AUGMENTATION OF L-DOPA-EVOKED DOPAMINE EFFLUX BY METHYLPHENIDATE: ROLE FOR THE D2 AUTORECEPTOR?

by

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Abstract

Using a 6-OHDA model of Parkinson's disease, we have preliminary evidence that L-DOPA-derived dopamine (DA) ceases to be released through conventional mechanisms of exocytosis under severe denervation. This may be problematic, as large, and possibly unregulated release of L-DOPA-derived DA would be expected to cause abnormal patterns of DA stimulation at the postsynaptic receptors, likely contributing to the development of dyskinesia.

This issue may be overcome with the Dopamine Transporter (DAT) blocker Methylphenidate (MPD). Ahn and Phillips observed that MPD augmented the L-DOPA-derived DA efflux in a manner consistent with the under-appreciated fact that MPD also acts as a vesicular monoamine transporter (VMAT2) agonist (Volz, 2008), thereby facilitating the sequestration of cytosolic DA into vesicles, where it could enter into a cycle of impulse-dependent release processes. The hypothesis that MPD may influence the sequestration and release of L-DOPA-derived DA into presynaptically-regulated mechanisms of exocytosis may have beneficial therapeutic implications.

Thus, the two major objectives of this thesis were first to assess whether L-DOPA-derived DA remained under presynaptic D2 autoreceptor regulation dependent on the severity of striatal denervation, and second, to investigate the mechanism(s) by which MPD may facilitate vesicular DA release, possibly by involvement of the D2 autoreceptor. L-DOPA was reverse-dialyzed into the intact and 6-OHDA lesioned dorsal striatum of the rat, followed by the reverse-dialysis of the D2 autoreceptor agonist and antagonist, Quinpirole and Eticlopride, or the VMAT2 inhibitor, Tetrabenazine. Although L-DOPA-evoked DA efflux remained under D2 autoreceptor control in the intact and moderately lesioned striatum, in the case of severe, 95% denervation, L-DOPA-evoked DA efflux was unaffected by D2 autoreceptor regulation or VMAT2 inhibition. However, despite the

apparent loss of autoreceptor regulation, a subsequent study found that inhibitory binding of the D2 autoreceptor by reverse-dialysis of Eticlopride into the severely denervated striatum prior to the administration of MPD, completely blocked MPD-induced augmentation of L-DOPA-derived DA.

These results implicate the D2 autoreceptor in a novel mechanism by which MPD can facilitate DA neurotransmission, and suggest that even under conditions of severe denervation, the presynaptic D2 autoreceptor may be manipulated pharmacologically to facilitate the exocytotic release of L-DOPA-derived DA.

Preface

The experiments in this thesis were designed by me under close guidance and input by Dr. Soyon Ahn, as well as important contributions from my supervisor, Dr. Anthony Phillips. I conducted all experiments, created the figures, as well as did the data analysis by myself, under the tutelage of Dr. Soyon Ahn. My colleagues Giada Vacca and Haiyan Zhou were also supportive in helping with my learning and conduction of the microdialysis experiments. The manuscript for this thesis was written by me, with feedback that I had incorporated from Dr. Phillips and Dr. Ahn.

Specifically, the unilateral 6-OHDA lesion surgery protocol was written by Dr. Ahn, with active input to the revisions of the surgery coordinates and protocol by Dr. Ahn and I. Setting up for and running the microdialysis experiments were taught by Dr. Ahn. I researched and designed the experimental protocol for the microdialysis studies, with close feedback by her expertise, as well as input from Dr. Tony Phillips. I independently set up and ran the microdialysis experiments under guidance of Dr. Ahn, Giada Vacca and Haiyan Zhou. The EZ chrome software analysis of the neurochemical peaks by HPLC was done largely by me, with occasional support by Dr. Ahn, Giada Vacca and Haiyan Zhou. Maintenance of the HPLC columns was done by Giada Vacca. I carried out the probe-verification histology, under the guidance of Haiyan Zhou. The post-mortem comparison of dopamine levels in the lesioned striatum were performed by me, with equal help by my colleagues, Dr. Ahn, Giada Vacca and Haiyan Zhou. The protocol for the post-mortem comparison of dopamine levels in the denervated striatum was researched and pioneered by Giada Vacca. Haiyan Zhou was indispensable in her effort in helping me care for the experimental subjects. I ran the statistical analysis independently, after receiving tutelage from Dr. Ahn. The manuscript, figures and data analysis were written and prepared by me, and diligently revised and edited by Dr. Phillips. Dr. Ahn also provided valuable input.

The work done in this thesis builds on a larger research initiative looking at the benefits of Methylphenidate and L-DOPA combination therapy in Parkinson's disease, spearheaded by Dr. Ahn and Dr. Phillips (Principal Investigator), with co-investigators including Giada Vacca, Haiyan Zhou and Kitty So and Christina Cheng in the past. All experiments were carried out in accordance with the Canadian Council of Animal Care, with approval by the Animal Care Committee at the University of British Columbia under protocol number A12-0096.

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5-HT	5-hydroxytryptamine / Serotonin
6-OHDA	6-hydroxydopamine
ADHD	Attention Deficit Disorder
ANOVA	Analysis of Variance
AP	Anteroposterior
ATP	Adenosine Triphosphate
BL	Baseline
D2R	Dopamine D2 receptor
DA	Dopamine
DAT	Dopamine Transporter
DDC	Dopa Decarboxylase
DOPA	Dihydroxyphenylalanine
DOPAC	3,4 dihydroxyphenylacetate
DMSO	Dimethylsulfoxide
DTBZ	Dihydrotetrabenazine
DV	Dorsoventral
CA	Calcium
CL	Chloride
CNS	Central Nervous System
COMT	Catechol-O-methyl Transferase
CSF	Cerebral Spinal Fluid
CYT	Cytosol

EDTA	Ethylenediaminetetraacetic acid
ENK	Enkephalin
ETI	Eticlopride
GABA	γ-Aminobutyric acid
GPCR	G-Protein Coupled Receptor
Gpi	Globus pallidus, internal segment
Gpe	Globus pallidus, external segment
HPLC	High Performance Liquid Chromatography
HVA	Homovanilllic Acid
IA	Intra Aural
K	Potassium
K_D	Dissociation Constant
K_i	Inhibition Constant
KO	Knock-out
L-DOPA	Levodopa
MAO-B	Monoamine Oxidase B
MFB	Medial Forebrain Bundle
ML	Mediolateral
MPD	Methylphenidate
MPP+	1-methyl-4-phenylpyridinium
MSNs	Medium Spiny Neurons
MT	Mitochondria
NA	Sodium

NT	Neurotransmitter
N.S.	Non significant
PD	Parkinson's Disease
PET	Positron Emission Topography
PNS	Peripheral Nervous System
QUIN	Quinpirole
RM ANOVA	Repeated Measures Analysis of Variance
SEM	Standard Error of the Mean
SERT	Serotonin Transporter
SN	Substantia nigra
SNc	Substantia Nigra pars compacta
SNr	Substantia Nigra pars reticulate
TH	Tyrosine hydroxylase
TBZ	Tetrabenazine
TTX	Tetrodotoxin
VMAT	Vesicular Monoamine Transporter

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I would also like to thank my friends and lab mates for embarking on this journey with me. In particular, I would like to thank **Dr. Soyon Ahn** for your everlasting patience and guidance. Not only did you teach me everything I needed to know, from microdialysis to statistics, you always took the time to give me insightful feedback every time I barged into your office with some crazy idea. I sincerely valued your mentorship. **Giada Vacca** and **Haiyan Zhou**, you two have been so thoughtful and have provided me with endless support, friendship, soup and cookies throughout this whole process. **Carine Dias** and **Fiona Choi**, I thank you for showing me the ropes when I first arrived as an undergrad! To my **friends outside of the lab**, what could I have done without my cheering squad?

Finally, I'd like to express my heartfelt thanks to my **parents**. You've made countless sacrifices for me, and have always encouraged me to strive to be the best I can be. It is to you that I dedicate this thesis.

1. Introduction

1.1 Overview

Parkinson's disease (PD) is one of the most predominant neurodegenerative disorders in the world, second only into Alzheimer's disease. It is characterized by the motor symptoms of bradykinesia, rigidity, resting tremor and postural instability, resulting from the degeneration of the substantia nigra and the ensuing deficiency in the dopamine neurotransmission necessary for control of voluntary movement. Parkinson's disease is highly age related, with 1 in 100 of people over age 60 being affected. The average age of onset is around 55-60 years of age, though patients in early in their mid-20s have been diagnosed. In Canada, approximately 100,000 Canadians have PD (according to the Parkinson's Society of Canada, 2003), with 5,500 new diagnoses each year. One million people in the United States have the disease, and by 2016, these numbers in Canada and the United States are expected to double. Within the next 30 years, diagnoses in individuals over the age of 65 are expected to rise from 11.6% to 23.6% of the population. Worldwide, the prevalence of the disease ranges from 0.015% to 12.5% (Chen and Tsai, 2010).

Given the long-term nature of the disease and relatively large portion of the population affected, Parkinson's disease has a huge economic burden. The worldwide cost for its medication alone is estimated to be US $11 billion per year (Parkinson's Society of Canada, 2003). In Canada, the estimated cost of the disease for society ranges from $2.5 to $5 billion annually, with the typical Canadian patient using approximately $1000 worth of medication per month.

1.2 Pathology of Parkinson's disease

As noted, Parkinson's disease (PD) is characterized by the motor symptoms of bradykinesia (asymmetrical onset), resting tremor and rigidity. To be clinically diagnosed with the disease, the

individual must have one of the following symptoms: resting tremors, bradykinesia, rigidity, loss of postural reflexes, flexed posture, or freezing phenomenon, in addition to either resting tremors or bradykinesia. However, Parkinson's disease is not just a motor disorder, as there are a plethora of additional behavioral symptoms such as constipation, sleep disturbances, dysphagia (difficulty swallowing), micrographia, etc. Psychological and cognitive problems also develop in later stages of the disease. Depression is the biggest secondary symptom associated with PD, in addition to cognitive impairment and autonomic dysfunction (Chen and Tsai, 2010). It is estimated that the prevalence of depression in PD patients lie between 10-45% (Chen and Tsai, 2010). As PD is a disease that primarily afflicts the older population, it is often manifested along with other systemic diseases such as diabetes mellitus, hypertension, coronary artery disease, cerebral vascular disease, spinal degenerative disease, orthopedic disorders, as well as other neurodegenerative disorders (Chen and Tsai, 2010).

The pathology of Parkinson's disease includes the presence of alpha synuclein, Lewy bodies in the neurons of the substantia nigra, and insufficient DA formation and transmission in the midbrain. The pathophysiological anomalies in PD – specifically, the Lewy bodies - appear in a specific sequence. First, they emerge in the dorsal motor nucleus (Schapira 2006). Then they emerge in the olfactory bulbs and nucleus, followed by the locus coeruleus to finally reach the substantia nigra pars compacta (Schapira, 2006). The location where Lewy bodies are found can therefore be used as a marker for disease progression. However, the way in which the presence of Lewy bodies may contribute to nigrostriatal degeneration is currently unclear.

Motor symptoms of Parkinson's disease result from the degeneration of the nigrostriatal pathway (Schapira et al, 2014). The factors that trigger the specific degeneration of these

dopaminergic neurons in idiopathic Parkinson's disease still remain to be elucidated. Apoptosis of the dopaminergic cells of the SNc causes insufficient dopamine activation of the striatum in the basal ganglia. It is estimated that by the time the early signs of PD motor symptoms are observable, the DA levels have already reduced to 60-70% of normal values. At this point, clinical diagnosis made. As the disease progresses, the denervation becomes increasingly severe, until almost 99% of the nigrostriatal dopaminergic neurons are lost. There is now a growing body of evidence that indicate that it is the degeneration of the axons, and not cell bodies, that is the main determinant of progression of the disease (Cheng et al, 2010).

In addition to the motor symptoms of Parkinson's disease, there are also the added complications of non-motor symptoms. These non-motor symptoms are present for the duration of the disease, with some of the non-motor symptoms manifesting prior to the appearance of the motor symptoms, while others show up in the advanced stages. These include, but are not limited to, depression, anxiety, hallucinations/delusions, cognitive impairment, sexual dysfunction, anxiety, apathy, nausea, dysphagia, sleep disorders, anhedonia, bowel problems and pain (Chaudhuri and Schapira, 2009). These non-motor cognitive and behavioral impairments of Parkinson's disease are treated individually according to symptoms but are frequently under-reported and therefore inadequately managed (Jankovic and Poewe, 2012).

The mean age of onset is observed from 55 to 65 years of age, though 5% of cases occur between 21-39 years, in addition to some rare occurrences of juvenile PD. The rate of prevalence increases with age. Parkinson's disease is observed in all populations around the world, but certain reports claim that it may be less commonly found in people of Asian and black descent than in white

people (Lau and Breteler, 2006). There is, however, a well-documented gender bias for men over women for exhibiting the disease.

There are two widely used characterizations of the disease: idiopathic or sporadic (without known genetic or environmental causes) and familial (genetically inherited). Approximately 90% of cases diagnosed are considered idiopathic.

1.2.1 Etiology: genetic and environmental risk factors

The exact causes of selective dopaminergic cell loss in PD are still unclear. However, mitochondrial dysfunction, oxidative stress and protein misfolding, inflammatory change leading to cell dysfunction and death by apoptosis or autophagy are a few recurring themes that seem to underlie PD pathogenesis (Schapira et al, 2014). PD patients are thought to develop the disease not through a single factor, but from several factors acting together, such as environmental risks, polygenic influences, or a combination of the two (Schapira, 2006). The idea is that genetic predisposition puts individuals at higher risk to react to environmental toxins to influence nigrostriatal degeneration. Parkinson's disease primarily affects the older population, as with advancing age, the natural decline in neuronal and mitochondrial function may increase the susceptibility of the substantia nigra to the polygenic influences and environmental risks that induce neurodegeneration.

Parkinson's disease is characterized into two types: idiopathic and familial. In idiopathic Parkinson's disease, the underlying themes - mitochondrial dysfunction, oxidative stress, inflammatory change and/or protein mishandling - are thought to be induced by non-genetic and environmental risk factors, though likely in interaction with susceptible genes (Lau and Breteler, 2006). Though Parkinson's disease is considered largely to be sporadic, there has been some

autosomal dominant and autosomal recessive genetic inheritance that has been traced. Familial Parkinson's disease accounts for 5-10% of the disease diagnoses. Monozygotic twin studies show that certain genetic predisposition may play a role in the disease by causing vulnerability to environmental risk factors. Overall, having a first-degree relative with Parkinson's disease increases the risk of the individual for obtaining the disease by approximately two- to three-fold. The gene studies have identified an emerging pattern in Parkinson's disease prognosis (Lin and Farrer, 2012). In early-onset PD, it is usually problems with mitochondrial and lysosomal regulation that are affected, while genetic mutations that affect synaptic transmission, vesicular and receptor recycling, as well as protein folding are observed in late-onset PD (Lin and Farrer, 2012). 6 genes have been identified as causing familial PD (Schapira, 2006). These include mutations in a-synuclein, parkin, UCHL1, DJ1, PINK1, LRRK2 (all following a Mendelian pattern of inheritance).

Here I will outline the genetic and environmental influences that have been identified so far in Parkinson's disease. Notice that all the environmental and genes that have been linked to PD seem to, in some way, have the ability to cause nigrostriatal cell death via the recurring themes of mitochondrial dysfunction, induction of oxidative stress, inflammatory change and/or the mishandling of proteins.

Genetic factors

a-synuclein (PARK 1): Some familial cases of PD show patients with PARK 1 mutations in the a-synuclein gene. Multiple repeats of the WT form of a-synuclein gene have also been observed in familial as well as early onset cases. In flies and rodents, overexpression of a-synuclein has been shown to induce some of the behavioral and pathological components of PD, such as loss of dopaminergic neurons, Lewy body-like inclusions and motor deficits (Feany and Bender, 2000). Even

in idiopathic, apparently sporadic PD, a-synuclein is a large component of the Lewy body aggregates observed. A-synuclein has been observed to mediate cytotoxicity, and deletion of a-synuclein in mice has been shown to confer protection against the parkinsonian neurotoxins MPTP and 6-OHDA (Alvarez-Fischer et al, 2008; Dauer et al, 2002). There are several hypotheses put forth to explain a-synuclein mediated cytotoxicity, including the disruption of membrane by harmful adduct formation, prevention of lysosomal protein degradation and mitochondrial dysfunction (Mosharov et al, 2009). Mosharov et al (2009) suggests that perhaps dopaminergic degeneration is specific to the SN, because of the brain region's particular susceptibility to high cytoplasmic Ca^{2+} levels, elevated cytoplasmic DA and a-synuclein expression. An interference of any of these 3 factors seems to rescue the neurons from cell specific degeneration (Mosharov et al, 2009).

Parkin (PARK 2): PARK 2 encodes parkin, an E3 ligase that ubiquitinates proteins for proteasomal destruction (Shimura et al, 2000). It is a protein that has been associated with the endoplastic reticulum, Golgi apparatus, synaptic vesicles and mitochondrial (Kubo et al, 2001; Shimura et al, 1999; Stichel et al, 2000). Parkin mutations have been linked to protein insolubility and aggregation, leading to ER stress and cell death (Imai et al, 2001). When Parkin was knocked out in mice, an increase in extracellular striatal DA, reduction in synaptic excitability and a mild motor deficit were measured (Schapira 2006). Parkin KO in flies lead to the develop muscle problems, mitochondrial deformities and apoptotic cell death (Greene et al, 2003). Parkin and PINKI together are critical for the regular turnover of the mitochondria by autophagy (Schapira 2006).

UCH-L1 (PARK 5): PARK 5 encodes for ubiquitin carboxyhydrolaze L1, an enzyme that hydrolyzes the C-terminus of ubiquitin to allow clearance of proteins. A missense mutation in UCH-

L1 was observed in 2 siblings with typical PD, although no further cases have been observed, throwing some doubt into the importance of UCH-L1 to Parkinson's disease (Schapira 2006).

PINK1 (Park 6): Mutations in PTEN-induced kinase 1 (PINK1), a mitochondrial kinase, are found in a familial recessive form of Parkinsonism that affects the 21-40 year age group (Schapira 2006). This mutation may create the disease pathogenesis by inducing an abnormal stress response and neurodegeneration from altered phosphorylation of PINK1's target proteins (Schapira 2006). Patients with PINK1 mutations usually have mitochondrial dysfunction as well as the presence of Lewy bodies (Schapira et al, 2011).

DJ-1 (Park 7): DJ-1 is a homodimeric protein involved in the cell's regulation of transcription, as well as its response to oxidative stress (Saracchi et al, 2014). It is also involved in the regulation of chaperones, proteases and mitochondria (Ariga et al, 2013; Saracchi et al, 2014). It is expressed in both neurons and astrocytes, and its activity is regulated by various forms of stresses, including oxidative stress (Ariga et al, 2013). Downregulation of DJ-1 in astrocytes makes the neurons more vulnerable to oxidative stress, as mitochondrial complex I and inflammatory responses are then improperly regulated, making the astrocytes lose their ability to protect the neurons (Ariga et al, 2013). DJ-1 has also been associated with DA synthesizing and sequestering proteins. Both homozygous and heterozygous mutations of the DJ-1 protein has been shown to reduce the activity of tyrosine hydroxylase, L-DOPA decarboxylase and the vesicular monoamine transporter 2 (Ariga et al,2013). Deletions and missense mutations of the DJ-1 gene are causative for a rare form of autosomal recessive PD (Puschmann 2013; Ariga et la, 2013). Inactivation of DJ-1 by excessive oxidation has also been reported in patients with sporadic PD and Alzheimer's disease (Ariga et al, 2013). Thus, DJ-1 in the CSF has been examined by several groups for its potential as a Parkinson's

biomarker, though consensus so far has been conflicted. Although total DJ-1 levels in the blood are the same between PD patients and controls, it has recently been discovered that Parkinson's patients have higher levels of its 4-hydroxy-2-nomenal-induced post-translational isoform than AD patients and control groups (Saracchi et al, 2014).

LRRK2 (PARK 8): LRRK2 gene mutations are the most common mutations observed so far in both familial and sporadic PD. The G2019S is the most common LRRK2 mutation, and has been reported in 2.8-6.6% of autosomal dominant PD families, and 2-8% of sporadic cases (Hernandez et al, 2005). It is not seen in other neurodegenerative diseases, Alzheimer's, or other Parkinsonism syndromes (Hernandez et al, 2005). It is thought that LRRK2, which has a Ras/GTPase domain and a kinase domain, is involved in cytoskeletal assembly and signal transduction (Schapira 2006). LRRK2 mutant brains show loss of SNC dopaminergic neurons, neurofibrillary tangles, abnormal tau deposits, Lewy body aggregations, and patients demonstrate the typical movement disorders of PD (Schapira 2006).

Environmental factors

1-Methyl 4-phenyl 1,2,3,6 tetrahydropyridine (MPTP) is one of the few exogenous toxins known to produce Parkinsonism in humans, primates and rodents. It is converted into MPP+ by astrocytes and transported into the DA neuron leading to targeted degeneration of the nigrostriatal pathway. The discovery of this toxin fueled the idea that there may be other toxins and environmental risks that may induce the disease pathogenesis. A rural residency with the possibility of increased exposure to the pesticides and herbicides used in farming, is correlated with an increase in PD incidence. Several of the pesticides and herbicides widely used in agriculture are able to cross the blood-brain barrier and cause a-synuclein aggregation and degeneration of the

nigrostriatal pathway. Dithiocarbamates, a compound that enhances MPTP toxicity has also been named as a risk factor (Zhang et al, 2003). In addition to toxins and pesticides, other environmental factors include vascular disease(NOT ENVIRONMENTAL), bacterial/viral infection, trauma, contaminated drinking water, exposure to organic solvents, carbon monoxide and carbon dioxide (Corrigan et al, 1998).

1.2.2 Treatment

Currently, there are no drugs or other therapeutic interventions that are disease-modifying or can "cure" Parkinson's disease. A disease-modifying therapy is one that is able to delay the progression of the disease, and have a beneficial effect on reversing the underlying pathophysiological process (Jankovic and Poewe, 2012). One of the issues contributing to this lack of success is that there is still uncertainty over the exact mechanisms by which the various genetic, environmental and other factors cause PD-related neurodegeneration. Another problem is the lack animal models that exhibit a progressively degenerative disease phenotype. Therefore, current treatments of PD are mainly neuroprotective, or work to alleviate symptoms of the disorder. The evidence supporting the effectiveness of available neuroprotective options, however, is limited. Below, I will describe the many the available therapy options currently available.

<u>Symptomatic treatments</u>

Dopamine agonists work by providing direct, supplemental stimulation at the dopamine receptors under conditions of low dopamine presence. They are usually the first symptomatic monotherapy to be prescribed during the initial stages of PD (Jankovic and Poewe, 2012). However, as DA agonists are less effective in managing motor symptoms than Levodopa (L-DOPA), a combination of both are usually needed after 2-5 years of monotherapy treatment. Adding DA

agonists to pre-existing L-DOPA therapy also seems to be beneficial, as a systemic review and meta-analysis of 15 clinical trials indicate that a combination of the two is more effective than L-DOPA alone in reducing the disease symptoms (Talati et al, 2009).

However, negative psychiatric side effects have been associated with the long term use of DA agonists (Talati et al, 2009). Levodopa (or L-DOPA) therapy is unparalleled in its effectiveness at managing the motor symptoms of PD. However, there has been debate about whether the prescription of L-DOPA therapy should be delayed as long as possible, because of the fear that it may be neurotoxic and able to fuel disease progression (Jankovic and Poewe, 2012). Evidence supporting this fear has been inconclusive, however, and the traditional practice of delaying L-DOPA therapy has been increasingly challenged (Jankovic and Poewe, 2012). However, there is wide-spread recognition that early use of L-DOPA may lead to early onset of L-DOPA-induced motor side effects such as motor fluctuations and dyskinesia. Thus, the decision of when to begin this therapy varies from patient to patient and depends on the individual's age and needs (Jankovic and Poewe, 2012).

When the L-DOPA-induced motor fluctuations do arise, however, various strategies and treatments can be considered to help the patient manage the symptoms. Apomorphine, deep brain stimulation, COM-T inhibitors, MAO-B inhibitors and DA agonists are all options, while amantadine, though modest in its benefits, is considered the best available treatment for dyskinesia (Jankovic and Poewe, 2012).

Acetylcholine and dopamine need to be in proper chemical balance for voluntary movement control. Therefore, as DA is low in Parkinson's disease, anticholinergics attempt to regulate this imbalance by decreasing acetylcholine levels. It may be administered as a monotherapy, or in

conjunction with L-DOPA or DA agonists (Miyasaki et al, 2002). Although there is some evidence that this may improve tremor in younger patients (Miyasaki et al, 2002), it may also produce adverse side effects such as an increased heart rate, memory impairment and hallucinations.

Neuroprotective strategies

MAO-B inhibitors and mitochondrial (MT) enhancers are therapies developed to attempt to confer neuroprotection to the nigrostriatal degeneration. MAO-B is the enzyme that metabolizes DA to its harmful radical species in the dopaminergic cytosol. Inhibitors of MAO-B therefore attempt to reduce the amount of cytotoxic metabolic species generated and may be prescribed prior to L-DOPA, to help delay L-DOPA-induced wearing off/motor fluctuations and decline. Reports on whether this combination treatment strategy is superior to L-DOPA alone are conflicted, however.

Various mitochondrial enhancers have also been investigated, such as coenzyme Q10 – a cofactor in the mitochondrial electron transport chain – and creatine, a nutritional supplement. Coenzyme Q10 demonstrated no statistical significance versus placebo in clinical trial, while creatine is projected to finish clinical trials in 2015 (Jankovic and Poewe, 2012).

1.2.3 The Basal Ganglia and neurodegenerative circuitry of Parkinson's disease

The basal ganglia consist of 5 subcortical nuclei found in the base of the forebrain. This structure is primarily associated with the control of voluntary movement, as well as procedural learning, cognition and emotion. The basal ganglia also play a large role in action selection (Friend and Kravitz, 2014).

The 5 subcortical nuclei of the basal ganglia are the striatum (which is made up of the caudate nucleus and putamen), globus pallidus, substantia nigra, nucleus accumbens and

subthalamic nucleus. The striatum receives input from multiple cortical regions including the prefrontal cortex and also receives an important afferent projection from the substantia nigra, which sends dopaminergic projections to the striatum via the nigrostriatal pathway. The internal segment of the globus pallidus (GPi) and substantia nigra pars reticulata (SNr) receives efferent projections from the striatum which exert inhibitory output control on the brainstem and thalamo-cortical neurons - areas that are critical for motor execution. Efferent projections from the dorsal striatum to the GPi/SNr can be divided into 2 pathways: the direct and indirect pathways. The striatal projections of the direct pathway are mainly composed of GABAergic medium spiny neurons (MSNs) that express the D1 dopamine receptor. These inhibitory neurons project directly onto the GPi and SNr, and release neuropeptides substance P, dynorphin as well as γAminobutyric acid (GABA).

The striatal MSNs of the indirect pathway express the D2 dopamine receptor and extend inhibitory projections to the external segment of the globus pallidus (GPe). In the indirect pathway, these neurons release GABA and enkephalin (Enk). Activation of the GPe then exerts inhibitory control over the STN to thereby cause STN-mediated disinhibition of the GPi and SNr (Smith et al, 1998). The direct and indirect pathway therefore exert functionally opposing and anatomically segregated action at the output ganglia, though recent work indicate that these pathways are not completely mutually exclusive (Wu et al, 2000; Fujiyama et al, 2011; Cazorla et al, 2014). The GPi and SNr then relay inhibitory output to areas of the brain that facilitate movement. As these two pathways exert opposing but coordinated control on the actions of the basal ganglia on movement, cognition and motivation, it is thought that an imbalance between these pathways underlie the pathophysiology of many brain and motor disorders, including Parkinson's disease.

DA activation of the striatum by the substantia nigra pars compacta causes an excitatory response via D1 receptors on the medium spiny neurons and an inhibitory response by activation of the D2 receptors. Activation of the dopaminergic projection from the substantia nigra pars compacta to the striatum thus allows for inhibitory control of the GPi and SNr by the direct and indirect pathways. Inhibition of the output ganglia removes its suppressive effects on the execution of motor events.

Neurodegeneration within the nigrostriatal pathway is the major factor leading to PD. This results in a deficiency in striatal DA activation of the direct and indirect pathway. Insufficient DA activation leads to greater excitation of the GPi/SNr in both pathways, and as a result, the output ganglia is able to exert a stronger inhibitory effect on areas that control the execution of movement. The consequence is hypokinesia and other motor manifestations of Parkinsonism.

1.2.4 Rodent models of Parkinson's disease

The best animal models for studying the neural mechanisms underlying PD are those that can mimic most closely the pathophysiological hallmarks of the disorder along with key behavioral phenotypes. Although none of the currently available models capture all the characteristics of PD, certain models do provide practical tools for evaluation of novel mechanisms and symptomatic treatments of the disease. These models have been highly useful over the last 2 decades to further our understanding of the disease's process, etiology, pathology and molecular mechanisms.

<u>6-OHDA and MPTP</u>

6-OHDA and MPTP are the most commonly used neurotoxin-generated models of PD. The degenerative action on the dopaminergic neurons by 6-hydroxydopamine (6-OHDA) and 1-methyl-4-

phenylpyridinium (MPP+) (the activate metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is regulated by the activity of DAT and VMAT2. The dopamine transporter mediates uptake of these substrates from the extracellular space into the cytosol where their toxic effects are exerted. DAT activity is therefore facilitative for MPP+ and 6-OHDA's neurodegenerative abilities. In contrast, VMAT2 plays a role in reducing the neurotoxicity of these substrates by sequestering them into vesicles. The levels of expression and function of these two transporters are thus crucial in the magnitude of parkinsonian syndromes generated by the toxins.

6-OHDA and MPTP can be used to generate bilateral or unilateral models of PD. Both are able to generate high levels of dopamine depletion. Bilateral lesions are better in that they are able to more closely proximate the neurodegenerative and behavioral manifestations of the human disease. The effects are also a more stable, as there is the reduced likelihood of compensatory sprouting of axons on the intact side. However, as bilateral lesions generate severe motor deficits, as well as a high risk of seizure and death, there is a preference for the unilateral lesion model. The unilateral approach has the additional benefit of providing within-subject controls, should the experimental design call for it. The degree of denervation produced by unilateral 6-OHDA administration can be tested by the administration of amphetamine or apomorphine to induce rotations contralateral to the lesion hemisphere (Iancu et al, 2005).

In order to produce denervation of the nigrostriatal pathway, 6-hydroxydopamine (6-OHDA) is directly injected into either the substantia nigra pars compacta or the medial forebrain bundle, as it does not cross the blood brain barrier. Interestingly, there have also been some indication of endogenous 6-OHDA found in the PD brain (Borah and Mohanakumar, 2012). Once in the brain, the compound is transported into the cell via the DA transporter (DAT), where it results in

intracellular oxidative stress, resulting in the death of the neuron (Jackson-Lewis et al, 2012). Systematic variation of the concentration of 6-OHDA can cause nigrostriatal dopamine lesions of varying levels of severity ranging from moderate to severe (Jackson-Lewis et al, 2012).

Nigrostriatal denervation in Parkinson's disease can be produced by administration of 6-OHDA into 3 regions: the medial-forebrain bundle, the substantia nigra pars compacta, and/or subregions of the caudate-putamen complex (Deumens et al, 2002). All 3 are able to give DA reductions that are chronic and severe (>95%) in the striatum. 6-OHDA lesions of the MFB usually produce a near total depletion of DA, whereas targeting the substantia nigra pars compacta and subregions of the caudate-putamen complex can be used generate more site-specific degeneration with varying degrees of severity(Deumens et al, 2002). Although 6-OHDA is effective in causing dopaminergic denervation, the animal model produced by this toxin is imperfect, as it is unable to produce a-synuclein or Lewy body aggregates.

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is the inactive neurotoxin precursor to MPP+, a metabolite that specifically kills dopaminergic neurons to produce Parkinsonism. Its role in Parkinson's disease was discovered after it was accidentally ingested by heroin addicts (Fahn, 1996). MPTP is able to replicate most of the hallmarks in PD, including oxidative stress, reactive oxygen species, energy failure, inflammation and possibly even the presence of Lewy bodies in higher mammals (Jackson-Lewis et al, 2012). Rats, however, seem to be less susceptible to systemic injection of this toxin (Chiueh et al, 1984), possibly because more of the toxin is metabolized before being able to cross the blood brain barrier (Kalaria et al, 1987). MPTP, an inactive lipophilic compound, is able to cross the blood brain barrier to be transported into astrocytes. There, it is metabolized to its reactive form, MPP+, by MAO-B. MPP+ is then released into the extracellular

space and pumped into the neuron by DAT to interfere with mitochondrial complex I to decrease cellular levels of ATP and induce neurodegeneration (Blum et al, 2001). Its ability to act as a substrate for VMAT2 also provokes the displacement of vesicular dopamine into the cytosol to produce cytotoxicity by oxidative dopamine free radicals (Blum et al, 2001). MPP+ is able to selectively target dopaminergic cells because of its high affinity for the dopamine transporter.

Pesticides/Herbicides

Pesticides and herbicides that have been used in animal models include paraquat and rotenone. Paraquat, an herbicide, is frequently used to study the process of Lewy body formation, as well as the role of a-synuclein in Parkinson's disease as it is able to induce these pathologies. However, consensus on whether this pesticide is a risk factor for Parkinson's disease, as well as whether it has any effect on nigrostriatal neurodegeneration has been speculative. Rotenone, an herbicide and insecticide, is able to cross the blood-brain barrier to reproduce almost all the hallmarks of PD, such as complex I blockade, Lewy-like body formation, oxidative stress and gastrointestinal dysfunction, as well as alpha-synuclein aggregation and Lewy body formation (Jackson-Lewis et al, 2012). However, rotenone has never been reported as a causative agent for PD in humans, nor does it cause nigrostriatal DA depletion (Jackson-Lewis et al, 2012).

Genetic models

Genetic manipulations are the newer techniques used to model Parkinson's disease in animals, as PD has traditionally been considered a non-genetic disorder. Animal models with mutations in a-synuclein, LRRK2, PINK1/Parkin and DJ-1, etc, are useful for elucidating the role of these compounds and how their genetic mutations induce familial forms of human PD. However,

the problem with genetic models is that familial Parkinson's disease only accounts for 10% of cases, and while overexpression or knockouts are frequently used to induce these models, and none of the proteins mentioned above are overexpressed or knocked out in human patients (Jackson-Lewis et al, 2012). Constituent knockout of many of these genes also do not produce strong levels of neurodegeneration (Jackson-Lewis et al, 2012).

1.3 Dopamine: from synthesis to release

DA is a neurotransmitter that is part of the catecholamine and phenethylamine family. It is synthesized in neurons and in cells of the adrenal glands. In the synthesis of DA, tyrosine is first metabolized from the essential amino acid, phenylalanine that crosses the blood brain barrier by carrier-mediated transport to enter the DA nerve terminals. Tyrosine is then converted into l-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase (TH), the rate limiting enzyme in DA biosynthesis, and its cofactor 6tetrahydrobiopterin (BH4). Dopa decarboxylase (DDC) then decarboxylates L-DOPA to form DA.

The newly synthesized DA first forms a transient, cytosolic pool, before being transported by the vesicular monoamine transporter 2 (VMAT2) into the vesicles to form a secondary, vesicular pool. Vesicular dopamine is then released in response to the presynaptic action potential. Dopamine is sequestered into 3 pools of vesicles including a readily releasable pool (RRP), recycling pool, and the reserve pool (Rizzoli and Betz, 2005). The readily releasable pool is comprised of vesicles docked to the active zone in the presynaptic terminal. These vesicles have their SNARE proteins in the readily-release conformation, and await neural stimulation to mediate exocytotic release of their vesicular DA. Only a few vesicles (1-2% of the total vesicle pool) comprise of the readily releasable pool at a given time (Rizzoli and Betz, 2005).

The recycling pool contains 5-20% of the total vesicle pool. They are not docked, and are found distributed throughout the cytosol. They constitute a pool of vesicles that stands by to replenish the readily releasable pool to maintain continuous release of transmitter during moderate stimulation (Rizzoli and Betz, 2005). This pool is replenished continuously by the newly recycled vesicles (Rizzoli and Betz, 2005). In certain cell preparations, the recycling pool also provides a source of DA for the reserve pool (Rizzoli and Betz, 2005).

The reserve pool refers to the pool of vesicles from which release is only possible after intense, usually non-physiological levels of stimulation (Rizzoli and Betz, 2005). It contains about 80-90% of vesicles in the presynapse. It is thought that only under intense, prolonged stimulation, does depletion of the recycling pool of vesicles reach levels that facilitate the mobilization and release of vesicles from the reserve pool, through mechanisms that remain to be specified (Rizzoli and Betz, 2005). Replenishment of the reserve pool accrues a large energetic demand, as it involves surface membrane and cisternae infolding. The reserve pool of vesicles and the recycling pool of vesicles are both non docked and distributed through the same areas in the cytosol of the nerve terminal. They are distinguishable from each other due to their different endocytotic pathways, mobility, and clustering (Rizzoli and Betz, 2005).

RIM proteins dock and prime synaptic vesicles at the active zone, the site of vesicle fusion. Simultaneously, RIM proteins recruit Ca^{2+} channels and attach them to the primed, synaptic vesicles so they form a single complex (Sudhof, 2013). The inward flow of Ca^{2+} during an action potential is then able to stimulate the immediately adjacent synaptogamin (located on the synaptic vesicles), to facilitate membrane fusion and exocytosis, inducing neurotransmitter release (See Fig 1. For a schematic of exocytotic DA release).

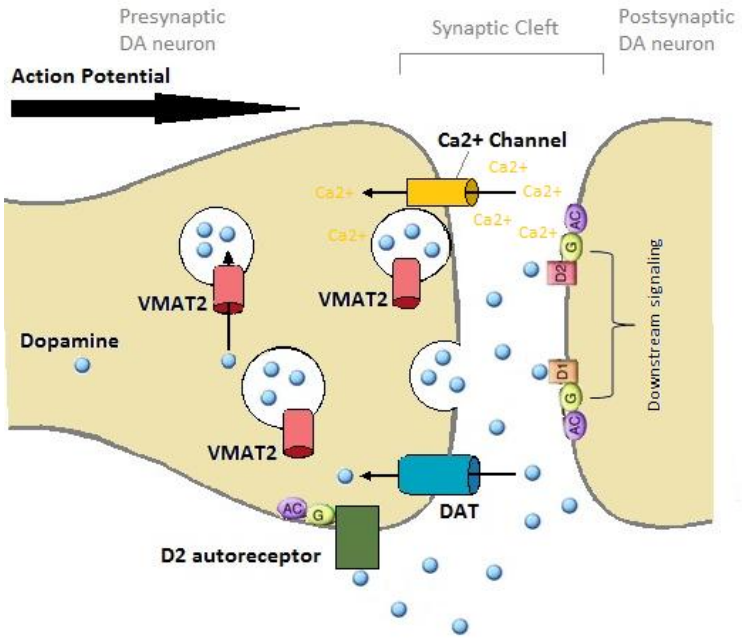


Fig 1. Conventional dopamine neurotransmission by mechanism of vesicular-mediated exocytosis. Dopamine (blue circle) is synthesized and uptaken and stored in the vesicles by the vesicular monoamine transporter (VMAT2). When an action potential reaches the terminal, the voltage-gated calcium channels to open, allowing Ca^{2+} to influx into the cell, inducing mechanisms of vesicular fusion with the presynaptic membrane that allow the secretion of vesicularly-stored dopamine into the extracellular space.

The nerve terminal has multiple ways to balance the speed and potency of neurotransmission (Blakely and Edwards, 2014). The potency of neurotransmission – which, for example, depends on the degree of vesicular filling - is especially important for monoamines, where the postsynaptic receptor may be located away from the presynaptic terminal (Blakely and Edwards, 2014), Various balancing factors need to be controlled and considered: with steeper electrochemical gradients across the vesicular membrane, more neurotransmitter gets packaged into the vesicles and thus released per vesicle exocytosis, but the amount of cytosolic DA available for refilling may then be depleted. With more shallow electrochemical gradients, fewer transmitters get packaged per vesicle, but more cytosolic DA will be available for refilling. The dopamine transporter (DAT) is also an important factor because active, fast, DAT reuptake provides a source of DA to replenish the pool of releasable transmitters. It is also important to note that DAT activity will decrease the length and duration of signaling to the postsynaptic cell (Blakely and Edwards, 2014). On the other hand, slow reuptake, or blockage of reuptake, will increase the activation of the postsynaptic receptors, but will eventually result in the depletion of the vesicular stores (Jones et al, 1998). Together these important functions of VMAT2 and DAT are critical in control of the rate and amplitude of neurotransmission.

Dopaminergic neurons have two patterns of firing. Tonic firing is the regular pacemaker discharge of DA from the presynaptic terminals that is independent of salient stimuli (Grace and Bunney, 1984). This contributes to the background-steady-state basal level of DA in the extracellular space. So-called background levels of extracellular background DA are tightly regulated by homeostatic mechanisms and changes to these background levels provoke compensatory mechanisms to restore normal levels (Grace 1991). Physiological basal levels of DA in the striatum

usually range between the 4-20 nM concentration, as measured by microdialysis and voltammetry (Parsons et al, 1991; Jones et al, 1998; Chen, 2005).

Phasic, stimulus-induced firing, on the other hand, can produce extracellular dopamine levels of the uM range (Chen, 2005). Phasic release occurs as a large, brief, transient release of DA in response to a train of neuron firing such as that induced by behaviorally relevant stimuli (Grace 1991). Phasic release from presynaptic terminals is large enough to activate the postsynaptic receptors to cause neurotransduction. However, this newly released DA is quickly exposed to reuptake into the presynapse by DAT and is not involved in homeostatic regulation of neurotransmitter function, Nevertheless, DAT and the aforementioned neurotransmission regulation methods act to quickly bring extracellular DA back to steady-state basal levels (Grace, 1991). The extraneuronal enzymes catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO-B) are also present to contribute to the extracellular DA regulation, breaking the neurotransmitter down into HVA and DOPAC respectively.

Intracellularly, cytosolic concentrations of DA in the untreated intact brain are also maintained at relatively consistent levels. Cytosolic concentrations of endogenous DA are <0.1 uM in cultured midbrain neurons (Mosharov et al, 2009) when measured by intracellular patch electrochemistry. These levels are maintained by tyrosine hydroxylase – the rate limiting enzyme – as well as MAO-B, VMAT2 and DAT activity. Cytosolic levels of DA are also affected by the dose of exogenous L-DOPA administered. 1 hour of 100 uM L-DOPA pretreatment of the cultured midbrain DA neurons induced an increase of cytosolic DA to 17.4 ± 1.7 μM (mean ± SEM)(Mosharov et al, 2009).

1.3.1 D2 autoreceptor and its regulation of dopamine neurotransmission

The DA D2 autoreceptor is part of the family of G-protein coupled receptors, found on the terminals and cell bodies of the presynaptic neuron (Cooper et al, 1996)(Fig 1). It is coupled to G_i and inhibits adenylate cyclase (Senogles, 1994) and can modulate ion channel activity (Sulzer et al, 2011). As it is located at a site adjacent to the DA release zone at the synaptic cleft, extracellular DA levels must be high enough to diffuse out of the synaptic cleft to reach the autoreceptor to enable its activation. D2 autoreceptor regulation is only known to act on impulse flow-dependent, vesicular-mediated forms of DA release (Imperato et al, 1985).

The D2 autoreceptor exerts control over many processes in the presynaptic dopaminergic cell. Rapidly and transiently, the D2 autoreceptor is able to inhibit firing through its control of K^+ channels (Lacey et al, 1987; Liu et al, 1994). Currents arising from $K_v1.2$ voltage-gated potassium channels are increased after D2 agonist activation of the autoreceptor, a mechanism that involves the $G_{B\gamma}$ subunit signaling and the physical coupling of the D2 autoreceptor to striatal $K_v1.2$-containing channels (Fulton et al, 2011). This serves to cause immediate hyperpolarization of the cell to dampen DA firing. The D2 autoreceptor can also modulate extracellular DA levels via slower processes that can last from minutes to hours. For example, activation of the DA autoreceptor by DA agonists decreases the amount of DA released by interacting with tyrosine hydroxylase to lower DA synthesis (Onali and Olianas, 1989; Strait and Kuczenski, 1986). Quinpirole, a potent D2 agonist, inhibits evoked dopamine release in a dose-dependent manner (Joseph et al, 2002). Activation of the D2 autoreceptor can also regulate DAT activity to cause an increase in the rate of DA reuptake (Meiergerd et al, 1993) and has the ability to regulate VMAT2 expression (Rothblatt et al, 1997). Stimulation of the D2 autoreceptor can activate many mechanisms to induce a downregulation of extracellular DA levels. The inhibitory feedback on DA release by the D2 autoreceptors is blocked by

D2 autoreceptor antagonists (Suzler et al, 2010). D2 antagonists enhance extracellular DA levels by potentiating DA neurotransmitter release (Imperato and Di Chiara, 1984; Suaud-Chagny et al, 1991), as well as inhibiting DA reuptake (Wu et al, 2002). In addition to D2 autoreceptors, heteroreceptors found on the dopamine cell bodies and terminals can also modulate DA release, though the mechanisms by which occurs are not well-understood (Sulzer et al, 2011).

1.3.2 Neuroadaptive changes in dopamine transmission following dopaminergic terminal loss

Interestingly, the partially denervated striatum can maintain a normal range of extracellular DA levels, despite a substantial loss of nigrostriatal neurons (Abercrombie et al, 1990; Garris et al, 1997). This is thought to be mediated by various compensatory mechanisms, including increased DA synthesis in the remaining DA neurons by elevated expression and activity of TH (Blanchard et al, 1995), increased DA release in the remaining dopamine neurons (Zigmond et al, 1984), larger quantas of DA per vesicle release (Garris et al, 1997), increased DA turnover by MAO-B (Zigmond et al, 1984), increased extracellular levels of glutamate to promote DA neurotransmission (Robelet et al, 2004) and reduced overall DA reuptake by downregulation of DAT (Bezard et al 2000). The uptake kinetics of DAT also seem to increase as denervation becomes more severe (Garris et al, 1997). Calabresi et al (1993) observed that in denervated DA slices, there was a large amount of spontaneously depolarizing postsynaptic potentials. This was not seen in normal DA slices. Taken together, these results demonstrate that major compensatory changes occur in the presynaptic cell while dopaminergic neurons are progressively lost in the striatum of Parkinson's patients.

After toxin-induced nigrostriatal lesions in animal models of PD, the physiological responses to dopamine receptor stimulation are also altered. There is an increased sensitivity of the DA D2

receptors that control the release of glutamate, as well as a reduction in the D1 receptor inhibition of the postsynaptic cell (Calabresi et al, 1993).

As PD progresses and denervation becomes increasingly severe, presynaptic changes are insufficient to compensate for the rapid decline in DA function (Zigmond and Stricker, 1980; Chen, 2005). However, changes occur in the postsynaptic DA receptors with respect to their physiological responses to dopamine stimulation (Calabresi et al, 1993). Receptor supersensitivity is seen, due to an upregulation in DA D2 receptor levels, along with an increased sensitivity of the DA D2 receptors that control the release of glutamate (Bezard and Gross, 1998). This upregulation of the D2 receptor has been widely observed in the striatum of PD patients even prior to any therapeutic treatments (Qin et al, 1994; Frohna et al, 1995, Calabresi et al, 1993; Betarbet and Greenamyre, 2004).There are conflicting reports of whether expression levels of the D1 receptor are increased (Narang and Wamsley, 1995) or decreased (Calbresi et al, 1993; Qin et al, 1994). This may be because the changes in the D1 receptor are more transient than the more stable changes in the D2 receptor (Araki et al, 2000). The levels and duration of these changes in D1 and D2 receptor regulation also differ across various parts of the striatum (Araki et al, 2000). These findings indicate that not only are compensatory mechanisms engaged, these neuroadaptive changes evolve over time as neurodegeneration becomes increasingly severe.

1.3.3 L-DOPA-derived dopamine release

L-DOPA (a dopamine precursor) is the most effective symptomatic treatment of Parkinsonian movement disorder on the market. When L-DOPA is administered systemically it crosses the blood brain barrier and is transported into the nerve terminal by L-type amino acid transporters (Sampaio-Maia et al, 2001) to increase dopamine concentration. It is converted to DA

by dopa decarboxylase (DDC) and its cofactor, pyridoxal phosphate, in both the CNS and PNS, bypassing the rate-limiting enzyme, tyrosine hydroxylase. As there is an abundance of DDC in the remaining DA terminals in the denervated striatum, exogenously administered L-DOPA can increase striatal DA in a dose-dependent manner (Lundblad et al, 2004), to beneficially supplement the diminished DA supply in the degenerating brain (Cooper et al, 1996). One of the attractiveness of using L-DOPA therapy is that L-DOPA is also an endogenously found as a product of L-tyrosine. The metabolic products of L-DOPA include norepinephrine and epinephrine, in addition to DA.

DA is synthesized from L-DOPA in the dopaminergic neurons. However, dopaminergic terminals are not the only site of release, as L-DOPA is also converted to dopamine by dopa decarboxylase-containing glial cells (Nakamura et al, 2000) as well as serotonergic (Arai et al, 1994) and GABAergic neurons (Melamed et al, 1980). It is thought that as dopaminergic denervation gets increasingly severe, these other terminals are able to provide DA to the L-DOPA treated striatum (Kostrzewa et al, 2005). Initially, this ability of L-DOPA to provide supplemental DA is quite effective. However, prolonged use of L-DOPA produces motor side effects such as dyskinesia that become very disabling. Despite these problems, L-DOPA remains the most commonly used treatment for PD, as few other treatments have been as effective in alleviating the parkinsonian motor symptoms

Although the mechanisms by which L-DOPA induces dyskinesia are not fully understood, it is believed that non-physiological, pulsatile stimulation of the postsynaptic DA receptors induces downstream changes that cause motor complications (Chase and Oh, 2000). Various labs report difficulty in observing dyskinesia following repeated injections of L-DOPA into the intact striatum (Mones, 1971; Nutt, 1990; Langston et al, 2000). This may reflect the fact that the intact striatum has fully functional regulatory mechanisms to maintain the extracellular DA levels. As discussed in

previous sections, extracellular synaptic DA levels are tightly regulated and maintained at relatively constant values by various mechanisms in the intact brain. However, as the brain undergoes further denervation, the decreased levels of regulatory proteins, such as VMAT2 and DAT, leads to significant disruption of key regulatory mechanisms. A weakened buffering system, along with L-dopa's short plasma half-life (60-90 minutes) (Nutt and Fellman, 1984; Huot et al, 2013) thus causes extracellular levels of DA to fluctuate. In the progressively denervated striatum in the late-stage of the disease, extracellular DA concentrations are increasingly dependent on exogenously administered L-DOPA. Because of this, the postsynaptic DA receptors experience non-continuous, non-physiological patterns of high and low DA stimulation (Chase and Oh, 2000). This in turn causes abnormal output to the rest of the basal ganglia leading to post synaptic changes that cause the motor complications observed with long-term L-DOPA therapy in Parkinson's disease.

In the normal striatum, L-DOPA evoked DA release occurs mainly through conventional exocytosis (Synder and Zigmond, 1990). However, despite extensive research into the changes to DA neurotransmission in the denervated striatum, surprisingly, there have been relatively few papers that have looked at the mechanism of L-DOPA-evoked DA release in the lesioned striatum. Abercrombie and Miller (1991) reported that LD-induced DA release is mediated by both impulse-dependent and impulse-independent release. Recent findings from our group support this observation. Specifically, our group has observed differences between the intact and denervated striatum in the mechanisms by which L-DOPA-derived DA was released (Ahn et al, unpublished). Reverse-dialysis of 1 uM of L-DOPA in the intact striatum was accompanied by only a small increase in extracellular DA levels. However, in the denervated striatum, a significant increase in extracellular DA relative to baseline was observed after the administration of L-DOPA. Another difference was that in the intact striatum, depletion of vesicular DA packaging by the VMAT 2 antagonist

Tetrabenizine (TBZ) attenuated L-DOPA-derived extracellular DA levels to values below basal concentration (Ahn et al, unpublished). However, in the 6-OHDA denervated striatum, inhibition of vesicular DA sequestration failed to affect the L-DOPA-induced extracellular DA signal (Ahn et al, unpublished). As vesicular sequestration of DA is necessary for impulse-dependent neurotransmission, the inability of TBZ to affect the DA efflux in the denervated striatum suggested that the mechanism of L-DOPA-derived DA release in the denervated striatum was different than that of intact striatum. To further confirm this, Ahn et al (unpublished) then observed that although Tetrodotoxin (TTX) and a Calcium-free solution could attenuate the L-DOPA-induced DA efflux in the intact striatum, these manipulations were unable to affect the L-DOPA-induced DA efflux in the severely denervated striatum. These findings gave rise to the conclusion that as the striatum undergoes increasing denervation, there is a significant shift from an impulse-dependent (vesicular) mechanism of release, to an impulse-independent (non-vesicular) mechanism of release (Ahn and Phillips, unpublished). Therefore in the denervated striatum, the significant, non-vesicular efflux of DA after L-DOPA administration may exacerbate the problem of abnormal high and low, pulsatile stimulation of the postsynaptic DA receptors.

From these experiments, it seems that VMAT2 activity is reduced in the denervated striatum. It is plausible that not only may this create problems with respect to abnormal L-DOPA-induced DA release, it may also have dire consequences on cell survival. A reduction in the vesicular sequestration of DA would cause more DA to accumulate in the cytoplasm. As cytosolic levels of DA increase in the presence of L-DOPA (Mashorov et al, 2009), the denervated striatum's reduced ability to sequester DA may become especially problematic. Elevated levels of cytosolic DA may then induce neurotoxicity by generating DA_{cyt}-derived free radicals, as it has been reported that the

number of TH+ neurons decline exponentially from the dose of L-DOPA administered (Masharov et al, 2009).

Despite these issues, L-DOPA remains unparalleled in its ability to alleviate the motor symptoms of PD. Thus, it would be highly beneficial to the patient's quality of life - as well as the economic burden – if the onset of its adverse effects could be postponed. Based on the above findings, we propose that this may be achieved by enhancing the vesicular sequestration of L-DOPA-derived DA. Potentiating VMAT2's ability to transport cytosolic DA into vesicles may be favourable to L-DOPA therapy in three ways: i) reduction of the surge of extracellular DA seen after the administration of L-DOPA in the denervated brain, ii) promotion of more "normal" impulse-dependent, vesicular DA release to overcome the negative consequences of abnormal high and low patterns of stimulation at the postsynaptic receptors, and finally iii) the reduction of neurotoxicity of L-DOPA by sequestration of radical-generating cytosolic DA into vesicles. Therefore, as a potential drug target for Parkinson's disease, it is important to have a thorough understanding of the mechanisms by which VMAT2 can be manipulated to increase vesicular DA sequestration. The work in this thesis thus focuses on uncovering a novel mechanism by which DAT may work to promote VMAT2 activity by way of the D2R autoreceptor.

1.4 The mechanism of action of VMAT2

The vesicular monoamine transporter 2 (VMAT2, gene *SLC18A2*) is a protein with 12 transmembrane domains that is found on the membrane of synaptic vesicles in nerve terminals to transport monoamines from the cytosol into vesicles. It is predominantly expressed in the neurons of the central nervous systems, whereas its isoform VMAT1 is preferentially expressed in the peripheral nervous system. The vesicular monoamine transporter is also found on endocrine cells,

and the tumors that come from these cells (Schafer et al, 2013). In the dopaminergic cell, VMAT2 fills the vesicles with cytosolic DA by using the energy derived from an H^+ electrochemical gradient produced by vesicular H^+-ATPase. The amine is then held in these vesicles, ready for vesicle-mediated release during neurotransmission (Fig 1). Three main factors influence vesicular filling: the cytosolic concentration of monoamine, H^+ electrochemical driving force across the vesicular membrane, and the intrinsic properties of the vesicular transporter itself (Blakely and Edwards, 2014).

Both the vesicular neurotransmitter transporter and plasma membrane transporter (that I will discuss in the upcoming sections) are *concentrative transporters* because they act by using the electrochemical energy derived from pre-existing ion gradients (especially Na^+), and the transmembrane potential, to concentrate substrates. DAT and VMAT2 are thus similar in that they depend on electrochemical gradients built by other pumps, but differ in that DAT acts by the co-transport of ions with substrates, whereas VMAT2 counter-transports monoamines and protons. Concentrative transporters must contain a gating mechanism to prevent substrates from simply diffusing down their concentration gradient. The binding sites on these transporters thus alternate between cytoplasmic or extracellular (or intravesicular) environments via conformational changes.

VMAT2 function is important for neurotransmission and cell survival. Without vesicular sequestration of DA by VMAT2, there is less impulse-dependent DA release, as this process depends on the exocytosis of vesicle-contained neurotransmitters (Fon et al, 1997; Colliver et al, 2000). In addition to the interference of neurotransmission, Pifl et al (2014) demonstrated that the blockage of VMAT2 by TBZ decreased the movement of the animal, as well as the number of dopamine neurons. Administration of reserpine, a VMAT2 substrate that causes vesicular DA to be displaced

into the cytoplasm, promoted striatal oxidative stress (Bilska et al, 2007) and oxidative cellular damage from DA-generated radicals (Fuentes et al, 2007). The packaging of DA into vesicles by the action of VMAT2 is therefore necessary for two purposes: the facilitation of neurotransmission as well as the prevention of intracellular neurotoxicity leading to cell death (Mosharov et al., 2003; Vergo et al, 2007).

1.4.1 Pharmacology of VMAT2

The monoamine transporter has a number of substrates, including the classical VMAT2 inhibitors, TBZ and reserpine. However, their kinetics, binding sites and effects on VMAT2 are slightly different.

Reserpine is an irreversible inhibitor of both VMAT2 and the peripheral VMAT1 (Paleacu, 2007). It binds the transporter at a high affinity site and a low affinity site, at 30pM and 25nM respectively (Sievert et al, 2007). The high affinity reserpine site is induced by the vesicular membrane's electrochemical proton gradient, while the low affinity site is present independently of this gradient (Sievert et al, 2007). Binding of this compound causes monoaminergic depletion from the vesicles in both the central and peripheral nervous system.

Tetrabenazine, on the other hand, is considered selective for VMAT2, as it has a high affinity for the VMAT2 isoform and close to negligible affinity for VMAT1. It is a reversible inhibitor, and compared to reserpine, has a quicker yet a shorter duration of action (Pletscher et al, 1962). Binding of TBZ to VMAT2 inhibits the uptake of monoamines from the cytosol into vesicles and causes a depletion of the already sequestered vesicular monoamines (Paleacu, 2007). Reches et al (1983) used HPLC to measure the levels of DA, DOPAC and HVA inside the striatal tissues after an acute injection of TBZ. They found that in the striatal tissues, DA levels initially spiked, followed by an

attenuation of 90%. DOPAC had a 4 fold increase at post 30 min compared to control values. From these observations, it is thought that TBZ's mechanism of action is as follows: the binding of TBZ induces a conformational change to VMAT2 that allows for the release of DA from the synaptic vesicles into the cytosol, where its large quantities exit the nerve terminal leaving excess amounts of free cytosolic DA to be converted into DOPAC (Reches et al, 1983). TBZ and its derivatives bind to VMAT2 via a single high-affinity site (Sievert et al, 2007). [^{3}H] dihydrotetrabenazine ([^{3}H] DTBZ), one such derivative, is frequently used in radioligand studies of the brain as a biomarker for VMAT2 and the monoaminergic neurons that contain it. It binds to VMAT2 with a K_D of 3nM (Sievert et al, 2007).

TBZ binds to VMAT2 via a different site than reserpine or ketanserin, a serotonin 5-HT_{2A} receptor ligand that also acts as a substrate for the transporter. Thus it is suggested that VMAT2 has 3 potential binding sites: the substrate/high-affinity reserpine site, the ketanserin site, and the Tetrabenazine site (Sievert et al, 2007). It is still uncertain at the moment whether these binding sites are distinct from one another, or have partially overlapping domains.

1.4.2 Relation of VMAT2 to Parkinson's disease

There is growing interest in VMAT2 because of its potential role in Parkinson's disease. VMAT2 gene mutation in humans induces a Parkinson-like condition (Rilstone et al, 2013), and VMAT2 defects may lead to DA neuronal death in PD, as found from PD patients and the brains of MPTP-administered monkeys (Pifl et al, 2014). An increasingly popular theory proposes that it is in fact the differential expression of VMAT2 that confers certain individuals with more susceptible to Parkinsonism (Uhl, 2004). PD patients showed 71-80% less striatal dihydrotetrabenazine (DTBZ)-binding, indicating lowered VMAT2 levels (Wilson et al, 1996; Pifl et al, 2014) and after isolating synaptic vesicles from the striatum of PD patients, the remaining VMAT2 were shown to have a

defect in the DA transport activity. Uptake of DA into the vesicular lumen was decreased by 87-90%, and uptake rate of DA per VMAT2 site was reduced by 53% in the caudate and 55% in the putamen respectively (Pifl et al, 2014). The defect in VMAT2 transport activity seems to be specific to PD patients (Pilf et al, 2014; Goldstein et al, 2013; Hornykiewicz, 1964) and it is postulated that this impairment in DA storage may result from diseaseinduced modifications to the VMAT2 protein itself (Pilf et al, 2014).

Caudle et al (2007) showed that a reduction in striatal VMAT2 activity has severe PD-related implications. A 5-10% expression of normal levels of VMAT2 in transgenic mice was associated with a decrease in DAT and TH expression, as well as a gradual increase in a-synuclein and toxic DA metabolite accumulation (Caudle et al, 2007). This generated a severe degeneration of the substantia nigra and resulted in PD-like motor deficits in the mice, including reduced locomotor activity that could be rescued by L-DOPA (Caudle et al, 2007).

In contrast, new evidence suggests that increasing VMAT2 expression and activity may have therapeutic benefits. Overexpression of VMAT2 by bacterial artificial chromosome (BAC) caused an increase in striatal DA content and the release of DA from striatal slices, as measured by HPLC and fast-scan cyclic voltammetry respectively (Lohr et al 2014). Remarkably, this enhancement of DA signaling by alteration of VMAT2 levels did not produce the negative effects of a hyperdopaminergic state (Lohr et al, 2014). Instead, transgenic mice overexpressing VMAT2 showed improvement in tests of anxiety and depression, as well as an increase in movement (Lohr et al, 2014). They also experienced less SNpc cell loss and DA terminal damage when MPTP was administered (Lohr et al, 2014). In humans, the induction of VMAT2 gain of function by promoter haplotypes corresponded

with a decreased risk of PD in women (Glatt et al, 2006). These results indicate that the promotion of vesicular sequestration of DA may be a valuable future target for PD.

The downregulation of VMAT2 in the human disease pathogenesis seems to be mimicked by experimental models of PD. There are many reports citing reduced DTBZ binding in the striatum following n 6-OHDA lesions of the rodent brain (Walker et al, 2013; Sossi et al, 2010; Strome et al, 2006) and the DTBZ radioligand is commonly used as an assessment of the number of nerve terminals remaining intact. However, it is unclear whether in 6-OHDA-lesioned striatums, VMAT2 levels decline in proportion to the loss of nerve terminals, or whether the density of VMAT2 in the remaining terminals is modified.

1.5 The mechanism of action of DAT

The dopamine transporter (DAT) is an 80-kDA protein composed of 12 transmembrane regions. It is part of the sodium and chloride-dependent family of transporters. It is found on plasma membrane of the axon and terminals fields of nigrostriatal dopamine neurons (Fig 1), as well as glial cells. It is also found on nerve terminals of striatum, nucleus accumbens, globus pallidus, cingulate cortex, olfactory tubercle, amygdala and midbrain (Ciliax et al, 1995). The DAT mediates the co-transport of a DA molecule along with 2 Na^+ and Cl^- ions, using the energy driven by the electrochemical gradient generated by the Na^+/K^+ ATPase pump from the extracellular space into the cytosol. The translocation of the DA and ions into the cell correspond with the simultaneous outward transport of K^+. As the inward transport of DA and net positive ions is highly dependent on a negative membrane potential, conditions that change the membrane potential, ion gradients or amount of ATP molecules can modulate the function of DAT, including its directional mode (Blakely, 2001). By such methods, DAT modulates the amount of DA in the synaptic cleft by reuptake of DA

into the presynaptic neuron. This plays two critical roles: termination of DA transmission (Amara and Kuhar, 1993; Giros and Caron, 1993) and regulation of the persistence of DA release by the presynaptic neuron (Chen and Reith, 2000). By exerting such vital influences on dopamine function, it is able to modulate locomotor activity, cognition and the reward system. DAT is important in the maintenance of normal levels of DA. Various homeostatic processes are activated when DAT expression and function are disrupted. DAT KO mice exhibit a 95% decrease in intracellular DA stores (Jones et al, 1998), and the deletion of DAT causes a 2-fold increase in the rate of DA synthesis (Egana et al, 2009).

DAT is a major target for various drugs of abuse, such as cocaine and amphetamine, as well as therapeutic agents (methylphenidate for ADHD and bupropion for depression). Various substrates, such as amphetamine and MPP+, are structurally similar to DA, and thus can be carried across the transporter into the cell. Other molecules, such as cocaine and methylphenidate have specific binding sites on the transporter. When they bind with DAT, instead of being carried over, they block the carrier's ability to reuptake dopamine.

In addition to DAT 's various substrates, Gonzalo Torres and colleagues have identified a vast number of proteins that also interact with DAT, including various presynaptic receptors, protein kinases and membrane traffickers (Shih et al, 2006). Though DAT is able to function as an isolated uptake mechanism, it is now recognized that DAT commonly forms various highly regulated, multi-protein complexes, due to a variety of protein-protein interactions. These interactions serve to influence the distribution, targeting, localization, trafficking and function of DAT (Egana et al, 2009).

Of immediate relevance to our work, is the hypothesis that DAT can also interact with various G-protein coupled receptors (Torres et al, 2006). This includes the DA D2 receptor (Torres et

al, 2006, Pei et al, 2004), where a physical bond between the presynaptic DA D2R and DAT has been reported. Other G-protein coupled receptors, such as trace amine receptors, may also be activated indirectly by DAT through the interaction with its substrates amphetamine, methamphetamine and 3,4-methylenedioxymethamphetamine (Miller et al, 2005). These indirect and direct interactions between DAT and G-protein coupled receptors trigger the induction of various intracellular signaling cascades.

Another interaction that is of particular interest, is the suggestion that DAT may physically connect to synaptic vesicles through the binding of VMAT2 (Egana et al, 2009; Torres et al, 2006). I will discuss the interaction between DAT and VMAT2, as well as DAT and the presynaptic D2R in greater detail in later sections.

1.5.1 Relation of DAT to Parkinson's disease

It is estimated that DAT levels decline by approximately 6-7% per decade in the normal, human striatum (Shih et al, 2006). In the remaining nigral neurons of PD patients, significantly lower levels of DAT mRNA are observed when compared to age and gender-matched controls (Uhl, 2004). These decreased levels of the transporter in the PD striatum appear to be to correlated with the intensity of the PD symptoms exhibited (Guttman et al, 1997). Thus radiotracers that measure DAT levels are used to evaluate dopamine terminal denervation and thereby track the stage of progression of PD (Stoessl, 2014). Positron emission topography (PET) studies have shown a major reduction in DAT levels even prior to the symptomatic manifestations of PD (Adams et al, 2005; Stoessl, 2014). Therefore, there is now growing interest in using sensitive and specific DAT radiotracers for the early diagnose of PD prior to the patient's exhibition of motor symptoms (at

which point 60% of the neurons would have already been lost to make a traditional clinical diagnosis.)

Theoretically, the reduction of DAT should be beneficial in compensating for the reduced levels of synaptic DA in the PD brain (Sossi et al, 2007), as there would be less DA clearance from the extracellular space by reuptake. However, this reduction in DAT may also be harmful, as DA turnover (by extracellular enzymatic degradation) may actually increase, leading to greater fluctuations in extracellular DA levels that may ultimately contribute to dyskinesia and the motor complications of PD (Sossi et al, 2005).

Down-regulation of DAT seen in human patients has also been reported in toxin-induced animal models of Parkinson's disease. In MPTP and 6-OHDA rodent models, DAT plays a critical in transport of toxins into the cytoplasmic compartment of the cell to induce neurodegeneration. Transgenic overexpression of DAT (to 130% of normal levels) in combination with MPTP treatment causes more than a 170% loss of DA neurons compared to wildtype levels of DAT (Uhl, 2004). In contrast, the KO of DAT confers MPTP resistance to mice (Uhl, 2004). As already noted, DAT levels decrease with the loss of dopaminergic terminals after 6-OHDA infusion. It seems that the severity of DAT depletion may affect the uptake kinetics of the remaining transporters. The > 70% loss of striatal DAT by unilateral 6-OHDA lesions resulted in the reduction of DA uptake activity by 25%, when assayed by [^{3}H] DA (Chotibut et al, 2012). Interestingly, the uptake dynamics of the remaining transporters were observed to increase as DAT depletion approached 99% (Chotibut et al, 2012).

1.5.2 Evidence for dopamine efflux by reversal of the DAT

DAT is thought to be an asymmetrical transporter whose conformational property typically favours influx over efflux. Its net flux, however, is controlled by transmembrane substrate gradients,

and also takes into account a potential secondary messenger system (Sulzer, 2005). Amphetamine is a substrate of DAT that is widely known to cause the efflux of DA from the vesicular stores and cytosol into the extracellular space. First, the transmembrane gradient of high AMPH and Na^+ concentrations on the outside allows facilitated diffusion to occur. Amphetamine uptake into the cell, as well as the cotransport of Na^+ and Cl^-, increases the probability of an inward conformation of DAT's binding site to face the cytosol, so that intracellular DA can then bind (Sulzer 2005). The intracellular phosphorylation of DAT then plays a role in mediating the facilitated exchange of DA into the extracellular space (Khalig et al, 2005). In addition to the slow process of facilitated exchange, DAT can also mediate the efflux of large quantities of DA from the cell in a channel-like manner in the presence of amphetamine (Khalig et al, 2005).

Reverse transport of DAT was once considered to occur only in the presence of amphetamine. However, it is now well recognized that DAT mediated DA release can occur under more physiological circumstances. The administration of weak bases that are not DAT substrates, such as chloroquine and methylamine, are able to collapse vesicular pH gradients, as well as mediate reverse transport through the DAT (Sulzer et al, 1993). Khoshbouei et al (2003) found that in the absence of AMPH, simply increasing the concentration of intracellular Na^+ can mediate DA efflux from the cell. Glutaminergic inputs from the subthalamic nucleus to the substantia nigra may also act to trigger the efflux of DA through reversal of the DAT (Falkenburger et al, 2001). Intriguingly, these findings indicate that DA efflux through reversal of the DAT may be possible in the absence of a drug.

1.5.3 Interactions between DAT and VMAT2

Recent evidence suggests that DAT and VMAT may work closely together to regulate the sequestration of DA. DAT transports the DA from the extracellular space into the cytosol, where the VMAT2 sequesters it into vesicles. Thus, the concentration of free cytosolic DA is regulated by the mutual actions of DAT and VMAT2.

In addition to their synergistic mode of action, other data suggest that these two transporters may actually have regulatory effects on each other. Egana et al (2009) found that overexpression of the vesicular protein synaptogyrin-3 in certain cell lines was associated with an increase in DAT activity. This effect on DAT was negated by the administration of the VMAT2 inhibitor, reserpine. In other studies, a decrease in VMAT2 expression also corresponded with a reduction of DAT levels in mice (Caudle et al, 2007).

To take this interaction further, it now appears that the synaptic vesicles may be coupled to DAT, by formation of a synaptogyrin-3, VMAT2 and DAT complex. Using co-immunoprecipitation experiments of heterologous cell lines and neurons from the mouse brain, Egana et al (2009) showed that the vesicle protein synaptogyrin-3 interacts with DAT. This interaction was similarly observed by fluorescence resonance energy transfer (FRET) microscopy in neonatal rat hippocampal neurons (Egana et al, 2009). In addition to synaptogyrin-3 and DAT coupling, separate assays have also found a co-immunoprecipitation between DAT and VMAT2. Pull-down assays showed that the amino terminus of DAT is bound to synaptic vesicles purified from brain tissue (Egana et al, 2009). Because DAT is located at the perisynaptic area (Hersch et al, 1997), away from the NT active zone of release, it is unlikely that DAT interacts with the readily releasable pool of vesicles. Instead, it is postulated that DAT may interact with the reserve and recycling pool vesicles, or the synaptic

vesicles that come into its proximity during endocytotic recycling (Egana et al, 2009). The observation of a close interaction of VMAT2 and DAT has important implications for our work as it may explain how DA could shift from the lumen of the vesicles into the extracellular space after administration of TBZ.

1.6 Methylphenidate

Methylphenidate (MPD) is widely recognized as a classical DAT blocker. It is licensed as Ritalin to treat attention-deficit hyperactivity disorder (ADHD) and narcolepsy. Methylphenidate can enhance extracellular DA concentrations in the brain by blocking reuptake into the presynaptic region of the DA cell. Because the DAT plays a critical role in modulating the length and potency of DA transmission, Methylphenidate has also been investigated for its potential as a Parkinson's disease therapy.

MPD has been clinically investigated for its benefit to voluntary movements, such as gait and freezing, in Parkinson's disease. These studies have examined it for its potential use as a monotherapy, or as a combination therapy with L-DOPA. The rationale behind the use of MPD in Parkinson's disease is as follows: Because MPD is able to selectively inhibit DAT reuptake in the striatum, the extracellular levels of DA released from the remaining nerve terminals in the denervated striatum should be "boosted" when MPD is administered by itself (Nutt et al, 2004). When given in conjunction with L-DOPA, MPD should potentiate L-DOPA's effect by preventing the clearance of LD-derived DA from the synapse (Nutt et al, 2004). In a clinical study by Pollak et al (2004), MPD administered alone demonstrated slight improvement in gait for patients in late-stages of Parkinson's disease. Studies by Nutt et al, (2007) and Auriel et al, (2009), however, indicated that MPD had no benefits on gait when administered as a monotherapy, but was beneficial when given

in conjunction with L-DOPA. Thus, the benefits of MPD as a monotherapy are currently unclear. However, it seems as though a combination of MPD and L-DOPA demonstrate higher therapeutic potential, and there is recognition that more rigorous, randomized, controlled clinical trials are needed to investigate this further (Devos et al, 2013).

1.6.1 Methylphenidate: more than a classical DAT blocker?

MPD's role as a DAT inhibitor is well recognized. However, novel findings by the Fleckenstein group indicate that MPD may have an additional mechanism of action. They propose that in addition to MPD's direct action as a DAT blocker, the drug may augment the activity of VMAT2. Using purified rat striatal vesicles, they showed that incubation of MPD caused an increase in [3H] DA vesicular uptake, as well as the binding of the VMAT2 ligand dihydrotetrabenazine (DHTBZ) in a dose-dependent manner (Sandoval et al, 2002).

Examining this in greater detail by separating the vesicular pool into two fractions (the cytosolic and membrane bound), Fleckenstein's lab reported that following administration of MPD, VMAT2-containing vesicles were redistributed from the synaptosomal membranes into the cytoplasm (Volz et al, 2008). VMAT2 kinetics and vesicular DA content in both vesicular fractions were also increased by MPD (Sandoval et al, 2002; Volz et al, 2007; Volz et al, 2008), as was quantal size, velocity and magnitude in K^+-stimulated DA release (Volz et al, 2008). As the total DA content in striatal tissues was not altered after MPD administration (Sandoval et al, 2002), the authors concluded that the increase in vesicular DA content was not due to a potentiation of DA synthesis, but by a redistribution of the cytosolic dopamine into the vesicles. These findings reveal *in vitro* the novel ability of MPD to manipulate the trafficking, DA sequestration and DA content of the vesicles used in impulse-dependent neurotransmission.

1.7 Rationale for experiments in the present thesis

Given the clinical interest in the use of MPD for treatment of Parkinson's disease, the Phillips' lab examined its possible therapeutic benefits when co-administered with L-DOPA. Using unilateral 6OHDA-lesioned rats, co-administeration of MPD with L-DOPA showed greater improvements in voluntary movement than L-DOPA therapy alone (Cheng, 2005; Ahn et al, unpublished). When the neurochemical effects of MPD were examined by microdialysis, Dr. Ahn observed that MPD produced a sustained elevation of DA efflux in the intact dorsal striatum, well beyond that produced by L-DOPA alone. While the most parsimonious explanation for the augmented extracellular DA signal is that reuptake inhibition prevents the clearance of DA from the synaptic space, it was unclear why this increase was sustained, as chronic reuptake inhibition should result in the gradual depletion of striatal DA (Sivam, 1995; Wilson et al, 1996; Gainetdinov et al, 1998).

We then became aware of the aforementioned work by the Fleckenstein group, who demonstrated that *in vitro*, MPD was able to increase VMAT2 activity and promote vesicular sequestration and depolarization-dependent release of DA (Sandoval et al, 2002; Sandoval et al, 2003; Volz et al, 2007; Volz et al, 2008). Testing this *in vivo*, Ahn et al (unpublished) found that reverse-dialysis of TBZ following the MPD-induced augmentation of extracellular DA efflux attenuated this value by 70%. As TBZ depletes the vesicular stores of DA, and prevents further vesicular sequestration, the interference of these processes would consequentially abolish vesicular-mediated neurotransmission (exocytosis) (Reches et al, 1983). Therefore, the attenuation of 70% of the MPD-augmentation of L-DOPA-evoked DA signal by TBZ suggested that this portion of the DA signal was induced by MPD-mediated vesicular neurotransmission. The residual 30% of the extraneuronal DA signal unaffected by TBZ was attributed to MPD's inhibition the dopamine

transporter (DAT). These hypotheses interpretations were examined further by the infusion of a calcium-free medium and Tetrodotoxin (TTX). Like TBZ, both the calcium-free medium and TTX were able to attenuate the majority of the MPD-augmented DA efflux, thereby providing further support that this component of the DA signal was contributed by vesicular-mediated release.

If Methylphenidate can promote vesicular sequestration and exocytotic release, then theoretically, these properties may be beneficial by ensuring that DA synthesized from L-DOPA is transported into vesicles where it can be released in an impulse-dependent, physiological manner. In 6-OHDA lesioned animals, we have data indicating that the L-DOPA-synthesized DA does not undergo transport into the vesicles via the vesicular monamine transporter (VMAT2), but instead is released from the cytosolic pool by unconventional mechanisms (see section 1.3.3). Under such conditions of severe DA denervation, the addition of MPD to L-DOPA treatment may be beneficial in a) allowing for more controlled, stimulus dependent neurotransmitter release b) reducing cytotoxicity from cytosolic DA accumulation, c) preventing the adverse consequences of this abnormal efflux (ie. dyskinesia). Behaviorally, the induction of vesicular-mediated release of the DA synthesized from L-DOPA may account for the superior motor responses in 6-OHDA lesioned animals when MPD was administered in conjunction with L-DOPA, as compared to animals given L-DOPA alone (Cheng, 2005; Ahn et al, unpublished). It is tempting to speculate that this may also be why MPD therapy does not induce hyperkinesia (Volkow et al, 2001) in humans, and why the combination therapy of L-DOPA and MPD was superior to L-DOPA alone in a clinical trial for Parkinson's disease (Nutt et al, 2004).

This thesis builds on these exciting observations by Dr. Ahn by investigating further the possible mechanism(s) by which MPD may induce these effects. A detailed literature review

revealed a link between DAT and the DA D2 autoreceptor. Specifically, the incubation of the striatal slices with the selective D2 receptor antagonist, Eticlopride, attenuated MPD induced upregulation of VMAT2 and vesicular release (Sandoval et al, 2002). The question remains as to how MPD may trigger the activation of this receptor. The simplest explanation would be that MPD's activation of the D2 receptor is indirect, through the augmented levels of extracellular DA as a result of reuptake inhibition. Theoretically, this would imply that the observed upregulation of vesicular-mediated neurotransmission in 6-OHDA lesioned rats is non-specific to MPD, and that this effect could be induced via agonist activation of the D2 autoreceptor by all classes of DAT inhibitors, as well as direct acting D2 agonists such as Quinpirole. Against this hypothesis, however, one must consider that *in vitro*, the DAT inhibitor, cocaine, failed to upregulate VMAT2 activity in the readily releasable pool of vesicles, nor could it increase stimulus-evoked DA release (Farnsworth et al, 2009). It is also difficult to reconcile the fact that the D2 receptor agonist Quinpirole would be expected to increase DA release, when D2 autoreceptor activation inhibits DA firing, thereby attenuating DA efflux (see Usiello et al, 2000 for review).

Accordingly, we favour the involvement of a direct interaction between DAT and the D2 autoreceptor that in turn may facilitate VMAT2-mediated vesicular DA sequestration and subsequent release. A growing body of evidence supports the concept of a close interaction between the D2 autoreceptor and DAT (Giros et al, 1996; Jones et al, 1999; Dickinson et al, 1999; Kimmel et al, 2001; Pei et al, 2004; Wu et al, 2002; Torres et al, 2006), as well as links between the DAT and D2R to VMAT2 (Patel et al, 2003; Caudle et al, 2007). As DAT and the DA D2S, the presynaptic D2 autoreceptor, are found in very close proximity in the striatum (100 A^{o})(Bolan et al, 2007), and DAT is known to form numerous protein-protein interactions (Egana et al, 2009), it is possible that a protein-protein interaction is induced between DAT and the D2 autoreceptor.

Therefore, we hypothesize that MPD-induces an interaction between the DAT and the D2 autoreceptor that allows it to facilitate the vesicular sequestration and release of DA in the striatum. The MPD-induced interaction between the DAT and the D2 autoreceptor may also initiate an intracellular signaling cascade that promotes exocytotic neurotransmission.

Modulation of DA neurotransmission by the presynaptic D2 autoreceptor is critical for cell survival (Olanow et al, 1998). This is undoubtedly affected negatively by the major compensatory changes to the function, activity and expression of the D2 autoreceptor that occur with progressive denervation in the 6-OHDA lesioned and human PD brain (Seeman and Niznik, 1990; Dragicevic et al, 2014). As such, we would expect these changes to affect L-DOPA-evoked DA neurotransmission, although neither the mechanism nor regulation of this release has been extensively documented. Preliminary studies by Ahn et al (unpublished) report that with severe denervation, L-DOPA-derived DA efflux ceases to be affected by the application of Tetrabenazine or a calcium-free medium. This implies that the efflux of DA is no longer mediated by impulse-dependent vesicular release mechanisms and as such, L-DOPA-derived DA efflux may cease to be regulated by the D2 autoreceptor. On the other hand, reports of heightened sensitivity and expression of the D2 autoreceptor in end stage Parkinson's disease (Dragicevic et al, 2014) may indicate a role for autoreceptor modulation of some remaining terminals in the control of DA efflux. Understanding the implications of these neuroadaptive changes on L-DOPA-derived DA regulation may provide valuable insight as to how the presynaptic neuron may be modulated to induce vesicular-mediated neurotransmission

Thus, the two objectives of this thesis were to:

i) Examine the effects of the selective DA D2 autoreceptor agonist Quinpirole and the antagonist Eticlopride on the ability of the D2 autoreceptor to regulate L-DOPA-evoked DA release in the intact striatum, and at various stages of 6-OHDA denervation

ii) Employ Quinpirole and especially Eticlopride to explore the role of the D2 autoreceptor in mediating MPD's effects on the augmentation of the L-DOPA derived DA efflux via vesicular sequestration of DA.

2. Methods

2.1 Unilateral 6-hydroxydopamine lesion rat model of Parkinson's disease

Male Sprague-Dawley rats (225-275g) from Charles River (St. Constant, Quebec, Canada) were used in all experiments. Upon arrival, animals were pair-housed and allowed to habituate in the colony room for a week prior to surgery. Post-surgery, animals were housed individually in Plexiglas cages with *ad libitum* access to food and water, in a colony maintained at 19-22^{O}C in reverse light cycle (7am-7pm dark).

4% isoflurane mixed with oxygen was used to induce animals (at 10 weeks of age) into a surgical plane of anaesthesia, after which 1.5-2.5% isoflurane (AErrane, Baxter Co., Ontario, Canada) was used to maintain this state throughout the surgery. Upon entering a surgical plane of anesthesia, animals were subcutaneously injected with 2 x 3mL saline solution and 0.05 mL/100g anafen. Animals were adjusted to flat skull position (incisor bar at -3.2 to -3.9mm) in the stereotaxic apparatus, while placed on a heating pad to maintain body temperature at 37 ^{o}C To lesion the nigrostriatal DA neurons, 6-OHDA (3ug/uL), prepared in 0.02% ascorbic acid, was injected unilaterally into the substantia nigra *pars compacta* (SNc) and the medial forebrain bundle (MFB) at 2 uL per site in the right hemisphere. The coordinates were determined by averaging the measurements from bregma (SNc: from bregma, 5.20mm AP, -2.00 mm ML; from dura, -7.10 mm DV; MFB: -4.16mm AP, -1.70mm ML, -7.90mm DV) derived from Paxinos and Watson (1997), with coordinates measured from the intra aural (IA) line (SNC: from IA, +3.80mm AP, -2.00mm ML, +2.00mm DV; MFB: +4.84mm AP, -1.70mm ML, +1.20mm DV). The inclusion of IA coordinates accounted for body weight variations larger than the size of the reference rodents used in Paxinos and Watson's Rat Brain Atlas. 6-OHDA was delivered into the SNC and MFB via a 30 gauge needle attached to PE10 Polyethylene Tubing (inner diameter .28mm, outer diameter .61mm) at 1uL/min.

After infusion into each site, the injection needle was left in place for an additional 4 minutes to allow for drug diffusion before being slowly raised from the brain. 19 gauge stainless steel guide cannulae (15mm) were then implanted bilaterally into the dorsal striatum (from bregma, +1.0 AP, +/-3.1 ML, -1.0 DV). Four screws were inserted into the skull to secure a head cap of dental cement (Jet Tooth Shade Powder mixed with Jet Liquid, Lang Dental Manufacturing Co, Wheeling, West Virginia, USA). Immediately post-surgery, animals were placed into a Plexiglas cage containing a heating pad to facilitate recovery and given easy access to food and water for an hour.

2.2 Drugs and preparation

6-hydroxydopamine hydrobromide (6-OHDA) and Tetrabenazine were obtained from Tocris Biosciences, Bristol, UK, and stored at -20^{o}C and +4^{o}C respectively. L-3.4-dihydroxyphenylalanine methyl ester (L-DOPA), (-)-Quinpirole hydrochloride, S-(-)-Eticlopride hydrochloride, L-ascorbic acid and dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich, Oakville, Ontario, Canada, with L-DOPA and Quinpirole stored at -20oC, and the rest of the drugs kept at room temperature. Methylphenidate hydrochloride (kept at room temperature) was a gift from Dr. Michael Krausz, Department of Psychiatry, UBC.

A fresh solution of 0.02% L-ascorbic acid solution was prepared by dissolving L-ascorbic acid in saline prior to the beginning of surgery. It was then aliquotted into vials of 6-OHDA (6 ug/uL) and stored in the dark at -20^{o}C until immediately before infusion. For microdialysis studies, L-DOPA, Quinpirole, Eticlopride, Methylphenidate hydrochloride and Tetrabenazine were prepared fresh daily in ACSF pH 6.5 containing 0.05% DSMO. An anti-oxidative solution made of .082% v/v HCL (2.0N), 100mg/100mL Na Metabisulfate and 10mg/100mL EDTA in Milli-Q water was used as a vehicle for L-DOPA to reduce L-DOPA's degeneration.

2.3 Microdialysis

Microdialysis probes included 4.0mm length of semipermeable membrane (240 uM outer diameter; 65,000 Dalton molecular weight cutoff; Filtral 12; Hospal, Neurnberg, Germany) connected to an inlet and outlet line of silica capillary tubing (inlet: 98 uM inner diameter, oulet: 74.5 uM inner diameter, Polymicro Technologies, Lisle, IL, USA). Pharmacological manipulations were adminstered into the probe through the inlet silica, and striatal dialysate was collected from the probes through the outlet silica.

Subjects were allowed 4 weeks of recovery from surgery before undergoing microdialysis experiments. Prior to implantation into the rat brain, the microdialysis probes were flushed at 1.1 uL/min for 20 minutes with artificial cerebrospinal fluid (147.0 mM NaCl, 1.2 mM CaCls, 3.0 mM KCl, 1.0 mM MgCl2, 10 mM sodium phosphate buffer, pH 7.4) using a 2.5 mL gas-tight syringe (SGE Analytical Sciences, Ottawa, Ontario, Canada) and a syringe pump (model 22; Harvard Apparatus, South Natick, MA, USA). Rats were then briefly immobilized by isoflurane (4% isoflurane mixed with oxygen for 4 minutes) to allow implantation of the probes into the striata via the guide cannulae. The flow rate was maintained at 0.2 uL/min while the rats remained overnight in the testing chambers with access to food and water. On the morning of test day, food was removed, testing chambers were covered to minimize external sensory disturbances and the flow rate was increased and maintained at 1.1 uL/min for the experiments. Dialysate samples were then collected at 10 min intervals and assayed for DA and its metabolites 3,4 dihydroxyphenylacetate (DOPAC), homovanillic acid (HVA) and 5-hydroxytryptamine (5-HT). After a stable baseline was established – the criteria being 4 samples within 10% variation – the pharmacological manipulations for the study were reverse-dialyzed into the striata, and samples continued to be collected every 10 minutes for

injection into the HPLC (see Fig 2. for the sequence of pharmacological manipulations). The subjects were maintained in reverse-light cycle for the duration of all experiments.

The basal DA concentrations (obtained during the assessment for a stable, pre-treatment baseline) were used as an indication of lesion severity. Subjects were grouped as severely lesioned (<0.5 nM basal DA), moderately lesioned (between 0.5 nM and 1 nM basal DA) and intact (6-OHDA-naïve striatum with basal DA levels > 2 nM).

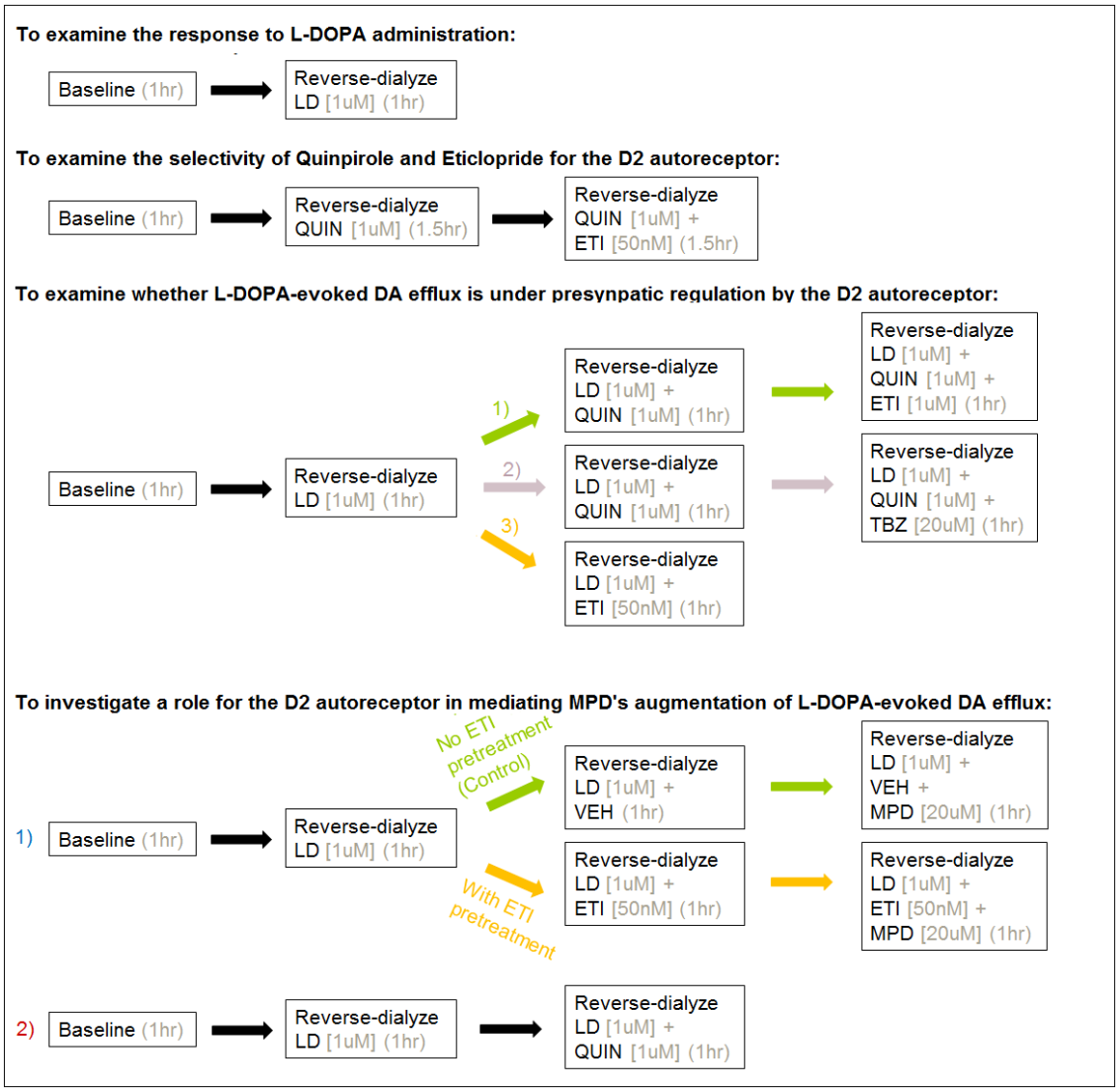


Fig 2. Experimental design of the microdialysis studies. The intact and 6-OHDA denervated dorsal striatum were examined for their response to L-DOPA administration. 1uM Quinipirole and 50nM Eticlopride was then confirmed for selectivity to the D2 autoreceptor. To examine whether L-DOPA-evoked DA efflux was under presynaptic regulation by the D2 autoreceptor, three separate studies were conducted. In the first study, Quinpirole was reverse-dialyzed after L-DOPA, after which Eticlopride was added. In the second study, reverse-dialysis of L-DOPA was followed by Quinpirole and Tetrabenazine. And in the final study, L-DOPA was followed by the reverse-dialysis of Eticlopride. The D2 autoreceptor was then investigated for its role in mediating MPD's augmentation of L-DOPA-evoked DA efflux. In the first study, after the reverse-dialysis of L-DOPA, the experimental subjects were randomly split into two groups. Group 1 (control) continued to receive L-DOPA, followed by MPD, while group 2 was given Eticlopride prior to MPD reverse-dialysis. In the second study, L-DOPA reverse-dialysis was followed by Quinpirole to observe whether direct activation of the D2 autoreceptor was sufficient to mimic MPD's augmentation of L-DOPA-evoked DA efflux. LD = L-DOPA, QUIN = Quinpirole, ETI = Eticlopride, TBZ = Tetrabenazine, VEH = Vehicle, MPD = Methylphenidate.

2.4 High performance liquid chromatography

To assay the striatal dialysates for levels of DA and its metabolites, four HPLC-electrical detection systems were used. The systems employed an ESA 582 pump (Chelmsford, MA), a Scientific Systems, Inc. (State College, PA) pulse damper, a Rheodyne manual injector (20 uL injection loop, Rhonert Park, CA), a TOSOH Biosep (Montgomeryville, PA) Super ODS TSK column (2 mm x 10 mm; 2uM particle), an Antec Leyden (Leyden, The Netherlands) Links system and an Antec Intro detector with a VT-03 electrochemical flowcell (Vapplied = +0.7 V). The mobile phase (70 mM sodium acetate, 40mg/L EDTA, 50mG/L sodium octyl sulfate, pH 4.0, 12% methanol) was pumped at a rate of 0.150mL/min – 0.165mL/min through the HPLC system. The chromatographic data was then analyzed with EZChrom Elite software to obtain the concentration of DA and its metabolites in concentration nM, as well as the relative changes in percent from baseline.

2.5 Data analysis and statistics

All data was expressed as concentration nM and subsequently presented as a difference from baseline. Each drug is reverse-dialyzed for an hour, by which time its effects on extracellular DA signal in the striatum had plateaued. The dialysate samples were collected and analyzed by HPLC every 10 minutes, providing a measure of absolute DA concentration every 10 minutes for the 60 minute duration of each drug phase. For each experimental subject, the absolute DA concentrations from the last three (pretreatment) dialysate samples were averaged to yield a baseline concentration. Subsequently, this baseline concentration was used to obtain a difference from baseline value (nM) at every 10 minute sample measured. The difference from baseline values of the last three dialysate samples (at the plateau) of every drug phase were then averaged to yield a final difference score (nM). Each of the three lesion groups (intact, moderately lesioned and severely lesioned) were analyzed separately for their response to the various drug treatments.

1-way repeated measure analysis of variance (1-way RM ANOVA) was consistently used for the majority of the experiments, as within group comparisons were made to the response to three or more drug conditions. Using a 1-way RM ANOVA, the final difference scores for each of the post-drug treatment conditions were compared for an effect of drug on the DA response, followed by the Holm Sidak test for all pairwise multiple comparisons.

There were two studies that did not use 1-way RM ANOVA. In the preliminary studies establishing an effect of L-DOPA on the DA signal, the final difference scores were compared by 1-WAY ANOVA, as within group comparisons were made between the responses to two drug conditions only (pre-drug baseline versus L-DOPA). In the studies examining the effect of Eticlopride on the DA response to Methylphenidate, the final difference scores were compared by 2-way RM ANOVA to make between group comparisons (Group 1: with Eticlopride pretreatment versus Group 2: without Eticlopride pretreatment) on the response to multiple (more than 3) drug conditions. Following the establishment of statistical significance, all pairwise multiple comparisons were made by the Holm Sidak test using a p value of < 0.05 as the standard for statistical significance.

2.6 Histology

Half of the experimental subjects were randomly selected to undergo histological verification of microdialysis probe placement. After the completion of the studies, the animals were deeply anaesthetized and decapitated. The brains were immediately removed and fixed in formaldehyde solution (20% w/v sucrose, 10% w/v neutral buffered formalin) for a minimum of 48 hours before being sectioned at 50uM at -80^{o}C for verification of probe placement into the striata. Placement was determined with reference to reference to the Paxinos and Watson stereotaxic atlas of the rat brain (Paxinos and Watson, 1997).

3. Results

3.1 D2 autoreceptor regulation of the increase in DA efflux following L-DOPA administration

3.1.1 Response of the intact striatum and the severely denervated striatum to L-DOPA administration

Basal levels of DA in the intact striatum of L-DOPA-naïve animals were 3.15 ± 0.52 nM (mean ± SEM) (absolute concentration), as compared to basal DA of 0.83 ± 0.05 nM (mean ± SEM) (absolute concentration) in the moderately lesioned striatum and 0.20 ± 0.04 nM (mean ± SEM) (absolute concentration) in the severely lesioned striatum. Accordingly, basal DA levels in the severely denervated striatum were approximately 6% of the normal levels of basal DA, and 26% of the normal in the moderately lesioned striatum. As the brain's ability to regulate DA undergoes changes after denervation of the nigrostriatal pathway, the response of the intact and denervated striatum were examined separately after the infusion of various pharmacological manipulations.

Every effort was made to ensure consistency with respect to handling and manipulation of the various groups of animals across the separate experiments. A 2-way ANOVA confirmed no statistical significance between the various groups of intact animals with respect to basal striatal DA levels and the DA response to 1 uM L-DOPA. Thus, to examine the DA efflux after L-DOPA administration, it was justifiable to pool the baseline and L-DOPA data across all the intact groups. A 1-way ANOVA then revealed that reverse-dialysis of 1 uM L-DOPA caused an increase in basal DA levels of 0.90 ± 0.14 nM (mean ± SEM) difference from baseline that were statistically significant ($F_{1,84} = 40.530$, $p < 0.001$, Holm Sidak test $p < 0.001$)(Fig 3AB).

Baseline and L-DOPA data from all severely lesioned groups were also pooled following confirmation that they did not differ significantly. The resulting value for the increase in DA efflux in the striatum following reverse dialysis of 1 uM L-DOPA was 0.39 ± 0.03 nM above baseline ($F_{1,66}$ =

149.197, $p < 0.001$, Holm Sidak test $p < 0.001$)(Fig 3CD). These results indicate that in both the intact and severely denervated striatum, L-DOPA administration causes a statistically significant increase in net efflux of DA into the extracellular space.

3.1.2 Selectivity of Quinpirole and Eticlopride for the D2 autoreceptor

Reverse-dialysis of 1 uM of the D2 agonist Quinpirole followed 60 min later by the addition of 50 nM of Eticlopride into the intact striatum resulted in changes in DA efflux (1-way repeated measure analysis of variance (ANOVA) ($F_{2,24} = 16.096$, $p < 0.001$) (Fig 4). Specifically, Quinpirole induced a significant reduction from basal DA levels (Holm Sidak test, $p = 0.07$) and Eticlopride reversed these effects (Holm Sidak test, $p < 0.001$) presumably by competitive binding to the D2 autoreceptor. In the intact striatum, 50 nM of Eticlopride was not only able to reverse the reduction in DA efflux caused by 1uM Quinpirole, but also elevated extracellular DA above normal basal levels (Holm Sidak test, $p = 0.025$). These results are consistent with the classic effects of D2 autoreceptor agonism and antagonism, and show that 1 uM Quinpirole and 50 nM Eticlopride are selective for the D2 autoreceptor.

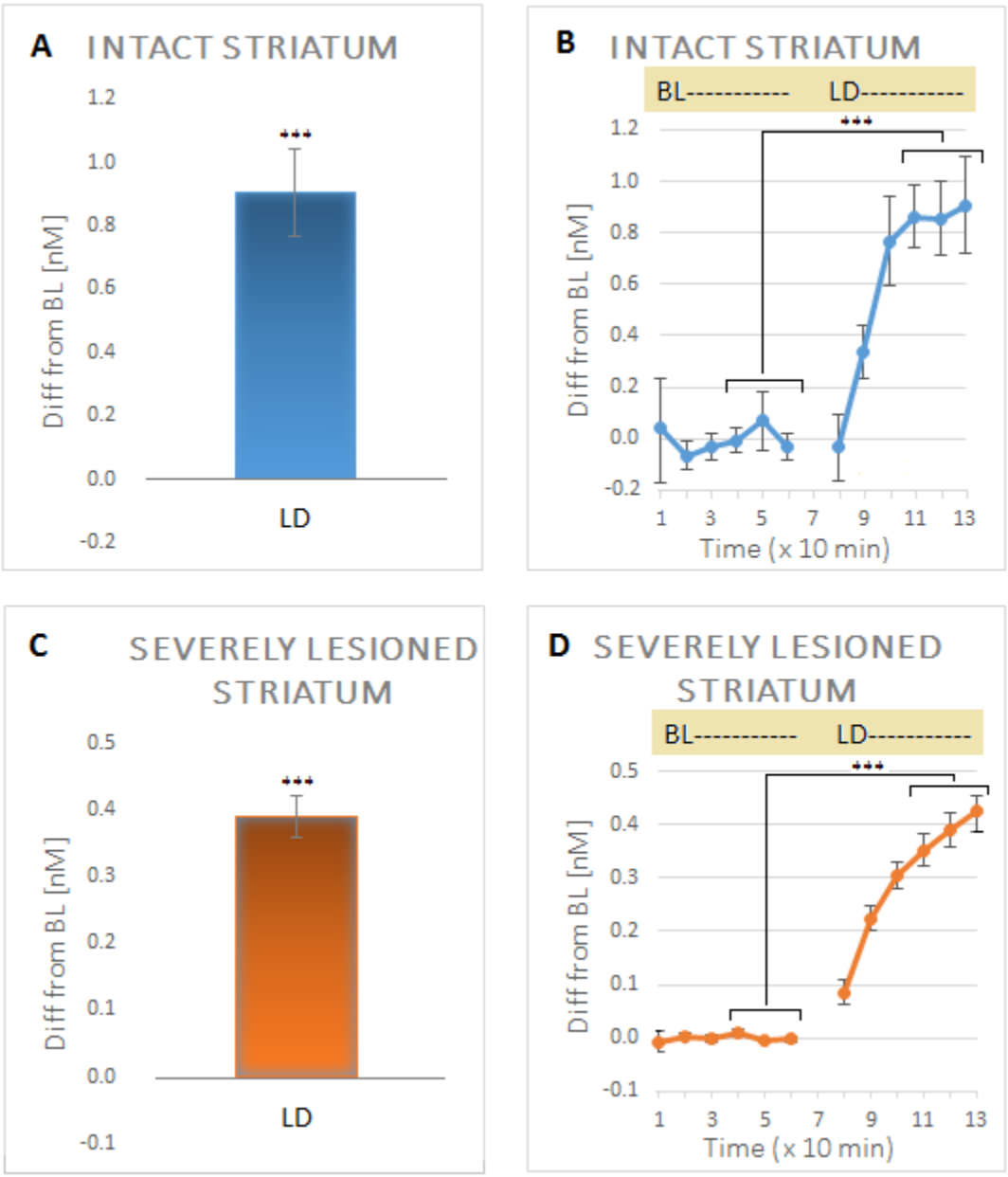


Fig 3. DA efflux in the intact and 6-OHDA denervated striatum after reverse-dialysis of 1 uM L-DOPA (60 min) as measured by HPLC analysis. Data were pooled from all groups of animals from the experiments conducted in this thesis. Bar graphs represent the mean increase in striatal DA efflux measured in last 3 dialysate samples of each drug manipulation (mean ± SEM) in **A.** the intact striatum (n=45) and **B.** the severely denervated striatum (n=35). **C + D.** Time-dependent changes in DA response to the pharmacological manipulations are plotted for the intact and severely denervated striatum respectively. Data points represent mean ± SEM of the dialysate samples collected at that 10 minute interval. LD = L-DOPA. *** $p \leq 0.001$ denotes significance to the pre-drug baseline.

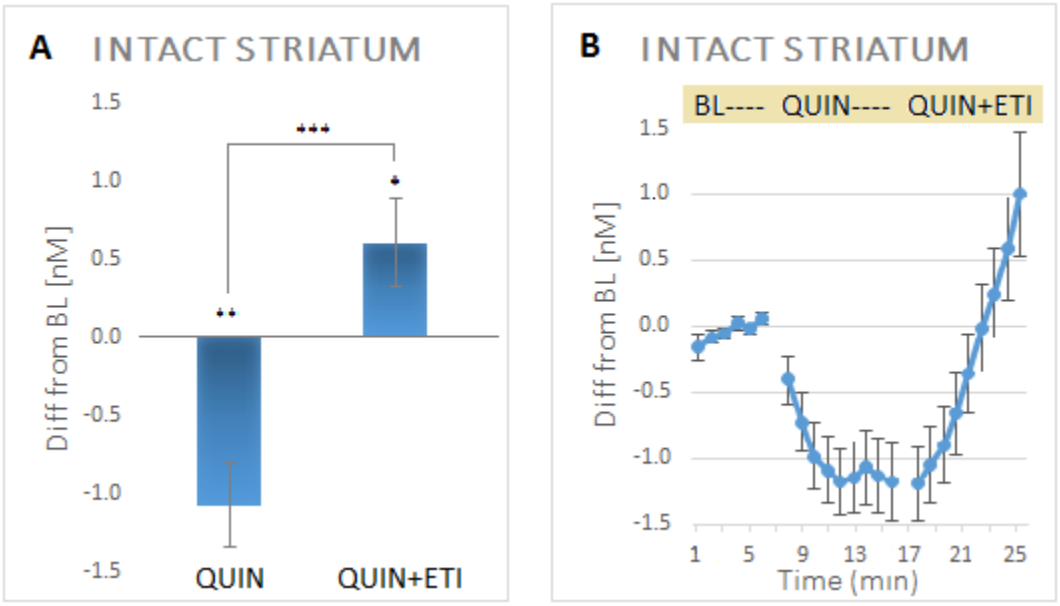


Fig 4. Changes in DA efflux in the intact striatum after the reverse-dialysis of 1 uM Quinpirole (90 minutes), followed by the reverse-dialysis of 1 uM Quinpirole + 50 nM Eticlopride (90 minutes) (n=12) as measured by HPLC analysis. **A**. Bar graphs represent the average of the striatal DA efflux values measured in last 3 dialysate samples of each drug manipulation (mean ± SEM) **B**. Time-dependent changes in DA response to these pharmacological manipulations expressed as concentration difference from baseline. Data points represent mean ± SEM of the dialysate samples collected at that 10 minute interval. BL = Baseline, QUIN = Quinpirole, ETI = Eticlopride. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ denotes significance to the pre-drug baseline, or when indicated, a particular drug treatment.

3.1.3 L-DOPA-derived DA neurotransmission is regulated by the D2 autoreceptor in the normal striatum, but not in the severely denervated striatum

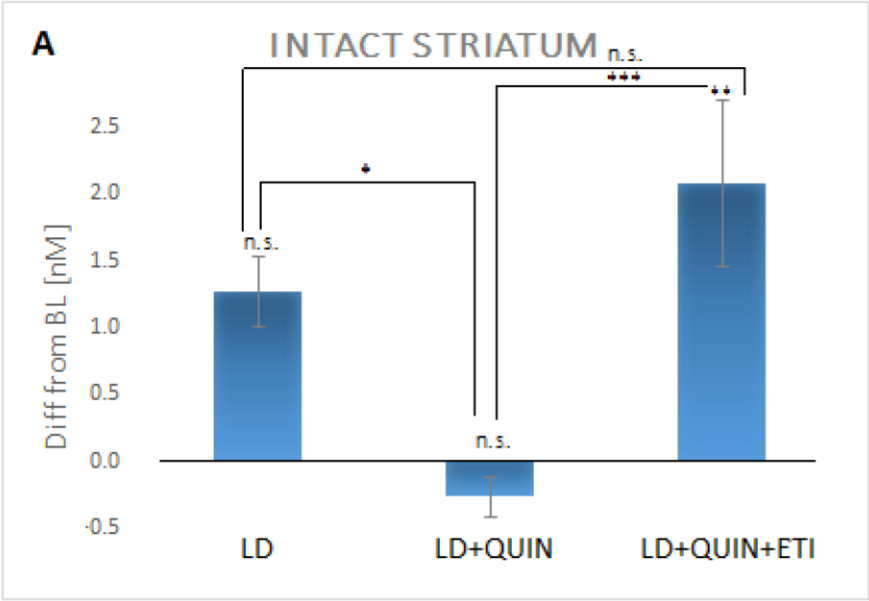
In the normal intact striatum (categorized by basal DA levels of > 2 nM), 1-way RM ANOVA revealed an effect of L-DOPA, Quinpirole and Eticlopride treatment on the striatal DA response within the intact group ($F_{3,39} = 8.857$, $p < 0.001$)(Fig 5A). Reverse-dialysis of L-DOPA caused an increase in extracellular DA (+1.08 ± 0.22 nM difference from baseline). Holm Sidak post-hoc analysis, however, showed that the rise in DA signal from L-DOPA treatment just failed to reach statistical significant ($p = 0.06$). Inclusion of Quinpirole in addition to L-DOPA resulted in a significant reduction of the L-DOPA-derived DA signal ($p = 0.021$) to slightly below baseline levels (-0.23 ± 0.12 nM diff from baseline). Finally, infusion of Eticlopride resulted in a marked increase in extracellular DA ($p < 0.001$) that reversed the attenuation caused by Quinpirole and eventually extracellular DA levels were attained that exceed those induced initially by L-DOPA alone.

1-way RM ANOVA confirmed statistical significant in the changes in DA efflux in response to the reverse dialysis of L-DOPA, Quinpirole and Eticlopride ($F_{3,9} = 10.695$, $p = 0.003$) in the moderately lesioned striatum (characterized by basal DA levels of 0.5 - 1.0 nM)(Fig 5B). 1 uM of L-DOPA reverse-dialyzed into the moderately lesioned striatum induced an increase in DA efflux of +0.36 ± 0.05 nM from baseline ($p = 0.018$). This effect of L-DOPA was then completely attenuated by Quinpirole ($p = 0.012$) to values very close to basal levels (-0.05 ± 0.05 nM difference from baseline). Finally, the addition Eticlopride reversed the inhibitory effect of Quinpirole ($p = 0.011$), and extracellular DA levels returned to those induced by L-DOPA alone.

In the severely lesioned striatum (categorized by basal DA levels of ≤ 0.5 nM), reverse-dialysis of 1 uM L-DOPA induced a statistically significant increase in DA levels (+0.45 ± 0.06 nM above baseline (1-way RM ANOVA: $F_{3,24} = 74.080$, $p < 0.001$, Holm Sidak test, $p < 0.001$)(Fig 5C). The

addition of 1 uM Quinpirole had no effect on the L-DOPA-derived increase in DA efflux ($p = 0.704$), and competitive inhibition at the D2 receptor by 50 nM Eticlopride provided no further effect on the L-Dopa induced increase in DA efflux ($p = 0.575$).

These results confirm the often observed significant increase in DA efflux following administration of L-DOPA into the dorsal striatum. Importantly, the present experiments show clearly that this L-DOPA-induced increase in DA efflux can be modulated by activation or inhibition of the D2 autoreceptor in the intact, normal striatum, and also in the moderately lesioned striatum, albeit to a lesser degree. However, in the severely denervated striatum the L-DOPA-derived increase DA efflux is impervious to D2 autoreceptor regulation.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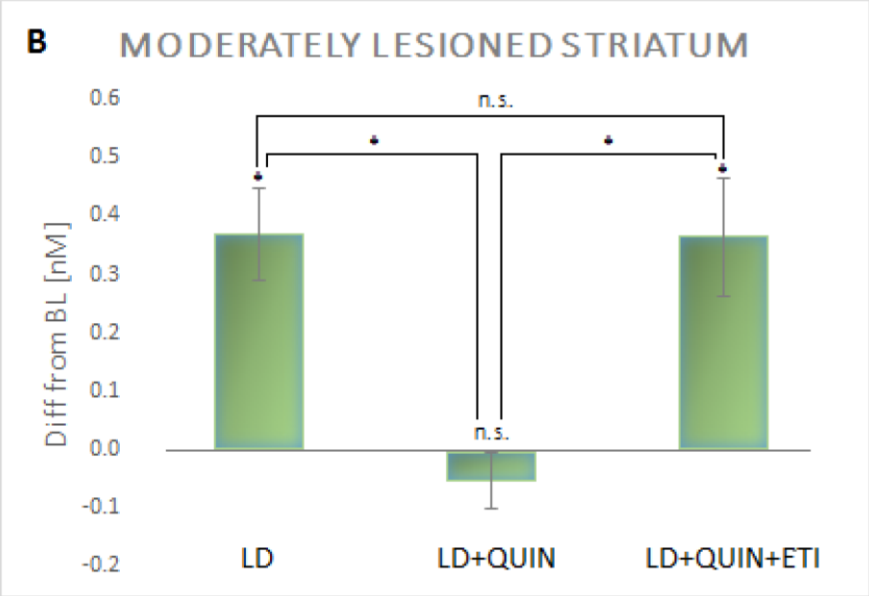
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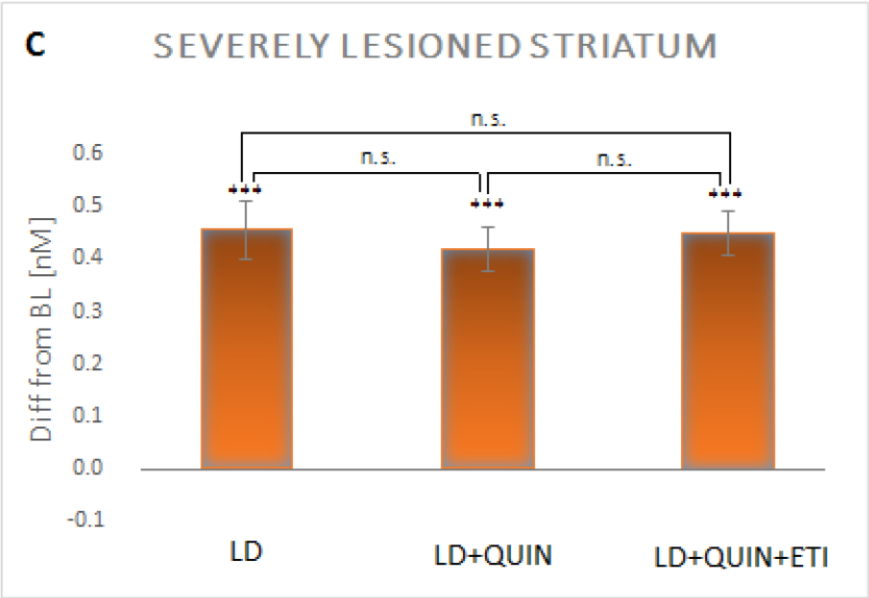


Fig 5. Effect of the direct D2 autoreceptor agonist Quinpirole (QUIN) followed and subsequent antagonism by Eticlopride on the L-DOPA-induced increases in DA efflux in the intact and 6-OHDA denervated striatum as measured by HPLC analysis. Changes in the striatal DA signal were measured after 1 uM L-DOPA was reverse-dialyzed (60 min), followed by 1 uM L-DOPA + 1 uM Quinpirole (60 min), and finally, 1 uM L-DOPA + 1 uM Quinpirole + 50 nM Eticlopride (60 min, all by reverse-dialysis). Bar graphs represent the average of the striatal DA response measured in the last 3 dialysate samples of each drug manipulation (mean ± SEM) in **A.** the intact striatum (n=14) **B.** the moderately denervated striatum (n=7) and **C.** the severely denervated striatum (n=10). LD = L-DOPA, QUIN = Quinpirole, ETI = Eticlopride. n.s. = non significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, denotes significance to the predrug baseline, or when indicated, a particular drug treatment.

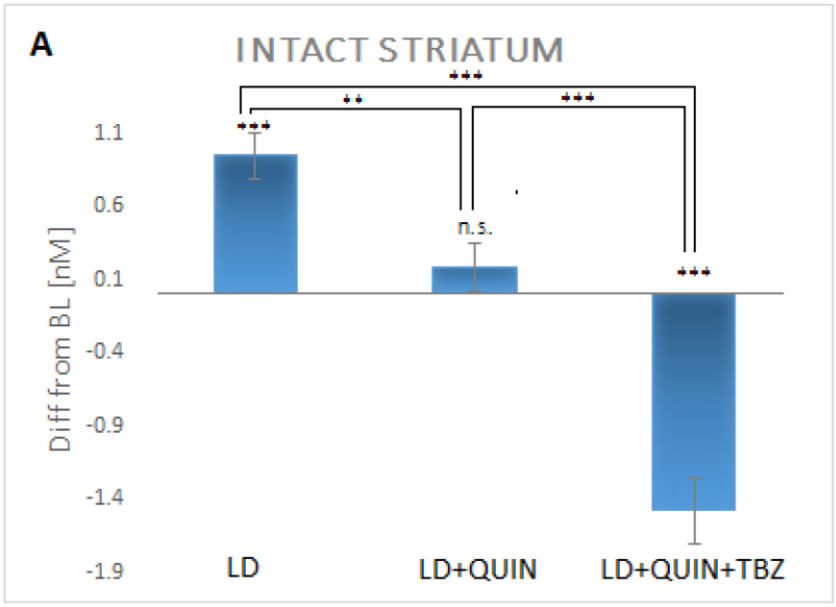
3.1.4 D2 autoreceptor regulation of L-DOPA-derived DA neurotransmission is attenuated by Tetrabenazine in the intact striatum, but not in the denervated striatum

Effect of pharmacological treatment on DA efflux in the intact striatum were analyzed by 1-way RM ANOVA (DA: $F_{3,42} = 31.605$, $p < 0.001$). As observed previously, reverse dialysis of 1 uM L-DOPA resulted in a significant increase in efflux of DA (+0.97 ± 0.16 nM difference from baseline, $p = 0.001$) that was completely reversed to basal levels by 1 uM Quinpirole (+0.18 ± 0.22 nM difference from baseline, $p = 0.008$)(Fig 6A). Administration of 20 uM Tetrabenazine to the striatum in the continued presence of L-DOPA, was accompanied by an immediate and pronounced augmentation of the extraneuronal DA signal, followed by the gradual attenuation to below basal levels recorded prior to L-DOPA treatment(1.48 ± 0.24 nM difference from BL, $p < 0.001$ when compared to basal levels of DA).

1-way RM ANOVA ($F_{3,30} = 37.352$, $p < 0.001$) confirmed that the sequential infusion of 1 uM L-DOPA, 1 uM QUIN and 20 uM Tetrabenazine also affected extracellular DA levels in the severely lesioned striatum (Fig 6B). L-DOPA induced an in extracellular DA efflux (+0.41 ± 0.05 nM difference from BL, $p < 0.001$) that was unaffected by the addition of Quinpirole (+0.51 ± 0.07 nM difference from BL; $p = 0.130$). The addition of 20 uM Tetrabenazine to L-DOPA and Quinpirole reversed the small augmentation of the DA signal by Quinpirole ($p = 0.05$), returning DA efflux to values observed with L-DOPA alone.

These results demonstrate that in the normal striatum, the L-DOPA-derived DA neurotransmission can be completely eliminated by Tetrabenazine mediated inhibition of VMAT2 and its effects on vesicular sequestration and depletion of the vesicular stores. However, in the severely denervated striatum, the L-DOPA-derived DA efflux is unaffected by either Tetrabenazine inhibition of the vesicular monoamine transporter (VMAT2), or pharmacological modulation of the

D2 autoreceptor. Together these findings suggest that in the severely denervated striatum, the increase in DA efflux resulting from L-DOPA treatment is not derived from vesicular-mediated neurotransmission.



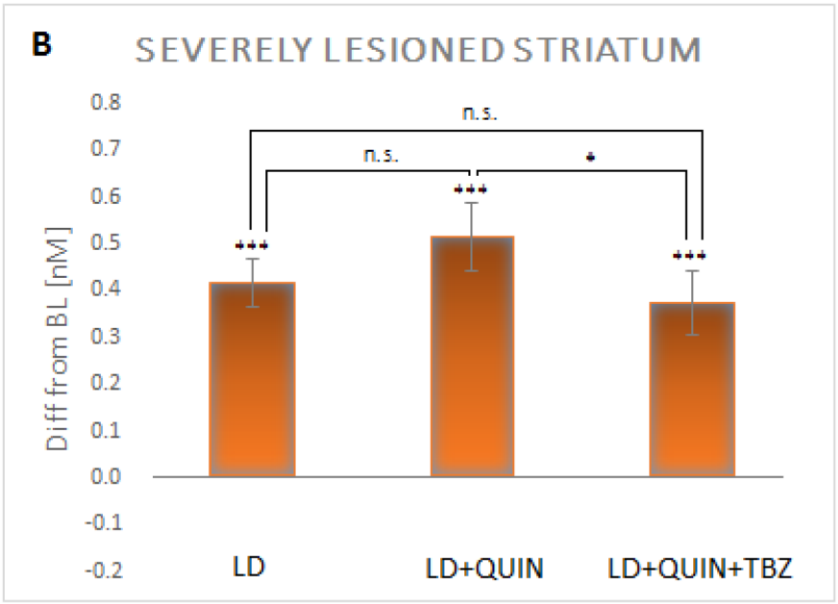


Fig 6. Effect of direct D2 autoreceptor agonism and VMAT2 inhibition on the L-DOPA-derived DA efflux in the intact and 6-OHDA denervated striatum as measured by HPLC analysis. Changes in the striatal DA signal were measured after 1 uM L-DOPA reverse-dialysis (60 min) followed by reverse-dialysis of 1 uM L-DOPA + 1 uM Quinpirole (60 min), and finally the reverse-dialysis of 1 uM L-DOPA + 1 uM Quinpirole + 20 uM Tetrabenazine (60 min). Bar graphs represent the average of the striatal DA response measured in the last 3 dialysate samples of each drug manipulation (mean ± SEM) in **A.** the intact striatum (n=15) and **B.** the severely denervated striatum (n=11). LD = L-DOPA, QUIN = Quinpirole, TBZ = Tetrabenazine. n.s. = non significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, denotes significance to the pre-drug baseline, or when indicated, a particular drug treatment.

3.1.5 D2 autoreceptor inhibition causes an augmentation of the L-DOPA-derived DA neurotransmission in the normal striatum, but has no effect in the denervated striatum

1-way RM ANOVA revealed a response of the intact brain to both L-DOPA and L-DOPA plus Eticlopride treatment (DA: $F_{2,3} = 10.826$, $p = 0.002$)(Fig 7A). L-DOPA induced an efflux of DA that increased extracellular levels by 0.70 ± 0.22 nM above baseline. This efflux however was not statistically significant ($p = 0.163$). The addition of Eticlopride to L-DOPA then resulted in large augmentation of the L-DOPA-derived DA efflux that was statistically greater than the efflux from L-DOPA alone ($p = 0.02$) at an increase of 2.52 ± 0.77 nM from baseline.

In the severely lesioned striatum, the DA response to the pharmacological treatments indicated a different profile (Fig 7B). The reverse-dialysis of 1 uM L-DOPA induced a statistically significant increase in DA efflux of 0.37 ± 0.10 nM (mean ± SEM) from baseline (1-way RM ANOVA, $F_{2,12} = 19.319$, $p < 0.001$, Holm Sidak test, $p < 0.001$). The addition of a selective D2 autoreceptor antagonist, Eticlopride (50 nM) to L-DOPA, however, did not cause a statistically significant change to the levels of extracellular DA derived from L-DOPA ($p = 0.656$).

While previous studies have investigated the use of Eticlopride on the L-DOPA-derived DA signal, the D2 autoreceptor antagonist had always been administered in the presence of Quinpirole. These results confirm that in the absence of Quinpirole, the administration of a D2 autoreceptor antagonist to the L-DOPA-derived DA efflux induces the expected augmentation of the extraneuronal DA signal, consistent with the effects of D2 autoreceptor inhibition. By contrast, the administration of Eticlopride to the L-DOPA-derived DA efflux did not produce any changes to the extracellular DA signal.

3.2 Role of the D2 autoreceptor in Methylphenidate's ability to increase L-DOPA-derived DA neurotransmission

3.2.1 Pre-inhibition of the D2 autoreceptor completely attenuates Methylphenidate's augmentation of the L-DOPA-derived DA efflux in the severely denervated striatum

1 uM of L-DOPA was administered into the striatum by reverse-dialysis, followed by the addition of either 50 nM Eticlopride or vehicle, after which 20 uM of MPD was administered. A 2-way RM ANOVA revealed an interaction between group x treatment (DA: $F_{3,36} = 5.029$, $p = 0.005$) in the intact group (Fig 8A) (Note: for reasons of visual clarity, only the statistical outcomes that had hypothetical implications were displayed on Fig 8A. For the complete list of statistical comparisons, refer to Table 2). I then compared two groups of intact (6-OHDA-naïve) animals given MPD – with or without pretreated with Eticlopride. The non Eticlopride-pretreated and Eticlopride-pretreated groups showed similar DA responses following 1 uM L-DOPA administration (0.62 ± 0.70 nM vs 0.70 ± 0.20 nM increase from baseline, respectively). This L-DOPA-induced DA increase did not differ significantly from basal levels of DA. Following 1 hour of L-DOPA reverse-dialysis, one of the intact groups received Eticlopride by reverse-dialysis along with L-DOPA. The other intact group continued to receive L-DOPA only. L-DOPA + Eticlopride caused an augmentation of L-DOPA-derived DA (+2.52 ± 0.68 nM from baseline) that differed greatly from the effects of L-DOPA alone, but were non significant (+0.30 ± 0.73 nM from baseline). In control subjects not treated with Eticlopride, MPD caused an 35.88 ± 2.13 nM augmentation of the L-DOPA-derived DA efflux ($p < 0.001$, within group comparisons), whereas the presence of Eticlopride induced an even larger augmentation of the L-DOPA-derived DA efflux by MPD (51.69 ± 5.01 nM, $p < 0.001$, within group comparisons). The Holm Sidak post-hoc test showed that there was a significantly greater DA response to MPD in the presence than in the absence of Eticlopride pretreatment ($p < 0.001$, between group comparisons). The greater than 20-fold increase in the DA signal from the Methylphenidate phase of the drug

treatment would obscure the ability to gain statistical insight from the much smaller, but still important changes to the DA signal after L-DOPA and L-DOPA+Eticlopride/vehicle administration. Thus, these first two phases of drug treatment were isolated from the Methylphenidate phase and examined separately by 2-way RM ANOVA. While the DA efflux post L-DOPA administration was non significant in either groups, Holm Sidak post hoc analysis confirmed that the addition of Eticlopride to L-DOPA caused a statistically significant increase both within group ($p = 0.008$) and between group ($p = 0.004$) (Fig 8B).

The response of the severely lesioned groups of animals to Eticlopride and MPD gave a slightly different profile (interaction of group x treatment: $F_{3,36}$ = (DA: $F_{3,36} = 3.014$, $p = 0.043$)(Fig 8C)(Note: for reasons of visual clarity, only the statistical outcomes that had hypothetical implications were displayed on Fig 8C. For the complete list of statistical outcomes, refer to Table 3). In the control group without Eticlopride pretreatment, L-DOPA caused a sustained increase in extracellular DA (+0.29 ± 0.08 nM from baseline) that was further augmented by the addition of MPD (+1.95 ± 0.76 nM from baseline, Holm Sidak test, $p < 0.001$). In the group pretreated with Eticlopride, L-DOPA caused a similar increase in extracellular DA (+0.38 ± 0.09 nM from baseline). The reverse dialysis of 50 nM Eticlopride in combination with 1 uM L-DOPA, did not alter extracellular levels of L-DOPA-derived DA (L-DOPA only: +0.38 ± 0.09 nM vs. L-DOPA+Eticlopride: 0.41 ± 0.09 nM difference from baseline, $p = 0.932$, n.s.). Note: This is different from the intact striatum where Eticlopride caused a massive augmentation of the L-DOPA-derived DA efflux. However, although Eticlopride did not influence the L-DOPA-derived DA efflux in the denervated brain, the addition of Eticlopride before and during the administration of a DAT blocker completely prevented the MPD augmentation of extracellular DA ($p = 0.002$, between group comparisons)(Fig 8C). As it was difficult to gain understanding of the response of the severely denervated striatum to

L-DOPA and L-DOPA+Eticlopride/vehicle in light of the huge variance from the MPD treatment, similar to for the intact brain, the first two phases of drug treatment were isolated from the Methylphenidate phase and examined by a separate 2-way RM ANOVA. 2-way RM ANOVA revealed that as there was no statistical difference between the two severely lesioned groups with respect to their basal levels of DA, response to L-DOPA and response to L-DOPA+Eticlopride/vehicle. The two groups were then combined to confirm that overall, there was a statistical increase in DA with the administration of exogenous L-DOPA ($p < 0.001$) that did not change statistically with the administration of L-DOPA+Eticlopride/vehicle ($p = 0.076$).

These results show that the D2 autoreceptor plays a role in MPD's ability to increase the L-DOPA-derived DA efflux in the denervated striatum, as pre-inhibition of the D2 autoreceptor is able to completely attenuate the MPD-induced increase in the DA signal.

3.2.2 D2 autoreceptor inhibition has no effect on the L-DOPA-derived DA signal in the denervated striatum

The previous experiments indicated a role for the D2 autoreceptor in mediating MPD's augmentation of the L-DOPA-derived DA efflux in the denervated brain. I then wanted to see if direct D2 autoreceptor agonism could induce an augmentation of the L-DOPA-derived DA signal similar to that of MPD.

The handling and manipulation of the various intact groups of animals used over the course of these experiments with respect was kept consistent, and no statistical difference was seen between their basal DA levels and DA responses to L-DOPA and Quinpirole. Therefore, their data was pooled and analyzed by 1-way RM ANOVA to see whether Quinpirole could augment the L-DOPA-derived DA signal. 1-way RM ANOVA indicated an effect of treatment (DA: $F_{2,56} = 30.594$, $p <$

0.001). Reverse-dialysis of 1 uM L-DOPA produced a significant increase 1.02 ± 0.15 nM difference from baseline that was not augmented, but instead significantly attenuated by the addition of 1 uM Quinpirole ($P < 0.001$)(Fig 9A).

Similarly, after combining the data from the severely lesioned animals, 1-way RM ANOVA showed an effect of treatment (DA: $F_{2,38} = 106.109$, $p < 0.001$). L-DOPA was able to induce an increase in the extracellular DA signal (0.43 ± 0.04 nM difference from baseline, $p < 0.001$). Holm Sidak post hoc analysis, however, confirmed that even in the denervated brain, direct stimulation of the D2 autoreceptor by Quinpirole could not cause a statistically significant augmentation of the L-DOPAderived DA signal ($p = 0.291$)(Fig 9B).

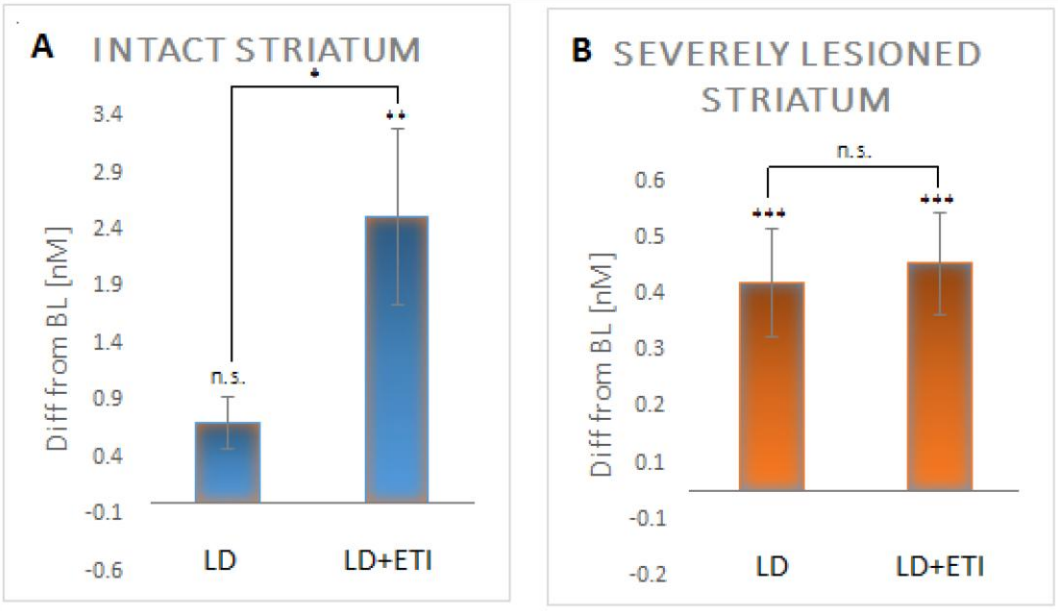


Fig 7. Effects of D2 autoreceptor inhibition on the L-DOPA-derived DA efflux in the intact and 6-OHDA denervated striatum. Bar graphs represent the average of the striatal DA response measured in the last 3 dialysate samples of each drug manipulation (mean ± SEM) in **A.** the intact striatum and **B.** the severely denervated striatum after 1 uM L-DOPA reverse-dialysis (60 min); subsequent reverse-dialysis of 1 uM L-DOPA + 50 nM Eticlopride. All results are expressed as a concentration difference from baseline in nM. LD = L-DOPA, ETI = Eticlopride. n.s. = non significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, denotes significance to the pre-drug baseline, or when indicated, a particular drug treatment.

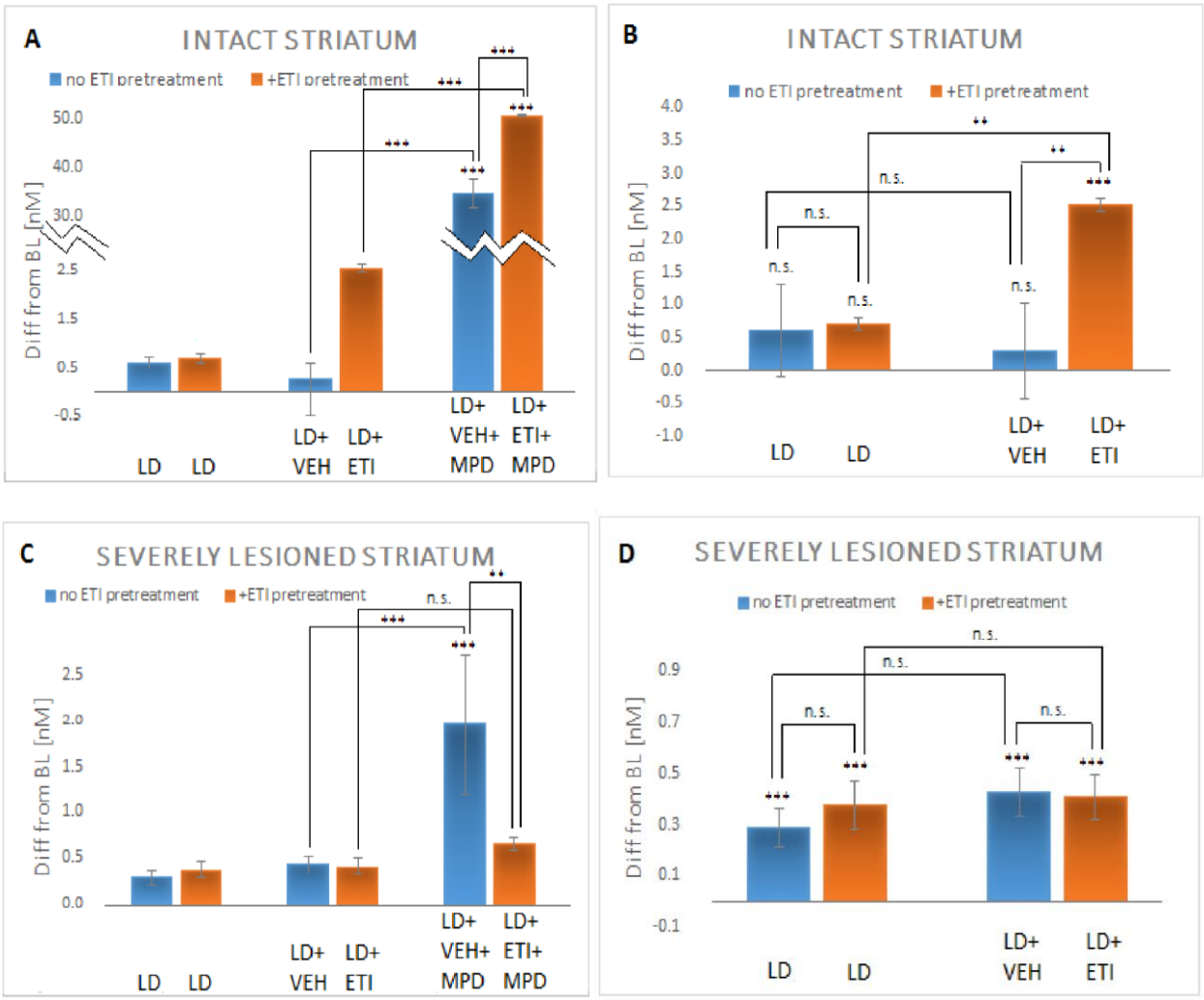


Fig 8. The effect of D2 autoreceptor antagonism on the Methylphenidate-induced augmentation of the L-DOPA derived DA efflux in the intact striatum and 6-OHDA denervated striatum. Note that 1 uM L-DOPA was administered via reverse dialysis throughout each experiment. Changes in the striatal DA signal were measured after 1 uM L-DOPA reverse-dialysis (60 min) followed by 50 nM Eticlopride (60 min or vehicle pretreatment (60 min). The final manipulation involved reverse-dialysis of 1 uM L-DOPA + 20 uM Methylphenidate (60 min) (blue), or 1 uM L-DOPA (60 min) followed by 1 uM L-DOPA + 50 nM Eticlopride (60 min) or vehicle, pretreatment prior to the addition of 20 uM Methylphenidate reversedialysis (60 min)(red). Bar graphs represent the average of the striatal DA response measured in the last 3 dialysate samples of each drug manipulation (mean ± SEM) in **A.** the intact striatum (N = 16: No ETI pretreatment n = 6, with ETI pretreatment n = 10) and **C.** the severely denervated striatum (N = 14: No ETI pretreatment n = 7, with ETI pretreatment n = 7). **B and D.** The DA response to the first two phases of drug treatment (LD followed by LD+ETI/VEH) were then isolated and analyzed via a separate 2-way RM ANOVA. LD = L-DOPA, VEH = Vehicle, ETI = Eticlopride, MPD = Methylphenidate. n.s. = non significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, denotes significance to the pre-drug baseline, or when indicated, a particular drug treatment.

Table 1. List of statistical outcomes for the effect of D2 autoreceptor antagonism on the Methylphenidate-induced augmentation of L-DOPA-derived DA efflux in the intact striatum. Results are from Holm Sidak post hoc analysis showing all pairwise multiple comparisons. ($F_{3,36} = 5.029$, $p = 0.005$ by 2-way RM ANOVA) (N = 16: No ETI pretreatment n = 6, with ETI pretreatment n = 10)

Comparisons of Drug Treatment within Group on Striatal DA levels					
for the group pretreated with ETI (Group 1):			**for the group not pretreated with ETI (Group 2):**		
Comparisons	**P**	**P < 0.05**	**Comparisons**	**P**	**P < 0.05**
LD+ETI+MPD vs. BL	<0.001	Yes	LD+VEH+MPD vs. BL	<0.001	Yes
LD+ETI+MPD vs. LD	<0.001	Yes	LD+VEH+MPD vs. LD	<0.001	Yes
LD+ETI+MPD vs. LD+ETI	<0.001	Yes	LD+VEH+MPD vs. LD+VEH	<0.001	Yes
LD+ETI vs. BL	0.810	No	LD+VEH vs. BL	0.958	No
LD+ETI vs. LD	0.814	No	LD+VEH vs. LD	0.995	No
LD vs. BL	0.818	No	LD vs. BL	0.999	No
Comparisons of Group within Drug Treatment on Striatal DA levels					
Comparisons	**P**	**P < 0.05**			
Group 1 vs. Group 2 at BL	0.969	No			
Group 1 vs. Group 2 at LD	0.980	No			
Group 1 vs. Group 2 at LD+ETI/VEH	0.529	No			
Group 1 vs. Group 2 at LD+ETI/VEH+MPD	<0.001	Yes			

group 1 = intact group pretreated with Eticlopride; group 2 = intact group not pretreated with Eticlopride

BL = Baseline; LD = L-DOPA; ETI = Eticlopride; VEH = Vehicle; MPD = Methylphenidate

Table 2. List of statistical outcomes for the effect of D2 autoreceptor antagonism on the Methylphenidate-induced augmentation of L-DOPA-derived DA efflux in the 6-OHDA severely denervated striatum. Results are from Holm Sidak post hoc analysis showing all pairwise multiple comparisons. ($F_{3,36}$ = 3.014, p = 0.043 by 2-way RM ANOVA) (N = 14: No ETI pretreatment n = 7, with ETI pretreatment n = 7)

Comparisons of Drug Treatment within Group on Striatal DA levels					
for the group pretreated with ETI (Group 1):			**for the group not pretreated with ETI (Group 2):**		
Comparisons	**P**	**P < 0.05**	**Comparisons**	**P**	**P < 0.05**
LD+ETI+MPD vs. BL	0.481	No	LD+VEH+MPD vs. BL	<0.001	Yes
LD+ETI+MPD vs. LD	0.863	No	LD+VEH+MPD vs. LD	<0.001	Yes
LD+ETI+MPD vs. LD+ETI	0.788	No	LD+VEH+MPD vs. LD+VEH	0.001	Yes
LD+ETI vs. BL	0.832	No	LD+VEH vs. BL	0.620	No
LD+ETI vs. LD	0.932	No	LD+VEH vs. LD	0.723	No
LD vs. BL	0.811	No	LD vs. BL	0.707	No
Comparisons of Group within Drug Treatment on Striatal DA levels					
Comparisons	**P**	**P < 0.05**			
Group 1 vs. Group 2 at BL	0.992	No			
Group 1 vs. Group 2 at LD	0.824	No			
Group 1 vs. Group 2 at LD+ETI/VEH	0.965	No			
Group 1 vs. Group 2 at LD+ETI/VEH+MPD	0.002	Yes			

group 1 = severely lesioned group pretreated with Eticlopride; group 2 = severely lesioned group not pretreated with Eticlopride

BL = Baseline; LD = L-DOPA; ETI = Eticlopride; VEH = Vehicle; MPD = Methylphenidate

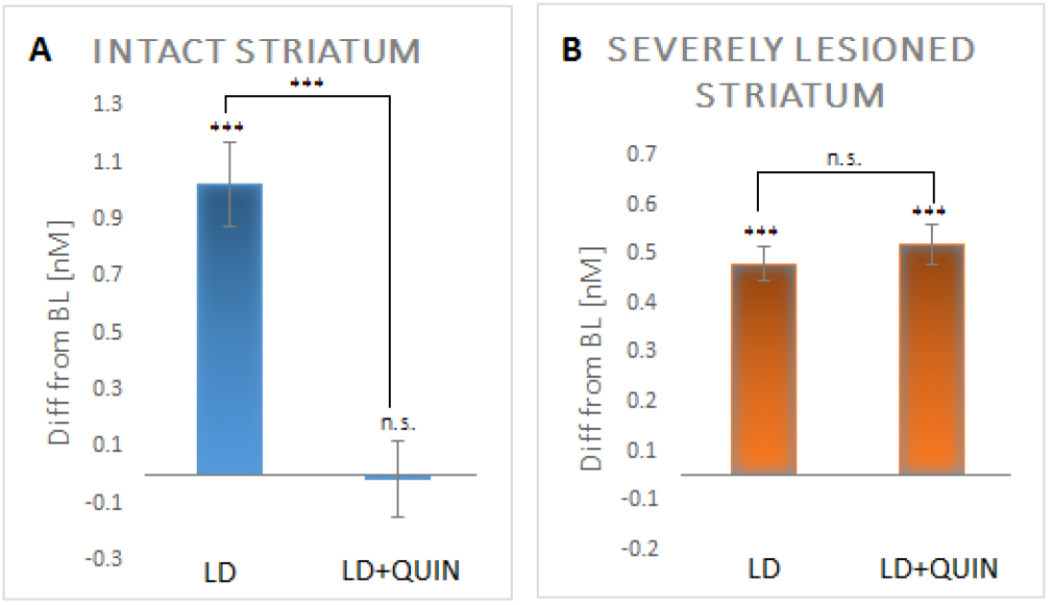


Fig 9. Effects of direct D2 autoreceptor agonism on the L-DOPA-derived DA efflux in the intact and 6OHDA denervated striatum as measured by HPLC analysis. Changes to striatal DA levels were measured after 1 uM L-DOPA was reverse dialyzed into the striatum (60 min), followed by the addition of 1 uM Quinpirole to the L-DOPA (reverse-dialysis, 60 min). Data was pooled from the various groups of animals used over the course of the experiments for this thesis. Bar graphs represent the average of the striatal DA response measured in last 3 dialysate samples of each drug manipulation (mean ± SEM) in **A.** the intact striatum (n=29) and **B.** the severely denervated striatum (n=21). LD = L-DOPA, QUIN = Quinpirole. n.s = non significant *** $p \leq 0.001$ denotes significance to the pre-drug baseline, or when indicated, a particular drug treatment.

4. Discussion

4.1 Relationship between striatal lesion severity and stages of Parkinson's disease

Three categories of striatal lesion severity were examined in this thesis: the normal (6-OHDA-naïve) striatum, the moderately denervated striatum and the severely denervated striatum. The intact, normal striatum measured basal DA levels of 3.15 ± 0.52 nM (mean ± SEM). Previous calibration data indicate a probe recovery of 40% for DA, and accordingly, the corrected estimate of the basal levels of DA in the intact dorsal striatum is 8 nM. This is similar to other studies, where *in vivo* sampling of the steady-state basal levels of DA in the striatum fall between 4-20 nM, when measured by microdialysis and voltammetry (Parsons et al, 1991; Jones et al, 1998; Chen, 2005). The moderately lesioned and severely lesioned striatum in our experimental subjects had uncorrected basal DA levels of 0.83 ± 0.05 nM (mean ± SEM) and 0.20 ± 0.04 nM (mean ± SEM), respectively. Corrected values were 2 nM and 0.5 nM. This corresponded to an approximate 74% reduction of normal basal DA in the moderately lesioned striatum, and an approximate 94% reduction in the severely denervated striatum. As Parkinson's disease is clinically diagnosed after DA levels in the striatum have reduced to 60-70% of normal values (Schapira et al, 2014), our 'moderately lesioned striatum' corresponds to the initial stages of the disease. Denervation becomes increasingly more severe as PD progresses, continuing until more than 95% of the dopaminergic neurons are lost (Schapira 2014; Bernheimer et al, 1973). And thus, using basal DA levels as an estimate of the extent of denervation, our 'severely denervated striatum', with a 94% reduction in basal DA, was used to model the end stages of the disease.

As discussed in section 1.3, nigrostriatal denervation induces changes to the ability of DA neurons to regulate intracellular and extracellular DA. Not only are compensatory mechanisms engaged, the nature of these compensatory mechanisms differ depending on the stage of the

disease and DA denervation (Narang and Wamsley, 1995). Therefore one cannot assume that the denervated striatum functions and responds to various pharmacological manipulations in the same way as the normal, intact striatum. Accordingly, lesion severity is an important variable in the present thesis and neurochemical and pharmacological data are analyzed according to this factor whether an individual subject was assigned to the intact, partially denervated and severely denervated striatum condition.

4.2 Selective manipulation of the D2 autoreceptor by Quinpirole and Eticlopride

In the intact striatum condition, the D2 autoreceptor agonist 1 uM Quinpirole caused a reduction in basal DA efflux. In contrast, administration of the D2 autoreceptor antagonist Eticlopride (50 nM) and reversed the effect of Quinpirole and DA efflux values rose above baseline measures. These effects are consistent with classical D2 autoreceptor agonism and antagonism, and seem to suggest that at the doses chosen, Quinpirole and Eticlopride are selective for the D2 autoreceptor.

The DA autoreceptor is a pre synaptic receptor that maintains extracellular levels of DA within an optimal physiological range consistent through a negative feedback loop. It is activated when extracellular DA levels are sufficiently large enough to diffuse out of the synaptic cleft to reach the autoreceptor to enable its activation. Activation of the autoreceptor then, by substrates, such as DA or Quinpirole, can inhibit vesicular mediated release of DA by reduction of the firing rate, synthesis and depolarization-evoked release of DA from the presynaptic dopaminergic terminals (Benkert et al, 1992; Lehmann et al, 1983). The ability of Quinpirole to attenuate extracellular l DA levels and decrease in locomotor activity has been attributed to binding preferentially to the presynaptic D2R over the postsynaptic D2R (Usiello et al, 2000). Also, at low doses, DA receptor

agonists have a 10-fold higher affinity for the D2 autoreceptor over its functionally different, post-synaptic isoform (Lehmann et al, 1983). Thus, our observed attenuation of basal levels of DA after Quinpirole administration is consistent with autoreceptor function, and indicates that at the dose employed it is selective for the presynaptic D2 autoreceptor under the present experimental conditions.

When synaptic dopamine concentrations are low, or when the D2 autoreceptor is inhibited, the autoreceptor engages mechanisms to enhance the extracellular DA levels including augmenting DA release (Imperato and Di Chiara, 1984) as well as inhibiting DA reuptake (Wu et al, 2002). Therefore, the ability of 50 nM of Eticlopride to increase the extracellular DA signal is consistent with the effects of antagonism at the D2 autoreceptor. Furthermore, as Eticlopride could antagonize the effects of Quinpirole, can reverse the attenuation of basal DA induced by D2 autoreceptor agonism, provides further indication that Eticlopride and Quinpirole can bind competitively at the same target. Indeed, it seems likely that Eticlopride and Quinpirole interact with the D2 autoreceptor at the same binding site, as both competitively inhibit [3H]spiperone binding to the receptor (Tang et al, 1994). Eticlopride has a higher affinity for the D2 autoreceptor (Ki = 0.09 nM)(Martelle and Nader, 2008) than Quinpirole (Ki = 1.5 nM)(Levant et al, 1992). Therefore it is not surprising that 50 nM Eticlopride was more potent than 1 uM Quinpirole to induce the D2 autoreceptor to mediate DA release.

While Quinpirole has high affinity for the D2 receptor, it is also partially selective for the D3 and D4 receptors (Rodrigo et al, 2011). Similarly, Eticlopride also has high specificity and affinity for the D2 receptor, but may induce less activity at the D3, D4 and alpha-adrenergic receptors (Martelle and Nader, 2008). It is challenging to distinguish the D2 receptor from the D3 and D4 receptors

because of the high sequence homologies (including at the binding site)(Platania et al, 2012), as well as the shared ability to inhibit basal DA levels upon activation (Tang et al, 1994). Therefore, one must recognize the possibility that the effects of 1 uM Quinpirole and 50 nM Eticlopride may be mediated in part by D3 and D4 receptors. However, even if this were the case, the predominant effects exerted by these drugs should be on the D2 autoreceptor, as Quinpirole and Eticlopride are much more selective for the D2R, and dopamine D3 and D4 receptors are present in much lower concentrations in the striatum (Booze and Willliams, 1995; Ariano et al, 1997; Khan et al, 1998).

4.3 L-DOPA-evoked DA release

L-DOPA caused a large efflux of DA in the intact and denervated striatum. These findings are consistent with many previous reports that exogenously administered L-DOPA can induce DA synthesis and enhanced release of DA in both the intact and 6-OHDA striatum. Using the same dose of L-DOPA, the magnitude of DA efflux was much larger in the intact striatum (0.9 nM) than the severely denervated striatum (0.4 nM), and this has been observed both *in vitro* (Sarre et al, 1994; Snyder and Zigmond, 1990; Snyder et al, 1990) and *in vivo* (Xu and Dluzen, 1996). Basal (L-DOPA-naïve) DA levels correspond to the extent of denervation (Xu and Dluzen, 1996, Snyder et al, 1990). Direct comparison of DA levels following L-DOPA administration in the intact brain with the severely denervated brain, revealed drug effects in the 6-OHDA lesion group that were 44% of the regular DA output in response to L-DOPA, despite basal values that were only 6% of the values measured in the intact brain. This discrepancy between the extent of denervation and the L-DOPA-derived DA output has also be reported by other studies (Sarre et al, 1994; Snyder and Zigmond, 1990, Snyder et al, 1990; Xu and Dluzen, 1996). Two possible explanations have been offered to explain this phenomenon: i) the remaining DA neurons compensate by operating at a higher level of activity to ensure elevated DA output, ii) the L-DOPA evoked DA observed after severe denervation may be

derived from non-dopaminergic sources (Zigmond et al, 1990). To these we propose a 3rd possibility: As vesicular storage of DA is compromised after 6-OHDA lesions (Spencer and Wooten, 1984), the DA synthesized from L-DOPA may bypass storage in vesicles and remain in a cytosolic pool from which it can directly efflux from the cell. We will discuss this possibility more thoroughly in the following section.

4.4 Presynaptic D2 autoreceptor regulation of L-DOPA-evoked DA release

As already described, in the intact striatum, L-DOPA caused a large increase in extracellular DA that in turn was reversed to pre-L-DOPA basal values by the D2 agonist Quinpirole. Furthermore, subsequent administration of Eticlopride a D2 autoreceptor antagonist, eliminated the Quinpirole-induced inhibition of L-DOPA mediated DA release. Separately, Eticlopride alone potentiated L-DOPA-evoked increase in DA efflux. The effect of Quinpirole on L-DOPA-derived DA is entirely consistent with classical effects of the autoreceptor mediated inhibition of DA release through mechanisms of hyperpolarization, which in turn would reduce action potentials, inhibition of synthesis and upregulation of clearance. Similarly, the effect of Eticlopride are predictable, on the basis of removal of autoreceptor-mediated inhibition, to increase electrical firing (Saud-Chagny et al, 1991) along with inhibition of reuptake (Wu et al, 2002). Together these findings suggests that in the intact brain, all of the L-DOPA-derived DA is under D2 autoreceptor regulation. Tetrabenazine interacts with VMAT2 to deplete DA from the vesicular stores and prevent further vesicular DA sequestration resulting in the inhibition of vesicular-mediated exocytosis. In the intact striatum, Tetrabenazine was able to reduce DA levels to below baseline in the presence of L-DOPA. This effect of a VMAT2 antagonist provides further support for the hypothesis that the L-DOPA-derived DA efflux that is presynaptically regulated by the D2 autoreceptor, is mediated by impulse dependent vesicular release mechanisms characteristic of physiological control of neurotransmitter release.

In the present thesis, significant variance was observed in DA efflux was observed in the intact striatum, after Eticlopride treatment despite a substantial sample size (n=14). This may be due to non-homogeneity of midbrain DA neurons as intrinsic control of firing as well as the responses to D2 autoreceptor regulation differ between subpopulations (Berretta et al, 2010; Liss and Roeper, 2010). However, D2 autoreceptor agonism by Quinpirole treatment alone in the same subjects did not display a similar magnitude of variance. As Eticlopride was mainly infused in the presence of Quinpirole in the present studies, perhaps the competitive agonist/antagonist binding caused shifts between autoreceptor mediated inhibition or activation of DA cell firing. Binding site saturation in the presence of both the D2 agonist and antagonist may also have influenced non D2 autoreceptor targets.

In the moderately lesioned brain, L-DOPA induced an increase in DA efflux (slightly smaller than that observed in the intact brain). As was also the case in the intact striatum, this increase was then attenuated by Quinpirole and then reversed again as Eticlopride potentiated this signal. These findings indicate that with partial denervation, the D2 autoreceptor still functions through classical feedback inhibition and that the remaining D2 autoreceptors are able to exert complete control over the L-DOPA evoked DA. Because the L-DOPA evoked DA could be completely attenuated by activation of the presynaptic D2 autoreceptor, this also indicates that despite substantial terminal loss (approximately 75%), the source of DA from exogenous L-DOPA came entirely from dopaminergic neurons.

A completely different picture was observed in the severely lesioned brain. L-DOPA again induced a significant increase in DA efflux, however this was unaffected by D2 autoreceptor agonism, antagonism or inhibition of VMAT2. These observations are supported by findings *in vitro*,

where although D2 autoreceptor antagonism could augment the L-DOPA-evoked DA signal in intact striatal slices, no change was observed in the 6-OHDA-lesioned striatum (Snyder and Zigmond, 1990). Therefore, we conclude that L-DOPA-derived DA in the severely lesioned striatum is mediated by non-conventional mechanisms of release, as it is unaffected by D2 autoreceptor regulation and is unaffected by disruption of vesicular-mediated neurotransmission.

Several explanations for this apparent reliance on non-conventional mechanisms of release come to mind , including compensatory alterations in D2 autoreceptor function(Dragicevic et al, 2014), insufficient levels of D2 autoreceptor (Seeman and Niznik, 1990), non-dopaminergic sources of DA transmitter release (Navailles et al, 2010). It is also feasible that after synthesis from L-DOPA, this pool of DA is not sequestered into vesicles due to low levels of VMAT2 in the severely lesioned brain (Caudle et al, 2007; Walker et al, 2013; Sossi et al, 2010; Strome et al, 2006). Vesicular storage would then be compromised (Stephen and Wooley, 1999) as low levels of VMAT2 expression are accompanied by greater displacement of DA into the cytosol (Patel et al, 2003). *In vivo* experiments also demonstrate that vesicular-mediated neurotransmission is significantly reduced following a 95% reduction of VMAT2 (Patel et al, 2003). As the D2 autoreceptor only influences vesicular-mediated neurotransmission (Saud-Chagny et al, 1991), a non-vesicular pool of DA would explain the lack of effect of both TBZ and the D2 autoreceptor modulation of L-DOPA derived DA efflux in the severely depleted striatum. Such a non-vesicular sequestered pool of L-DOPA-derived DA is also supported by observations of increased DOPAC efflux in comparison to the intact brain (Zu and Dluzen, 1996) and by the absence of inhibition of DA efflux by a calcium-free medium (Ahn et al, unpublished).

How then, can one explain the mechanism(s) responsible for DA efflux in the severely denervated striatum? Conventionally, two types of release have been described: Exocytotic release mediated via vesicular mechanisms and carrier-mediated release through the reversal of the DAT (Giros et al, 1996; Patel et al, 2003). The efflux observed following severe lesions is unlikely to be mediated through the DAT, as treatment with a DAT blocker failed to inhibit the efflux. Indeed when Dr. Ahn examined the effect of MPD to the L-DOPA-derived DA efflux in the severely denervated striatum, DA efflux was augmented, not attenuated. Furthermore, treatment with both the VMAT2 antagonist Tetrabenazine as well calcium depletion both had significant effects of reversing the dopamine efflux augmented by MPD, without influencing the increase due to L-DOPA alone. This indicates that while the component of L-DOPA-evoked DA efflux augmented by MPD is likely mediated by mechanisms of vesicular-mediated release (exocytosis), DA from L-DOPA alone is effluxing from the cell through mechanisms that are not disrupted by inhibition of DAT reversal or exocytosis. Thus, having eliminated a role for DAT in the efflux of L-DOPA derived DA, one is still left with questions regarding the mechanism of release. Snyder and Zigmond (1990) have also reported that L-DOPA administration induced DA efflux that was calcium-independent and not carrier-mediated in striatal slices. However, this has not been examined further and the mechanism of release is still unknown.

In a related study using vitro stimulation of striatal slices, Zigmond and Snyder (1990) reported that DA from L-DOPA administration was released by the intact striatum via two mechanisms, with 56% being Ca^{2+}-dependent (exocytosis), and the other portion being Ca^{2+} - and DAT – independent. In the 6-OHDA-denervated brain, the release appeared to be mainly Ca^{2+} - and DAT – independent. This is supported by other *in vivo* findings from our lab by Ahn et al (unpublished), and now by the findings reported in the present thesis examining the ability of the

D2 autoreceptor and Tetrabenazine to manipulate the L-DOPA-derived DA efflux. We conclude that L-DOPA-mediated neurotransmission is exocytotic and under D2 autoreceptor control in the intact brain and also under conditions of partial degeneration. However, in the advanced stages of denervation, the D2 autoreceptor can no longer regulate L-DOPA-derived DA. Knowledge of these denervation-dependent changes to the ability to regulate L-DOPA DA outflow may have useful clinical applications.

L-DOPA-evoked DA can also be modulated by MPD (Ahn et al, unpublished), an effect that may involve a role for the D2 receptor (Volz et al, 2007). Dr. Ahn observed that MPD augmented the exocytotic release of L-DOPA-derived DA, a finding that is complimented by the present data showing that exocytotic L-DOPA evoked DA release is fully regulated by presynaptic D2 autoreceptor functions. Therefore, we wanted to see if there was a role for the D2 autoreceptor in mediating the effects of MPD on L-DOPA mediated DA efflux. As our previous results showed that the D2 autoreceptor's ability to modulate L-DOPA-evoked DA is very different between the intact and denervated striatum, we would not be surprised to find differing results between these two conditions.

In the intact striatum, MPD alone induced an augmentation of the L-DOPA-derived DA signal, thereby confirming Dr. Ahn's findings (unpublished). Pretreatment by Eticlopride caused a further augmentation of this signal in the presence of MPD. In the Eticlopride-free group, the magnitude of MPD-induced increase in DA efflux was approximately 35 nM, similar to that observed by Dr. Ahn (unpublished). Dr. Ahn observed an approximate 70% reduction in the MPD-induced DA signal after treatment with Tetrabenazine (unpublished), consistent with its disruption vesicular-mediated release, with the remaining 30% possibly reflecting inhibition of reuptake. In general

terms, this means that of the 35 nM increase caused by MPD, 25 nM may be mediated by MPD acting as a VMAT2 agonist (Sandoval et al, 2003; Volz et al, 2007) thereby facilitating vesicular release, leaving the residual 10 nM increase as a product of DAT blockade. Surprisingly, a combination of L-DOPA/ Eticlopride/MPD/ induced an extracellular signal of approximately 51 nM DA, approximately 16 nM above L-DOPA/vehicle/MPD alone. These data indicate that not only did Eticlopride pretreatment fail to affect MPD's effects on both vesicular-neurotransmission and reuptake inhibition, it also to facilitated an additional increase in DA efflux, above the 2 nM contribution from direct D2 autoreceptor antagonism alone. In the intact brain, the fact that Eticlopride, a D2 autoreceptor antagonist, failed to block MPD's augmentation of L-DOPA-derived DA strongly argues against a role for the D2 autoreceptor in this effect. This conclusion receives further support from the observation that direct D2 autoreceptor activation by Quinpirole failed to augment the L-DOPA-derived DA signal in a manner similar to that produced by MPD.

In the severely lesioned striatum, MPD alone induced a significant augmentation of the L-DOPA-evoked DA signal. Remarkably, pretreatment with Eticlopride completely prevented the augmentation by MPD of the L-DOPA-evoked DA signal. As the D2 autoreceptor is exclusive to the presynaptic dopaminergic neuron, these results confirm that this increase in extracellular DA efflux induced by MPD comes from a dopaminergic source. In light of the significant attenuation of the MPD effects by direct D2 autoreceptor antagonism, the effect of a selective D2 autoreceptor agonist was examined separately. However Quinpirole, alone had no effect on the L-DOPA-evoked DA signal in the severely denervated brain.

We note that in the severely lesioned striatum, there was a large variance in DA response to L-DOPA/MPD. This may be due to the large variances in individual ability to maintain high

extracellular DA levels with denervation greater than 95% (Robinson et al, 1990), and a larger sample size is needed to minimize this. Interestingly, the severely lesioned striatum produced an extraneuronal DA signal that was approximately 5% of the magnitude of the signal produced by the intact striatum in the presence of L-DOPA and MPD. As the severely lesioned striatum contains approximately 5% of the number of DA terminals as the intact striatum, the magnitude of the signal thus seems proportional to the number of remaining DA terminals. This is likely due to the correlational decline in VMAT2 and DAT densities with denervation. Finally, the ability of D2 autoreceptor antagonism to completely attenuate MPD's augmentation of DA signal provides optimism that despite 95% denervation, the remaining autoreceptors can be modulated to provide sufficient regulation of the L-DOPA-evoked DA signal. This may be because instead of declining in function and density with terminal denervation, the D2 autoreceptor has widely been observed to be upregulated in function, expression and sensitivity in the severely denervated rodent model (Dragicevic et al, 2014) and human PD brain (Seeman and Niznik, 1990).

In summary, in the intact brain, the failure of either a D2 antagonist to influence the MPD augmentation of L-DOPA-derived DA efflux, nor a D2 agonist to mimic the MPD augmentation, eliminates the involvement of D2 autoreceptors in this phenomenon. However the situation appears to be very different in the severely denervated striatum, as Eticlopride pretreatment completely blocked MPD's augmentation of the L-DOPA-derived DA efflux. The fact that direct activation of the D2 autoreceptor by Quinpirole failed to duplicate the effect of MPD suggests that the interaction between MPD and the D2 autoreceptor may be restricted to a confirmation induced by binding by an antagonist.

4.5 A model for the D2 autoreceptor-dependent mechanism by which Methylphenidate upregulates vesicular-mediated dopamine neurotransmission

In the severely denervated brain, the D2 autoreceptor is clearly implicated in the clinically-significant augmentation of DA efflux observed following co-administration of L-DOPA and MPD. The most parsimonious way that MPD could engage with the autoreceptor would be through the elevated levels of extrasynaptic DA due to reuptake inhibition (Seeman and Madras, 1998; Volkow et al, 2002; Volz, 2008). However, our observations do not support this model. Instead, we propose that MPD binds to DAT, to induce a coupling between the DAT and the D2 autoreceptor. This then may induce an intracellular signaling cascade that ultimately augments DA efflux through facilitation of VMAT2 activity, vesicular sequestration and release. Prior inhibition of the D2 autoreceptor with a direct antagonist would thereby interrupt the potential for the D2 autoreceptor and the DAT to interact (Fig 10). The failure of direct D2 autoreceptor activation, to mimic the effects of MPD also point to a unique role for direct interaction between DAT blockade and D2 autoreceptor blockade in facilitating the effects of L-DOPA on DA efflux.

The necessity for a direct interaction between the DAT and the D2 autoreceptor to facilitate vesicular-mediated DA release may also explain findings by the Fleckenstein group. They reported in many experiments that although both MPD and Quinpirole could increase vesicular sequestration of DA and vesicular DA content (Sandoval et al 2002; Sandoval et al 2003; Volz et al, 2007; Volz et al, 2008), Quinpirole was unable to induce a similar increase in stimulus-evoked DA neurotransmission (Volz et al, 2008). This may be explained by the absence of a DAT-D2 autoreceptor interaction in the presence of Quinpirole alone.

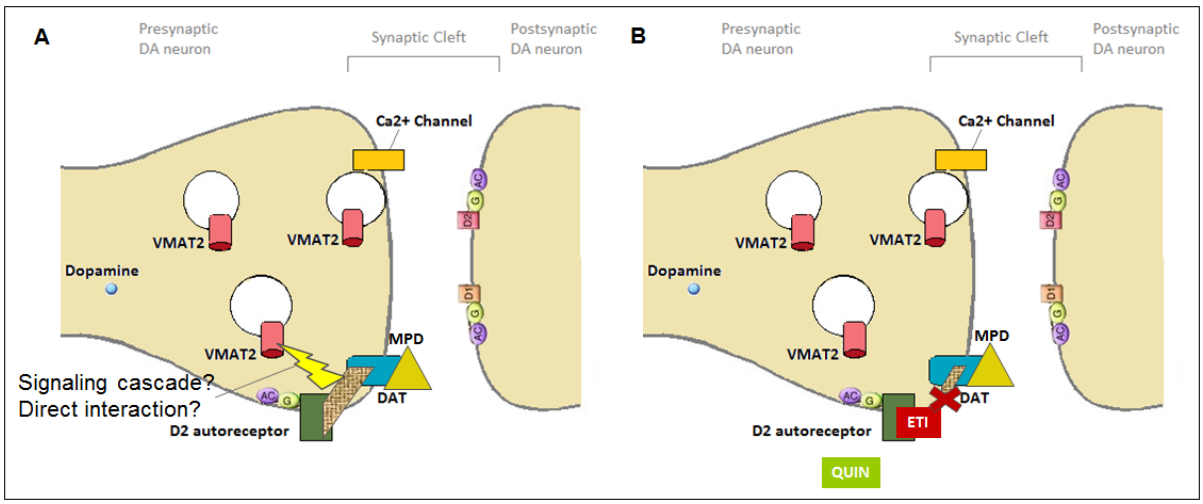


Fig 10. Proposed model for the D2 autoreceptor-dependent mechanism by which MPD upregulates vesicular-mediated dopamine neurotransmission. **A.** MPD (yellow triangle) binds to DAT to induce an interaction (brown bar) between DAT and the D2 autoreceptor. The DAT and D2 autoreceptor interaction subsequently promotes the upregulation of VMAT2 activity and vesicular-mediated release of DA by way of a signaling cascade (or direct interation with VMAT2). **B.** A DAT-D2 autoreceptor interaction is necessary to facilitate MPD's augmentation of the L-DOPA-evoked DA efflux, as prior inhibiton of the D2 autoreceptor by Eticlopride was able to prevent the MPD-induced DA augmentation, possibly by impeding an MPD-induced interaction between DAT and the D2 autoreceptor. Alternatively, the necessity for a DAT-D2 autoreceptor interaction may explain why direct activation of the D2 autoreceptor by Quinpirole was insufficient to induce a similar augmentation of the L-DOPA-evoked DA efflux. ETI = Eticlopride, QUIN = Quinpirole.

DAT is known to rely primarily on post-translational modifications to respond quickly to changes in the extracellular environment (Schmitt et al, 2010). DAT function is regulated by various means, such as substrate/inhibitor binding, presynaptic GPCRS, intracellular signaling cascades, and protein-protein interaction (Schmitt et al, 2010). It is highly documented that DAT and the D2 autoreceptor tightly regulate one another, with alterations in the functions of one protein inducing changes in the functions of its partner (Giros et al, 1996; Jones et al, 1999; Dickinson et al, 1999; Kimmel et al, 2001; Patel et al, 2003; Pei et al, 2004; Torres et al, 2006). As DAT and the D2 autoreceptor are located close to one another (<100 A^{o}), the physical interaction is highly plausible (Schmitt et al, 2010). Significantly, a physical bond has been documented between DAT and the D2 autoreceptor in the striatum *in vitro* (Lee et al, 2007). Here, DAT was shown to bind directly to the D2 autoreceptor through its N-terminus, and the autoreceptor's third intracellular loop (Lee et al, 2007). Interaction with the third intracellular loop induces a variety of intracellular signaling mechanisms (Fukunaga and Shioda, 2012), including calcium/CaM-dependent signaling implicated in regulating neurotransmitter exocytosis (Fukunaga and Shioda, 2012). Although much is still unknown about the conditions related to the binding between DAT and the D2 autoreceptor in the striatum (Lee et al., 2007), it is intriguing to note that once the physical interaction has occurred, application of D2 autoreceptor agonists and antagonists do not cause a dissociation (Lee et al, 2007). Although the ability of MPD to induce a DAT-D2 autoreceptor coupling has yet to be described, certain conditions may facilitate the physical interaction between the DAT and D2 autoreceptor. *In vivo* studies of the human brain, suggest such an interaction occurred during certain memory tasks and magnetic resonance imaging (Schmitt et al, 2010).

Given Dr. Ahn's (unpublished) finding that MPD induced vesicular-mediated neurotransmission in both the intact and 6-OHDA denervated striatum, it is unclear why in the

present studies, Eticlopride failed to block MPD's effects in the intact striatum. Perhaps, this was related to drug dosage. In the intact striatum, the concentration of 50 nM Eticlopride may have inhibited too few of the D2 autoreceptors. With a much higher concentration of 20 uM MPD (of a 10^3-fold magnitude), a significantly larger proportion of the DAT population would have been occupied by MPD and subsequently formed DAT-D2 autoreceptor interactions with the remainder of Eticlopride-unoccupied D2 autoreceptors. In contrast, because of a much lower D2 autoreceptor density, 50 nM Eticlopride may have been sufficient to cause saturation and inhibition of a D2 autoreceptor-DAT interaction in the severely denervated striatum. Alternatively, the enhanced of sensitivity and function of the D2 autoreceptor caused by severe denervation (Dragicevic et al, 2014) may account for the success of Eticlopride in preventing the MPD induced augmentation of L-DOPA mediated DA efflux.

We believe that a unique, intrinsic characteristic of MPD underlies its ability to induce an increase of vesicular NT release, as this is not observed in all DAT blockers (Farnsworth et al, 2009). Volz et al (2008) agree in part with this suggestion, but also attribute MPD's uniqueness to its dual ability to engage both the D2 receptor and the muscarinic receptor. They suggest that heightened extrasynaptic DA levels from reuptake inhibition is sufficient to activate the D2 autoreceptor to promote the sequestration of vesicular DA (Volz, 2008). However, MPD's abilities to bind the muscarinic receptor is necessary for exocytosis of the sequestered DA (Volz et al, 2008), though this connection is currently unclear. The results in our experiments do not refute this possibility, and more work is needed to investigate MPD's mechanism of DA release.

4.6 Limitations and future directions

The results in this thesis were derived exclusively from microdialysis experiments. Microdialysis samples the neurochemical changes in the extracellular space, and does not provide a direct measure of the intracellular changes, limiting our abilities to make direct inferences regarding vesicular DA sequestration and release. Also, the striatum is innervated by various types of cells in addition to dopaminergic neurons, and the neurochemical efflux from these sources may contribute to the dialysate samplings of the extracellular mileu of striatal tissue. Finally, as samples are acquired over a 10 min interval, this limits the time resolution and prevents measurement of more transient changes in DA efflux.

The results of our studies have led us to propose a model by which the MPD may engage the D2 autoreceptor to promote vesicular DA release. More work is needed to assess the validity of this mechanism, including biochemical assays to gain further understanding of MPD-induced interaction between the DAT and D2 autoreceptor. We propose that it is important for these assays to look for this interaction in the intact striatum and 6-OHDA Parkinson's model striatum in the presence and absence of exogenous L-DOPA, as post translational modifications and interactions at the DAT and D2 autoreceptor are highly dependent on denervation and environmental conditions (Chotibut et al, 2012; Dragicevic et al, 2014).

Our results demonstrate that there is a shift in the mechanism of L-DOPA-evoked DA release between early and late stage Parkinson's disease. In early stages of Parkinson's disease, L-DOPA-evoked DA still seems to be released through conventional mechanisms of presynaptically regulated exocytosis, while with severe denervation, the striatum lacks the ability to release DA in this manner. Thus, MPD's ability to promote vesicular-mediated DA release may confer greater

therapeutic benefits in advanced Parkinson's disease, and less so in the early stages of the disease. Future preclinical and clinical studies examining MPD's therapeutic benefits on the motor symptoms of Parkinson's disease should therefore make a distinction between the stages of Parkinson's disease in its subjects. It is tempting to speculate that the inconsistencies in MPD's therapeutic benefits in clinical trials may be, in part, due to this lack of distinction.

4.7 Conclusion

Compensatory changes to the regulation machinery of the presynaptic dopamine neuron have been documented extensively in studies of Parkinson's disease. However, much less is known about the mechanism by which L-DOPA evokes DA release in the degenerating striatum, and whether under conditions of severe 6-OHDA denervation, the release of this L-DOPA-evoked DA can be modulated. Here, we provide novel insights from *in vivo* experiments to whether L-DOPA-derived DA neurotransmission is under presynaptic D2 autoreceptor control in both the normal striatum, as well as in striatum in models of early and advance of Parkinson's disease.

Our results suggest that L-DOPA-evoked DA efflux in the intact striatum operates under full presynaptic control. However, the ability of the D2 autoreceptor to modulate L-DOPA mediated DA efflux in an early stage and late stage model of Parkinson's disease differ dramatically. With 75% loss of dopaminergic terminals, the remaining D2 autoreceptors were still able to exert full control over the release of DA after L-DOPA administration. However, once denervation approaches 95%, the DA efflux from L-DOPA ceases to be released by conventional exocytosis, nor does it seem to efflux through DAT reverse-transport. Most importantly, we found that this L-DOPA-evoked DA efflux ceases to be controlled by presynaptic regulatory mechanisms. The inability of the

presynaptic neuron to regulate or sequester this increase in DA efflux has immediate implications for the adverse response to L-DOPA therapy at advanced stages of Parkinson's disease.

In this context, the novel findings of Ahn et al (unpublished) showing that L-DOPA-derived DA can be pharmacologically manipulated to be released by vesicular-mediated neurotransmission takes on added significance. In particular these findings may have important therapeutic applications, especially when L-DOPA-derived DA efflux in the denervated striatum does not appear to be under physiological regulation. The mechanisms by which Methylphenidate may activate vesicular mediated release are unclear. In this thesis, we described initial investigations of possible mechanism, and found *in vivo* evidence for a role of the presynaptic D2 autoreceptor.

Finally, as MPD has affinity at various binding sites on non-dopaminergic and serotonin neurons (Leonard et al, 2004), and vesicular-mediated release of L-DOPA-derived DA is widely believed to come from these other non-dopaminergic terminals under conditions of severe denervation (Prinz et al, 2013; Navailles et al, 2010), the origin of the MPD-induced augmentation of L-DOPA derived DA efflux observed by Ahn et al (unpublished), remained to be specified. Here, we confirm that despite 95% loss of dopaminergic terminals, the source of vesicular DA efflux originates from the presynaptic DA neuron.

The findings in this thesis that i) the pool of DA synthesized from L-DOPA in an end stage model of Parkinson's disease is no longer under regulatory presynaptic control and ii) there is a novel D2 autoreceptor-dependent mechanism by which this non-controlled pool of L-DOPA synthesized DA may be pharmacologically manipulated into a controllable, vesicular pool, present important new therapeutic considerations for improving the clinical treatment of Parkinson's disease.

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