to degeneration in PD.

## **1.2.6 DOPAMINE SEQUESTRATION IN PARKINSON'S DISEASE**

Deficits in the vesicular sequestration of dopamine have the potential to increase oxidative stress and protein modifications that could impair neuronal functioning and increase vulnerability to degeneration. There is significant evidence that VMAT2 is dysfunctional in PD. Miller *et al.* (1999) identified a decrease in VMAT2 immunoreactivity in the DAergic terminals of postmortem brains from PD patients (Miller et al., 1999). Additional studies have isolated synaptic vesicles from the remaining terminals from post-mortem tissue from PD patients and shown that VMAT2 rate of uptake is impaired in PD tissue compared to healthy age-matched controls, which suggests that in addition to a loss of VMAT2 protein, the remaining VMAT2 has impaired

functioning (Pifl et al., 2014). There is an increase in the ratio of dopamine metabolites to dopamine in the striatum of tissue from patients with PD, which suggests an increase in dopamine turnover (Pifl et al., 2014). Additionally, an increase in the ratio of DOPAL to dopamine has been identified in post-mortem tissue from Parkinson's patients (Goldstein et al., 2011; Goldstein et al., 2013). Studies have indicated a decrease in the amount of glycosylated VMAT2 with normal aging, which is thought to result in a decrease in vesicular localization of VMAT2 (Cruz-Muros et al., 2008) and may contribute to age being a risk factor for PD.

Mutations in the gene for VMAT2 (*SLC18a2*) have been controversially linked to PD. Early reports from PD cohorts were unable to detect any single nucleotide polymorphisms in *SLC18a2* associated with developing the disease (C. Glatt et al., 2006). However, a recent report identified two low-activity variants in VMAT2 that may be associated with an increased risk of developing the disease (Xiong et al., 2016). There is also evidence of a systemic deficiency in VMAT2 in PD patients as shown by decreased VMAT2 mRNA in circulating platelets of PD patients, which suggests a systemic down-regulation of VMAT2 involved in PD pathogenesis (Sala et al., 2010).

Previous work has also shown that the sequestration of dopamine is impaired downstream of PD associated environmental factors (Qi et al., 2014), and many studies have identified dysregulation of dopamine sequestration and release downstream of genetic factors including misfolded  $\alpha$ -synuclein (Guo et al., 2008; Larsen et al., 2006; Mosharov et al., 2006), VPS35 variants (Cataldi et al., 2018), and LRRK2 variants (Melrose et al., 2010; Yue et al., 2015). In addition, the dopamine quinone has also been shown to interact with PD associated chaperone protein DJ-1 (Girotto et al., 2012), and dopamine and its metabolic products have also been shown to interact with  $\alpha$ -synuclein (Bisaglia, Greggio, et al., 2010; Jinsmaa et al., 2016; Mor et al., 2017; Outeiro et al., 2009), which can cause downstream deficits in chaperone mediated autophagy (Martinez-Vicente et al., 2008), decreased glucocerebrosidase activity (Burbulla et al., 2017), damage to synaptic vesicles (Plotegher et al., 2017), and mitochondrial dysfunction (Burbulla et al., 2017; Di Maio et al., 2016). Previous experiments have suggested dopamine mediates  $\alpha$ -synuclein-induced toxicity both in cell culture (Bisaglia, Tosatto, et al., 2010) and animal modeling experiments (Ulusoy et al., 2012), and other recent studies have implicated calcium as a susceptibility factor mediating DAergic neuron vulnerability to  $\alpha$ -synuclein (Mosharov et al., 2009). The cumulation of these studies demonstrate that impairment in dopamine sequestration is a convergent mechanism downstream of a variety of initiating factors, and that dopamine can have a feedback effect on PD-associated pathogenic mechanisms, which may contribute to the propagation of pathogenic mechanisms.

While impairments in dopamine sequestration appear to be involved in the development of PD, single nucleotide polymorphisms in the promoter region for VMAT2 that result in increased transcription have been identified that are associated with a decrease in PD risk, which suggests that increased VMAT2 is protective against developing Parkinson's (Brighina et al., 2013; C. E. Glatt et al., 2006; Yang et al., 2015). These data suggest that the sequestration of dopamine within synaptic vesicles is necessary to maintain neuronal health, and points to VMAT2 as a potential therapeutic target for PD.

### **1.2.7 PARKINSON'S DISEASE TREATMENTS**

There is no cure for PD, and we have yet to discover a disease-modifying therapeutic intervention that would slow or halt disease progression. Because diagnosis occurs late in disease progress, after many of the neurons have already undergone degeneration, it is imperative to

develop earlier biomarkers of the disease. Additional research is needed to understand PD pathogenesis and identify novel therapeutic interventions. Despite the lack of disease-modifying therapeutic interventions, there are a number of treatments that alleviate the motor symptoms of PD. These therapies work by targeting the deficiency in DAergic transmission in the striatum resulting from nigrostriatal degeneration. These therapies can act pre-synaptically by increasing the production of dopamine, post-synaptically by activating dopamine receptors, or on the larger basal ganglia structure by restoring basal ganglia activity through deep brain stimulation.

Diagnosis of PD follows the evaluation of motor and non-motor symptoms and whether the motor symptoms improve by supplementing with L-DOPA – the precursor to dopamine (Rizek et al., 2016). L-DOPA supplementation was developed to restore striatal DAergic transmission (Barbeau, 1969) and is administered with carbidopa to prevent L-DOPA's conversion in the periphery, allowing for maximal effect in the brain and minimal side effects (Marsden et al., 1973). While L-DOPA is effective at alleviating the motor symptoms of PD, it has a wearing-off effect over time and loses efficacy (Pahwa & Lyons, 2009). L-DOPA supplementation has been used in combination with other interventions that target the dopamine system including inhibitors of the enzyme responsible for degrading dopamine (monoamine oxidase) or dopamine receptor agonists. However, these other pharmacological interventions may cause unfavorable side-effects (Bonuccelli et al., 2009; Riederer & Laux, 2011), necessitating the development of novel therapeutic interventions.

Due to the limitations of pharmacological-mediated symptomatic relief, alternative therapeutic interventions have been developed. However, these alternatives are invasive and include deep brain stimulation (Deuschl et al., 2006; Schuepbach et al., 2013) or gene therapy (Axelsen & Woldbye, 2018; Coune et al., 2012). Deep brain stimulation has been implemented to

regulate the activity of basal ganglia circuitry, which has been beneficial in alleviating the motor symptoms of PD (Fang & Tolleson, 2017). Gene therapy efforts have include the administration of a virus overexpressing AADC – the enzyme responsible for converting L-DOPA to dopamine (Christine et al., 2019). Other gene therapy efforts have targeted neurotrophic factors believed to maintain the health of neurons (Axelsen & Woldbye, 2018) and enzymes in non-DAergic populations, such as glutamic acid decarboxylase, targeted to the subthalamic nucleus to restore basal ganglia activity (Niethammer et al., 2018).

Many of the therapeutic interventions targeting the dopamine deficiency work to increase the amount of dopamine produced in the brain. However, if the hypothesis that dopamine contributes to the degeneration of DAergic neurons in PD is true, these therapies may jeopardize the health of the remaining neurons. Given the toxic potential of cytosolic dopamine and the deficiency in dopamine sequestration in PD patients (Miller et al., 1999; Pifl et al., 2014; Sala et al., 2010), it is possible that the treatment of PD motor symptoms with L-DOPA could result in a further increase in dysregulated dopamine. This hypothesis has been tested with inconclusive results. Primary cultures derived from heterozygote VMAT2 knock-out animals showed increased cell death from L-DOPA (Kariya et al., 2005), however when tested in VMAT2 heterozygote animals L-DOPA did not result in increased toxicity (Reveron et al., 2002). There is evidence from animal studies demonstrating that adding the overexpression of VMAT2 into the viral preparation improves the efficacy of gene therapy interventions increasing dopamine production (Sun et al., 2004), which suggests that targeting VMAT2 either through genetic or pharmacological methods may be beneficial in the treatment of PD.

### **1.2.8 MODELS OF PARKINSON'S DISEASE**

Cellular and animal models of PD have been employed to understand pathogenic mechanisms associated with the disease process as well as test therapeutic interventions before moving to clinical trials. While *in vitro* experiments have been instrumental in understanding the pathogenic mechanisms involved in PD, there are many limitations to cell culture models of PD (Falkenburger & Schulz, 2006). Many of the cell lines used are non-neuronal, and neuronal cell lines are immortalized, which alters the characteristics of the cells. In addition, cell culture experiments lack the microenvironment surrounding neurons in the brain, including non-neuronal support cells like glia, which may contribute to disease pathogenesis, and the network in which the neurons exist. As a more faithful neuronal model, primary neuronal cultures have been utilized, however these neurons are derived from embryos and the contribution of age in the pathogenesis of PD is difficult to model in these cultures. Patient-derived induced pluripotent stem cells (iPSCs) have been implemented to study pathogenic mechanisms in a system believed to more directly replicate disease (Xiao et al., 2016).

Model organisms ranging from yeast to *C. elegans* (Cao et al., 2005; Cooper, 2006), zebrafish (Fitzmaurice et al., 2013), *Drosophila melanogaster* (Auluck et al., 2002; Feany & Bender, 2000), rodents (Cannon et al., 2009; Jackson-Lewis & Przedborski, 2007), and non-human primates (Emborg, 2007)have been utilized to study PD. Gene editing has led to the development of animal models that harbor PD-associated mutations, however genetic models have traditionally failed to replicate parkinsonian degeneration. Animals that harbor PD-associated genetic mutations include point mutations in  $\alpha$ -synuclein or LRRK2, gene duplication or triplications in  $\alpha$ -synuclein, and deletions of genes such as PINK1, Parkin, and DJ-1. In addition, delivery of fibrillar  $\alpha$ -synuclein directly into the brain can be used to initiate the aggregation of  $\alpha$ -synuclein and induce parkinsonian degeneration (Luk et al., 2012). These models have been extensively reviewed for their strengths and limitations (Williams-Gray et al., 2018). There are many possible explanations for why these models fail to replicate PD in animals including species differences in gene expression or organelles, compensatory mechanisms that emerge as a result of developing in the absence of a critical gene, and the lack of environmental components necessary to initiate disease pathogenesis.

Conditional (i.e. tissue-specific) and inducible (i.e. time-dependent) models of gene expression manipulation have been utilized to circumvent the traditional problems resulting from global genetic changes. Gene therapy techniques have been implemented to manipulate gene expression through exogenous overexpression or through a decrease in gene product through RNA interference (Fischer, 2016; Van der Perren et al., 2014). These models allow for targeting of gene manipulation to specific neuronal populations.

Specific DAergic toxicants such as methamphetamine (Larsen et al., 2002), MPTP (Smeyne & Jackson-Lewis, 2005), or 6-hydroxydopamine (Tranzer & Thoenen, 1967, 1972) have been used to induce DAergic degeneration, however the degeneration does not replicate the process of degeneration seen in PD. The rapid onset of parkinsonian motor symptoms following administration of an opioid contaminated with MPTP helped to uncover pathogenic mechanisms of degeneration in PD. MPTP is lipophilic and will readily cross the blood brain barrier. MPTP is converted to MPP+ by MAO within glial cells. MPP+ is extruded from glial cells and taken up by DAergic neurons as a substrate for DAT. MPP+ interacts with complex I of the electron transport chain in mitochondria, resulting in oxidative stress and mitochondrial dysfunction that can cause neurodegeneration. In this way, MPP+ acts as a specific DAergic neuron toxin. MPTP has been

used to study parkinsonian degeneration in animal models, and while it does not replicate neurodegeneration well in rats, it is used successfully in primates and mouse lines (Meredith & Rademacher, 2011; Porras et al., 2012).

Non-genetic models of PD include exposure to environmental toxicants that have been identified as risk factors in developing PD. These models include exposure to paraquat (McCormack et al., 2002), TCE (Gash et al., 2008), and rotenone (Cannon et al., 2009; Drolet et al., 2009). Paraquat's contribution to PD pathogenesis, and its ability to replicate PD in animal models, has been controversial (Berry et al., 2010; Miller, 2007). In terms of replicating disease progression, perhaps the best toxin model for PD is the systemic administration of the organic pesticide rotenone which specifically replicates features of PD (Cannon et al., 2009). Rotenone, an organic pesticide and complex I inhibitor, has also been identified as an environmental exposure linked with developing PD (Tanner et al., 2011). Rotenone can be used to reproducibly model PD in rat models through chronic, systemic injection (Cannon et al., 2009; Drolet et al., 2009) and in *Drosophila* models (Bayersdorfer et al., 2010; Coulom & Birman, 2004; Lawal et al., 2010). Although rotenone is administered peripherally, it results in specific DAergic neurodegeneration in the nigrostriatal pathway as well as replicating the pathological hallmark of Lewy Body inclusions.

#### **1.3 PREVIOUS ANIMAL MODELS MANIPULATING DOPAMINE HOMEOSTASIS**

Previous groups have developed *in vitro* and *in vivo* models to study the potential of dopamine to act as an endogenous toxin. Experiments in cell culture demonstrate that toxicity induced by the toxin rotenone is exacerbated by dysregulation of dopamine sequestration (H. Choi et al., 2005; Dukes, 2005; H. Q. Liu et al., 2005). In addition, the toxin rotenone has been shown to impair dopamine sequestration (Watabe & Nakaki, 2007, 2008). Furthermore, evidence from cell culture suggests that toxicity induced by methamphetamine and rotenone is mediated by dopamine (Sai et al., 2008). *In vivo* studies have shown that methamphetamine results in increased oxidized dopamine (LaVoie & Hastings, 1999), and recent work has shown that amphetamine's toxicity is dependent upon the action of VMAT loading amphetamine into synaptic vesicles, thereby depleting the vesicular proton pool, and displacing dopamine into the cytosol (Freyberg et al., 2016).

Although VMAT2 knock-out mice die shortly after birth (Wang et al., 1997), experiments performed in heterozygote VMAT2 knock-out animals show increased susceptibility to DAergic toxins (Gainetdinov et al., 1998). Additionally, hypomorph animals that express 5% of normal VMAT2 levels show age-dependent DAergic degeneration (Caudle et al., 2007), degeneration in the locus coeruleus (Taylor et al., 2014) as well as the replication of other non-motor PD symptoms (Taylor et al., 2009). Introducing cytosolic dopamine to non-DAergic neurons without proper sequestration machinery results in neurodegeneration (Chen et al., 2008), which demonstrates that unregulated cytosolic dopamine is sufficient to cause neurotoxicity in striatal neurons.

### **1.3.1 MODELS OF DECREASED DOPAMINE SEQUESTRATION**

As mentioned above, while VMAT2 knock-out mice die a few days after birth, heterozygote knock-out mice have been instrumental in understanding the interaction between decreased VMAT2 expression and environmental exposures. Heterozygote knock-out mice show increased susceptibility to DAergic toxins (Gainetdinov et al., 1998). In addition, a hypomorph mouse exists that expresses 5% of the normal levels of VMAT2. These animals, referred to as VMAT2-lo mice, show evidence of dysregulated dopamine through a decrease in VMAT2 protein expression and increased presence of dopamine metabolites (Caudle et al., 2007). Despite the potential of developing compensatory mechanisms to manage the decrease in VMAT2 expression, these animals show an age-dependent degeneration of DAergic cell bodies in the SNpc as well as a loss of DAergic terminals in the striatum. Interestingly, while this decrease in VMAT2 is ubiquitously experienced throughout the body, the neurodegeneration seen is specific to the SNpc and locus coeruleus (Taylor et al., 2014). A systemic decrease in VMAT2 expression in this model also replicates non-motor features of PD (Taylor et al., 2009). This model shows that impairments in DAergic sequestration, when experienced over a lifetime, can result in specific neurodegeneration mimicking the degeneration seen in PD.

## **1.3.2 MECHANISMS OF TOXICITY**

Many *in vitro* and *in vivo* experiments have been performed to understand the processes by which cytosolic dopamine acts as an endogenous neurotoxin. Dopamine, when injected into the brain, results in neurotoxicity mediated by intracellular dopamine oxidation (T. G. Hastings, Lewis, D.A., Zigmond, M.J., 1996). There is significant evidence that dopamine interacts with  $\alpha$ -

synuclein to cause neurotoxicity (Burbulla et al., 2017; Mor et al., 2017; Outeiro et al., 2009; Ulusoy et al., 2012). Experiments performed in the VMAT2-lo mice by Ulusoy et al. demonstrated that  $\alpha$ -synuclein mediates the neurotoxicity induced by decreased VMAT2 expression (Ulusoy, Bjorklund et al. 2012). This neurodegeneration is dependent on the production of dopamine and can be rescued by decreasing TH expression and therefore dopamine synthesis. These data suggest that interactions between dopamine and  $\alpha$ -synuclein mediate neurodegeneration.

Interestingly, during the initial generation of the VMAT2-lo mouse model, it was observed that the animals did not express  $\alpha$ -synuclein and the age-dependent neurodegeneration was seen only once  $\alpha$ -synuclein was reintroduced. Additional studies have investigated how dopamine interacts with  $\alpha$ -synuclein to cause neurotoxicity. Mor *et al.* identified the sequence on  $\alpha$ -synuclein where dopamine binds and demonstrated that neurodegeneration can be rescued in the A53T mouse model and *C. Elegans* by preventing dopamine and  $\alpha$ -synuclein interactions through mutations in the binding sequence (Mor et al., 2017). Dopamine-modified  $\alpha$ -synuclein has been shown to prevent the chaperone-mediated autophagy of multiple substrates (Martinez-Vicente et al., 2008) and dopamine-modified  $\alpha$ -synuclein binds to the mitochondrial protein TOM20, resulting in a loss of protein import and consequential mitochondrial dysfunction (Di Maio et al., 2016). Recent experiments have also identified a reduction in glucocerebrosidase activity by direct interaction following dopamine oxidation (Burbulla et al., 2017).

## **1.3.3 MODELS OF ENHANCED DOPAMINE SEQUESTRATION**

Experiments performed *in vitro* have demonstrated the protective ability of VMAT2 overexpression against PD-associated toxicants (Munoz et al., 2012). VMAT2 overexpression as a therapeutic target has been tested in a genetic mouse model that has a three-fold increase in
VMAT2 protein expression. These animals show protection against the DAergic toxin MPTP as well as protection against methamphetamine-induced neurotoxicity (K. M. Lohr et al., 2014; K. M. Lohr et al., 2016; K. M. Lohr et al., 2015). In addition, in a *Drosophila* model with overexpression of the *Drosophila* homolog of VMAT2, there is protection against toxin-induced DAergic neurodegeneration (Lawal et al., 2010). In addition, cells transfected to overexpress VMAT2 showed protection against L-DOPA induced toxicity (Mosharov et al., 2009). These experiments suggest that targeting VMAT2 activity would be a promising therapeutic intervention for PD. An increase in VMAT2 expression or activity would provide the dual therapeutic benefit of not only improving the motor symptoms by restoring dopaminergic transmission, but also protecting neuronal health by decreasing toxic cytosolic dopamine-derived compounds.

# 2.0 MATERIALS AND METHODS

# **2.1 CELL CULTURE**

Cell culture experiments were performed in RCSN-3 cells: an immortalized dopaminergic neuronal cell line derived from the substantia nigra of an adult rat (Paris, 2008). RCSN-3 cells were obtained through a material transfer agreement with the University of South Florida and the University of Chile and maintained in media (DMEM, 10% BS, 2.5% FBS, 40mg/l GS) in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO2.

# **2.2 TRANSFECTION PROTOCOL**

Predesigned small-hairpin ribonucleic acid (shRNA) constructs targeting rat vesicular monoamine transporter 2 (VMAT2) were obtained from Invitrogen and the shRNA construct demonstrating the best knock-down efficiency was chosen for viral production (Invitrogen, catalog number 10336022). A scramble control shRNA construct was provided from Invitrogen and used for *in vitro* experiments. Cells were transfected with a construct for green fluorescent protein (GFP) and shRNA constructs using Lipofectamine LTX and Plus Reagent and collected at 48 hours post-transfection for RNA and protein analysis.

## 2.3 CELL SURVIVAL ASSAY

Cell survival was analyzed using a Cellometer (Nexcelom Biosciences) to determine the number of GFP + cells at 24, 48, and 72 hours post-transfection.

# **2.4 IMMUNOCYTOCHEMISTRY**

Immunocytochemistry was used to visualize protein immunoreactivity. Cells were plated on poly-d-lysine coated coverslips before transfection. At the desired timepoint, cells were washed with sterile phosphate buffered saline (PBS) before being fixed with 4% paraformaldehyde. Following fixation, cells were washed in PBS before blocking (10% normal donkey serum, 0.3% Triton-X) for one hour at room temperature. Following blocking, cells were incubated in primary antibody solution (1% normal donkey serum, 0.3% Triton-X) for 24 hours at 4°C. After primary incubation, cells were washed in sterile PBS before incubation with secondary antibody solution (1% normal donkey serum, 0.3% Triton-X) for 2 hours at room temperature. After secondary incubation, cells were washed in sterile PBS before coverslips were mounted onto slides with gelvatol. Primary antibodies used for immunocytochemical analysis are listed in Table 2. Imagining was performed on an Olympus IXB1 confocal microscope, and intensity measurements was performed in Fluoview software by circling regions of interest (ROIs) were drawn around GFP-positive cells.

## **2.5 RNA ANALYSIS**

RNA was isolated utilizing the RNeasy Mini Kit (Qiagen catalog number 74104). For qtPCR analysis, cDNA was generated with the iScript cDNA Synthesis Kit (Bio-rad catalog number 1708890).

## **2.6 VIRAL VECTORS**

The oligonucleotide encoding the shRNA for VMAT2 is targeted against the following nucleotide sequence: [TCA][ACA][GTT][ATG][TTT][GCC][TTC][TCC][AGC].

The oligonucleotide encoding the shRNA construct with optimal VMAT2 knock-down (Invitrogen, catalog number 10336022) was annealed into the BamHI/EcoRI sites of pAAV-D(+)-U6-siRNA-CMV-GFP (a gift from Drs. Qing Bai and Edward Burton at the University of Pittsburgh). Adeno-associated (AAV) viruses (serotype 2) expressing the shRNA pAAV vector were produced by the Penn Vector Core (University of Pennsylvania). The shVMAT2 viral vector (AAV2-shVMAT2) expresses the shRNA construct under the human U6 promoter with bicistronic expression of eGFP under the CMV promoter with a titer of  $3.56 \times 10^{12}$  genome copies/mL.

A previously established universal shRNA control virus was used for control experiments (Zharikov et al., 2015).

The knock-down resistant VMAT2 construct expresses human VMAT2 with four silent mutations and a c-terminal myc-DDK tag. Site-directed mutagenesis was performed to construct the shRNA resistant VMAT2 (kdrVMAT2) construct utilizing NEBaseChanger Site-Directed Mutagenesis Kit (New England BioLabs catalog number E0554S). The nucleotide sequence in the

knock-down resistant VMAT2 viral vector is: [TCT][ACG][ATC][ATG][TTC][GCC][TTC][TCC][AGC]. This construct was cloned into pZac2.1 plasmid and the AAV2-kdrVMAT2 virus was produced by the Penn Vector Core with a titer of 1.73 x 10<sup>13</sup> genome copies/mL.

A control virus overexpressing GFP (AAV2-GFP) was ordered from Penn Vector Core with a titer of  $1.816 \times 10^{13}$  genome copies/mL.

For rescue experiments, all viruses were diluted to an equal titer (2.00 x  $10^{12}$  genome copies/mL) in sterile PBS.

Virus	Starting Titer	Vector Name
AAV2-shVMAT2	3.56e12 GC/mL	AAV2.U6.shR.VMAT2.CMV.eGFP.SV40
AAV2-shControl	1.53e13 GC/mL	AAV2.U6.shR.Scr.CMV.eGFP.SV40
AAV2-GFP	1.816e13 GC/mL	AAV2.CMV.PI.eGFP.WPRE.bGH
AAV2-VMAT2	1.556e13 GC/mL	AAV2.CMV.PI.hVMAT2wt-mycDDKtag.SV40
AAV2-kdrVMAT2	1.72e13 GC/mL	AAV2.CMV.PI.hVMAT2kdr-mycDDKtag.SV40

Table 1. SUMMARY OF VIRUSES

# **2.7 ANIMALS**

Adult ( $\geq$  3 months) male and female Lewis rats were obtained from Envigo (Indianapolis, Indiana, USA). Rats were singly housed in temperature-controlled conditions under a 12:12 light-

dark cycle with ad libitum access to food and water. All experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

## 2.8 STEREOTAXIC SURGERY

Stereotaxic surgery was performed to deliver virus to the brain. Animals were placed under isoflurane anesthesia (2-4%) via Somnosuite (Kent Scientific) and tested for proper anaesthetization based on response to toe- and tail-pinch. Once properly anesthetized, animals were placed on a stereotax and fitted with ear bars. Animals received continuous oxygen and 2-4% isoflurane throughout the duration of the surgery and were kept on a heated surface to maintain body temperature. The animal's head was shaved and treated with betadine before an incision was made lengthwise along the scalp to expose the skull. Bregma was identified and coordinates for Bregma were recorded. To inject into the substantia nigra, the following coordinates were used from Bregma: AP: -5.8mm, ML: +/-2.2mm, DV: -8.4mm (from skull). To inject into the ventral tegmental area, the following coordinates were used from Bregma: AP: -5.0mm, ML: +/-0.8mm, DV: -8.4mm (from skull). Once the coordinates in the anterior-posterior and medio-lateral dimensions were found, a small hole was made in the skull to allow the needle to be lowered into the brain to -8.4mm in the dorsal-ventral dimension. The virus kept cold until the point of infusion. A 10uL Hamilton Syringe (7635-01) with a Hamilton 1.25in, 30-gauge, 45° beveled needle (7803-07) was used to deliver the virus. The virus was infused at a rate of 0.2uL/minute to deliver 2uL of virus over a ten minute-period. After the infusion period, the needle was left in place for five minutes to account for virus diffusion. Once the needle was removed, the hole in the skull was filled with bone wax and the scalp was sutured. The animal was kept on a heated surface while

recovering from anesthesia and closely monitored. Animals received analgesia in the form of ketoprofen (3.0mg/kg s.c.) with the first dose pre-surgery and 2x/day for three days following surgery.

# **2.9 SACRIFICE**

Animals were deeply anesthetized with a mixture of phenytoin and pentobarbital (Henry Schein, Beuthanasia-D) prior to sacrifice by transcardial perfusion with phosphate buffered saline followed by 4% paraformaldehyde. The brains were harvested and submerged in 30% sucrose prior to sectioning on a freezing-stage microtome (ThermoFisher HM 450 sliding microtome) at 35µm. Fixed brain sections were maintained in cryoprotectant at -20°C until analysis. For biochemistry, animals were sacrificed at 8 weeks post-transduction via transcardial perfusion with phosphate buffered saline (PBS). Following PBS perfusion, the striatal tissue was dissected, weighed (12-30mg), and rapidly frozen on dry ice. Tissues were stored at -80°C until used for the analyses of free and protein-bound cysteinyl catechols and parent catechol levels.

# 2.10 BEHAVIORAL ANALYSIS

Deficits in dopamine-mediated behaviors were evaluated by two previously established tests of dopamine-mediated deficits - the postural instability test and the cylinder test (Woodlee, Kane et al. 2008). For the postural instability test, an animal was held with both back paws lifted off the surface and one forepaw restrained. The unrestrained forepaw was placed on a board covered with sandpaper marked with distances at 0.5cm intervals and the animal was held at a 45degree angle to the surface. The animal's nose was placed at 0cm and the animal was moved forward parallel to the surface until the animal took a corrective step with the forepaw placed on the board. The distance the animal had been moved was measured by the placement of its nose along the marked sandpaper. The distance the animal moved before the corrective step was triggered is referred to as the distance to trigger in centimeters. Each day the test was performed in triplicate for each forepaw. For the cylinder test, the animal was placed in a clear cylinder and in a dimly-lit room and allowed to explore for a 5-minute period. During this time, the investigator left the room and the animal was recorded on video camera. The videos were scored to record the number of times the animal used each forepaw during rearing behavior in order to determine paw preference during the exploratory behavior. The cylinder test was repeated three times across a two-week period for each animal to aggregate total number of paw touches.

# 2.11 ROTENONE

Rotenone was prepared at 2.8mg/mL in 2% DMSO and 98% miglyol. Vehicle solution was prepared at 2% DMSO and 98% miglyol. Rotenone was prepared fresh every three days and dosed intraperitoneally once per day while alternating sides of the abdomen to decrease risk of peritonitis. In the endpoint study, animals were dose daily until motor endpoint was reached. Endpoint was determined by analyzing the animal's posture, coordination, and mobility. In the acute study, animals were dosed for five days and sacrificed 24h after the last injection.

## 2.12 HISTOLOGY

Histology was performed on 35µm brain sections maintained in cryoprotectant at -20°C. Immunohistochemistry was performed on free-floating sections before mounting on glass slides for imaging. Sections were washed in PBS before blocking (10% NDS, 0.3% Triton-X) for 1 hour at room temperature. Following blocking, sections were incubated in primary antibody solution (1% NDS, 0.3% Triton-X) for 48 hours at room temperature. After primary antibody incubation, sections were washed in PBS before fluorescently-tagged secondary antibody incubation (1% NDS, 0.3% Triton-X) for 2 hours at room temperature. Following secondary antibody incubation, sections were washed in PBS before mounted and coverslipped on slides using gelvatol.

3,3-Diaminobenzidine (DAB) was also utilized to visualize protein. In the DAB protocol, sections were pretreated with hydrogen peroxide to block endogenous peroxidase activity to reduce non-specific signal. Blocking and primary antibody incubations were the same as previously described. Following primary incubation, sections were treated with a biotinylated secondary and then exposed to a solution containing avidin-biotin complex. Tissue was then treated with DAB in a solution containing hydrogen peroxide before the reaction was stopped with PBS washes. Following PBS washes, the sections were mounted onto slides and put through a progressive dehydration protocol before coverslipping using permount.

# 2.13 PROXIMITY LIGATION ASSAY

Proximity ligation assay (PLA) was performed following a traditional immunohistochemistry protocol. Sections were incubated in PLA primary antibodies (in 1% NDS)

overnight at 4°C. After primary PLA incubation, sections were washed in PBS and mounted on slides. PLA probe solutions (Duolink; Sigma Aldrich catalog numbers: DUO92001, DUO92001, DUO92004, DUO92005) were made with AB diluent and Blocking solution obtained from Sigma and sections were incubated for 1 hour at 37°C. Following probe incubation, sections were washed in Wash Buffer A before incubation in Ligation solution (Ligation 5X, Ligase, diH2O) for 45 minutes at 37°C. Following Ligation, sections were washed in Wash Buffer A before incubation, sections were washed in Amplification solution (Amplification, polymerase, diH2O) for 100 minutes at 37°C. Following Amplification, cells were washed in Wash Buffer B before drying and coverslipping with gelvatol. PLA was visualized with orange (Duolink; Sigma Aldrich catalog number DUO92007-30RXN) and far-red (Duolink; Sigma Aldrich DUO92013-30RXN) kits.

# **2.14 ANTIBODIES**

Antibodies used for immunohistochemical analysis are listed in the following table:

Host	Target	Company	Catalogue #	Dilution
Rabbit	4HNE	Abcam	AB46545	1:500
Mouse	3NT	Santa Cruz	SC-32757	1:500
Sheep	TH	Millipore	AB1542	1:2000
Mouse	TH	Millipore	MAB318	1:2000
Rabbit	TH	Millipore	AB152	1:2000
Goat	VMAT2	Santa Cruz	SC-7721	1:1000
Rabbit	VMAT2	Abcam	ab70808	1:1000
Mouse	GFP	Millipore	MAB3580	1:2000
Rabbit	GFP	Abcam	ab183734	1:2000
Rabbit	pThr73-Rab10	Abcam	ab230261	1:1000
Mouse	Мус	Cell Signaling	9B11	1:2000
Rabbit	Tom20	Santa Cruz	sc-11415	1:1000
Mouse	α-syn	BD biosciences	610787	1:2000
	Nissl	Thermo	N21483	1:500
Mouse	DDK	Origene	TA50011-100	1:1000
Rabbit	pSer129-syn	Abcam	AB1253	1:2000
Mouse	MAP2	Millipore	MAB378	1:2000
Mouse	LRRK2	UC Davis	N241A/34	1:500
Rabbit	pSer1292-LRRK2	Abcam	AB203181	1:500

# Table 2. SUMMARY OF ANTIBODIES

Host	Target	Wavelength	Company	Dilution
Donkey	Rabbit	488	ThermoFisher	1:500
		568	ThermoFisher	1:500
		647	ThermoFisher	1:500
Donkey	Mouse	488	ThermoFisher	1:500
		568	ThermoFisher	1:500
		647	ThermoFisher	1:500
Donkey	Sheep	350	ThermoFisher	1:500
		488	ThermoFisher	1:500
		555	ThermoFisher	1:500
		568	ThermoFisher	1:500
		647	ThermoFisher	1:500
Donkey	Goat	488	ThermoFisher	1:500
		568	ThermoFisher	1:500
		647	ThermoFisher	1:500
Donkey	Rabbit	680	Licor	1:500
		800	Licor	1:500
	Mouse	680	Invtirogen Molecular Probes	1:500
		680	Licor	1:500
		800	Licor	1:500

Fluorescent secondary antibodies

## 2.15 UNBIASED STEREOLOGY

Stereology was initially performed on serial sections from the midbrain following a protocol adapted from Tapias *et al.* 2013 (Tapias et al., 2013). Briefly, tissue was immunolabeled for TH, MAP2, and DAPI and imaged at 20x on a Nikon90i fluorescent microscope in the Center for Biologic Imaging at the University of Pittsburgh. Images were analyzed in Nikon Elements software counting the number of dopaminergic neurons determined by overlap between DAPI, MAP2, and TH within a region of interest defining the SNpc as previously described (Tapias et al., 2013). Subsequent experiments were performed with a modified method of stereology utilizing serial sections and overlap between TH and fluorescent Nissl (NeuroTrace 647, Life Technologies). These images were obtained on an Olympus BX61VS slide scanning microscope, and analysis was performed in Nikon Elements software. Previous studies have reported equivalent counts performed between the two methods (De Miranda et al., 2019).

# 2.16 BIOCHEMISTRY

Striatal tissue was homogenized in 0.1 M perchloric acid. Homogenates were centrifuged (34,000xG for 20min) to separate the acid precipitated protein from the acid-soluble components and stored at -80 until analysis. The acid-soluble component was filtered (Spin-X centrifuge tube filter, 0.22µm; Costar) and analyzed by HPLC-ED (high performance liquid chromatography with electrochemical detection). The protein pellet was washed by resuspension and re-centrifugation in 0.1 M perchloric acid containing 0.2 mM sodium bisulfite and then subjected to acid hydrolysis as described (T. G. Hastings & Zigmond, 1994). The catechol moieties were extracted with

alumina and then the cysteinyl catechol derivatives were analyzed using HPLC-ED (Waters Alliance 2695 HPLC system with 2465 Electrochemical detector; Microsorb-MV column (C18; 4.6 x 250 mm; Agilent)). The mobile phase contained 0.05 M sodium phosphate, 0.01 M citric acid, 0.01 M sodium acetate, 0.32 mM sodium octyl sulfate, and 0.1 mM EDTA, 10% (vol/vol) methanol, and was titrated to pH 2.5 with HCl. Compounds were identified and quantified by their elution positions and peak are under the curve in comparison to catechol standards.

### 2.17 MICROSCOPY

Images of sections prepared for immunohistochemistry and PLA were taken on an Olympus IXB1 confocal microscope and analyzed in Fluoview or Image J software. Images to visualize DAB immunostaining were taken on an Olympus BX61VS microscope and analyzed for pixel saturation in CellSens software. Intensity measurements were obtained in Fluoview software by circling ROIs around TH-positive neurons in the non-transduced hemisphere and TH- and GFP-positive neurons in the transduced hemisphere while blinded to the protein of interest. Intensity values of the protein of interest were compared between transduced and non-transduced TH-positive neurons. Number of objects analysis was performed in Nikon Elements software by circling ROIs around TH-positive neurons in the non-transduced hemisphere and TH- and GFP-positive neurons in the transduced hemisphere while blinded to the interest. Intensity values of the protein of objects analysis was performed in Nikon Elements software by circling ROIs around TH-positive neurons in the non-transduced hemisphere and TH- and GFP-positive neurons in the transduced hemisphere while blinded to the immunostaining for the protein of interest. Within ROIs, a threshold was applied to the protein of interest in order to achieve unbiased counts of objects. Stereological and montage images were taken on a Nikon 90i slide scanning microscope (Center for Biological Imaging at the University of Pittsburgh) or an Olympus BX61VS slide scanning microscope and analyzed in Nikon Elements software. Striatal

terminal analysis was performed by imaging on an Odyssey IR scanner by Licor and optical density measurements were performed in Odyssey software.

# 2.18 STATISTICS

Unless otherwise noted, each data point in each graph corresponds to the mean value for an individual animal. In every graph, the mean value and standard error of the mean is displayed. Statistical tests were performed in Prism GraphPad. Paired t-tests were used to evaluate within animal protein immunoreactivity, neurochemical measurements, neuronal counts, and behavior. Unpaired t-tests were used to compare protein and RNA between shVMAT2 and shControl *in vitro* treatments, and to compare protein immunoreactivity and neuronal counts between treatment groups in rescue experiments. One-way ANOVAs with Tukey's post-hoc test was performed to evaluate time-points following AAV2-shVMAT2, for human and rat mRNA analysis in rescue *in vitro* experiments, and for protein immunoreactivity and neuronal counts in AAV2-VMAT2 and rotenone *in vivo* experiments.

# 2.19 STUDY APPROVAL

Experimental approval was obtained through the Institutional Animal Care and Use Committee at the University of Pittsburgh.

# 3.0 ESTABLISHING AN ANIMAL MODEL OF VIRAL-MEDIATED shRNA INTERFERENCE OF VMAT2 EXPRESSION

Aim 1a: Characterize the impact of decreased vesicular sequestration of dopamine on dopaminergic neuronal health in the substantia nigra pars compacta, and investigate the mechanisms by which this impairment causes nigrostriatal dopaminergic neurons to become vulnerable to degeneration.

# **3.1 RATIONALE**

We hypothesized that because dysfunctional vesicular sequestration of dopamine may contribute to the pathogenesis of PD, that the packaging of dopamine within synaptic vesicles would be a targetable mechanism that modulates the health of DAergic neurons. Our objective was to construct an improved *in vivo* model of impaired vesicular DAergic sequestration by targeting VMAT2 expression. Utilizing gene therapy, our model targets VMAT2 expression through smallhairpin ribonucleic acid interference. Compared to previous genetic models, the acquired loss of VMAT2 expression is progressive with onset in adulthood, reducing the development of compensatory mechanisms, and would be able to be used in mouse or rat models. The viralmediated delivery can be injected directly into the brain targeting specific brain areas rather than having global knock-down, and this also allows for unilateral injections with an internal control on the contralateral hemisphere. The time course of these experiments is much faster than the 19 months required in the VMAT-lo mice, and the acquired loss of VMAT2 prevents the occurrence of compensatory mechanisms that arise during development. Collectively, the viral-mediated method of decreasing VMAT2 expression would produce a model that can be used to be understand dopamine-mediated pathogenic mechanisms of degeneration *in vivo* as well as a model to test novel therapeutic interventions.

To circumvent the potential compensatory mechanisms seen in genetic models, we developed an *in vivo* model of acquired dysregulation of dopamine sequestration through viralmediated interference of VMAT2 expression. This model would allow for direct targeting of viralexpression, and therefore protein knock-down, to a specific neuronal population. Our goal was to replicate the findings seen in previous animal models that dysregulation of dopamine sequestration is neurotoxic, and expand analysis of pathogenic mechanisms.

## **3.2 RESULTS**

# 3.2.1 RNA INTERFERENCE OF VMAT2 EXPRESSION in vitro

Expression of VMAT2 was targeted by small-hairpin ribonucleic acid (shRNA) interference in a DAergic neuronal cell line (RCSN-3) derived from the midbrain of an adult rat. The shRNA against nucleotides 617-643 of VMAT2: was targeted rat TCAACAGTTATGTTTGCCTTCTCCAGC (Figure 1a). Transfections with shVMAT2 were performed with co-administration of GFP to identify transfected cells, and efficiency of shVMAT2-mediated knock-down was quantified compared to cells transfected with a scramble shRNA (shControl). Analysis of VMAT2 protein immunoreactivity at 48 hours post-transfection demonstrated a 41.4% decrease compared to shControl transfected cells (unpaired t-test, n=37 cells in shControl treatment and 29 cells in shVMAT2 treatment, p<0.0001) (Figure 1b-c). Analysis of VMAT2 mRNA performed at the same time-point identified a 46.0% decrease compared to shControl transfected cells (paired t-test, n=4 experimental replicates, p=0.0057) (Figure 1d). These data demonstrate the efficacy of shRNA targeting in decreasing VMAT2 expression at both the mRNA and protein level. Given the success of knock-down achieved in vitro, this construct was picked for translation into in vivo experiments.



Figure 1. RNA interference of VMAT2 expression in vitro.

A. shRNA target against rat VMAT2 B. Representative 60x confocal images of immunocytochemistry in RCSN-3 cells co-transfected with shControl and GFP (top) or shVMAT2 and GFP (bottom) immunostained for GFP (green), VMAT2 (red), and DAPI (blue). C. Quantification of VMAT2 immunoreactivity from GFP expressing cells demonstrated a 41.4% loss of VMAT2 protein in shVMAT2 transfected cells compared to shControl transfected cells (unpaired t-test, n=37 cells in shControl treatment and 29 cells in shVMAT2 treatment, p<0.0001). D. VMAT2 mRNA normalized to GAPDH analyzed by qtPCR was decreased by 46.0% in shVMAT2 transfected cells compared to shControl transfected cells (unpaired to shControl transfected cells (paired t-test, n=4 experimental replicates, p=0.0057).

# 3.2.2 VIRAL-MEDIATED RNA INTERFERENCE in vivo

Following *in vitro* confirmation of VMAT2 knock-down at both the mRNA and protein level, the shVMAT2 insert was cloned into an AAV-U6 vector with bicistronic expression of shVMAT2 under the U6 promoter, and GFP under the CMV promoter (Figure 2a). Following sequence confirmation, the shVMAT2 plasmid was sent to Penn Vector Core and an adenoassociated virus (AAV serotype 2) was produced. The AAV2-shVMAT2 virus was validated for purity and the titer was determined to be  $3.56 \times 10^{12}$  genome copies/mL. A previously established control virus (AAV2-shControl) (Zharikov et al., 2015) that introduces a scrambled shRNA sequence that does not target anything in the rat genome under the U6 promoter with GFP expression under the CMV promoter was obtained from Penn Vector Core and used at a titer equivalent to the targeting viral vector at  $3.56 \times 10^{12}$  genome copies/mL (Figure 2b).



## Figure 2. Viral plasmid maps of AAV2-shControl and AAV2-shVMAT2.

A. The viral preparation uses an adeno-associated virus (serotype 2) that expresses the shVMAT2 construct under the human U6 promoter with bicistronic expression of enhanced green fluorescent protein (eGFP) under the CMV promoter to identify virally-transduced neurons. The AAV2-shVMAT2 virus was administered *in vivo* through unilateral injections of 2µL volume targeting the DAergic neurons in the SNpc of adult Lewis rats. Six weeks post-viral transduction, there was robust GFP immunoreactivity in tyrosine hydroxylase (TH) immunoreactive neurons in the SNpc indicating efficient viral transduction of TH immunoreactive (TH-positive) neurons (Figure 3a-b). In male animals, at six weeks post-transduction, VMAT2 immunoreactivity was decreased by 50.5% within AAV2-shVMAT2 transduced TH-positive neurons compared to non-transduced TH-positive neurons from the contralateral hemisphere (paired t-test, n=5 animals, p=0.0104) (Figure 3c top).

In female animals, at six weeks post-transduction VMAT2 immunoreactivity was decreased by 39.2% within AAV2-shVMAT2 transduced TH-positive neurons compared to non-transduced TH-positive neurons from the contralateral hemisphere (paired t-test, n=5 animals, p=0.0008) (Figure 3c bottom). In male animals, there was a modest 13.4% decrease in TH immunoreactivity within transduced TH-positive neurons compared to non-transduced TH-positive neurons from the contralateral hemisphere (paired t-test, n=5 animals, p=0.0243) (Figure 3d top). However, there was no significant difference in TH immunoreactivity within transduced TH-positive neurons in the contralateral hemisphere of female animals (paired t-test, n=5 animals per treatment group, p=0.4742) (Figure 3d bottom).



Figure 3. AAV2-shVMAT2 decreased VMAT2 expression in male and female rat substantia nigra.

A. Representative image following unilateral AAV2-shVMAT2 injection to the SNpc demonstrating viral transduction, identified by GFP (green), VMAT2 (purple), and TH-positive (blue) neurons. B. Representative 90x magnification confocal images of transduced TH-positive neurons in the SNpc with corresponding non-transduced SNpc in the contralateral hemisphere. C. Quantification of VMAT2 immunoreactivity within AAV2-shVMAT2 transduced TH-positive neurons compared to non-transduced neurons in the contralateral hemisphere in males (top) (paired t-test, n=5 animals, p=0.0104) and females (bottom) at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.0008). Each data point represents the average value for an individual animal calculated from at least 100 neurons per animal. D. Quantification of TH immunoreactivity within AAV2-shVMAT2 transduced TH-positive neurons in the contralateral hemisphere in males (top) (paired t-test, n=5 animals, p=0.0243) and females (bottom) at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.4742). Each data point represents the average value for an individual animal calculated from at least 100 neurons compared to non-transduced neurons in the contralateral hemisphere in males (top) (paired t-test, n=5 animals, p=0.0243) and females (bottom) at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.4742). Each data point represents the average value for an individual animal calculated from at least 100 neurons per animal.

To confirm that the decrease in VMAT2 expression following AAV2-shVMAT2 was specific to the knock-down virus, and not a product of off-target effects of the virus itself, VMAT2 and TH were analyzed in neurons transduced with the scramble control virus. Analysis of TH-positive neurons transduced with AAV2-shControl virus demonstrated no significant difference in VMAT2 (paired t-test, n=5 animals, p=0.5642) or TH immunoreactivity (paired t-test, n=5 animals, p=0.1696) within transduced TH-positive neurons compared to non-transduced TH-positive neurons in the contralateral hemisphere (Figure 4a-c).





A. Representative 90x magnification confocal images of transduced TH-positive neurons in the SNpc AAV2shControl with corresponding non-transduced SNpc in the contralateral hemisphere. B. Quantification of VMAT2 immunoreactivity within AAV2-shControl transduced TH-positive neurons compared to non-transduced neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.5642). Each data point represents the average value for an individual animal calculated from at least 100 neurons per animal. C. Quantification of TH immunoreactivity within AAV2-shControl transduced TH-positive neurons compared to nontransduced neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.1696). Each data point represents the average value for an individual animal calculated from at least 100 neurons per animal.

# **3.2.3 STRIATAL DOPAMINE DYSREGULATION**

While a decrease in VMAT2 was observed in the cell bodies in the SNpc, the majority of VMAT2 would be expected to be in the striatal terminals. Due to the extensive arborization of nigrostriatal DAergic neurons, the terminals are diffusely spread throughout the striatum. In order to better visualize VMAT2 in the diffuse terminals, DAB immunostaining was performed because the reaction is able to be titrated to amplify the signal. In addition to a loss of VMAT2 protein in the TH-positive neuronal cell bodies of the SNpc, there was a corresponding loss of VMAT2 protein in the dorsal striatum as visualized by DAB immunostaining (Figure 5a). Visually, this decrease can be seen throughout the dorsal striatum. Quantification of VMAT2 immunoreactivity in the dorsal striatum demonstrated a 44.0% loss compared to the contralateral non-transduced hemisphere (paired t-test, n=5 animals, p=0.0012) (Figure 5c).

There was a slight decrease in TH expression within AAV2-shVMAT2 transduced cell bodies, however, in the striatal terminals the loss of TH immunoreactivity was more dramatic. A focal lesion in TH immunostaining in the dorsolateral striatum of the transduced hemisphere was observed (Figure 5b) that corresponded with a 28.1% loss of TH immunoreactivity compared to the contralateral non-transduced hemisphere (paired t-test, n=5 animals, p=0.0215) (Figure 5d). The loss of TH in the striatum was greater than the observation within transduced cell bodies in the SNpc, and the focal loss of TH mimics the staining pattern seen in models of PD, which is suggestive of nigrostriatal degeneration.



#### Figure 5. AAV2-shVMAT2 decreased VMAT2 expression in rat striatum.

A. Representative image of VMAT2 protein visualized by DAB in the striatal terminals following unilateral AAV2shVMAT2 injection (transduced hemisphere identified by notch in cortex) at 6 weeks. B. Representative image of TH protein visualized by DAB in the striatal terminals following unilateral AAV2-shVMAT2 injection (transduced hemisphere identified by notch in cortex). C. Quantification of VMAT2 immunoreactivity in the dorsal striatum of the transduced hemisphere compared to the internal control non-transduced contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.0012). Each data point represents the average value for an individual animal calculated from 3 striatal sections per animal. D. Quantification of TH immunoreactivity in the dorsal striatum of the transduced hemisphere compared to the internal control non-transduced contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.0215). Each data point represents the average value for an individual animal calculated from 3 striatal sections per animals, p=0.0215). Each data point represents the average value for an individual animal calculated from 3 striatal sections per animal. To confirm that the differences in VMAT2 and TH immunostaining in the striatum observed following AAV2-shVMAT2 were specific to VMAT2 knock-down, analysis was performed in the striatum following AAV2-shControl. Analysis of VMAT2 immunoreactivity in the striatum following AAV2-shControl transduction revealed uniform VMAT2 throughout the terminals (Figure 6a). Additionally, analysis of TH immunoreactivity in the striatum following AAV2-shControl transduction and uniform TH throughout the terminals (Figure 6b).



## Figure 6. AAV2-shControl did not impact VMAT2 expression in striatum.

A. Representative image of VMAT2 protein visualized by DAB in the striatal terminals following unilateral AAV2shControl injection (non-transduced hemisphere identified by hole in cortex). B. Representative image of TH protein visualized by DAB in the striatal terminals following unilateral AAV2-shControl injection (non-transduced hemisphere identified by hole in cortex).

Given the decrease in VMAT2 expression observed in the striatum following VMAT2 knock-down, we investigated dopamine neurochemistry to determine whether dopamine handling had been dysregulated. Neurochemistry was performed at 4, 8, and 12 weeks post-transduction in freehand dissected dorsal striatum to measure dopamine and its metabolites. Compared to the nontransduced hemisphere, dopamine was decreased in the transduced hemisphere by 41.7% at 4 weeks (paired t-test, n=4 animals, p=0.0085), 50.4% at 8 weeks (paired t-test, n=4 animals, p=0.0433), and by 78.5% at 12 weeks post-transduction (paired t-test, n=3 animals, p=0.0391) (Figure 7a). Dopamine turnover was measured at each timepoint by calculating the ratio of DOPAC to dopamine and compared to ratios from the non-transduced contralateral hemisphere. This ratio is a measure of enzymatic degradation and an increase in the ratio is indicative of increased cytosolic dopamine metabolism. At 4 weeks post-transduction there was a nonsignificant trend towards increased dopamine turnover (paired t-test, n=4 animals, p=0.0676), at 8 weeks post-transduction dopamine turnover was significantly increased by 64.7% compared to the non-transduced hemisphere (paired t-test, n=4 animals, p=0.0338), and at 12 weeks posttransduction dopamine turnover was increased by 244.2% (paired t-test, n=3 animals, p=0.0293) (Figure 7b). Protein cysteinyl-dopamine adducts, measured as an index of dopamine oxidation, were evaluated at each timepoint. Compared to the non-transduced hemisphere, at 4 weeks posttransduction there was no difference in the amount of cysteinyl-dopamine adducts in the transduced hemisphere (paired t-test, n=4 animals, p=0.2417), at 8 weeks post-transduction the amount of cysteinyl-dopamine adducts were increased by 27.4% in the transduced hemisphere compared to the non-transduced hemisphere (paired t-test, n=4 animals, p=0.0209), and at 12 weeks post-transduction (paired t-test, n=3 animals, p=0.8363) (Figure 7c right). The decrease in

dopamine with corresponding increase in dopamine metabolism suggests an increase in cytosolic dopamine as a result of VMAT2 knock-down.



#### Figure 7. AAV2-shVMAT2 resulted in dysregulated striatal dopamine neurochemistry.

A. Levels of dopamine at 4, 8, and 12 weeks post-transduction in non-transduced and AAV2-shVMAT2 transduced hemispheres. Measurements from the transduced striatal hemisphere were compared internally to the non-transduced contralateral hemisphere. Each data point represents the value per animal. (4 weeks paired t-test, n=4 animals, p=0.0085; 8 weeks paired t-test, n=4 animals, p=0.0433; 12 weeks paired t-test, n=3 animals, p=0.0391). B. Dopamine turnover as measured by a ratio of DOPAC:dopamine at 4, 8, and 12 weeks post-transduction in non-transduced and AAV2-shVMAT2 transduced hemispheres. Measurements from the transduced striatal hemisphere were compared internally to the non-transduced contralateral hemisphere. Each data point represents the value per animal. (4 weeks paired t-test, n=4 animals, p=0.0676; 8 weeks paired t-test, n=4 animals, p=0.0338; 12 weeks paired t-test, n=3 animals, p=0.0293). C. Dopamine oxidation as measured by cysteine-modified dopamine at 4, 8, and 12 weeks post-transduction in non-transduced and AAV2-shVMAT2 transduced hemispheres. Measurements from the transduced hemispheres. Measurements from the transduced transduced transduced and AAV2-shVMAT2 transduced hemisphere. Each data point represents the value per animal. (4 weeks post-transduction in non-transduced and AAV2-shVMAT2 transduced hemispheres. Measurements from the transduced striatal hemisphere were compared internally to the non-transduced and AAV2-shVMAT2 transduced hemispheres. Measurements from the transduced striatal hemisphere were compared internally to the non-transduced contralateral hemisphere. Each data point represents the value per animal. (4 weeks paired t-test, n=4 animals, p=0.2417; 8 weeks paired t-test, n=4 animals, p=0.0209; 12 weeks paired t-test, n=3 animals, p=0.8363).

## **3.2.4 DEFICITS IN DOPAMINE-MEDIATED BEHAVIORS**

It is known that the motor symptoms of PD result from a loss of dopamine transmission in the striatum. To determine whether the focal lesion in striatal TH terminals, and dysregulation of dopamine sequestration seen in figure 5, resulted in motor deficits, two dopamine-mediated behavioral tests in animals that received either unilateral injections of AAV2-shVMAT2 or AAV2shControl and a group of animals that received AAV2-shVMAT2 injections with AAV2shControl injections in the contralateral hemisphere. Behavioral tests were performed at 20 weeks post-transduction.

The postural instability test measures an animal's ability to take a corrective step when placed off balance and has previously been shown to measure a parkinsonian motor deficit rescuable by dopamine receptor agonist (Cannon et al., 2009). In the treatment group that received unilateral AAV2-shVMAT2 injections, the forepaw contralateral to knock-down showed a 74.3% increase in distance to trigger compared to the unaffected forepaw which consistently performed at 2.5cm distance to trigger (paired t-test, n=4 animals, p=0.0029). In animals that received unilateral AAV2-shControl injections, the contralateral forepaw showed a non-significant trend towards increase in distance to trigger compared to the unaffected forepaw (paired t-test, n=4 animals, p=0.0592). In animals that received bilateral viral injections, the forepaw contralateral to AAV2-shVMAT2 showed a 76.2% increase in distance to trigger compared to the got paired to trigger compared to the forepaw contralateral to the forepaw contralateral to AAV2-shControl (paired t-test, n=4 animals, p<0.0001) (Figure 8a).

As an additional measure, forepaw use was measured during the cylinder test, which has similarly shown decreases in rearing behavior in parkinsonian conditions able to be rescued with dopamine receptor agonist (Cannon et al., 2009). Paw use was counted from three trails each lasting 5 minutes each. Animals receiving unilateral AAV2-shVMAT2 injections showed a nonsignificant decrease in use of the forepaw contralateral to knock-down (paired t-test, n=4 animals, p=0.2163). Animals receiving unilateral AAV2-shControl injections showed a non-significant decrease in use of the forepaw contralateral to knock-down (paired t-test, n=4 animals, p=0.6487). In the animals receiving bilateral viral injections, animals showed a preference for the forepaw ipsilateral to AAV2-shVMAT2 (contralateral to AAV2-shControl), and rarely used the affected forepaw contralateral to AAV2-shVMAT2 during rearing behavior (paired t-test, n=4 animals, p=0.0301) (Figure 8b).





A. Postural instability test was performed at 20 weeks post-transduction in animals with unilateral AAV2-shVMAT2 or AAV2-shControl injections, or bilateral injections with AAV2-shVMAT2 in one hemisphere and AAV2-shControl injections in the contralateral hemisphere. Ipsilateral paw refers to the paw on the same side of the body as the AAV2-shVMAT2 injection (or AAV2-shControl in middle group), contralateral paw refers to the paw contralateral to and therefore affected by the AAV2-shVMAT2 injection (except in middle group where contralateral paw is affected by AAV2-shControl) (paired t-test, n=4 animals for each treatment, \*\*p<0.01, \*\*\*p<0.001). Eat data point represents the average distance to trigger for each forepaw in an individual calculated over three trials. D. Cylinder test was performed in the same animals as Figure 10a to measure number of paw touches during rearing observed from three 5 minute trials (paired t-test, n=4 animals for each treatment, \*\*p=0.0301). Each data point represents the number of paw touches for each paw in an individual animal summed over three 5 minute trials.

# 3.2.5 DOPAMINERGIC NEUROTOXICITY

The deficits in dopamine-mediated behaviors observed in Figure 8 have been observed in PD animal models (Cannon et al., 2009). The focal loss of TH in the dorsal striatum and the presence of dopamine-mediated behavioral deficits are suggestive of nigrostriatal degeneration. To determine whether VMAT2 knock-down resulted in a loss of DAergic neurons in the SNpc, a semi-automated fluorescent method of DAergic stereology was performed. This method counts TH-positive neurons by identifying overlap between TH, a neuronal marker microtubule associated protein 2 (MAP2), and the nuclear marker DAPI demonstrated that following six weeks of viral transduction, there was a loss of 40.8% of DAergic neurons in the transduced SNpc compared to the contralateral non-transduced hemisphere (paired t-test, n=5 animals, p=0.0177) (Figure 9a-b). Stereology performed in female animals under the same experimental conditions demonstrated a similar amount of neurodegeneration with 52.5% fewer DAergic neurons in the transduced hemisphere (paired t-test, n=5 animals, p=0.0046) (Figure 9c).


Figure 9. AAV2-shVMAT2 resulted in dopaminergic neurotoxicity.

A. Representative image of SNpc. Stereological dopaminergic neuronal counts in the SNpc were performed by identifying overlap of neuronal marker MAP2 (red), nuclear marker DAPI (blue), and dopaminergic neuronal marker TH (green). B. Quantification of TH-positive neuronal counts at 6 weeks post-transduction. Number of TH-positive neurons from the transduced hemisphere were compared to the internal control non-transduced contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.0177). Each data point represents the total number of TH-positive neurons in an individual animal calculated from serial sections. C. Quantification of TH-positive neuronal counts in female animals at 6-weeks post-transduction. Number of TH-positive neurons from the transduced hemisphere were compared to the internal control non-transduced contralateral hemisphere were compared to the internal control non-transduced contralateral hemisphere animals at 6-weeks post-transduction. Number of TH-positive neurons from the transduced hemisphere were compared to the internal control non-transduced contralateral hemisphere (paired t-test, n=5 animals, p=0.0046). Each data point represents the total number of TH-positive neurons in an individual animal calculated from serial sections.

Furthermore, the extent of DAergic degeneration following VMAT2 knock-down was modulated by the time-point at which animals are sacrificed post-transduction. Figure 10 demonstrates a time-point dependent loss of DAergic neurons over time. At 4 weeks posttransduction, no neurodegeneration was observed. However, at 8 weeks and 12 weeks posttransduction, there was a significant loss of DAergic neurons compared to the 4-week time-point (One-way ANOVA with Tukey's post-hoc test, n=3 animals per time-point, \*p<0.05). These data show that the neurodegeneration of DAergic neurons is the result of the progressive loss of VMAT2 over time, with the 4-week time-point being too early to observe a significant loss of DAergic neurons.



Figure 10. Extent of AAV2-shVMAT2-induced neurotoxicity was modulated by time-point.

A. Stereological counts of TH-positive neurons at three time-points following AAV2-shVMAT2 transduction expressed as the percent of TH-positive neurons in the transduced hemisphere neurons compared to the contralateral non-transduced hemisphere (One-way ANOVA with Tukey's post-hoc test, n=3 animals per time-point, p<0.05, \*\*p<0.01). To confirm that the significant loss of DAergic neurons observed at 6 weeks following AAV2-shVMAT2 transduction was specific to VMAT2 knock-down, DAergic neuron stereology was performed in animals that received injections of the AAV2-shControl virus. At 6 weeks post-transduction, TH-positive neurons transduced with AAV2-shControl showed a non-significant loss of TH immunoreactive neurons, which was consistent with previous reports (Zharikov, Cannon et al. 2015) (paired t-test, n=5 animals, p=0.2054) (Figure 11a).



Figure 11. AAV2-shControl did not result in dopaminergic neurotoxicity.

A. Number of TH-positive neurons in the SNpc following AAV2-shControl viral transduction compared to the contralateral non-transduced hemisphere at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.2054). Each data point represents the number of TH-positive neurons calculated from serial sections

### **3.2.6 OXIDATIVE DAMAGE**

To begin to examine mechanisms associated with neurodegeneration following VMAT2 knock-down, specific pathogenic mechanisms and indices of neuronal degeneration were investigated. Because cytosolic dopamine is susceptible to processes that generate reactive oxygen species and oxidative stress, tissue was evaluated for the presence of oxidative damage. Protein modifications, 3-nitrotyrosine (3NT) and 4-hydroxynoneal (4HNE), are markers of oxidative damage and have classically been used as an index of oxidative stress within neurons (Pennathur et al., 1999; Sulzer et al., 2000). Decreased VMAT2 immunoreactivity resulted in a 26.7% increase in the amount of 4-HNE within transduced TH-positive neurons as compared to non-transduced TH-positive neurons (paired t-test, n=9 animals, p=0.0424) at 6 weeks post-transduction (Figure 12a,c). Similarly, there was a 27.6% increase in the amount of 3NT within transduced TH-positive neurons as compared to non-transduced TH-positive neurons (paired t-test, n=9 animals, p=0.0424) at 6 weeks post-transduction (Figure 12a,c). Similarly, there was a 27.6% increase in the amount of 3NT within transduced TH-positive neurons (paired t-test, n=9 animals, p=0.027) at 6 weeks post-transduction (Figure 12b,d). The increase in both 4HNE and 3NT levels suggests increased oxidative stress and oxidative modification of macromolecules.



#### Figure 12. AAV2-shVMAT2 resulted in oxidative damage.

A. Representative 90x confocal images of TH-positive (blue) virally-transduced GFP-expressing (green) neurons in the SNpc immunostained to identify 4-hydroxynonenal (4HNE) (yellow). B. Representative 90x confocal images of TH-positive (blue) virally-transduced GFP-expressing (green) neurons in the SNpc immunostained to identify 3nitrotyrosine (3NT) (purple). C. Quantification was performed by counting the number of 4HNE positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shVMAT2 transduced neurons were compared to internal control contralateral non-transduced neurons at 6 weeks post-transduction (paired t-test, n=9 animals, p=0.0424). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal. D. Quantification was performed by counting the number of 3NT positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shVMAT2 transduced neurons were compared to internal control contralateral non-transduced neurons at 6 weeks post-transduction (paired t-test, n=9 animals, p=0.0424). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal. D. Quantification was performed by counting the number of 3NT positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shVMAT2 transduced neurons were compared to internal control contralateral non-transduced neurons at 6 weeks post-transduction (paired t-test, n=9 animals, p=0.0027). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal. To confirm that the oxidative damage seen in AAV2-shVMAT2 transduced neurons was a result of VMAT2 knock-down and not a result of viral transduction, analysis of oxidative damage was performed in animals that received AAV2-shControl injections. Within AAV2-shControl transduced TH-positive neurons there were no significant differences in the amount of 4HNE (paired t-test, n=3 animals, p=0.351) (Figure 13a) or 3NT (paired t-test, n=5 animals, p=0.1973) (Figure 13b) compared to non-transduced TH-positive neurons in the contralateral hemisphere.



Figure 13. AAV2-shControl did not result in oxidative damage.

A. Quantification was performed by counting the number of 4HNE positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shControl transduced neurons were compared to internal control contralateral non-transduced neurons at 6 weeks post-transduction (paired t-test, n=3 animals, p=0.3531). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal. B. Quantification was performed by counting the number of 3NT positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shControl transduced neurons were compared to internal control contralateral non-transduced neurons at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.1973). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal.

# 3.2.7 INDUCTION OF LRRK2 ACTIVITY

The presence of oxidative damage following a loss of VMAT2 is indicative of oxidative stress, and we have recently shown that oxidative stress can induce activation of the PD-associated kinase LRRK2 (Di Maio et al., 2018). We hypothesized that the loss of VMAT2 and subsequent accumulation of cytosolic dopamine may induce LRRK2 activity due to the production of oxidative stress. To investigate this, we utilized a recently developed PLA that can be used to quantify the amount of autophosphorylated LRRK2 at serine 1292 (pSer1292-LRRK2), which is an indirect measure of LRRK2 activity (Di Maio et al., 2018). The PLA signal of pSer1292-LRRK2 was increased within transduced TH-positive neurons by 28.4% compared to nontransduced neurons (paired t-test, n=4 animals, p=0.025) (Figure 14a,c left). As an additional measure of LRRK2 activity, the amount of phosphorylated Rab10 at threonine 73 (pThr73-Rab10) - a LRRK2 substrate - can be quantified. VMAT2 knock-down resulted in a 23.5% increase in the amount of pThr73-Rab10 within transduced TH-positive neurons as compared to non-transduced neurons (paired t-test, n=4 animals, p=0.0313) (Figure 14b,d left). These results were also replicated in female animals following VMAT2 knock-down, which demonstrated a 28.3% increase in autophosphorylated LRRK2 PLA immunoreactivity (paired t-test, n=4 animals, p=0.0225) (Figure 14c right) and a 39.0% increase in the amount of pThr73-Rab10 (paired t-test, n=6 animals, p=0.0091) (Figure 14d right). Analysis was performed within transduced TH-positive neurons and compared to non-transduced neurons.



#### Figure 14. AAV2-shVMAT2 resulted in increased LRRK2 activity.

A. Representative 90x confocal images of TH-positive (blue) virally-transduced GFP-expressing (green) neurons in the SNpc immunostained to identify activated LRRK2 by PLA (red). B. Representative 90x confocal images of TH-positive (blue) virally-transduced GFP-expressing (green) neurons in the SNpc immunostained to identify pThr73-Rab10 (blue). C. Quantification of LRRK2 PLA immunoreactivity within transduced TH-positive neurons was compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere in male animals (left) (paired t-test, n=6 animals, p=0.0489) and female animals (right) at 6 weeks post-transduction (paired t-test, n=4 animals, p=0.0225). Each data point represents the average immunoreactivity of PLA signal in a TH neuron collected from at least 75 neurons per animal. D. Quantification was performed by counting the number of pThr73-Rab10 positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shVMAT2 transduced neurons were compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere in male animals (left) (paired t-test, n=4 animals, p=0.0313) and female animals (right) at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.0091). Each data point represents the average number of objects/TH neuron area number of objects in a TH neuron for the contralateral hemisphere in male animals (left) (paired t-test, n=4 animals, p=0.0313) and female animals (right) at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.0091). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal.

Given the data in Figure 13, which demonstrated no increases in indices of oxidative damage following AAV2-shControl, we would hypothesize that there would be no induction of LRRK2 activity in the absence of oxidative stress in AAV2-shControl transduced neurons. Analysis of the amount of pThr73-Rab10 as an index of LRRK2 activity demonstrated no significant difference in the amount of pThr73-Rab10 within AAV2-shControl transduced TH-positive neurons compared to non-transduced neurons from the contralateral hemisphere (paired t-test, n=4 animals, p=0.2416) (Figure 15a-b).



Figure 15. AAV2-shControl did not induce LRRK2 activity.

A. Representative 90x confocal images of TH-positive (red) virally-transduced GFP-expressing (green) neurons in the SNpc with pThr73-Rab10 (blue). B. Quantification was performed by counting the number of pThr73-Rab10 positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shControl transduced neurons were compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=4 animals, p=0.2416). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal.

# 3.2.8 FORMATION OF ABERRANT α-SYNUCLEIN

The pathological hallmark of PD is the formation of protein aggregates, called Lewy bodies, that contain aberrant forms of  $\alpha$ -synuclein. Although measurements of total  $\alpha$ -synuclein immunoreactivity were not increased within transduced neurons (paired t-test, n=3 animals, p=0.4396) (Figure 16a,d), phosphorylated  $\alpha$ -synuclein at serine 129 (pSer129-syn) was increased by 42.9% within transduced TH-positive neurons as compared to non-transduced neurons (paired t-test, n=3 animals, p=0.014) (Figure 16b,e). Pathogenic forms of α-synuclein, including pSer129synuclein and dopamine-modified  $\alpha$ -synuclein, can interact with TOM20 – a protein localized to the mitochondrial membrane responsible for protein import – and this interaction results in a loss of protein import and subsequent mitochondrial impairment (Di Maio et al., 2016). This interaction between TOM20 and α-synuclein can be measured by PLA as previously described (Di Maio et al., 2016). The interaction between  $\alpha$ -synuclein and TOM20 was increased by 90.9% within transduced TH-positive neurons compared to non-transduced neurons (paired t-test, n=3 animals, p=0.0249) (Figure 16c,f). Although there were no significant increases in total  $\alpha$ -synuclein, the increase in pSer129-syn and the increase in α-synuclein:TOM20 interaction suggests pathogenic forms of α-synuclein were present following VMAT2 knock-down.





Figure 16. AAV2-shVMAT2 resulted in the formation of aberrant α-synuclein formation.

A. Representative 90x confocal images of TH-positive (blue) virally-transduced GFP-expressing (green) neurons in the SNpc immunostained to identify  $\alpha$ -synuclein (yellow). B. Representative 90x confocal images of TH-positive (blue) virally transduced GFP-expressing (green) neurons in the SNpc immunostained to identify pSer129-syn (purple). C. Representative 150x confocal images of TH-positive (white) virally-transduced GFP-expressing (green) neurons in the SNpc immunostained to identify an interaction between  $\alpha$ -synuclein and mitochondrial protein import protein TOM20 (red). D. Quantification of  $\alpha$ -synuclein immunoreactivity within AAV2-shVMAT2 transduced THpositive neurons were compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=3 animals, n.s.). Each data point represents the average immunoreactivity for each animal calculated from at least 50 neurons per animal. E. Quantification of pSer129-syn immunoreactivity within AAV2-shVMAT2 transduced TH-positive neurons was compared to internal control nontransduced TH-positive neurons from the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=3 animals, p=0.014). Each data point represents the average immunoreactivity for each animal calculated from at least 75 neurons per animal. F. Quantification of TOM20: $\alpha$ -synuclein PLA compared to non-transduced neurons at 6 weeks post-transduction (paired t-test, n=3 animals, p=0.0249). Analysis performed in animals that received AAV2-shControl injections revealed no differences in the in the amount of total  $\alpha$ -synuclein (paired t-test, n=4 animals, p=0.2807) (Figure 17a) or pSer129-syn (paired t-test, n=4 animals, p=0.5988) within AAV2-shControl transduced TH-positive neurons as compared to non-transduced TH-positive neurons in the contralateral hemisphere (Figure 17b).



Figure 17. AAV2-shControl did not result in the formation of aberrant α-synuclein.

A. Quantification of  $\alpha$ -synuclein immunoreactivity within AAV2-shControl transduced TH-positive neurons were compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=4 animals, p=0.2807). Each data point represents the average immunoreactivity for each animal calculated from at least 50 neurons per animal. B. Quantification of pSer129-syn immunoreactivity within AAV2-shControl transduced TH-positive neurons was compared to internal control non-transduced TH-positive neurons was compared to internal control non-transduced TH-positive neurons was compared to internal control non-transduced TH-positive neurons from the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=4 animals, p=0.5988). Each data point represents the average immunoreactivity for each animal calculated from at least 50 neurons per animal.

# 3.2.9 RESCUE BY EXOGENOUS VMAT2

To confirm that AAV2-shVMAT2-mediated neurodegeneration was specific to the loss of VMAT2, a second virus was constructed to reintroduce VMAT2 expression. This virus reintroduces human VMAT2 through a shVMAT2-resistant construct. This construct, first tested in vitro, expresses human VMAT2 with a myc-DDK tag, which contains only one nucleotide difference in the region targeted by the shVMAT2 construct. To induce resistance, four additional nucleotide base changes were introduced that are silent mutations, which result in the same amino acid sequence translated from a nucleotide sequence that is not recognized by the shVMAT2, thereby making it knock-down resistant (kdrVMAT2) (Figure 18a). Co-transfection of RCSN-3 cells with shVMAT2 and kdrVMAT2 was able to rescue cell survival compared to cells transfected with an empty vector (EV) or wild type VMAT2 (wtVMAT2) (Figure 18b). However, cotransfection with shVMAT2 and kdrVMAT2 restored VMAT2 immunoreactivity. Co-transfection of RCSN-3 cells with shVMAT2 and wild-type VMAT2 wtVMAT2 was unable to rescue VMAT2 immunoreactivity, however co-transfection with shVMAT2 and kdrVMAT2 restored VMAT2 immunoreactivity (Figure 18c). Analysis of human and rat mRNA revealed that co-transfection with shVMAT2 and kdrVMAT2 resulted in increased human VMAT2 mRNA, but not rat VMAT2 mRNA (One-way ANOVA with Tukey's post-hoc test, n=4-6 experimental replicates, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001) (Figure 18d-e).

Collectively, these data demonstrate that the knock-down resistant construct can reintroduce VMAT2 at both the mRNA and protein level in cells. Following *in vitro* confirmation of rescue, a virus was made to confirm rescue *in vivo* (Figure 19).







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### Figure 18. Rescue of VMAT2 expression by reintroducing exogenous VMAT2 expression in vitro.

A. shRNA target and human construct with silent mutations to generate a knock-down resistant VMAT2. B. Cell survival of RCSN-3 cells transfected with empty vector (EV), shControl, shVMAT2, wild type VMAT2 (wtVMAT2), or knock-down resistant VMAT2 (kdrVMAT2). C. Representative images of immunocytochemistry performed in RCSN-3 cells transfected with EV, shControl, shVMAT2, wtVMAT2, or kdrVMAT2 with immunolabeling for GFP (green), VMAT2 (red), and DDK (white). D. Human VMAT2 mRNA normalized to GAPDH analyzed by qtPCR (one-way ANOVA with Tukey's post-hoc test, n=4-6 experimental replicates, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001). C. Rat VMAT2 mRNA normalized to GAPDH analyzed by qtPCR (one-way ANOVA with Tukey's post-hoc test, n=3-6 experimental replicates, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001).



### Figure 19. Viral plasmid map of knock-down resistant VMAT2 overexpression virus.

A. Overexpression of human VMAT2 with silent mutations conferring knock-down resistance with a myc-DDK tag under the CMV promoter.

In an effort to restore VMAT2 expression through exogenous expression, animals received co-injections of AAV2-shVMAT2 and a control virus overexpressing GFP (AAV2-GFP), or AAV2-shVMAT2 and AAV2-kdrVMAT2 (Figure 20a). Immunohistochemistry was performed to detect TH, GFP, and VMAT2 immunoreactivity in each treatment group (Figure 20b). DAergic neurons expressing GFP in the AAV2-shVMAT2 + AAV2-kdrVMAT2 treatment group showed increased VMAT2 immunoreactivity compared to GFP+ DAergic neurons in the AAV2-shVMAT2 + AAV2-GFP treatment group (unpaired t-test, n=5-7 animals per treatment group, p=0.008) (Figure 20c). These data demonstrate that, while the AAV2-shVMAT2 virus knocks-down endogenous VMAT2 expression, exogenous VMAT2 expression was achieved through the knock-down resistant construct in AAV2-kdrVMAT2.



b.





### Figure 20. Rescue of VMAT2 expression by reintroducing exogenous VMAT2 expression in vivo.

A. Representative images following unilateral AAV2-shVMAT2 + AAV2-GFP injection (left panel) or AAV2shVMAT2 + AAV2-kdrVMAT2 (right panel) to the SNpc (SN) demonstrating viral transduction, identified by GFP (green), VMAT2 (purple), and TH-positive (blue) neurons. B. Representative 90x magnification confocal images of transduced (middle panel: AAV2-shVMAT2 + AAV2-GFP; bottom panel: AAV2-shVMAT2 + AAV2-kdrVMAT2) TH-positive neurons. GFP (green), TH (blue), VMAT2 (purple). C. Quantification of VMAT2 immunoreactivity within transduced TH-positive neurons expressed as a percentage of non-transduced neurons from the internal control contralateral hemisphere at 6 weeks post-transduction (unpaired t-test, n=5-7 animals, p=0.008).

# **3.2.10 RESCUE OF TOXICITY**

Following the data in Figure 20 that demonstrated the ability to reintroduce VMAT2 expression, we investigated whether this reintroduction of VMAT2 expression was able to protect against VMAT2 knock-down induced DAergic neurodegeneration. Stereology was performed to count the number of DAergic neurons in the transduced and non-transduced hemispheres of each treatment group (Figure 21a). Overlap between TH and Nissl was used to identify a DAergic neuron, and the AAV2-shVMAT2 + AAV2-kdrVMAT2 treatment group showed protection against neurodegeneration in comparison to the AAV2-shVMAT2 + AAV2-GFP treatment group (unpaired t-test, n=5-7 animals per treatment group, p=0.0146) (Figure 21b). These data demonstrate that reintroduction of VMAT2 expression by AAV2-kdrVMAT2 is able to protect against AAV2-shVMAT2 mediated DAergic degeneration.



Figure 21. Rescue of dopaminergic toxicity by reintroducing exogenous VMAT2 expression.

A. Representative images of SNpc for stereological dopaminergic neuronal counts performed by identifying overlap of neuronal marker Nissl (purple) and dopaminergic neuronal marker TH (blue) showing viral transduction by GFP (green). B. Quantification of TH-positive neuronal counts. Number of TH-positive neurons from the AAV2-shVMAT2 + AAV2-GFP treatment group was compared to AAV2-shVMAT2 + AAV2-kdrVMAT2 treatment. at 6 weeks post-transduction (unpaired t-test, n=5-7 animals, p=0.0377). Each data point represents the total number of TH-positive neurons in an individual animal calculated from serial sections.

# 3.2.11 OXIDATIVE DAMAGE IN RESCUE

We investigated the pathogenic mechanisms associated with AAV2-shVMAt2 knockdown induced neurodegeneration within the rescue cohort in order to determine whether these pathogenic mechanisms were also protected against following VMAT2 reintroduction. Analysis of oxidative damage demonstrated a trend toward decreased amount of 4HNE from TH-positive neurons transduced with AAV2-shVMAT2 and AAV2-kdrVMAT2 compared to TH-positive neurons transduced with AAV2-shVMAT2 and AAV2-GFP (Unpaired t-test, n=5 animals per treatment, p=0.0818) (Figure 22). The amount of 4HNE is expressed as a percentage of the amount within transduced TH-positive neurons compared to non-transduced TH-positive neurons. The average value for the AAV2-shVMAT2 and AAV2-GFP treatment group is 114.57%, which suggests a modest increase in the amount of 4HNE within the knock-down transduced neurons. Within the AAV2-shVMAT2 and AAV2-kdrVMAT2 treatment group, the average value was 81.76%, which suggests a slight decrease in the amount of 4HNE within the transduced neurons.



Figure 22. Analysis of oxidative damage in rescued substantia nigra.

A. Quantification was performed by counting the number of 4HNE positive objects and controlling for TH neuron area. Each data point represents the percent of the number of objects/TH neuron area within transduced neurons divided by the number of objects/TH neuron area from non-transduced TH-positive neurons in the internal control contralateral hemisphere at 6 weeks post-transduction (Unpaired t-test, n=5 animals per treatment, p=0.0818).

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Analysis of LRRK2 activity demonstrated no difference in the amount of pThr73-Rab10 within TH-positive neurons transduced with AAV2-shVMAT2 and AAV2-GFP compared to neurons transduced with AAV2-shVMAT2 and AAV2-kdrVMAT2 (unpaired t-test, n=4-5 animals per treatment, p=0.6017) (Figure 23). The amount of pThr73-Rab10 is expressed as a percentage of the amount within transduced TH-positive neurons compared to non-transduced TH-positive neurons. The average value for the AAV2-shVMAT2 and AAV2-GFP treatment group is 115.64%, which suggests a modest increase in the amount of pThr73-Rab10 within the knock-down transduced neurons. Within the AAV2-shVMAT2 and AAV2-kdrVMAT2 treatment group, the average value was 106.8%, which suggests a mild protection against the knock-down induced increase in the amount of pThr73-Rab10.



Figure 23. Analysis of LRRK2 activity in rescued substantia nigra.

A. Quantification was performed by counting the number of pThr73-Rab10 positive objects and controlling for TH neuron area. Each data point represents the percent of the number of objects/TH neuron area within transduced neurons divided by the number of objects/TH neuron area from non-transduced TH-positive neurons in the internal control contralateral hemisphere at 6 weeks post-transduction (Unpaired t-test, n=4-5 animals per treatment, p=0.6017).

### **3.3 DISCUSSION**

Viral-injections of AAV2-shVMAT2 and AAV2-shControl resulted in robust transduction of DAergic neurons in the SNpc, with the six weeks post-transduction time-point chosen for analysis as determined by minimizing the time-point with significant loss of VMAT2 expression and the presence of neurodegeneration. Through viral-mediated RNA interference of VMAT2 expression, a decrease in VMAT2 immunoreactivity was observed in DAergic cell bodies in the SNpc (Figures 3) and within striatal terminals (Figures 5). However, following control injections with a scrambled-shRNA sequence (AAV2-shControl), there was no difference in VMAT2 immunoreactivity observed in the SNpc (Figure 4) or striatum (Figure 6).

While AAV2-shVMAT2 resulted in a mild loss of TH immunoreactivity within the remaining transduced neurons in the SNpc (Figure 3), there was a focal TH lesion in the dorsolateral striatum indicative of nigrostriatal neurodegeneration (Figure 5). Analysis in the SNpc reveals no neurodegeneration observed at four weeks post-transduction (Figure 10), however beginning at six weeks there was neurodegeneration observed (Figure 9) that was maintained at later time points (Figure 10). The focal lesion in the striatum was absent in AAV2-shControl transduced animals (Figure 5), and there was no neurodegeneration observed in the SNpc present following AAV2-shControl (Figure 11).

In rodent models of PD, including the rotenone model, there are motor deficits that correspond with the nigrostriatal neurodegeneration observed (Cannon et al., 2009). Two dopamine-mediated behaviors, the postural instability test and cylinder test, were evaluated following AAV2-shControl and AAV2-shVMAT2 to determine whether the nigrostriatal neurodegeneration observed resulted in motor deficits. AAV2-shVMAT2 resulted in an increase in the distance to trigger on the postural instability test as well as a decrease in paw use during

rearing behavior in the cylinder test (Figure 8). However, AAV2-shControl did not result in an increase in distance to trigger or any differences in paw use during the cylinder test (Figure 8).

To confirm that VMAT2 knock-down resulted in dysregulated dopamine handling, dopamine and its metabolic products were measured in dissected striatum. There was a decrease in the amount of dopamine beginning at four-weeks post-transduction, although there were no differences in dopamine turnover or dopamine oxidation at this time-point (Figure 7). The decrease in dopamine continued at eight- and twelve-weeks post-transduction (Figure 7). Beginning at eight-weeks post-transduction, there was an increase in dopamine turnover observed that continues at twelve-weeks post-transduction (Figure 7). At the eight-week post-transduction time-point, there was also an increase in the amount of protein cysteinyl-dopamine adducts, which is an index of oxidized dopamine (Figure 7). The decrease in dopamine with no differences in dopamine turnover or oxidation at the four-week time-point suggests that this time-point precedes the oxidative damage of cytosolic dopamine and the pathogenic mechanisms associated with nigrostriatal neurodegeneration. However, it is also possible that at this earlier time-point, these processes are happening but the neurons are able to combat the oxidative stress with endogenous antioxidant defenses.

To determine whether the increase in cytosolic dopamine following VMAT2 knock-down resulted in oxidative damage, two markers of oxidative damage – the amount of 4-hydroxynonenal (4HNE) and 3-nitrotyrosine (3NT) – were evaluated. There was an increase in both the amount of 4HNE and 3NT following AAV2-shVMAT2 (Figure 12) however, there were no differences in the amount of 4HNE or 3NT following AAV2-shControl (Figure 13). Recently, we have demonstrated an increase in LRRK2 activity as measured by PLA for autophosphorylated LRRK2 in idiopathic PD (Di Maio et al., 2018). The increase in the amount of autophosphorylated LRRK2
corresponds with an increase in the phosphorylation of LRRK2 substrate Rab10. Analysis of LRRK2 activity in this study revealed an increase in both the autophosphorylation PLA and amount of pThr73-Rab10 following AAV2-shVMAT2 (Figure 14), which was not seen following AAV2-shControl (Figure 15). This is the first report of a cytosolic dopamine-mediated increase in LRRK2 activity.

The formation of aberrant  $\alpha$ -synuclein is also a key pathologic hallmark of PD. Previous models have demonstrated an increase in α-synuclein following VMAT2 knock-down (Caudle et al., 2007), however we did not observe a significant increase in total  $\alpha$ -synuclein following AAV2shVMAT2 (Figure 16). Interestingly, the neurodegeneration observed in the VMAT2-lo expressing mice was contingent upon the animals expressing  $\alpha$ -synuclein, suggesting that an interaction between dopamine and  $\alpha$ -synuclein specifically mediates the pathogenic neurodegenerative process (Caudle et al., 2007; Colebrooke et al., 2006). Although we did not observe an increase in α-synuclein following AAV2-shVMAT2, we detected an increase in both the amount of pSer129-syn and the association between  $\alpha$ -synuclein and TOM20, which has been shown to block mitochondrial import and only occurs with pathogenic forms of α-synuclein, including dopamine-modified a-synuclein (Di Maio et al., 2016) (Figure 16). Both of these observations suggest the presence of aberrant forms of a-synuclein, possibly including dopaminemodified  $\alpha$ -synuclein (Di Maio et al., 2016). It is likely that although the total amount of monomeric a-synuclein did not change in our model, VMAT2 knock-down resulted in aberrant asynuclein that likely contributed to the neurodegenerative process.

Importantly, we demonstrated that VMAT2 expression could be rescued through reintroduction of exogenous VMAT2 utilizing a second virus expressing human myc-ddk tagged VMAT2 with silent mutations, resulting in a construct resistant to the shRNA in AAV2-shVMAT2 (Figure 19-20). In addition to rescuing VMAT2 expression, we also observed protection against neurodegeneration with reintroduction of VMAT2 (Figure 21). We attempted to measure pathogenic mechanisms of degeneration in rescued neurons, but were unable to detect significant differences in 4HNE (Figure 22) or pThr73-Rab10 (Figure 23).

The quantification of protein immunoreactivity following knock-down was performed only in neurons expressing both TH and GFP in order to ensure that analysis was only performed in virally-transduced SNpc DAergic neurons. However, it has been well documented that DAergic neurons lose their phenotype and stop expressing TH when they are undergoing stress (Reis et al., 1978; Sauer & Oertel, 1994). It is possible that some of the GFP-positive and TH-negative neurons in the SNpc had once been DAergic neurons expressing TH but lost their phenotype. It is also possible that the GFP-positive and TH-negative neurons were non-DAergic neurons to begin with and instead neurons from the relatively small (~29%) non-DAergic (GABAergic) neuronal population in the SNpc found in rats (Nair-Roberts et al., 2008). For this reason, GFP-positive-TH-negative neurons were excluded from analysis, which may have resulted in conservative estimates of knock-down.

While there was a trend towards more neurodegeneration observed over time, it failed to reach significance (Figure 10). The low n in each group of this experiment may explain the lack of significance, however it is also possible that there is a ceiling-effect of degeneration being reached. The viral injections transduce only a subset of SNpc DAergic neurons, therefore the remaining TH-positive neurons counted at later time-points may predominantly consist of non-transduced neurons. The half-life of VMAT2 is unknown, and the method of knock-down employed in this study depends upon blocking the translation of new VMAT2 protein. In this way, a progressive loss of VMAT2 is achieved as new VMAT2 is blocked from being transduced and

old VMAT2 is degraded over time. Because of this, individual neurons may be affected differently by the viral-mediated knock-down based on the amount and age of VMAT2 being expressed at the time of transduction. In addition, it is important to note that the tissue was handled differently in the time-point cohort from other experiments. In other experiments, the animals were perfusion fixed with 4% PFA. In the time-point cohort, the animals were perfused only with phosphate buffered saline before the brain was removed and the striatum was dissected for HPLC analysis. Following striatal dissection, the remainder of the brain was post-fixed in 4% PFA. It is possible that the difference in handling of the tissue of this cohort impacted the immunostaining of the tissue.

There are many possible explanations for why we were unable to detect significant differences in pathogenic mechanisms in the rescue experiments. In the knock-down experiments, the animals received  $2\mu$ L of the full titer ( $3.56 \times 10^{12}$  genome copies/mL) of virus, resulting in  $7.12 \times 10^9$  genome copies being delivered. However, in the rescue experiments a total of  $2\mu$ L were delivered consisting of an equal mix of the two viruses. In addition, the viruses were diluted to  $2.0 \times 10^{12}$  genome copies/mL to deliver equal amounts of each virus. This resulted in  $2.0 \times 10^9$  genome copies of each virus being delivered. Therefore, one possibility is that as a result of fewer genome copies of the virus being delivered in these experiments, we were replicate the modest changes observed in the knock-down experiments.

Another possibility is due to these experiments requiring injections of two viruses simultaneously, and an inability to determine whether neurons being analyzed had been transduced by both viruses. Due to technical limitations, including antibody host combinations, and imaging limitations, including the number of channels able to be imaged at one time, we were unable to perform an experiment to identify neurons expressing both GFP from the knock-down virus and myc-ddk tag from the rescue virus. As such, transduced neurons were identified by GFP expression with the assumption that all GFP+TH+ neurons had been transduced by both the knock-down and rescue viruses. In the AAV2-GFP + AAV2-shVMAT2 group, GFP-positive neurons may have been transduced only by AAV2-GFP.

Another consideration of the rescue experiments is that the rescue virus expresses the knock-down resistant construct under the CMV promoter, and the knock-down virus expresses GFP also under the CMV promoter. There is the potential for competition of expression with two genes under the same promoter, which may have resulted in an unequal expression of one of the constructs over the other. To address whether both viruses were expressed, an additional cohort could be used to evaluate dopamine biochemistry following VMAT2 reintroduction to demonstrate that the exogenous VMAT2 introduced by the knock-down resistant virus was able to protect against the accumulation of cytosolic dopamine. Because of the limitations listed here, the analysis of pathogenic mechanisms was abandoned following preliminary analysis reported here that was unable to detect significant differences.

In order to better evaluate the mechanism by which reintroducing VMAT2 is protective against degeneration, other experiments and analyses could be performed. Due to fewer viral particles being introduced, it is possible that differences in pathogenic mechanisms may have been observed had the analysis been performed at a longer time-point past 6 weeks post-transduction. In order to administer the full amount of viral particles as earlier experiments, two surgeries could have been performed where animals received the knock-down virus first followed by a second surgery introducing the knock-down resistant virus. This experiment was not performed as we were concerned with the consequences of two invasive surgeries on the health of the animals, and we were concerned that two surgeries would cause an increased inflammatory response and already transduced neurons may more resistant to secondary transduction.

# 4.0 VIRAL-MEDIATED shRNA INTERFERENCE OF VMAT2 EXPRESSION IN THE VENTRAL TEGMENTAL AREA

Aim 1b: Characterize the impact of decreased vesicular sequestration of dopamine on dopaminergic neuronal health in the ventral tegmental area, and investigate possible mechanisms by which this population is resistant to degeneration in Parkinson's disease.

### **4.1 RATIONALE**

The DAergic neurons in the VTA appear to be less affected than the DAergic neurons in the SNpc in PD. We hypothesized that because the VTA is less affected in PD, that the DAergic neurons in the VTA would be resistant to the effects of VMAT2 knock-down. Previous studies have shown that mice deficient in VMAT2 show no degeneration in the VTA (Taylor et al., 2014). Our objective was to knock-down VMAT2 expression within DAergic neurons in the VTA and evaluate neurodegeneration, as well the PD-associated pathogenic mechanisms evaluated in the SNpc. If differences between the pathogenic mechanisms were observed between the VTA and the SNpc, this may suggest mechanisms to focus on as potential therapeutic targets.

### 4.2 RESULTS

### 4.2.1 VMAT2 KNOCK-DOWN IN VENTRAL TEGMENTAL AREA

To determine whether the consequences of VMAT2 knock-down on neuronal health were specific to DAergic neurons in the SNpc, the AAV2-shVMAT2 virus was injected targeting the VTA – a population of DAergic neurons adjacent to the SNpc. VTA DAergic neurons transduced with AAV2-shVMAT2 demonstrated a 43.06% loss in VMAT2 immunoreactivity compared to non-transduced neurons from the contralateral hemisphere (paired t-test, n=4 animals, p=0.0451) (Figure 24a).



Figure 24. AAV2-shVMAT2 expression in ventral tegmental area.

A. Quantification of VMAT2 immunoreactivity within AAV2-shVMAT2 transduced TH-positive neurons within the VTA compared to non-transduced neurons in the contralateral hemisphere in males at 6 weeks post-transduction (paired t-test, n=4 animals, p=0.0451).

## 4.2.2 DOPAMINERGIC TOXICITY IN THE VENTRAL TEGMENTAL AREA

The DAergic neurons in the VTA appear to be less affected in PD, which suggests there is an inherent difference in these neuronal populations that confers either enhanced vulnerability in the SNpc, or resistance against neurodegeneration in the VTA (Alberico et al., 2015). Stereological counts of TH-positive neurons were performed in the VTA following AAV2-shVMAT2 injections to determine whether knock-down of VMAT2 resulted in DAergic degeneration. Counts of THpositive neurons revealed a 50.6% loss of TH-positive neurons in the transduced hemisphere compared to the non-transduced contralateral hemisphere (paired t-test, n=6 animals, p=0.0111) (Figure 25a). However, counts of NISSL+ neurons revealed no significant difference in the number of neurons in the AAV2-shVMAT2 transduced hemisphere compared to the contralateral nontransduced hemisphere (paired t-test. n=6 animals, p=0.9188) (Figure 25b).



Figure 25. Stereological counts in ventral tegmental area following AAV2-shVMAT2.

A. Quantification of TH-positive neuronal counts. Number of TH-positive neurons from the transduced hemisphere were compared to the internal control non-transduced contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.0111). Each data point represents the total number of TH-positive neurons in an individual animal calculated from serial sections. B. Number of Nissl-positive neurons from the transduced hemisphere compared to the internal control non-transduced contralateral hemisphere at 6 weeks post-transduced hemisphere neurons from the transduced hemisphere compared to the internal control non-transduced contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.9188).

## 4.2.3 OXIDATIVE DAMAGE IN THE VENTRAL TEGMENTAL AREA

Oxidative damage was analyzed in AAV2-shVMAT2 transduced neurons in the VTA. There was no difference in the amount of 4HNE (paired t-test, n=6 animals, p=0.7150) (Figure 26a) or 3NT (paired t-test, n=6 animals, p=0.7929) within transduced TH-positive neurons in the VTA compared to non-transduced TH-positive neurons (Figure 26b).



Figure 26. Analysis of oxidative damage in ventral tegmental area.

A. Quantification was performed by counting the number of 4HNE positive objects and controlling for TH neuron area of neurons in the VTA. Number of objects/TH neuron area from AAV2-shVMAT2 transduced VTA neurons were compared to internal control contralateral non-transduced neurons at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.7150). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal. B. Quantification was performed by counting the number of 3NT positive objects and controlling for TH neuron area of neurons in the VTA. Number of objects/TH neuron area from AAV2-shVMAT2 transduced VTA neurons were compared to internal control contralateral non-transduced neurons area from AAV2-shVMAT2 transduced VTA neurons were compared to internal control contralateral non-transduced neurons at 6 weeks post-transduced neurons at 6 weeks post-transduced to internal control contralateral non-transduced neurons at 6 weeks post-transduced to internal control contralateral non-transduced neurons at 6 weeks post-transduced to internal control contralateral non-transduced neurons at 6 weeks post-transduced to internal control contralateral non-transduced neurons at 6 weeks post-transduction (paired t-test, n=5 animals, p=0.7929). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal.

## 4.2.4 LRRK2 ACTIVITY IN THE VENTRAL TEGMENTAL AREA

The activity of LRRK2 was investigated in AAV2-shVMAT2 transduced neurons in the VTA. While there was a modest 14.8% increase in LRRK2 PLA intensity (paired t-test, n=6 animals, p=0.0142) (Figure 27a), there was no significant difference in the amount of pThr73-Rab10 within transduced neurons compared to non-transduced TH-positive neurons (paired t-test, n=4 animals, p=0.3449) (Figure 27b).



Figure 27. Analysis of LRRK2 activity in ventral tegmental area.

A. Quantification of LRRK2 PLA immunoreactivity within transduced TH-positive neurons in the VTA was compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.0142). B. Quantification was performed by counting the number of pThr73-Rab10 positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-shVMAT2 transduced neurons in the VTA were compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=4 animals, p=0.3449). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal.

### 4.2.5 ABERRANT $\alpha$ -SYNUCLEIN IN THE VENTRAL TEGMENTAL AREA

The presence of aberrant  $\alpha$ -synuclein was evaluated within AAV2-shVMAT2 transduced neurons in the VTA. The amount of total  $\alpha$ -synuclein was not significantly different between AAV2-shVMAT2 transduced and non-transduced TH-positive neurons in the VTA (paired t-test, n=6 animals, p=0.4690) (Figure 28a). Additionally, there was no difference in the amount of pSer129-syn within AAV2-shVMAT2 transduced ventral tegmental TH-positive neurons compared to non-transduced TH-positive neurons (paired t-test, n=6 animals, p=0.0837) (Figure 28b).



Figure 28. Analysis of α-synuclein in ventral tegmental area.

A. Quantification of α-synuclein immunoreactivity within AAV2-shVMAT2 transduced TH-positive neurons in the VTA were compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.4690). Each data point represents the average immunoreactivity for each animal calculated from at least 100 neurons per animal. B. Quantification of pSer129-syn immunoreactivity within AAV2-shVMAT2 transduced TH-positive neurons was compared to internal control non-transduced TH-positive neurons from the contralateral hemisphere at 6 weeks post-transduction (paired t-test, n=6 animals, p=0.0837). Each data point represents the average immunoreactivity for each animal calculated from at least 75 neurons per animal.

#### **4.3 DISCUSSION**

The population of DAergic neurons in the VTA, which are adjacent to the SNpc, appear to be less affected in PD (Alberico et al., 2015). We investigated whether TH-positive VTA neurons were resistant to the toxic effects of AAV2-shVMAT2. AAV2-shVMAT2 transduced TH-positive neurons in the VTA demonstrated a significant decrease in VMAT2 expression (Figure 24). While DAergic stereology reveals a loss of TH-positive neurons in the VTA, NISSL-positive counts demonstrated no significant differences in the total number of neurons in the transduced hemisphere compared to the non-transduced hemisphere (Figure 25). Additionally, analysis of PDassociated pathogenic mechanisms revealed no oxidative damage (Figure 26), a slight increase in LRRK2 autophosphorylation PLA signal but no difference in the amount of pThr73-Rab10 (Figure 27), and no formation of aberrant  $\alpha$ -synuclein (Figures 28). Our results replicate what has previously been seen in mice deficient in VMAT2 expression that displayed no degeneration in the VTA (Taylor et al., 2014), and suggest that the DAergic neurons in the VTA possess a decrease in vulnerability to degeneration compared to the DAergic neurons in the SNpc.

Viral treatment	Viral titer (genome copies/mL)	Number of animals	Male/ Female	Time post-viral transduction	Figures	Analyses
AAV2-shVMAT2	3.56x10 <sup>12</sup>	5	Male	6 weeks	3, 5, 9, 14, 16	Nigral VMAT2 and TH, striatal VMAT2 and TH, nigral stereology, pRab10, α- synuclein
AAV2-shVMAT2	3.56x10 <sup>12</sup>	6	Female	6 weeks	3, 9, 14	Nigral VMAT2 and TH, stereology, LRRK2 PLA
AAV2-shVMAT2	3.56x10 <sup>12</sup>	4 4 3	Male	4 weeks 8 weeks 12 weeks	7, 10	Nigral VMAT2, neurochemistry, stereology
AAV2-shVMAT2	1.78x10 <sup>12</sup>	9	Male	6 weeks	12	Oxidative damage
Ipsilateral AAV2-shVMAT2 Contralateral AAV2-shControl	3.56x10 <sup>12</sup> 1.53x10 <sup>13</sup>	4	Male	20 weeks	8	Dopamine-mediated behaviors
Unilateral AAV2-shVMAT2 Unilateral AAV2-shControl	3.56x10 <sup>12</sup> 1.53x10 <sup>13</sup>	4 4				
AAV2-shVMAT2 + AAV2-GFP AAV2-shVMAT2 + AAV2-kdrVMAT2	$2.0x10^{12} \mbox{ and } 2.0x10^{12}  2.0x10^{12} \mbox{ and } 2.0x10^{12}  10^{12} $	7 5	Male	6 weeks 6 weeks	20 – 23	
AAV2-shControl	3.56x10 <sup>12</sup>	5	Male	6 weeks	4, 6, 11, 13, 15, 17	Nigral VMAT2 and TH, striatal VMAT2 and TH, stereology, pRab10
AAV2-shVMAT2 (VTA)	3.56x10 <sup>12</sup>	6	Male	6 weeks	24 – 28	Nigral VMAT2, stereology, pRab10, α- synuclein

### Table 3. SUMMARY OF ANIMALS USED IN SPECIFIC AIM 1 ANALYSES

# 5.0 ESTABLISHING AN ANIMAL MODEL OF VIRAL-MEDIATED OVEREXPRESSION OF VMAT2

Aim 2: Investigate the therapeutic potential of targeting dopamine handling as an intervention in Parkinson's disease models.

### **5.1 RATIONALE**

Given the neurotoxic consequences of cytosolic dopamine observed in our experiments and previously reported (Caudle et al., 2007; Chen et al., 2008; Lawal et al., 2010), we hypothesized that enhanced vesicular sequestration would be protective against DAergic degeneration in a model of PD. Additionally, a number of studies have identified variants in *SLC18a2* that decrease the likelihood of developing PD, which is believed to be a result of gainof-function mutations. These variants are the result of single nucleotide polymorphisms found in the promoter region of *SLC18a2*, which result in a decreased odds ratio of developing PD (Brighina et al., 2013; C. E. Glatt et al., 2006; Yang et al., 2015). Analysis within subpopulations of the group studied in one experiment demonstrated an odds ratio as low as 0.38 for women homozygous for the gain-of-function polymorphisms (C. E. Glatt et al., 2006). These polymorphism, when introduced in a cell culture system, result in increased transcription (C. E. Glatt et al., 2006; Lin et al., 2005). While an increase in the transcription of *SLC18a2* is an indirect measure of the amount of VMAT2 present, these studies suggest that increased sequestration of dopamine may be protective against the development of PD. These studies demonstrating the protective nature of VMAT2 suggest that targeting VMAT2 may have therapeutic viability for PD. Targeting VMAT2 in PD treatment may provide the dual-benefit of slowing disease protection by maintain neuronal health through a decrease in cytosolic dopamine, and by alleviating the motor symptoms by increasing dopamine transmission.

Previous experiments in murine models demonstrated that overexpression of VMAT2 is protective against DAergic toxins (K. M. Lohr et al., 2014). However, the MPTP metabolite MPP+ is selectively transported into DAergic neurons by DAT and then sequestered into synaptic vesicles by VMAT2. The overexpression of VMAT2 therefore is likely to confer protection against MPTPinduced DAergic neurotoxicity because of enhanced sequestration of MPP+ into synaptic vesicles, preventing MPP+ from causing mitochondrial dysfunction. Additionally, work in a *Drosophila* model has shown the protective potential of VMAT overexpression against rotenone-induced toxicity (Lawal et al., 2010).

Our goal was to translate previous research demonstrating a protective effect of VMAT2 overexpression against parkinsonian degeneration into the well-established rodent rotenone model of PD (Cannon et al., 2009). This model was chosen because rotenone, when administered systemically, results in specific DAergic degeneration and replication of other features of PD (Cannon et al., 2009). Rotenone, similar to MPP+, results in neurotoxicity due to its inhibition of complex I of the electron transport chain in mitochondria. However, in contrast to MPTP which results in specific DAergic degeneration due to MPP+ being a substrate for DAT and VMAT2, rotenone is works independently of dopamine homeostasis machinery and, due to its lipophilic nature, affects mitochondrial functioning ubiquitously throughout the brain and body. Despite rotenone affecting all cells in the body, it results in specific DAergic degeneration. MPTP is rarely implicated in the development parkinsonism beyond the case during which it was discovered as a

contaminant in the production of a synthetic opioid (Langston et al., 1983). Therefore, it is unlikely to contribute to the pathogenesis of PD. However, rotenone has been widely used as an organic pesticide, and exposure to rotenone results in an increased risk of developing PD (Tanner et al., 2011). In testing the therapeutic potential of VMAT2 overexpression in the rotenone model, we believe the results would be more directly translational to PD.

### 5.2 RESULTS

### 5.2.1 VIRAL-MEDIATED OVEREXPRESSION OF VMAT2 in vivo

To investigate the therapeutic potential of VMAT2 overexpression for PD, an adenoassociated virus (AAV serotype 2) overexpressing myc-DDK tagged human wild-type VMAT2 (AAV2-wtVMAT2) was obtained from Penn Vector Core (Figure 29).



### Figure 29. Viral plasmid map of VMAT2 overexpression virus.

A. Overexpression of human VMAT2 with a myc-DDK tag under the CMV promoter.

This construct was previously tested for the rescue experiments in Figure 18, and demonstrated the ability to express VMAT2 *in vitro*. Analysis of viral efficacy was performed on two animals sacrificed at 5 weeks post-transduction. Analysis of VMAT2 immunoreactivity demonstrated co-localization of VMAT2 with myc-DDK tag, indicative of exogenous VMAT2 expression (Figure 30a). Quantification revealed a 113.1% increase in VMAT2 immunoreactivity within TH-positive AAV2-wtVMAT2 transduced neurons compared to non-transduced TH-positive neurons from the contralateral hemisphere (Figure 30b) (no stats performed, n=2 animals).



Figure 30. Viral-mediated overexpression of VMAT2.

A. 60x magnification confocal images of AAV2-wtVMAT2 transduced TH-positive neurons in the SNpc with corresponding non-transduced SNpc in the contralateral hemisphere. Virally-transduced neurons were identified by the presence of Myc-DDK tag immunoreactivity. B. Quantification of VMAT2 immunoreactivity within AAV2-wtVMAT2 TH-positive neurons compared to non-transduced TH-positive neurons in the contralateral hemisphere at 5 weeks post-transduction. Each data point represents the average intensity of VMAT2 immunoreactivity per animal calculated from at least 50 neurons.

## 5.2.2 THERAPEUTIC POTENTIAL OF VMAT2 OVEREXPRESSION IN THE CHRONIC ROTENONE MODEL OF PARKINSON'S DISEASE

The rotenone model of PD was chosen due to its ability to consistently reproduce features of PD following systemic administration (Cannon et al., 2009). As a preliminary experiment to test the protective effect of VMAT2 overexpression, all animals received rotenone injections and no vehicle group was included because each animal contains a within-animal control – the contralateral non-transduced hemisphere – and comparisons could be made to a control overexpressing treatment group that received AAV2-GFP injections. Animals received daily intraperitoneal injections of rotenone (2.8mg/kg prepared in 2% DMSO and 98% miglyol) starting 5 weeks post-transduction of AAV2-wtVMAT2 (n=7 animals) or AAV2-GFP (n=3 animals) as a control. Once motor endpoint was reached, animals were sacrificed by transcardial perfusion and tissue was collected for analysis. A timeline of the experimental design is outline in Figure 31.



#### Figure 31. Experimental outline of VMAT2 overexpression in chronic rotenone model.

A. Animals received unilateral injections of AAV2-wtVMAT2 (n=7 animals) or AAV2-GFP (n=3 animals) targeting the SNpc. After 5 weeks of viral transduction, 2 animals that received AAV2-wtVMAT2 were sacrificed and the remaining animals received daily rotenone injections (2.8mg/kg) were administered until motor endpoint. Postural instability testing was performed daily. At motor endpoint, animals were sacrificed by transcardial perfusion with 4% paraformaldehyde and the brains were harvested. Brains were post-fixed with 4% paraformaldehyde before moving to 30% sucrose before sectioning and tissue analysis.

Overexpression of VMAT2 did not extend the survival of animals compared to GFP overexpression (Figure 32a). Additionally, animals in both treatment groups lost weight at the same rate (Figure 32b), and postural instability testing revealed no difference in distance to trigger in the forepaw contralateral to AAV2-wtVMAT2 compared to AAV2-GFP (Figure 32c). These data demonstrate that overexpression of VMAT2 was unable to protect against the development of motor symptoms and general toxicity induced by rotenone exposure.



Figure 32. Effect of VMAT2 overexpression on rotenone-induced parkinsonism.

A. Survival curve following rotenone administration for AAV2-VMAT2 (n=5 animals) and AAV2-GFP (n=3 animals) animals. B. Weight loss following rotenone administration for AAV2-VMAT2 and AAV2-GFP animals. C. Postural instability test values for the affected forepaw (i.e. contralateral to AAV2-VMAT2 or AAV2-GFP) following rotenone administration.

To ensure that the AAV2wtVMAT2 virus was able to successfully overexpress VMAT2, immunohistochemical analysis in the SNpc following rotenone exposure was performed. We were able to identify myc-ddk tagged VMAT2 expression within TH-positive neurons following AAV2-wtVMAT2, which confirms viral efficacy (Figure 33a bottom). In addition, robust GFP immunoreactivity was observed in TH-positive neurons following AAV2-GFP (Figure 33a top). Based on this data, we concluded that the AAV2-wtVMAT2 virus was able to successfully increase the expression of VMAT2 within DAergic neurons.

## a. AAV2-GFP



#### Figure 33. Viral expression following rotenone exposure.

A. Analysis of GFP and VMAT2 expression in SNpc of chronic rotenone model of animals that received AAV2-GFP (top) or AAV2-VMAT2 (bottom). Representative images showing viral transduction either by GFP (green) or Myc-tag (yellow) in TH-positive neurons (blue) and VMAT2 (red) expression.

To quantify the extent of VMAT2 overexpression achieved by AAV2-wtVMAT2, immunohistochemical analysis was performed. Quantification of VMAT2 immunoreactivity following rotenone exposure revealed a significant increase in VMAT2 immunoreactivity within TH-positive AAV2-wtVMAT2 transduced neurons compared to non-transduced TH-positive neurons from the contralateral hemisphere (One-way ANOVA with Tukey's post-hoc test, n=3-7 animals per treatment, \*p<0.05) (Figure 34a-b). There were no differences in VMAT2 expression within the AAV2-GFP treatment group.



Figure 34. Analysis of VMAT2 expression in substantia nigra of chronic rotenone model.

A. Representative 60x magnification confocal images of transduced TH-positive neurons in the SNpc with corresponding non-transduced SNpc in the contralateral hemisphere. B. Quantification of VMAT2 immunoreactivity within AAV2-GFP and AAV2-VMAT2 transduced TH-positive neurons compared to non-transduced neurons in the contralateral hemisphere. (One-way ANOVA with Tukey's post-hoc test, n=3-7 animals, \*p<0.05). Each data point represents the average value for an individual animal calculated from at least 100 neurons per animal.

To determine whether the overexpression of VMAT2 was able to protect against rotenoneinduced DAergic degeneration, stereological counts of TH-positive neurons in the SNpc were performed. Analysis of DAergic neurotoxicity was performed by identifying overlap between TH and the neuronal marker NISSL (Figure 35a). While the counts of TH-positive neurons reflected equivalent rotenone-induced degeneration to previous studies (De Miranda et al., 2019), the number of TH-positive neurons did not significantly differ in the AAV2-wtVMAT2 treatment group compared to the AAV2-GFP treatment group (unpaired t-test, n= 3-5 animals per treatment, p=0.1928) (Figure 35b). The counts from all treatment groups suggest degeneration in TH-positive neurons as a result of rotenone treatment, however we were unable to detect protection within the hemisphere that received AAV2-wtVMAT2.



Figure 35. Analysis of dopaminergic neurotoxicity in chronic rotenone model.

A. Representative images of SNpc for stereological dopaminergic neuronal counts performed by identifying overlap of neuronal marker Nissl (purple) and dopaminergic neuronal marker TH (blue) showing viral transduction by GFP (green) or Myc (green). Top: AAV2-GFP, bottom: AAV2-VMAT2. B. Quantification of TH-positive neuronal counts. Number of TH-positive neurons from AAV2-GFP treatment group compared to AAV2-VMAT2 treatment following rotenone exposure. (One-way ANOVA with Tukey's post-hoc test, n=3-5 animals per treatment, n.s.). Each data point represents the total number of TH-positive neurons calculated from serial sections.

In addition to evaluating nigrostriatal neurodegeneration by performing stereological counts in the SNpc, the striatal terminals were evaluated. Analysis of striatal terminals revealed stereotypic rotenone-induced focal lesions by the loss of TH immunoreactivity in the dorsolateral striatum in both the AAV2-GFP (Figure 36a left) and AAV2-wtVMAT2 (Figure 36a right) treatment groups. Quantification revealed no significant differences in TH immunoreactivity between treatment groups (unpaired t-test, n=3-5 animals per treatment, p=0.5219) (Figure 36b) or the transduced and non-transduced hemispheres from both treatment groups (One-way ANOVA, n=3-5 animals per treatment, n.s.) (Figure 36c). These results, demonstrating no protection against terminal degeneration by VMAT2 overexpression, correlate with the stereological data demonstrating no protection against the loss of cell bodies.


Figure 36. Analysis of striatal terminals in chronic rotenone model.

A. Representative images of TH expression in the striatum following AAV2-GFP and rotenone (left) or AAV2-VMAT2 and rotenone (right). B. Quantification of striatal TH immunoreactivity in the transduced hemisphere expressed as a percent of the non-transduced contralateral hemisphere. (Unpaired t-test, n=3-5 animals per treatment, p=0.5219). Each data point represents the average value per animal calculated from serial striatal sections. C. Raw values of TH immunoreactivity from AAV2-GFP and contralateral non-transduced hemisphere and AAV2-VMAT2 and contralateral non-transduced hemisphere. (One-way ANOVA with Tukey's post-hoc test, n=3-5 animals per treatment, n.s.). Each data point represents the average value of immunoreactivity per animal calculated from 12 serial striatal sections.

### 5.2.3 ANALYSIS OF ABERRANT α-SYNUCLEIN IN CHRONIC ROTENONE MODEL

Although there were no significant differences at the level of nigrostriatal toxicity, analysis was performed to determine if neurons overexpressing VMAT2 were protected against the previously-established rotenone-induced pathogenic accumulation of  $\alpha$ -synuclein (Cannon et al., 2009). There was no significant difference in the immunoreactivity of  $\alpha$ -synuclein in the AAV2-wtVMAT2 overexpression treatment group compared to the AAV2-GFP treatment group (unpaired t-test, n=3-4 animals per treatment group, p=0.3660) (Figure 37a-b). In addition, there were no significant differences between the transduced and non-transduced neurons from each treatment group (Figure 37c) (One-way ANOVA with Tukey's post-hoc test, n=3-4 animals per treatment group, n.s.). Although there were no significant differences in the amount of  $\alpha$ -synuclein within transduced neurons, there was a trend towards decreased  $\alpha$ -synuclein within AAV2-wtVMAT2 transduced neurons.



Figure 37. Analysis of α-synuclein in chronic rotenone model.

A. Representative 60x confocal images of TH-positive (red) virally-transduced GFP- or Myc-expressing (green) neurons in the SNpc immunostained for  $\alpha$ -synuclein (white) B. Quantification of  $\alpha$ -synuclein immunoreactivity within transduced TH-positive neurons expressed as a percentage of the immunoreactivity from the internal control non-transduced TH-positive neurons in the contralateral hemisphere (unpaired t-test, n=3-4 animals per treatment, p=0.3660). Each data point represents the average immunoreactivity for each animal calculated from at least 100 neurons per animal. C. Raw values of  $\alpha$ -synuclein immunoreactivity from AAV2-GFP transduced and non-transduced TH-positive neurons and AAV2-VMAT2 transduced and non-transduced neurons. Each data point represents the average immunoreactivity from at least 100 neurons.

# 5.2.4 ANALYSIS OF LRRK2 ACTIVITY IN CHRONIC ROTENONE MODEL

In addition to α-synuclein, the activity of LRRK2 was evaluated by quantifying the amount of pThr73-Rab10 to determine whether overexpression of VMAT2 was protective against rotenone-induced induction of LRRK2 activity (Di Maio et al., 2018). Results showed there was no significant difference in the amount of pThr73-Rab10 in animals that received AAV2-VMAT2 compared to AAV2-GFP (Figure 38a-b) (One-way ANOVA with Tukey's post-hoc test, n=3-4 animals per treatment group, n.s.).



Figure 38. Analysis of LRRK2 activity in AAV2-VMAT2 transduced substantia nigra in chronic rotenone model.

A. Representative 60x confocal images of TH-positive (white) virally-transduced Myc-tagged (green) neurons in the SNpc with pThr73-Rab10 (red). B. Quantification was performed by counting the number of pThr73-Rab10 positive objects and controlling for TH neuron area. Number of objects/TH neuron area were compared between non-transduced and AAV2-GFP or AAV2-VMAT2 transduced neurons (One-way ANOVA with Tukey's post-hoc test, n=3-4 animals per treatment group, n.s.). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal.

# 5.2.5 THERAPEUTIC POTENTIAL OF VMAT2 OVEREXPRESSION IN THE ACUTE ROTENONE MODEL OF PARKINSON'S DISEASE

It is possible that there were no significant differences in toxicity or pathogenic mechanisms observed following chronic rotenone regimen because the analysis was performed at endpoint, at which a ceiling may have been reached. In order to determine whether VMAT2 overexpression is protective during the progression of the disease, an acute rotenone regimen consisting of 5 days of 2.8 mg/kg rotenone or vehicle was tested (Figure 39). This model has shown the induction of pathogenic mechanisms that precedes neurodegeneration (Rocha et al., 2019), making it a useful method for evaluating the progression of pathogenic mechanisms.



#### Figure 39. Experimental outline of VMAT2 overexpression in acute rotenone model.

A. Animals received unilateral injections of AAV2-wtVMAT2 (n=8 animals) or AAV2-GFP (n=7 animals) targeting the SNpc. After 5 weeks of viral transduction, animals received daily rotenone injections (2.8mg/kg) or vehicle (2% DMSO and 98% miglyol) were administered for five days. Postural instability testing was performed daily. Animals were sacrificed 24h after the last injection by transcardial perfusion with 4% paraformaldehyde and the brains were harvested.

Within the AAV2-wtVMAT2 treatment group, acute rotenone exposure showed motor deficits by increased distance to trigger on the postural instability test compared to animals that received vehicle treatment (Figure 40). However, there were no differences in performance on the postural instability test between the VMAT2 overexpression affected and unaffected forepaw in the rotenone treatment group (Figure 40).



Figure 40. Analysis of dopamine-mediated behaviors in AAV2-wtVMAT2 animals in the acute rotenone model.

A. Postural instability test was performed daily with three trials averaged for each forepaw.

Analysis of VMAT2 expression with AAV2-GFP and AAV2-wtVMAT2 transduced THpositive neurons expressed as a percentage of VMAT2 immunoreactivity in non-transduced THpositive neurons from the contralateral hemisphere demonstrated no significant differences in the amount of VMAT2 immunoreactivity between treatment groups (One-way ANOVA with Tukey's post-hoc test, n=3-4 animals per treatment, n.s.) (Figure 41a). However, by evaluating the raw values from all treatment groups, in both the rotenone and vehicle treated groups, the amount of VMAT2 within AAV2-wtVMAT2 transduced neurons appeared to trend towards increased expression over the other conditions (Figure 41b).





A. Quantification of VMAT2 immunoreactivity within AAV2-GFP and AAV2-VMAT2 transduced TH-positive neurons expressed as a percentage of VMAT2 immunoreactivity in non-transduced TH-positive neurons from the contralateral hemisphere following rotenone (2.8mg/kg) or vehicle administration. (One-way ANOVA with Tukey's post-hoc test, n=3-4 animals per treatment, n.s.). Each data point represents the average value for an individual animal calculated from at least 100 neurons per animal. B. Quantification represented as raw VMAT2 immunoreactivity intensity levels (arbitrary units) for non-transduced and AAV2-wtVMAT2 and AAV2-GFP transduced TH-positive neurons following vehicle and acute rotenone (2.8mg/kg daily for 5 days) administration (Two-way ANOVA with Bonferroni's post-hoc test, n=3-4 animals per treatment, n.s.).

Although neurodegeneration is not observed in the SNpc in this acute rotenone exposure model, the striatal terminals were analyzed because it is possible that neurodegeneration begins in the terminals and proceeds through a dying-back of neurons into the SNpc. Analysis of striatal terminals revealed uniform TH immunoreactivity throughout the striatum of all four treatment groups (Figure 42a). Quantification revealed a significant difference in TH immunoreactivity between both the AAV2-GFP + vehicle and the AAV2-GFP + vehicle treatment group compared to the AAV2-VMAT2 + rotenone group (Figure 42b). However, this appears to be a result of decreased TH expression within the AAV2-GFP transduced hemispheres independent of vehicle or rotenone treatment (Figure 42c) and not a reflection of AAV2-wtVMAT2-mediated protection (Figure 42d).



Figure 42. Analysis of striatal terminals in acute rotenone model.

A. Representative images of striatal TH expression following unilateral AAV2-GFP or AAV2-VMAT2 and acute rotenone or vehicle. Top left: AAV2-VMAT2 + Vehicle; Bottom left: AAV2-VMAT2 + Rotenone; Top right: AAV2-GFP + Vehicle; Bottom right: AAV2-GFP + Rotenone. B. Quantification of striatal TH immunoreactivity in the transduced hemisphere expressed as a percent of the non-transduced contralateral hemisphere. (One-way ANOVA with Tukey's post-hoc test, n=3-5 animals per treatment, \*p<0.05). Each data point represents the average value per animal calculated from serial striatal sections. C. Quantification of striatal TH immunoreactivity in the non-transduced and AAV2-GFP transduced hemispheres of vehicle and rotenone treated animals. (One-way ANOVA with Tukey's post-hoc test, n=3-4 animals per treatment group, n.s.) Each data point represents the average value per animal calculated from serial striatal sections. D. Quantification of striatal TH immunoreactivity in the non-transduced and wtVMAT2 transduced hemispheres of vehicle and rotenone treated animals. (One-way ANOVA

with Tukey's post-hoc test, n=3 animals per treatment group, n.s.) Each data point represents the average value per animal calculated from serial striatal sections.

# 5.2.6 ANALYSIS OF LRRK2 ACTIVITY IN THE ACUTE ROTENONE MODEL

Pathogenic mechanisms associated with PD were evaluated to determine whether overexpression of VMAT2 was able to protect against rotenone-mediated induction of these mechanisms. As an initial experiment, LRRK2 activity was evaluated by quantifying the amount of pThr73-Rab10 within the AAV2-wtVMAT2 and rotenone treatment group. Analysis of LRRK2 activity within AAV2-VMAT2 transduced TH-positive neurons following acute rotenone exposure demonstrated no difference in the amount of pThr73-Rab10 (paired t-test, n=6 animals, p=0.9932) (Figure 43a). Due to there being no significant difference in the amount of pThr73-Rab10 within neurons overexpressing VMAT2, the rest of the treatment groups were not analyzed.



### Figure 43. Analysis of LRRK2 activity in acute rotenone model.

A. Quantification was performed by counting the number of pThr73-Rab10 positive objects and controlling for TH neuron area. Number of objects/TH neuron area from AAV2-VMAT2 transduced neurons were compared to internal control non-transduced TH-positive neurons in the contralateral hemisphere (paired t-test, n=6 animals, p=0.9932). Each data point represents the average number of objects in a TH neuron collected from at least 50 neurons per animal.

# 5.2.7 ANALYSIS OF $\alpha$ -SYNUCLEIN IN THE ACUTE ROTENONE MODEL

As an additional measure of pathogenic mechanisms of degeneration, the amount of total  $\alpha$ -synuclein was measured between all treatment groups. Quantification revealed no significant differences in  $\alpha$ -synuclein immunoreactivity between the four treatment groups (One-way ANOVA with Tukey's post-hoc test, n=3-5 animals per treatment group, n.s.) (Figure 44a).



### Figure 44. Analysis of α-synuclein in acute rotenone model.

A. Quantification of  $\alpha$ -synuclein immunoreactivity within transduced TH-positive neurons (One-way ANOVA with Tukey's post-hoc test, n=3-5 animals per treatment, n.s.) Each data point represents the average immunoreactivity for each animal calculated from at least 100 neurons per animal.

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## Table 4. SUMMARY OF ANIMALS USED IN SPECIFIC AIM 2 ANALYSES

Viral treatment	Rotenone/Vehicle	Viral titer (genome copies/mL)	Number of animals	Male/ Female	Time post-viral transduction	Figures	Analyses
AAV2-VMAT2 AAV2-GFP	Rotenone Rotenone	1.556e13 1.816e13	7 3	Male	5-7 weeks	30 – 38	Survival, body weight, postural instability test, nigral VMAT2, striatal TH, dopaminergic stereology, a-synuclein, LRRK2 activity
AAV2-VMAT2 AAV2-VMAT2 AAV2-GFP AAV2-GFP	Rotenone Vehicle Rotenone Vehicle	1.556e13 1.556e13 1.816e13 1.816e13	5 3 4 3	Male	6 weeks	40 – 44	Postural instability test, nigral VMAT2, striatal TH, dopaminergic stereology, α-synuclein, LRRK2 activity

### **5.3 DISCUSSION**

The experiments in aim 2 were performed to evaluate the therapeutic potential of VMAT2 overexpression in the rotenone rat model of PD. We were able to successfully overexpress VMAT2 through viral-mediated introduction of human wild type myc-ddk tagged VMAT2 (Figures 29-30, 33-34). In an initial experiment, the therapeutic potential of VMAT2 overexpression was tested with unilateral injections of AAV2-wtVMAT2 compared to animals receiving unilateral injections of a virus overexpressing GFP (AAV2-GFP) as a control (Figure 31). All animals received rotenone injections until motor endpoint was reached, at which point animals were sacrificed and the brains were analyzed.

The overexpression of VMAT2 did not impact survival of animals receiving rotenone injections, nor did it protect against loss of body weight following rotenone administration (Figure 32). The overexpression of VMAT2 was not able to protect against rotenone-induced motor deficits as evaluated by postural instability test (Figure 32). Analysis of neurodegeneration revealed no differences in the number of TH-positive neurons between AAV2-GFP and AAV2-wtVMAT2 transduced animals (Figure 35), and while the analysis of TH terminals in the striatum revealed the characteristic rotenone-induced lesion in the dorsolateral striatum, there were no hemispheric differences demonstrating viral-mediated protection against terminal degeneration (Figure 36).

Analysis of total α-synuclein showed no significant differences between AAV2-GFP and AAV2-wtVMAT2 transduced neurons (Figure 37). Additionally, analysis of the amount of pThr73-Rab10 demonstrated no significant differences between AAV2-GFP and AAV2-wtVMAT2 (Figure 38). It is possible that the inability to detect protection against nigrostriatal degeneration, motor deficits, and induction of pathogenic hallmarks of PD may be due to the

potency of rotenone as a toxin. Since analysis was performed once all animals had reached motor endpoint, it is possible that a ceiling-effect was reached in which the rotenone induced toxicity beyond what was able to be protected against. Additionally, this initial experiment lacked a vehicle injection treatment group.

In order to better evaluate the therapeutic potential of VMAT2 overexpression, a second experiment was run in which animals received acute rotenone exposure in the form of five injections across five days (Figure 39). The acute dosing paradigm of rotenone implemented in Figures 39 - 44 was developed to study pre-degenerative processes (Rocha et al., 2019). Although DAergic degeneration has not been observed in the acute dosing paradigm of rotenone at this timepoint, analysis of PD-associated pathogenic mechanisms of degeneration has revealed the formation of aberrant  $\alpha$ -synuclein in the acute model (Rocha et al., 2019). Based on the results in Figures 32 - 38, we hypothesized that we were unable to detect differences due to analysis being performed at the modeled endpoint of PD. As a limitation of the methods of analysis, we are only able to measure differences captured at the time of the animal's death, which may not be indicative of the process leading to degeneration. Thus, the acute dosing paradigm utilized in Figures 40 - 44 allowed us to evaluate differences during the degenerative process, rather than after the animals had achieved a functional parkinsonian endpoint.

Analysis of motor deficits failed to reveal VMAT2 overexpression mediated protection in the postural instability test compared to GFP overexpression (Figure 40). Analysis of the amount of VMAT2 detected a non-significant increase in the amount of VMAT2 within AAV2-wtVMAT2 transduced neurons (Figure 41). The low n in each treatment group likely prevented these results from reaching significance. Although a loss of DAergic neurons in the SNpc has not been observed in this acute dosing paradigm, we evaluated TH expression in the striatum to determine if there was a loss of terminal innervation preceding the loss of cell bodies in the SNpc. Analysis of TH terminals revealed focal lesions in the dorsolateral striatum, and quantification of TH intensity within the dorsal striatum showed significant differences between AAV2-GFP treated animals and AAV2-wtVMAT2 treated animals (Figure 42). However, when the raw intensity values were evaluated, it appears that the difference observed was due to a loss in the AAV2-GFP transduced hemisphere independent of rotenone exposure, suggesting the protection was not due to VMAT2 overexpression mediated protection (Figure 42). Additionally, analysis of total  $\alpha$ -synuclein and the amount of pThr73-Rab10 failed to show differences between AAV2-GFP and AAV2-wtVMAT2 transduced neurons (Figures 43 – 44).

The inability to observe AAV2-VMAT2-mediated protection against rotenone-induced toxicity may be due to a number of factors. The amount of VMAT2 overexpression is difficult to assess. While we can quantify a percent-increase in VMAT2 expression within the TH-positive cell bodies in the SNpc, it is difficult to assess the amount of VMAT2 overexpression within the terminals in the striatum. Due to the extensive arborization of SNpc DAergic neurons, the overexpressed VMAT2 may be distributed diffusely across the terminals, making quantitative analysis by immunohistochemistry difficult. In addition, the overexpression of VMAT2 at the protein level does not necessarily mean that the VMAT2 is functional or localized to the synaptic vesicles. For this reason, a pharmacological VMAT2 activator may have more therapeutic potential than gene therapy-mediated overexpression of VMAT2 expression. Drugs that increase VMAT2 activity include dacarbazine (Lawal et al., 2014), buproprion (Rau et al., 2005), cocaine (Brown et al., 2001), dopamine receptor agonists (J. G. Truong, Newman, et al., 2004; Jannine G. Truong et al., 2003), apomorphine (J. G. Truong, Hanson, et al., 2004), and methylphenidate (Sandoval et al., 2002). While several of these drugs are used clinically to treat other diseases, they

may not be suitable for treatment of PD. However, new compounds can be synthesized by studying the structure and function of how these drugs enhance VMAT2 activity.

Another possible explanation for the lack of protection may be due to rotenone's action at the electron transport chain in mitochondria, which makes it a potent inducer of oxidative stress (Betarbet, 2000; Sherer, 2003). In our understanding of PD, we hypothesize that cytosolic dopamine may contribute to or exacerbate the oxidative stress and neuronal dysfunction involved in the pathogenesis of the disease by interacting with or inducing other dysfunctional processes. However, cytosolic dopamine is only one factor of many involved in the pathogenic degeneration of neurons in PD, therefore the decrease in cytosolic dopamine may not be sufficient to protect against the other toxic processes occurring, especially in the rotenone model.

Other animal models that demonstrated a protective effect of VMAT2 overexpression utilized specific DAergic toxins that are substrates for DAT and VMAT2 including MPP+ and methamphetamine (K. M. Lohr et al., 2014; K. M. Lohr et al., 2016; K. M. Lohr et al., 2015). In these models, it is likely that the overexpression of VMAT2 allowed for increased sequestration within synaptic vesicles, thereby conferring protection by isolating the toxicants. Additionally, these animals demonstrate a 3-fold increase in VMAT2 expression in homogenate (K. M. Lohr et al., 2014), whereas we observe approximately a 2-fold increase in VMAT2 expression within transduced neurons at the viral titer and time-point used. Additionally, *Drosophila* that overexpress VMAT have demonstrated protection against rotenone-induced toxicity. These *Drosophila* have a 2-fold increase in VMAT expression (H. Y. Chang et al., 2006). It is possible that our viralmediated method of overexpressing VMAT2 may show protection in other toxin models.

### **6.0 CONCLUSIONS**

It is a long-standing hypothesis that dopamine contributes to the degeneration of nigrostriatal DAergic neurons seen in PD. Until recently, analysis of the gene (SLC18a2) for VMAT2 within PD cohorts was unable to conclude that variants in SLC18a2 result in increased likelihood of PD (C. E. Glatt et al., 2006). A recent study published by Xiong et al. in 2016 suggests an association between low-activity promoter haplotypes in SLC18a2 and PD (Xiong et al., 2016). Furthermore, rare mutations in SLC18a2 result in infantile parkinsonism (Jacobsen et al., 2016; Padmakumar et al., 2019; Rilstone et al., 2013). Although VMAT2 variants are infrequently identified in PD, it is possible that cytosolic dopamine contributes to the propagation of disease process through further oxidative stress and interactions with PD associated mechanisms from post-mortem tissue identified of degeneration. Analysis decreased VMAT2 immunoreactivity (Miller et al., 1999) and activity (Goldstein et al., 2013; Pifl et al., 2014) in the striatum, and analysis from circulating platelets of PD patients identified decreased VMAT2 mRNA (Sala et al., 2010), suggesting a systemic deficiency of VMAT2 in PD.

The hypothesis that dopamine has the potential to act as an endogenous neurotoxin has been tested previously in genetic animal models. While VMAT2 knock-out mice die shortly after birth (Wang et al., 1997), experiments performed in heterozygote VMAT2 knock-out mice show increased susceptibility to DAergic toxins (Gainetdinov et al., 1998). Additionally, VMAT2 hypomorph mice that express 5% of the normal amount of VMAT2 show specific, age-dependent, parkinsonian DAergic degeneration, as well as the replication of other features PD (Caudle et al., 2007; Taylor et al., 2014; Taylor et al., 2009). Overexpression of DAT results in an increase in dopamine metabolism associated with a loss of TH-positive neurons in the SNpc, motor deficits, and increased susceptibility to DAergic toxins (Masoud et al., 2015). Furthermore, Chen et al. demonstrated that introducing the plasma membrane dopamine transporter into striatal neurons that lack VMAT2 resulted in oxidative damage and neurodegeneration, providing evidence that dopamine without proper sequestration can induce non-DAergic neurotoxicity (Chen et al., 2008). These findings have been replicated in *Drosophila* deficient in VMAT, which show fewer DAergic neurons and increased vulnerability to pesticide-induced neurodegeneration (Lawal et al., 2010). It is important to mention that work published by Isingirini et al. did not detect degeneration following the conditional loss of VMAT2 (Isingrini et al., 2017). These animals showed a decrease in total movement as early as 8 weeks following knock-out, as well as a significant decrease in food and water intake and weight loss at 16 weeks, which corresponds with a decrease in survival. It is possible that the bilateral loss of VMAT2 resulting in decreased movement, feeding, and drinking caused animals to succumb due to dehydration or starvation before neurodegeneration was observed.

The DAergic neurons in the VTA appear to be less affected in PD, and much research has been dedicated to understanding why this population of DAergic neurons is resistant to degeneration (Alberico et al., 2015). The VTA neurons have different gene expression profiles compared to SN neurons, which may explain the difference in degeneration in PD (Chung et al., 2005; Greene et al., 2005), including different responses to toxicants (Chung et al., 2005; Phani et al., 2010). Two important differences are in VMAT2, which is higher in rat VTA compared to the SN (Nirenberg et al., 1996), and  $\alpha$ -synuclein, which is higher in human SN compared to VTA (Solano et al., 2000). Given the evidence that cytosolic dopamine and  $\alpha$ -synuclein interact to cause neurodegeneration (Ulusoy et al., 2012), the decrease in cytosolic dopamine and decrease in  $\alpha$ synuclein may explain the VTA's relative protection against degeneration. In addition, the two neuronal populations have different glial populations, which may mediate sensitivity to degeneration (Kostuk et al., 2019). Interestingly, data suggests that a subpopulation of VTA neurons co-express the vesicular glutamate transporter 2 (VGLUT2), which enhances vesicular loading of dopamine, which may play a protective role for these neurons (Hnasko et al., 2010). In mice deficient in VMAT2, no degeneration is observed in the VTA (Taylor et al., 2014), and in the rotenone model of PD, the VTA is relatively spared (Betarbet, 2000). Our data demonstrating protection against the induction of PD-associated pathogenic mechanisms of degeneration, and parkinsonian degeneration, replicate previous these findings, though there are many possible explanations for the specific mechanisms that confer protection.

Although the contribution of dopamine to the pathogenesis of PD has been controversial, there is a wealth of *in vitro* and *in vivo* literature demonstrating toxic consequences of dysregulated cytosolic dopamine. The results of this study demonstrate that dopamine dysregulation acquired in adulthood is sufficient to induce parkinsonian degeneration in a novel rodent model employing gene therapy techniques. The method of knock-down employed in this study has both strengths and limitations. This model utilizes viral-mediated delivery of shRNA targeting expression of VMAT2. Gene therapy techniques allow for local changes in gene expression, rather than ubiquitous changes following genetic or pharmacological manipulation of protein expression or activity, which can minimize side effects. The use of an adeno-associated virus (AAV serotype 2) allows for targeting of specific neuronal populations, specifically the DAergic neurons in the SNpc, for which AAV2 viruses have a high proclivity to transduce (Burger et al., 2004). This approach also provides the benefit of unilateral injections with an internal control on the contralateral hemisphere. Compared to traditional knock-down or knock-out genetic models where there is the potential for animals to develop with compensatory mechanisms that may obscure the

consequences of the loss of protein, viral-mediated RNA interference of protein expression allows for an acquired loss in adulthood.

The AAV2-shVMAT2 virus, which has bicistronic expression of the shRNA construct and GFP, allows for the identification of transduced neurons due to GFP's solubility and ability to fill the neuronal cell body. The choice of the human U6 promoter was based on previous work that established it as a good promoter to drive expression of the shRNA construct (Roelz et al., 2010). The CMV promoter, which drives GFP expression, was chosen because it results in high expression. The scrambled control virus is a universal control virus that was previously characterized to show minimal toxicity (Zharikov et al., 2015), and as adeno-associated viruses have shown to have tolerability and are safe, the viral injections in these studies did not result in any changes in body weight or survivability (data not shown). Only a single viral injection is required to achieve long-lasting effects, as the shRNA construct continues to express once a neuron is transduced. However, shRNA constructs run the risk of off-target effects if the knock-down target is similar to a sequence found in other proteins.

However, the injection of a virus into the brain requires an invasive surgery that can cause minimal physical damage to the tissue as a result of the injection itself. For this reason, it is vital to use precise stereotaxic coordinates to minimize tissue damage while maximizing the transduction of targeted neurons. There is the potential for variability between animals due to an individual animal's anatomy or differences due to the investigator's ability to be consistent with injections. Slight differences in the injection site could result in variability of number of transduced neurons. In effort to minimize the variability between animals, each experiment was performed with the contralateral non-transduced hemisphere serving as a within-animal control. Due to the invasive nature of stereotaxic surgeries, in all experiments, statistical analysis was limited by small sample size. Cohort size was chosen to minimize unnecessary animal use, however as a result of small cohort size, statistical tests were compromised by the loss of any animals unable to be included in analysis. Survival of the animal following stereotaxic surgery was influenced by a variety of factors. Older animals displayed increased sensitivity to isoflurane anesthesia, which occasionally resulted in death during surgery, and anatomical variability resulted in the possibility of blood vessels in the path of the needles, which could result in hemorrhage. In addition, technical limitations including surgeon error or equipment (i.e. needle and syringe) failure resulted in failure of viral transduction. Due to these limitations, it is possible that small sample size prevented analyses from reaching statistical significance.

Collectively, the viral-mediated method of decreasing VMAT2 expression produces a model that can be used to be understand dopamine-mediated pathogenic mechanisms of degeneration *in vivo*. This novel model of dysregulated dopamine sequestration has demonstrated that cytosolic dopamine is sufficient to induce PD associated pathogenic mechanisms of neurodegeneration, including the activation of LRRK2, which has previously been unreported *in vivo*. Our data provides further evidence that dopamine may participate in the pathogenesis of PD. The increase in cytosolic dopamine observed in PD (Pifl et al., 2014) may result from decreased VMAT2 expression (Miller et al., 1999; Sala et al., 2010), however, there are other factors that may impair the vesicular sequestration of dopamine. For instance, the ability of VMAT2 to load dopamine into synaptic vesicles depends upon an accumulation of protons within the synaptic vesicle mediated by the vacuolar-type (v-type) ATPase (Knoth et al., 1981). If there is a deficiency in ATP production, potentially downstream of mitochondrial dysfunction, among many other consequences to neuronal functioning, the v-type ATPase would be unable to load synaptic vesicles with protons and therefore dopamine would not be sequestered.

Cytosolic dopamine can cause neuronal dysfunction in a number of ways, many of which interact and can cause the propagation of dysfunctional processes. Previous work has shown that the sequestration of dopamine is impaired downstream of PD associated environmental factors (Qi et al., 2014), and many studies have identified dysregulation of dopamine sequestration and release downstream of genetic factors including misfolded  $\alpha$ -synuclein (Guo et al., 2008; Larsen et al., 2006; Mosharov et al., 2006), VPS35 variants (Cataldi et al., 2018), and LRRK2 variants (Melrose et al., 2010; Yue et al., 2015). Dopamine and its metabolic products have also been shown to interact with  $\alpha$ -synuclein (Bisaglia, Greggio, et al., 2010; Jinsmaa et al., 2016; Mor et al., 2017; Outeiro et al., 2008), decreased glucocerebrosidase activity (Burbulla et al., 2017), damage to synaptic vesicles (Plotegher et al., 2017), and mitochondrial dysfunction (Burbulla et al., 2017; Di Maio et al., 2016).

Direct injections of dopamine into the striatum resulted in DAergic degeneration that was able to be rescued by antioxidants (T. G. Hastings, Lewis, D.A., Zigmond, M.J., 1996). The cytosolic metabolism of dopamine can generate reactive oxygen species and the highly reactive dopamine quinone (Stokes et al., 1999). The dopamine quinone can directly impair mitochondrial functioning by opening the mitochondrial permeability transition pore (Berman, 1999) or interacting with the mitochondrially localized antioxidant selenoprotein glutathione peroxidase 4 (GPx4) (Hauser et al., 2013), and dopamine-modified  $\alpha$ -synuclein can block protein import by interacting with the mitochondrial-membrane localized protein TOM20 (Di Maio et al., 2016). In addition, DOPAL-modified  $\alpha$ -synuclein has been shown to cause synaptic vesicles to leak dopamine, providing another mechanism by which PD-associated mechanisms of degeneration may increase cytosolic dopamine and propagate dysfunction (Plotegher et al., 2017). Previous

experiments have suggested dopamine mediates  $\alpha$ -synuclein-induced toxicity both in cell culture (Bisaglia, Greggio, et al., 2010) and animal modeling experiments (Ulusoy et al., 2012), and other recent studies have implicated calcium as a susceptibility factor mediating DAergic neuron vulnerability to  $\alpha$ -synuclein (Mosharov et al., 2009). It is clear that dopamine and  $\alpha$ -synuclein have a complicated bi-directional relationship in which dysregulation of either dopamine or  $\alpha$ -synuclein can cause dysregulation of the other, and generate a propagative cycle of dysfunction. For this reason, we hypothesize that in our VMAT2 knock-down model, decreasing  $\alpha$ -synuclein expression may protect against neurodegeneration.

In addition to  $\alpha$ -synuclein, other PD-associated factors have a bi-directional relationship with dopamine. For instance, LRRK2-mediated phosphorylation of endocytosis-associated protein auxilin results in fewer synaptic vesicles and an increase in oxidized dopamine (Nguyen & Krainc, 2018). However, our data demonstrated an increase in LRRK2 activation following a loss of VMAT2, which we believe to be due to oxidative stress. An induction of LRRK2 activity, and the subsequent phosphorylation of LRRK2 substrates, can cause trafficking deficits, which may result in the accumulation of proteins due to endolysosomal deficits (Clague & Rochin, 2016). Rab proteins are involved in a number of vital trafficking processes, therefore the loss of Rab functioning resulting from an increase in LRRK2 activity may have many downstream consequences on additional pathways (Steger et al., 2016). Additionally, the dopamine quinone has also been shown to interact with PD associated chaperone protein DJ-1 (Girotto et al., 2012). The cumulation of these studies demonstrate that impairment in dopamine sequestration is a convergent mechanism downstream of a variety of initiating factors, and that dopamine can contribute to the propagation of pathogenic mechanisms. The accumulation of cytosolic dopamine may participate in the development of several dysfunctional processes that feed in to one another and propagate additional dysfunction. This suggests that any initial dysfunction – whether it's protein aggregation, endolysosomal deficits, mitochondrial dysfunction, or dopamine oxidation – can initiate a self-propagating cycle of the dysfunctional processes that are characteristic of PD and cause degeneration. Given that the DAergic neurons in the SNpc have a higher baseline oxidative stress (Guzman et al., 2010), higher energy demand (Pissadaki & Bolam, 2013), and broad unmyelinated axonal arborizations (Matsuda et al., 2009), it is likely that these neurons are vulnerable to any disruptions in normal functioning.

Given the vulnerability of SNpc DAergic neurons due to the toxic consequences of cytosolic dopamine, the vesicular sequestration of dopamine is an attractive therapeutic target. Despite there being precedent for gene therapy-mediated overexpression of a protein as a therapeutic intervention for treating PD (Axelsen & Woldbye, 2018), and animal studies suggesting that the inclusion of VMAT2 in a gene therapy cocktail would increase efficacy of gene therapy treatments targeting dopamine homeostasis (Sun et al., 2004), we were unable to establish VMAT2 overexpression-mediated protection against rotenone-induced neurodegeneration. We were, however, able to successfully demonstrate viral-mediated overexpression of VMAT2, although the extent of overexpression may have been insufficient to confer protection against nigrostriatal degeneration. While our results suggest that overexpression of VMAT2 may be an ineffective method by which to treat PD, pharmacological activators of VMAT2 may have more therapeutic viability. Encouragingly, a number of drugs have been identified to be VMAT2 enhancers (Lawal et al., 2014), which would be an impermanent and less-invasive way of targeting VMAT2 activity compared to gene therapy. Future research to understand how VMAT2

expression, or the vesicular sequestration of dopamine generally, can be enhanced would provide vital information for potential therapeutic interventions. Interventions that enhance the vesicular sequestration of dopamine would provide the dual benefit of removing dopamine from the cytosol and therefore minimizing cytotoxic processes, as well as increasing the amount of dopamine within synaptic vesicles therefore restoring the amount of dopamine released during transmission. These two benefits could alleviate the motor symptoms while also halting the progression of the disease by arresting the propagation of dysfunction.

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