LEVODOPA-INDUCED DYSKINESIA – FROM ABERRANT PRESYNAPTIC SIGNALING TO MALADAPTIVE POSTSYNAPTIC PLASTICITY

By

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ABSTRACT

LEVODOPA-INDUCED DYSKINESIA – FROM ABERRANT PRESYNAPTIC SIGNALING TO MALADAPTIVE POSTSYNAPTIC PLASTICITY

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Parkinson's disease (PD) is a neurodegenerative disorder that arises following the death of dopamine (DA) neurons in the substantia nigra pars compacta (SNc). DAergic signaling from these neurons is required for proper signaling of the basal ganglia, a circuit that regulates habitual motor behaviors. As the SNc degenerates, DA signaling to the hub of the basal ganglia—the striatum—is drastically reduced. This progressive loss results in the development of parkinsonian motor symptoms, including bradykinesia, tremor, and gait problems. To treat these symptoms, the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) can be administered to reintroduce DA signaling in the striatum. Unfortunately, chronic treatment with L-DOPA inevitably leads to the development of new motor symptoms, called L-DOPA-induced dyskinesia (LID), in the majority of PD patients. LID development is a complex, multifaceted process. The aim of this dissertation is to elucidate the mechanism of LID by studying abnormal presynaptic signaling and the aberrant postsynaptic striatal plasticity induced in dyskinesia. First, we found that DA release from serotonin (5-HT) cells of the dorsal raphe nucleus (DRN) is a critical contributing factor to LID in a rat model of PD, and that regulation of DRN neurons blocks LID development. We showed this by using recombinant adeno-associated virus (rAAV) to express the DA autoreceptor $D2R_S$ in DRN neurons, giving them ability to regulate abnormal DA release. Treatment with rAAV-$D2R_S$ blocks LID development by decreasing DA efflux into the striatum. Second,

we have characterized a novel postsynaptic molecular driver of LID, Nurr1. Nurr1 has been identified in genetic screens to be significantly upregulated in dyskinetic animal models. Therapies aimed at increasing Nurr1 are currently being investigated for PD, as the transcription factor is required for the health and long-term maintenance of the DA cells that degenerate in the disease. This dissertation provides evidence that Nurr1 plays a direct role in LID development. Viral expression of Nurr1 in the striatum can induce severe LID in a rat strain that is resistant to LID. Additionally, we showed that LID-associated Nurr1 expression is induced by direct stimulation of the pro-movement pathway of the basal ganglia. Finally, we determined that Nurr1 expression causes changes in both the activity and morphology of striatal medium spiny neurons (MSNs). We have shown that, independent of L-DOPA administration, Nurr1 causes altered striatal activity that mimics activity changes seen in dyskinetic animals. Ectopic Nurr1 expression also causes L-DOPA-independent decreases in dendritic spines. As dendritic spine plasticity is a hallmark of LID, our data suggests that Nurr1 plays a direct role in these maladaptive changes. Together, this dissertation provides compelling evidence for both presynaptic and postsynaptic mechanisms of LID development.

This dissertation is dedicated in memory of
Dr. Sudhish Mishra
A truly wonderful scientist and friend

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PREFACE

At the time of writing this dissertation, the three chapters describing my research are in preparation for publication. Chapter 2 has been submitted for publication, and accepted to *Acta Neuropathologica Communications* pending minor revisions. Chapters 3 and 4 will be compiled into a single manuscript and submitted in the near future.

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KEY TO ABBREVIATIONS

5-HT	5-Hydroxytryptamine, serotonin
6-OHDA	6-Hydroxydopamine
α-syn	Alpha synuclein
AADC	Aromatic amino acid decarboxylase
AIM	Abnormal involuntary movement
AOLs	Axial, orolingual, and limb AIMs
BDNF	Brain derived neurotrophic factor
BFP	Blue fluorescent protein
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CREB	cAMP responsive element binding protein
D1	Dopamine receptor type 1 -- direct pathway specific
D2	Dopamine receptor type 2 -- indirect pathway specific
$D2R_S$	Dopamine autoreceptor
DA	Dopamine
DAT	Dopamine transporter
DBS	Deep brain stimulation
dMSN	Medium spiny neuron of the direct pathway
DOPAC	3,4-dihydroxyphenylacetic acid

DREADD	Designer receptor exclusively activated by designer drugs
DRN	Dorsal raphe nucleus
Dyn	Dynorphin
Enk	Enkephalin
ERK1/2	Extracellular signal-regulated protein kinase
FAS	Forepaw adjusting steps
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
GI	Gastrointestinal
GPCR	G-protein coupled receptor
GPe	Globus pallidus, external segment
GPi	Globus pallidus, internal segment
HPLC	High-performance liquid chromatography
I.P.	Intraperitoneal
IEG	Immediate early gene
IHC	Immunohistochemistry
iMSN	Medium spiny neuron of the indirect pathway
ITR	Inverted terminal repeat
L-DOPA	3,4-dihydroxy-L-phenylalanine, levodopa
LFP	Local field potential
LID	Levodopa-induced dyskinesia
LTD	Long term depression
LTP	Long term potentiation

MAO-B	Monoamine oxidase B
MAPK	Mitogen-activated protein kinase
MFB	Medial forebrain bundle
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSK-1	Mitogen- and stress-activated kinase 1
MSN	Medium spiny neuron
NBRE	NGFI-B response element
NMDA	N-methyl-D-aspartate
NR4A1	Nur77
NR4A2	Nurr1
NR4A3	NOR-1
NurRE	Nur-responsive element
PD	Parkinson's disease
PKA	Protein kinase A
rAAV	Recombinant adeno-associated virus
RXR	Retinoid X receptor
S.C.	Subcutaneous
SERT	Serotonin transporter
SNc	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TH	Tyrosine hydroxylase
UPDRS	Unified Parkinson's Disease Rating Scale

VMAT2	Vesicular monoamine transporter

Chapter 1: Introduction

Parkinson's disease

I. History and impact

In 1817, an English surgeon published his characterization of a significant motor disorder which he had observed in six people—three of his patients, as well as three strangers he solely observed from a distance. The seminal work "An essay on the shaking palsy" by James Parkinson is the first medical characterization of the now-termed Parkinson's disease (PD). In his essay, Parkinson describes what are now recognized as the hallmark symptoms of the disease. He defined the shaking palsy as:

> "Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured." (Parkinson, 2002)

This characterization was expanded 55 years later by Jean-Martin Charcot, who suggested naming the disease after the English surgeon. Charcot's characterizations helped separate PD from other neurological disorders and defined the basis of the primary motor symptoms arising from slowness rather than weakness or paralysis (Charcot, 1872, 1879; Gelfand, 1989). Parkinson's recognition of a markedly distinct disease along with Charcot's clinical characterization paved the way for decades of research to understand and treat PD.

PD is the second most common neurodegenerative disease in the world, affecting approximately 1% of adults over the age of 60 (de Lau & Breteler, 2006). The

Parkinson's Foundation Parkinson's Prevalence Project projects that the number of PD patients in North America could nearly double by 2030, with over one million people living with the disease (Marras et al., 2018). Patient care and lost wages of both patients and familial caregivers placed a staggering economic burden in the United States of about $14 billion in 2010 (Johnson et al., 2013; Kowal et al., 2013). With the population shifting to a more aged demographic and projected doubling of PD patients, the associated economic strain demands focused research for better care of PD patients.

II. Clinical presentation

A PD diagnosis requires the presentation of cardinal motor symptoms of the disease—termed parkinsonism. Parkinsonism describes the motor symptoms that can be caused by PD. These are the presence primarily of bradykinesia (slowness of movement), along with either a resting tremor or passive joint rigidity—both in some cases (Postuma et al., 2015). While the majority of PD cases are sporadic, there are familial forms of PD and mutations in a number of genes, such as *parkin, LRRK2,* and *SNCA*, linked to the disease (Klein & Westenberger, 2012; Mhyre et al., 2012). PD cases can be separated from other disorders that exhibit parkinsonism by evaluating further criteria, such as responsiveness to pharmacotherapies and non-motor symptoms like anosmia. While PD is traditionally considered a motor disorder, there are numerous non-motor symptoms that can become very debilitating, including gastrointestinal (GI) issues and other autonomic dysfunction, dementia, and depression (Gelb et al., 1999). Interestingly, some of these non-motor symptoms, such as anosmia and GI problems can arise years prior to the motor symptoms required for diagnosis (Mantri et al., 2018).

Though the non-motor symptoms are now deservedly being recognized, the bulk of research and therapeutic efforts have focused on the hallmark motor symptoms of PD. Motor symptoms include a resting tremor, bradykinesia, akinesia, rigidity, and postural instability. Many parkinsonisms are not exclusive to PD. Tremors, for instance, can be common with other disorders, such as dementia with Lewy bodies and multiple system atrophy (Wenning et al., 1995; Louis et al., 1997). Therefore, a positive diagnosis of PD typically requires the presence of multiple motor symptoms in combination with one another. Tremors are the presence of a 3-6 Hz frequency tremor of the limb while at rest. Tremor prevalence in patients ranges from 69%-100% (Hoehn & Yahr, 1967; Martin et al., 1973; Gelb et al., 1999). Bradykinesia—slow, halting movement—affects roughly 77% to 98% of patients (Hoehn & Yahr, 1967; Martin et al., 1973; Gelb et al., 1999). Along with bradykinesia, many patients struggle to initiate movement due to akinetic 'freezing' as well as rigidity in the joints. Slow movements and progressive halts and hesitations are also seen in PD (Hoehn & Yahr, 1967). These symptoms are why the disease originally garnered the name *paralysis agitans*, which Charcot later disputed as these symptoms are not due to weakness or paralysis. Postural instability typically occurs later in disease progression, with only 37% of patients with a disease duration of 5 years or less showing severe postural instability (Hoehn & Yahr, 1967; Gelb et al., 1999).

III. Neuropathology of Parkinson's disease

There are two major pathological hallmarks of PD: the presence of Lewy bodies and cell loss in the substantia nigra pars compacta (SNc). These pathological markers are evaluated *post mortem* and their presence confirms the PD diagnosis.

Lewy bodies are intracellular inclusions associated with PD that were first described by Frederich Heinrich Lewy in 1912 (Lewy, 1912; Goedert et al., 2013). Lewy bodies are comprised of neurofilaments, ubiquitin, and aggregated proteins found in remaining neurons of PD-affected brain regions (Goldman et al., 1983; Lowe et al., 1988; Pollanen et al., 1993; Olanow et al., 2009). One protein, alpha synuclein (α-syn), is the main component of these inclusions and has been studied extensively in relation to PD (Spillantini et al., 1997). α-Syn pathology is seen in both the brain and peripheral nervous system of PD patients (Braak et al., 2003; Braak et al., 2006). Multiple mutations in, as well as multiplications of, the α-syn encoding gene, *SNCA,* are associated with familial cases of PD, and are thought to impact the disease through changes in protein function and increased α-syn aggregation (Polymeropoulos et al., 1997; Conway et al., 1998; Kruger et al., 1998; Chartier-Harlin et al., 2004; Burre et al., 2012). Changes in α-syn expression are also observed in sporadic cases of PD, further suggesting its involvement in the disease process (Neystat et al., 1999; Grundemann et al., 2008). α-Syn pathology impacts nearly every cellular pathway that has been linked to PD etiology, making it one of the best validated molecular markers in the pathogenesis of PD (Benskey et al., 2016). While the long-standing hypothesis has been that α-syn aggregates are directly neurotoxic, some research has suggested that the toxicity may be linked to α-syn loss-of-function as the protein is sequestered into the aggregates (Gertz et al., 1994; Gorbatyuk et al., 2010; Kanaan & Manfredsson, 2012).

The other key hallmark of pathology and the basis for the disease's cardinal motor symptoms is loss of SNc neurons and their projections to the striatum. SNc neurons are dopamine (DA) producing neurons that project to the striatum, where DAergic signaling

regulates learned motor behaviors and goal-oriented behaviors (Lanciego et al., 2012). Anatomically, cell loss can be seen in the lack of pigment in the SNc, arising from the loss of neuromelanin—a byproduct of DA metabolism—as the neurons degenerate (Dickson; Meiser et al., 2013). It was first proposed that disruption of the basal ganglia—a group of interconnected nuclei which includes the striatum and regulates functions including voluntary motor control—may contribute to PD motor symptoms in the 1870s (Meynert, 1871; Engelhardt, 2013). Damage to the SNc was first reported in the 1920s by a number of French researchers conducting research evaluating midbrain pathology in PD (reviewed in (Goetz, 2011)). *Post mortem* evaluation of advance stage PD patients' brains show severe neuronal loss in the SNc, with the caudal and ventrolateral tiers being most susceptible (Damier et al., 1999; Double et al., 2010; Jellinger, 2012). There is a corresponding loss of SNc projections throughout the caudate and putamen (striatum), which is correlated with dysfunctional DA metabolism (Kish et al., 1988; Gallagher et al., 2011). Decreased DA content due to this degeneration was also observed in both the striatum and SNc of PD patients (Ehringer & Hornykiewicz, 1960; Hornykiewicz, 1963; Hornykiewicz, 2002). It is clear that neurodegeneration begins long before the motor symptoms present, as nigrostriatal projections in the striatum are reduced roughly 50% one year after diagnosis, and that innervation is 70-90% depleted five years post-diagnosis (Kordower et al., 2013). With such dramatic levels of degeneration occurring before motor symptoms arise allowing for diagnosis, efforts are underway to identify biomarkers of the disease to achieve earlier diagnosis (Pagan, 2012; Barber et al., 2017; Frosini et al., 2017; Khodadadian et al., 2018; Nielsen et al., 2018).

Treating the motor symptoms of Parkinson's disease

While the benefit of hindsight shows the occasionally outlandish nature of early PD treatments—such as bloodletting and vibrating chairs—some pharmacological approaches taken by these pioneering clinicians hinted at the underlying cause of the motor symptoms (Goetz, 2011). One of the first established treatments for PD tremor was with belladonna alkaloids, which are now known to be anticholinergic agents and assist in balancing basal ganglia signaling (Price & Merritt, 1941; Weiner et al., 2013; Fahn, 2015). Charcot advocated for these types of treatment, along with the rye-based ergot fungus, which acts on DA receptors and is the basis for some current DA-agonist therapies such as bromocriptine (Lieberman & Goldstein, 1985; Schiff, 2006; Goetz, 2011). Though their mechanism was not originally understood, we now know that these first pharmacotherapies worked by affecting the underlying cause of PD motor symptoms: DA dysfunction and imbalance in the basal ganglia.

I. Anatomy of the basal ganglia

The basal ganglia is a series of nuclei that creates a complex circuit which regulates voluntary and habitual motor behavior, and goal-directed behaviors (Engelhardt, 2013). It is comprised of five of highly interconnected subcortical structures which, when properly coordinated, regulate these motor behaviors with output signals from the thalamus to the cortex (see Figure 1.1A for diagram of basal ganglia structures and signaling) (Lanciego et al., 2012; Calabresi et al., 2014). The central hub of the basal ganglia is the striatum. In humans and non-human primates, the striatum is compartmentalized into two nuclei, the caudate and putamen. In rodent models used in PD research, the striatum is a single nucleus. The putamen is the unit involved in basal

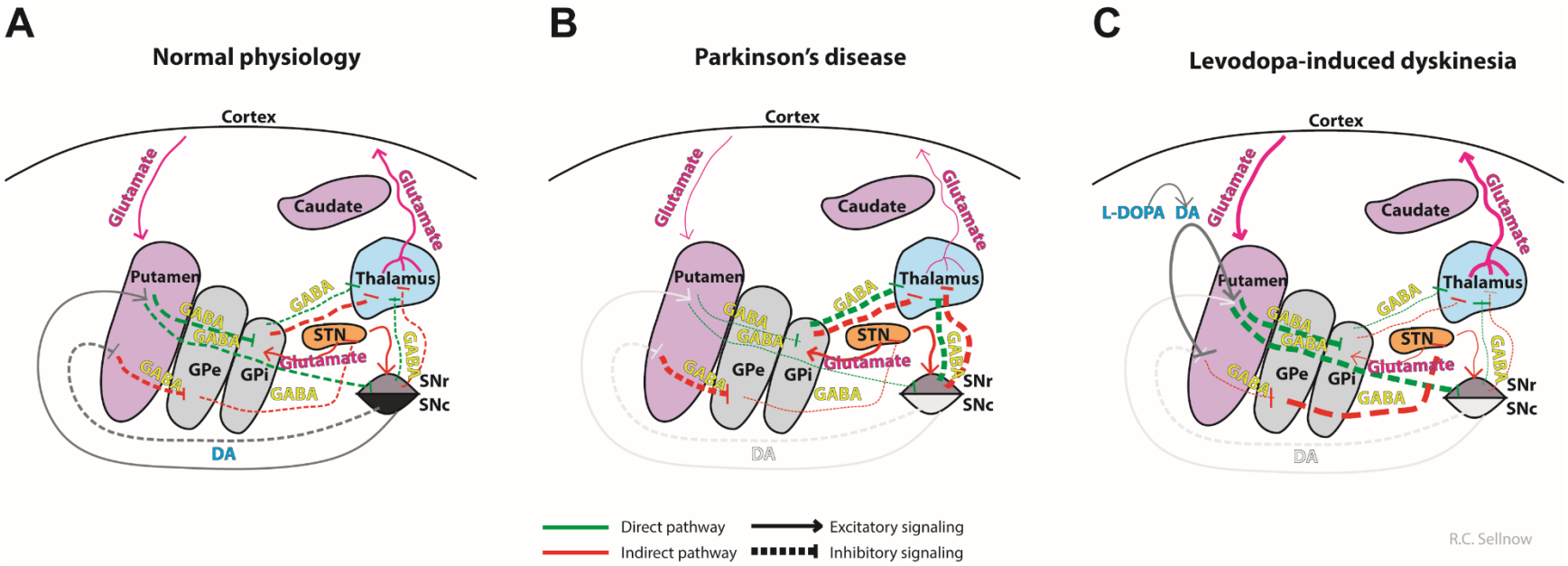

Figure 1.1 Schematic of basal ganglia signaling.

(A) Basal ganglia signaling pathways achieve proper motor control through the balance of the direct (green) and indirect (red) pathways under normal physiological conditions. (B) In Parkinson's disease, DAergic signaling from the SNc is lost, causing an imbalance in the basal ganglia and a decrease in excitatory signaling from the thalamus. (C) In L-DOPA induced dyskinesia, the imbalance in the basal ganglia is tipped to favor the direct pathway, promoting hyperexcitatory signaling from the thalamus.

Abbreviations: DA=dopamine; GABA=γ-aminobutyric acid; L-DOPA=levodopa; GPe=globus pallidus, external segment; GPi=globus pallidus, internal segment; STN=subthalamic nucleus; SNr=substantia nigra pars reticulata; SNc=substantia nigra pars compacta

ganglia, with inputs from the cortex and DAergic efferents from the SNc. Striatal signaling regulates the rest of the basal ganglia nuclei, which include the globus pallidus internal and external segments (GPi, GPe, respectively) and the substantia nigra pars reticulata (SNr), a part of the substantia nigra separate from the SNc. GPe efferents project to the subthalamic nucleus (STN), which itself modulates both the SNr and the GPi. Basal ganglia signaling inevitably modulates thalamic signaling to the cortex, either directly or indirectly by affecting the signaling of basal ganglia output nuclei—GPi and SNr—both of which project directly to the thalamus. The regulated output signal from the thalamus to the cortex determines the functional movement output (Fisone et al., 2007).

II. Dopaminergic regulation of basal ganglia signaling

Proper modulation of thalamic signaling to the cortex requires an intricately fine-tuned series of signals throughout the basal ganglia. This is achieved through DAergic modulation of the two signaling pathways of the basal ganglia—termed the direct and indirect pathways (Fisone et al., 2007; Calabresi et al., 2014). These two pathways are classically thought to work in opposition with one another to balance basal ganglia signaling and thus balance movement. The direct pathway is canonically known as generating pro-movement signaling, and the indirect pathway sends anti-movement signals (Ghiglieri et al., 2010; Calabresi et al., 2014). These distinct pathways begin in the striatum and its main population of neurons, medium spiny neurons (MSNs). MSNs comprise ~95% of the striatum, the rest being made up by cholinergic and GABAergic interneurons (Kawaguchi et al., 1995; Tepper & Bolam, 2004). Their dendrites are studded with synaptic structures called spines, which are dynamic and altered in normal

neuronal processes as well as in disease states (Maiti et al., 2015). MSNs themselves utilize the inhibitory neurotransmitter γ-aminobutyric acid (GABA).

The split between the two pathways begins with these neurons and is dependent on which of the two principle DA receptors each neuron expresses. Direct pathway MSNs (dMSNs) primarily express DA type 1 (D1) receptors, while indirect pathway neurons (iMSNs) express the DA type 2 (D2) isoform (Gerfen et al., 1990; Surmeier et al., 1996). Though D1 and D2 are the most predominant receptors, there are additional isoforms, being the D5 (a D1-type) and the D3 and D4 isoforms (D2-types) (Beaulieu & Gainetdinov, 2011). The receptor classes are defined by how they impact intracellular cyclic adenosine monophosphate (cAMP) levels—D1-type receptor stimulation causes an increase in cAMP, while D2 activation results in the opposite (Beaulieu & Gainetdinov, 2011). The majority of research, however, has been focused on the two predominant isoforms, D1 and D2.

The final result of direct pathway signaling is increased excitatory glutamatergic signaling from the thalamus to the cortex, promoting movement. Beginning in the striatum, dMSNs send inhibitory GABAergic signals to both the GPi and the SNr. The efferents of both of these nuclei are also GABAergic, and thus dMSN signaling inhibits their inhibitory signals to the thalamus. This disinhibition allows for excitatory glutamatergic signals to be sent from the thalamus to the cortex, promoting movement (Calabresi et al., 2014).

The indirect pathway—named such as its projections take a less direct route to the thalamus—also begins with GABAergic MSNs, these expressing the D2 isoform DA receptor. iMSNs first project to the GPe, inhibiting its signaling. The GPe projects to and

inhibits the STN with GABAergic signaling. This signal dampens STN excitatory glutamatergic signaling to the GPi, which outputs (as in the direct pathway) inhibit thalamic signaling. iMSN inhibition of the GPe lifts inhibitory signals to the STN, thus increasing GPi GABAergic signaling to the thalamus, leading to reduced glutamate release on the cortex from the thalamus. The end result is the dampening or inhibition of movement (Calabresi et al., 2014).

The balance of signaling between these two pathways is paramount for successful motor control. The key to keeping the indirect and direct pathways working together in coordination is DA signaling from the SNc (Rangel-Barajas et al., 2015). Projections from the SNc release DA onto striatal MSNs, differentially affecting each pathway due to their unique receptor expression. All DA receptors are G-protein coupled receptors (GPCR) but D1 signaling is mediated via the coupling with excitatory Gα subunits such as $G\alpha_{olf}$ and $G\alpha_{s}$, whereas D2 receptors couple with inhibitory $G\alpha_{i}$ (Corvol et al., 2004; Neve et al., 2004). This results in increased activity in direct pathway neurons with DA signaling due to an increase in cAMP signaling cascades, with the opposite effect in indirect pathway neurons. Tonic firing of SNc neurons during movement and goal-directed behaviors tune the final output signal of the basal ganglia by affecting direct and indirect MSN activity levels (Hyland et al., 2002).

In PD patients, the progressive loss of SNc projections in the striatum inevitably results in impaired DA signaling, and thus basal ganglia dysregulation (Figure 1.1B). Loss of DA signaling results in reduced activity of the pro-movement direct pathway, with simultaneous disinhibition and altered firing patterns of the indirect pathway, causing complications with initiating smooth and controlled movements (Raz et al., 2000).

Accordingly, decreased direct pathway signaling has been demonstrated in parkinsonian animal models, where dMSN firing rate has been shown to be reduced following DA depletion in mice (Ryan et al., 2018). This imbalance is the physiological root of the motor symptoms in PD.

III. L-DOPA: the beginnings of the 'gold standard' therapy

Once the loss of DA in the striatum of PD patients was confirmed in the late 1950s (Ehringer & Hornykiewicz, 1960; Hornykiewicz, 1963; Hornykiewicz, 2002), therapies aimed at correcting basal ganglia imbalance quickly moved to the forefront. It was in the late 1960s that George Cotzias published impressive results of treating parkinsonisms by administering high-doses of the DA precursor, 3,4-Dihydroxy-L-phenylalanine, also called levodopa or L-DOPA (Cotzias et al., 1967; Cotzias et al., 1969). This seminal discovery remains the most impactful development in PD treatment history, and L-DOPA—while not disease modifying—is still the most effective treatment for alleviating the motor symptoms of the disease.

L-DOPA was first isolated from the *Vicia faba* bean in 1913 and chemically synthesized in 1921, following closely the first chemical synthesis of DA (Barger & Ewins, 1910; Guggenheim, 1913; Waser & Lewandowski, 1921; Fahn, 2015). L-DOPA was first administered to patients with PD and parkinsonisms by pharmacologist Oleh Hornykiewicz and his clinical partner Walther Birkmayer in 1961 (Birkmayer & Hornykiewicz, 1961; Fahn, 2015; Lees et al., 2015). Hornykiewicz's previous work had identified that DA is lost in both the striatum and SNc of PD patients, prompting the movement to use L-DOPA as a therapy (Ehringer & Hornykiewicz, 1960; Hornykiewicz, 1963; Hornykiewicz, 2002). Their study of 20 patients using L-DOPA doses from 50-

150mg showed improvements in bradykinesia—but not rigidity—up to three hours following administration (Birkmayer & Hornykiewicz, 1961). Over the years, the two continued treating PD patients with weekly or biweekly administration of 25mg L-DOPA in over 200 cases, but results were less promising than the initial study, with nearly one third of patients gaining no benefit from treatment (Birkmayer & Hornykiewicz, 1964; Fahn, 2015). Multiple other studies using low doses of L-DOPA and D,L-DOPA (now known to be a less effective formulation) were similarly disappointing (Hornykiewicz, 2002; Fahn, 2015; Lees et al., 2015). Along with lackluster motor improvement, a variety of GI side effects, including nausea and vomiting, occurred with L-DOPA treatment (for review, see (Fahn, 2015)).

Despite growing skepticism, Cotzias moved forward with the high-dose treatment regimen of D,L-DOPA and published his findings 1967 (Cotzias et al., 1967). By slowly increasing the total daily dose to avoid the development of side effects, significant motor benefits in all patients began to arise with 12g daily doses. Cotzias continued to administer L-DOPA in conjunction with the peripheral DOPA decarboxylase inhibitor alpha-methyl-dopa hydrazine (carbidopa), which allowed for lower doses of L-DOPA to achieve motor benefits without as significant GI side effects (Cotzias et al., 1969). Confirmatory trials by other groups quickly began and found similar success using high-doses of L-DOPA (Fahn, 2015).

Intriguingly, the leap Cotzias made to significantly higher doses of L-DOPA was not intended to replenish lost DA in PD, but to address the loss of neuromelanin in the SNc. He hypothesized that the depigmentation itself was a cause of the disease, and proposed using L-DOPA to promote neuromelanin deposition in the brain (Cotzias et al.,

1964). Without this bold approach, L-DOPA therapy may not have been pursued further for PD treatment for decades.

IV. L-DOPA therapy and mechanism of action

Exogenously supplied L-DOPA successfully ameliorates PD motor symptoms by replenishing lost DA content in the basal ganglia. In normal physiologic conditions, DA synthesis begins with the rate-limiting step of tyrosine being converted into L-DOPA by the highly regulated enzyme tyrosine hydroxylase (TH) (Daubner et al., 2011). L-DOPA is then metabolized into DA by the enzyme aromatic amino acid decarboxylase (AADC), the physiologic final step in DA synthesis (Blaschko, 1942; Meiser et al., 2013). When given exogenously, L-DOPA is taken up by L-type amino acid transporters and can be converted to DA indiscriminately by any neurons expressing AADC, which includes any remaining SNc neurons (Uchino et al., 2002; del Amo et al., 2008; Hinz et al., 2016). DA is then packaged into vesicles by the vesicular monoamine transporter 2 (VMAT2) (Alter et al., 2013) and released on striatal MSNs, reinstating the lost balance of the direct and indirect pathways.

The GI side effects initially seen with L-DOPA therapy stems from DA neurons in the gut. There is a significant population of DA neurons in the enteric nervous system that regulate gut motility and are responsible for half of DA production that occurs in the body (Hernandez et al., 1987; Lambert et al., 1991; Eisenhofer et al., 1997; Li et al., 2004). Thus, without intervention, large amounts of exogenously supplied L-DOPA can be taken up and metabolized peripherally, before it can cross the blood-brain barrier. In turn, this requires larger doses of L-DOPA to achieve effective levels in the brain. This massive increase in DA concentration and signaling in the enteric system causes the

uncomfortable GI side effects, such as nausea, vomiting, and loss of appetite. To counteract this, peripheral decarboxylase inhibitors such as carbidopa are used in combination with L-DOPA therapy to block peripheral metabolism of L-DOPA, allowing for higher bioavailability in the brain which reduces the required therapeutic dose (Barbeau et al., 1972; Nutt et al., 1985).

In addition to carbidopa, other enzymatic inhibitors are commonly used adjunct with L-DOPA to improve its efficacy. Catechol-O-methyltransferase (COMT) inhibitors (entacapone and tolcapone) and monoamine oxidase B (MAO-B) inhibitors (rasagiline, selegiline, safinamide) are both used to inhibit DA breakdown into inactive metabolites (Goetz et al., 2005; Fox et al., 2011; Meiser et al., 2013). COMT inhibitors are exclusively used as an adjunct therapy, while MAO-B inhibitors can be used as initial monotherapies for early stage and mild PD patients (Stayte & Vissel, 2014).

Despite its therapeutic strength, L-DOPA is often not immediately prescribed, and instead other pharmacotherapies (discussed below) are employed with the intent to manage or delay the onset of L-DOPA side effects (Hubble, 2002). In fact, these side effects can become so debilitating that many clinicians, patients, and caregivers are hesitant to initiate therapy. This pervasive fear has led to a phenomenon known as “L-DOPA phobia” which ultimately fails patients who could benefit dramatically from L-DOPA therapy (Kurlan, 2005; Titova et al., 2018). One of these side effects include motor fluctuations caused during wearing-off of L-DOPA efficacy, which is observed in nearly half of PD patients after only two years of L-DOPA therapy (Ahlskog & Muenter, 2001). Motor fluctuations are seen when patients alternate between periods of therapeutic benefit (“on” state) and a reemergence of PD symptoms as the drug wears

off and the symptoms are no longer suppressed (“off” state) (Quinn, 1998). The most debilitating side effect is the emergence of abnormal involuntary drug-induced dyskinesia (discussed below in detail). Some studies have shown that even early treatment with DA agonists does not prevent L-DOPA side effects once it is inevitably introduced, while others suggest certain agonists may at least delay their onset (Hauser et al., 2007; Katzenschlager et al., 2008; Fox et al., 2011; Chondrogiorgi et al., 2014).

V. Other dopamine-based therapies

A number of DA receptor agonists are standardly employed to treat PD motor symptoms, either prior to L-DOPA therapy in early stage patients or in conjunction with L-DOPA as the disease progresses (Goetz et al., 2005; Fox et al., 2011; Stayte & Vissel, 2014). DA-agonist therapies aim to delay or prevent the onset of L-DOPA side effects, such as motor fluctuations seen in the wearing-off stage of the drug, and abnormal involuntary movements, such as dyskinesia. These therapies can be used as monotherapies, typically before L-DOPA therapy begins, or in combination with L-DOPA to better manage symptoms in advanced stages of the disease (Goetz et al., 2005). There are a dozen different DA agonists that have been evaluated clinically, with strong evidence supporting that most of these can effectively manage PD motor symptoms (Fox et al., 2011). Some, such as ropinirole, pramipexole, bromocriptine, and cabergoline have been shown to delay the onset of motor fluctuations and dyskinesia (Fox et al., 2011). Additionally, a subset of DA agonists (pramipexole, ropinirole, rotigotine, apomorphine, and pergolide) can manage motor fluctuations, but there is little evidence supporting that any clinical tested DA agonist treat dyskinesia (Fox et al., 2011).

VI. Non-dopaminergic therapies

Pharmacotherapies that do not directly affect DAergic signaling are currently being used and investigated clinically with the intent of increasing L-DOPA efficacy, reduce side-effects, and/or modify disease progression (Goetz et al., 2005; Fox et al., 2011; Stayte & Vissel, 2014). Anticholinergics, one of the first efficacious treatment strategies discovered for PD, are still occasionally used today to achieve balance between cholinergic and dopaminergic signaling in the striatum (Katzenschlager et al., 2003). These drugs, however, often result in neuropsychiatric side effects such as hallucinations, and therefore are not used as commonly (Katzenschlager et al., 2003; Sprenger & Poewe, 2013). N-methyl-D-aspartate (NMDA) receptor antagonists are used to treat L-DOPA side effects (amantadine) and have shown improvement in cognition (memantine) (Verhagen Metman et al., 1998; Stayte & Vissel, 2014). Adenosine A_{2A} receptor antagonists have are also in clinical trials, showing increased "on" L-DOPA period and decreased "off" period, but with increased time with dyskinesia (Stayte & Vissel, 2014).

A number of surgical interventions are also employed by clinicians to treat their patients, including deep brain stimulation (DBS), targeted lesioning, cell transplantation, and gene therapy. DBS, the preferred surgical approach, involves the implantation of a stimulating electrode in basal ganglia nuclei—commonly the thalamus, STN, or GPi—to promote proper signaling (Goetz et al., 2005; Fox et al., 2011; Moldovan et al., 2015). Targeted lesions, such as thalamotomy and pallidotomy, can be useful for managing L-DOPA motor complications, but are used much less frequently in modern therapy (Jankovic & Aguilar, 2008; Fox et al., 2011; Moldovan et al., 2015). Cell transplantation

has been investigated as an experimental therapy clinically since the mid-1980s, with varying results (Bjorklund & Kordower, 2013; Boronat-Garcia et al., 2017). While a subpopulation of patients experience decades of symptomatic relief from fetal ventral mesencephalic grafts in the striatum, another subpopulation of grafted patients see no benefit or loss of any benefit in a few years, with up to half experiencing major motor side effects (Olanow et al., 2003; Fox et al., 2011; Kefalopoulou et al., 2014; Boronat-Garcia et al., 2017). Many clinical trials using gene therapy for Parkinson's disease have been conducted, with some studies aiming to maximize the benefits of L-DOPA treatment by supplementing with DAergic factors, and others attempting to alter disease progression by delivering neuroprotective factors to diseased brain regions (Stayte & Vissel, 2014). These trials, while safe and well tolerated, have shown little improvement from current treatment options (Polinski, 2016).

L-DOPA-induced dyskinesia

Abnormal dyskinetic movements were first described in Cotzias' initial reports of L-DOPA use in PD patients (Cotzias et al., 1967; Cotzias et al., 1969). Significantly more debilitating than on-off fluctuations, L-DOPA-induced dyskinesia (LID) are a near inevitability with long term therapy, with reports ranging from 40-80% of patients developing them within 5 years of chronic L-DOPA treatment, and 80-90% after 10 years (Lesser et al., 1979; Lees & Stern, 1983; de Jong et al., 1987; Ahlskog & Muenter, 2001; Hauser et al., 2007; Manson et al., 2012; Turcano et al., 2018).

I. Features of LID

LID are a series of motor symptoms—distinct from the primary PD symptoms—that include excessive and abnormal hyperkinetic limb movements, abrupt jerking movements of the limbs, head, and neck (chorea and ballism), and sustained involuntary muscle contractions (dystonia). LID most commonly occur as peak-dose dyskinesia, where the symptoms arise when L-DOPA levels are at their highest in the plasma. Chorea is the most common component of LID and typically seen during peak-dose dyskinesia. This can be significantly debilitating, in some patients growing severe enough to affect daily living (Quinn, 1998; Bastide et al., 2015). Dystonia is often painful and can occur both during peak-dose dyskinesia and during a phenomenon known as diphasic dyskinesias (Quinn, 1998; Bastide et al., 2015).

LID is evaluated clinically using a number of rating scales that monitor abnormal involuntary movements (AIMs). Those most commonly used include the AIM scale, part IV of the Unified Parkinson's Disease Rating Scale (UPDRS), the Clinical Dyskinesia Rating Scale, and the Rush Dyskinesia Rating Scale. The AIM scale in particular is noted as being one of the best tools for evaluating LID clinically (Colosimo et al., 2010). The AIM scale assesses severity of 10 dyskinetic movements in the face and mouth, limbs, and trunk. Each is given a severity score from 0-4 (Guy et al., 1976). The AIM scale is usefully clinically as it gives both a global AIM score as well as distinguishes between body areas (Colosimo et al., 2010). Researchers have adapted the AIM scale for use in non-human primate and rodent models of LID (Petzinger et al., 2001; Lundblad et al., 2002; Tan et al., 2002; Steece-Collier et al., 2003).

II. Clinical management strategies

Once LID develop, clinicians have few effective options to manage them. The most straightforward approach is to reduce L-DOPA dose, but this can result in ineffective management of PD symptoms. It is widely thought that the pulsatile delivery method of oral L-DOPA and subsequent extreme peaks and valleys in DA concentration in the brain is a main contributor to LID (Chase, 1998; Nutt, 2007). To combat this, an extended release formulation can be prescribed (Fox et al., 2011). These controlled release formulations, while as efficacious as other formulations, may still carry the same risk for LID, and even increase the risk in advance stage patients (Fabbrini et al., 2010; Manson et al., 2012). Other approaches include constant L-DOPA/carbidopa infusion using an intestinal gel (Duodopa), which has been clinically shown to reduce LID (Antonini et al., 2016; Wirdefeldt et al., 2016). This requires surgery, and is thus more expensive and opens the risk for surgery related complications (Manson et al., 2012). Some gene therapy approaches have also been used to promote DA signaling in the striatum by delivering DAergic factors TH, AADC, and TH-regulating cofactor GTP-cyclohydrolase 1 (CH1) (Palfi et al., 2018). DBS in the STN or GPi has also been shown to help reduce LID and lead to lower doses of L-DOPA, thus helping reduce LID (Anderson et al., 2005; Sankar & Lozano, 2011). While a number of pharmacotherapies have been tested preclinically, amantadine is the only drug that has translated well clinically and been approved for LID management (Wolf et al., 2010; Ory-Magne et al., 2014; Hauser et al., 2017; Pahwa et al., 2017).

III. LID risk factors

L-DOPA dose and disease duration are the two strongest predictors of LID development (Grandas et al., 1999; Manson et al., 2012). Longer disease duration caries a greater risk for LID development, which could be due to a number of factors such as increasing disease severity (typically requiring higher L-DOPA doses) and differing ages of onset (Grandas et al., 1999; Manson et al., 2012; Nicoletti et al., 2016). In fact, patients diagnosed before age 40 show much higher LID incidence (up to 90% in five years) than those diagnosed after age 60 (26% incidence with 60-69 years age of onset) (Kostic et al., 1991; Schrag et al., 1998; Kumar et al., 2005; Manson et al., 2012). Additionally, higher daily doses of L-DOPA, regardless of adjunct DA agonist therapy, are associated with higher rates of LID (Grandas et al., 1999; Fahn, 2005; Manson et al., 2012).

Genetic risk factors for LID have also been examined, and a number of mutations in genes associated with familial PD, such as *parkin* and *LRRK2*, have been associated with higher LID risk (Dekker et al., 2003; Lesage et al., 2008; Nishioka et al., 2010). These mutations may not, however, be directly linked to LID as genetic forms of PD typically have earlier onset, which itself is a major LID risk factor. A number of studies have examined LID-linked polymorphisms in non-genetic (idiopathic) PD. A range of genes have been implicated, including members of DA metabolism and signaling pathways (D1, D2, D3, and D4 receptors, the dopamine transporter (DAT)) and genes non-DAergic genes (opioid receptors, brain derived neurotrophic factor (BDNF), MAO-B, COMT) (Oliveri et al., 1999; Bialecka et al., 2004; Strong et al., 2006; Carta et al.,

2008; Foltynie et al., 2009; Monville et al., 2009; Lee et al., 2011; Prashanth et al., 2011; Rieck et al., 2012; Purcaro et al., 2018).

LID etiology has been a focus of PD research since their initial observations. Understanding LID development is imperative to achieving more effective symptom management and improve the quality of life for patients. It is understood that the mechanisms underlying LID are multifaceted. The following sections will discuss the current understandings of LID etiology based on preclinical and clinical findings.

IV. Modeling LID for preclinical research

Our current understanding of LID has been dramatically influenced by the use and characterization of animal models of LID. Non-human primates and rodents (including mice and rats) are the most commonly utilized models, as LID can be established, monitored, and modulated using a variety of techniques in these animals. The first steps to a model of LID begin with a model of PD, most typically generated by selectively lesioning the SNc with neurotoxicants (Jackson-Lewis et al., 2012). 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is widely used to create PD-like lesions in non-human primates and mice (rats have shown resistance to MPTP toxicity) (Jackson-Lewis et al., 2012). The toxic properties of MPTP were inadvertently discovered when patients developed PD-like symptoms and pathology after taking synthetic opioids contaminated with MPTP (Langston et al., 1996). 6-Hydroxydopamine (6-OHDA) is also used regularly to induce SNc lesions in animals (Jackson-Lewis et al., 2012). An analog to DA, 6-OHDA is readily taken up by DAergic neurons, where its metabolism overwhelms the neuron with reactive oxygen species, leading to degeneration (Tieu, 2011). Lesion techniques using either of these neurotoxicants are able to cause near

complete degeneration of the nigrostriatal system, which is useful for modeling LID as dyskinesia development requires at least 90% striatal DA depletion (Ulusoy et al., 2010; Francardo et al., 2011). Following lesioning, dyskinesia can be induced in these animals with acute or chronic treatment with different DA agonists or L-DOPA (Lundblad et al., 2004; Bastide et al., 2015; Johnston & Fox, 2015). LID severity is assessed using the AIM scale discussed above. These models of a complex behavior are a powerful tool for better understanding LID etiology, as well as conducting experiments that would not be feasible in human patients.

V. Setting the stage for LID: dopamine depletion-induced changes

It is well established that L-DOPA “priming” is an essential step in LID development. Priming occurs when the DA deprived parkinsonian striatum encounters a sudden reintroduction of non-physiological DA signaling from either L-DOPA or DA agonist treatment (Jenner, 2008). DA depletion in the basal ganglia due to the disease process itself is required for priming. Significant changes occur in striatal MSN physiology both in PD patients as well as animal models. Increased D1 receptor presentation at the cell membrane is seen in MSNs following DA denervation, suggesting altered trafficking and increased sensitivity (Aubert et al., 2005; Guigoni et al., 2007; Berthet et al., 2009). DA depletion also increases expression of $G\alpha_{olf}$ in both post-mortem tissue and parkinsonian models, indicating that increases in D1 receptor coupling with $G\alpha_{olf}$ contribute to hypersensitization (Corvol et al., 2004; Alcacer et al., 2012). Striatopallidal neurons are significantly affected by alterations in glutamatergic synapses from cortical inputs, which drive indirect pathway signaling (Picconi et al., 2004; Day et al., 2006). DA-depletion does not induce similar changes in glutamatergic synapses of striatonigral

neurons of the direct pathway (Day et al., 2006). Additionally, overall dendritic spine density on MSNs is reduced in parkinsonian animal models (Zhang et al., 2013; Fieblinger et al., 2014). Changes to MSNs go beyond spine plasticity, as drastic dearborization of both striatopallidal and striatonigral MSNs dendrites occurs following DA denervation (Zaja-Milatovic et al., 2005; Deutch et al., 2007; Fieblinger & Cenci, 2015).

The drastic morphological and physiologic changes in the basal ganglia that occur due to DA depletion set the stage for LID induction. When the hypersensitized striatum is reintroduced to non-physiological DA signaling with L-DOPA or DA agonist therapy, the system becomes primed with further morphological and physiologic changes that inevitably lead to LID. Therapeutic efficacy of L-DOPA is lost in LID, as basal ganglia signaling is pushed out of balance once again, with striatal hypersensitivity driving aberrant pro-movement signals (Figure 1.1C).

VI. Dopamine receptor signaling in LID

Stimulating DA receptors with either exogenous L-DOPA therapy or DA direct agonists in the denervated striatum of PD patients and animal models results in dyskinesia-promoting signaling. The bulk of research has focused on changes in D1 receptor signaling, as specific dMSN stimulation with receptor agonists can induce L-DOPA-comparable severity dyskinesia (Rascol et al., 2001). Optogenetic stimulation of dMSNs also produces severe dyskinesia, further suggesting that D1 activation rather than DA itself plays a key role in the behavior (Ryan et al., 2018). D1 stimulation leads to downstream activation of the cellular signaling gene protein kinase A (PKA), which phosphorylates a number of downstream effectors (Neve et al., 2004). Inhibiting PKA

activity reduces LID in rodents, indicating that chronic D1 signaling and the PKA cascade are involved in the behavior (Lebel et al., 2010). Selectively inactivating downstream effectors of PKA in dMSNs is also able to reduce LID severity, further implicating the direct pathway signaling as being an instigator for LID (Bateup et al., 2010).

The research of D2 receptor signaling has been less involved than D1 signaling, but it is clear that these receptors also play a role in LID. This is showcased by the fact that D2 agonism alone primes the striatum and can induce dyskinesia on its own (Gomez-Mancilla & Bédard, 1992; Blanchet et al., 1993; Pollack & Yates, 1999; Drake et al., 2013; Chondrogiorgi et al., 2014). D2 receptor expression is upregulated following DA-depletion, and this is not reverted following L-DOPA therapy (Aubert et al., 2005; Guigoni et al., 2007). Recordings of iMSNs show that L-DOPA reduces their firing rate, indicating a decrease in indirect pathway signaling during LID (Ryan et al., 2018). Together, the research on DA receptors in LID has shown that abnormal signaling and receptor dynamics in both pathways occurs during LID.

VII. Presynaptic mechanisms of LID: the serotonin hypothesis

It is now understood that at the time of diagnosis and as PD patients begin DAergic therapy, dramatic cell loss in the SNc and DA terminals in the striatum has already occurred (Kordower et al., 2013). Despite this, L-DOPA therapy is still efficacious for symptom relief, even as the disease advances and nearly all nigrostriatal neurons are lost. The obvious question arises: if DAergic innervation of the striatum is lost, what neuronal population is processing L-DOPA and releasing DA into the striatum? While there are certainly compensatory mechanisms that stave off motor symptoms until

severe DA loss (Blesa et al., 2017), the leading hypothesis is that 5-hydroxytryptamine (5-HT, or serotonin) neurons of the dorsal raphe nucleus (DRN) become the primary site of DA release into the basal ganglia following L-DOPA administration.

The DRN is a bilateral brainstem nucleus comprised of a heterogeneous population of neurons. It contains roughly half of the 5-HT neurons of the rat and human brain (Jacobs & Azmitia, 1992). It is also comprised of GABAergic and a small population of DAergic neurons (Belin et al., 1983; Descarries et al., 1986). Highly collateralized 5-HT outputs from the DRN provide a primary source of 5-HT signaling in the forebrain, with target structures including the amygdala, cortex, nucleus accumbens, and the striatum (Waselus et al., 2011). DRN target structures suggest 5-HTergic influences a wide variety of behaviors, from stress and anxiety to motor control.

A key component to the 5-HT hypothesis of LID is the fact that 5-HT neurons are able to convert L-DOPA into DA. 5-HTergic neurons express AADC, the enzyme which converts L-DOPA to DA. It has been previously shown that exogenous L-DOPA is indeed taken up by 5-HT neurons and converted into DA (Arai et al., 1995). Furthermore, imaging data has suggested that the majority of striatal DA release in advanced PD patients comes from 5-HT neurons (Roussakis et al., 2016). Thus, the 5-HT system is pivotal for efficacious L-DOPA therapy as the DAergic system is progressively lost. Further studies, however, additionally implicate DRN 5-HT neurons in dyskinesia development.

5-HTergic DRN projections moderately innervate the striatum under normal physiologic conditions, where 5-HT signaling is thought to affect goal-directed behaviors and voluntary movement (Imai et al., 1986; Waselus et al., 2011; Mathur & Lovinger, 2012).

In parkinsonian animal models, 5-HTergic innervation of the striatum increases substantially (Maeda et al., 2003; Hiromasa Yamada et al., 2007; Rylander et al., 2010). This hyperinnervation occurs rapidly, with observable changes only two weeks following DA denervation in animal models (Maeda et al., 2003). Additional studies suggest that L-DOPA treatment itself also contributes to 5-HTergic axonal terminal sprouting in the striatum (Rylander et al., 2010). Recently, virally overexpressed BDNF was shown to induce serotonin fiber sprouting in the rat striatum, which correlated with more severe AIMs (Tronci et al., 2017). Together, this evidence suggests that 5-HT hyperinnervation allows for excess DA release in the striatum from DRN neurons.

Importantly, while DRN neurons can synthesize and release DA, they cannot effectively regulate DA release, as they do not express DAergic regulatory factors such as DAT or the dopamine autoreceptor ($D2R_S$). These factors are essential to proper modulation of DA signaling from DAergic neurons (Ford, 2014). Without this regulation, it is hypothesized that 5-HT neurons release DA in an unregulated fashion in the striatum. This unregulated release contributes to the pulsatile levels of L-DOPA in the system due to intermittent oral dosing that has been shown to drive LID (Cenci & Konradi, 2010). This dyskinesia-inducing mechanism for LID is supported by data showing that ablating the DRN, or modulating its activity with 5-HT agonists, can eliminate or reduce LID in animal models (Carta et al., 2007; Eskow et al., 2009; Bezard et al., 2013; Politis et al., 2014; Ghiglieri et al., 2016; Meadows et al., 2017).

The rationale for this theory of dyskinesiogenesis has prompted numerous clinical studies utilizing 5-HT agonists to treat LID. In practice, many of these drugs have fallen short, either negating anti-parkinsonian efficacy of L-DOPA or causing a worsening of

PD symptoms (Kannari et al., 2002; Iravani et al., 2006; Olanow et al., 2009; Cheshire & Williams, 2012). Despite disappointment in clinical trials, the rationale for this presynaptic mechanism of LID warrants further research in order to better understand how 5-HT neurons are involved in LID genesis. DAergic signaling in the denervated striatum is widely accepted to induce multiple postsynaptic changes that lead to LID, and thus regulating the release of DA following L-DOPA administration is an attractive approach for LID management.

VIII. Postsynaptic LID-associated gene expression

A variety of gene expression changes are associated with LID presentation. Changes in the extracellular signal-regulated protein kinase 1 and 2 (ERK1/2) signaling pathway and its downstream targets have been heavily researched in LID. Canonical D1 receptor signaling induces increases in cAMP following activation of PKA, which promotes transcription by the cAMP responsive element binding protein (CREB) (Cole et al., 1994; Konradi et al., 1994). Cross talk between glutamatergic NMDA receptors and D1 activation also promotes ERK1/2 phosphorylation (Girault et al., 2007). Following DA denervation and L-DOPA treatment, however, ERK1/2 activation (phosphorylation) and signaling occurs in dMSNs in an NMDA-independent manner (Gerfen et al., 2002; Fiorentini et al., 2013). The activation of the ERK1/2 cascade promotes transcription of a number of genes through its downstream effectors CREB and mitogen- and stress-activated kinase 1 (MSK1). Chronic activation of ERK1/2 leads to changes in a number of transcripts that have been found to be involved in LID. A transcriptomic study of differential expression following L-DOPA therapy and LID

induction has confirmed this, showing increases in transcripts such as *FosB*, *Junb*, *Arc*, and *Narp* (Charbonnier-Beaupel et al., 2015).

One notable category of expression changes is the increase in immediate early gene (IEG) expression with LID. IEGs are expressed throughout the brain and are rapidly induced following stimulus and can be induced by ERK1/2 cascades. Neuronal IEGs are heavily involved in synaptic plasticity that is required for a variety of functions, such as learning and memory (Okuno, 2011). The IEG *FosB* and its spliced isoform ΔFosB are one of the hallmark molecular markers of LID, and is upregulated following ERK1/2 signaling (Feyder et al., 2016). Both isoforms are highly upregulated in MSNs of dyskinetic rodent and non-human primate models of LID (Andersson et al., 1999; Pavón et al., 2006; Cenci & Konradi, 2010) and this has been confirmed in post-mortem PD patient tissue (Tekumalla et al., 2001; Lindgren et al., 2011). Higher endogenous expression of ΔFosB protein correlates with more severe dyskinetic behavior (Feyder et al., 2016). While ΔFosB and other genes show elevated expression with LID, it is important to differentiate between gene expression that is simply a consequence of the behavioral phenotype and those that play an active role in the process. This can be achieved by modulating the levels of specific genes in LID animal models. Accordingly, researchers have shown that ΔFosB is actively involved in LID development, as virally-mediated overexpression of ΔFosB exacerbates LID, and silencing the transcript can reduce behavior severity (Andersson et al., 1999; Engeln et al., 2016).

FosB is not the only IEG overexpressed in LID. Other IEGs including the cytoskeleton-associated protein Arc, transcription factor Zif268, and scaffolding protein Homer-1a have also been shown to become overexpressed in LID (Sgambato-Faure et al., 2005;

H. Yamada et al., 2007; Ebihara et al., 2011). Arc and Homer-1a are both associated with D1, but not D2, receptor activation (Sgambato-Faure et al., 2005; H. Yamada et al., 2007). Homer-1a is an example of a molecular marker that does not appear to play a causative role in LID, as LID can be reduced without reducing Homer-1a expression (Jimenez et al., 2009).

A handful of studies have investigated the expression signatures associated with LID in rat and mouse models. These have offered insights into molecular networks involved in LID. One study comparing dyskinetic to non-dyskinetic rats showed dysregulation of calcium signaling genes and synaptic plasticity (Konradi et al., 2004). Another study instead compared rats treated acutely with L-DOPA with chronically treated animals. There were 16 genes that were commonly regulated in both treatment groups (El Atifi-Borel et al., 2009). These shared genes are involved in a number of cellular mechanisms, including signal transduction, phosphorylation, and gene transcription and translation. There were a greater number of enhanced transcripts in the animals treated chronically with L-DOPA, and the researchers postulated that these changes were involved in the induction of structural changes in MSNs leading to LID (El Atifi-Borel et al., 2009).

While most of the omic-level characterizations of LID have looked in the striatum globally, there have been some efforts to elucidate specific changes in the dMSNs versus iMSNs (Heiman et al., 2014). Distinguishing between the two basal ganglia pathways is important, as there are differential changes in D1- and D2- dependent signaling in LID, as discussed above. One study by Dr. Paul Greengard and colleagues captured the translational profile of mouse dMSNs and iMSNs in response to chronic L-

DOPA (Heiman et al., 2014). Over 4,500 genes were differentially translated in dMSNs of severely dyskinetic animals. Some of the primary pathways affected by the expression changes included the mitogen-activated protein kinase (MAPK)/ERK1/2 pathway and actin cytoskeleton regulation. These results are indicative of the known increase in ERK1/2 signaling in LID, as well as morphological changes in MSNs (Heiman et al., 2014). Of the identified genes most highly correlated with higher L-DOPA doses and more severe LID, only one had been previously associated with LID (the *Trh* gene) (Cantuti-Castelvetri et al., 2010). This alone shows the importance of large-scale expression studies in LID, as many of the other targets may not otherwise have been identified as being involved. Other severe LID-associated genes that were upregulated in dMSNs included *Fosl1* (a member of the Fos family)*, Ire3* (an ERK1/2 dephosphorylation inhibitor), and *Nr4a2* (or *Nurr1*, a required transcription factor in DAergic neurons) (Heiman et al., 2014). Differential expression in iMSNs following severe LID induction was markedly less drastic, with only 415 genes showing changes (Heiman et al., 2014). These findings reaffirm previous work displaying heightened activity of dMSNs in LID with simultaneous decreases in iMSN activity (Fieblinger et al., 2014; Ryan et al., 2018).

IX. Aberrant striatal plasticity in LID

The magnitude of gene expression changes occurring with LID are thought to induce maladaptive changes in the physiology and morphology of MSNs. Numerous studies have characterized some of these changes in both human patients with LID as well as animal models.

It is classically thought that LID results following a substantial decrease in neuronal firing from the GPi, allowing for heightened excitatory signaling from the thalamus. This has been supported in PD patients with recordings of the GPi taken during operations for DBS implantations or pallidotomies. Single cell recordings monitoring individual GPi neurons show a reduction in firing rate in the “on” period with dyskinesia induced by apomorphine (Merello et al., 1999; Levy et al., 2001; Lee et al., 2007). However, this mechanism is challenged by the fact that lesions of the GPi can both improve PD motor symptoms and attenuate LID (Calabresi et al., 2014; Krishnan & Pisharady, 2017).

A recent study in mice recorded from individual dMSNs or iMSNs identified optogenetically to determine firing changes following lesion and with LID (Ryan et al., 2018). This study showed a dramatic reduction in firing rats of dMSNs following denervation, with a marked increase in LID-expressing mice (Ryan et al., 2018). This agrees with the classical assumption of overactivity of the direct pathway in LID (Calabresi et al., 2014). The researchers also observed a decrease in iMSN firing with LID, further implicating a striking imbalance between direct and indirect pathway signaling as a driver of LID (Ryan et al., 2018).

One of the most established electrophysiological hallmarks of LID is the loss of bidirectional plasticity in MSNs following LID development. Bidirectional plasticity allows for the strengthening and pruning back of synapses depending on neuronal activity, and is important for many processes including motor learning and memory (Graybiel, 1995). Synaptic changes are influenced by long term potentiation (LTP), which causes reinforced synaptic connections with repeated stimuli, and long-term depression (LTD), which decreases synaptic strength (Citri & Malenka, 2008). LTP can be induced in

MSNs with repetitive high-frequency stimulation (Calabresi et al., 1992; Charpier & Deniau, 1997). However, following DA denervation, MSNs lose LTP capabilities in animal models, resulting in unilateral plasticity only (Centonze et al., 1999; Picconi et al., 2003; Paillé et al., 2010). Therapeutic (non-LID-inducing) doses of L-DOPA can restore both LTP and LTD in lesioned animals. However, rats that develop dyskinesia display a reestablishment of LTP but not LTD on L-DOPA (Picconi et al., 2003). This suggests DA-induced strengthening of potentially maladaptive synapses that cannot be pruned back, promoting abnormal movements.

The original studies identifying the loss of bidirectional plasticity did not differentiate between the direct and indirect pathways. Differences between the two pathways have been looked at in a more recent study using transgenic mice (Thiele et al., 2014). Indeed, the researchers found unilateral plasticity following DA denervation, with dMSNs expressing LTD and iMSNs expressing LTP (Thiele et al., 2014). However, they observed differential plasticity between the two pathways in dyskinetic animals, with dMSNs only expressing LTP and iMSNs capable of only LTD (Thiele et al., 2014). The changes with LID in each pathway are opposite to their response following denervation, supporting the hypothesis of basal ganglia imbalance in both PD and LID.

As aberrant synaptic plasticity has been shown to influence LID through electrophysiology alterations, a number of studies have worked to identify a morphological basis for these changes. Specifically, studies assessing the density and morphology of dendritic spines on MSNs have shown that there are dramatic changes following DA denervation as well as LID induction (Fieblinger & Cenci, 2015). Dendritic spines are highly dynamic structures that are the site of synapse formation from many

input nuclei. Spines on MSNs primarily form excitatory glutamatergic synapses with inputs from the cortex and thalamus (Lacey et al., 2005; Ding et al., 2008). Alterations in spine density and their morphology can influence many neuronal processes as well as be indicative of some disease states (Maiti et al., 2015). Accordingly, studies have shown changes in synaptic architecture of MSNs in dyskinetic animals. It is established that striatal DA depletion leads to an overall loss of MSN dendrites and dendritic spines, and thus a loss of synaptic connections (Zhang et al., 2013; Suárez et al., 2014). Some studies suggest this loss only occurs in iMSNs (Fieblinger et al., 2014; Nishijima et al., 2014). Drastic morphology changes are again observed following L-DOPA treatment, though there has been some variability presumably due to study design. In one study that did not differentiate between dMSNs and iMSNs, an overall increase in spine density, as well as an increase in mature mushroom-type spines (Zhang et al., 2013). Studies that have differentiated the pathways have a general consensus that LID is associated with an increase in iMSN spine density with a concomitant decrease in dMSN spines (Fieblinger et al., 2014; Nishijima et al., 2014; Suárez et al., 2014). These spines are the post-synaptic targets for innervating neurons from the motor cortex and other structures, and changes in the synaptic connectivity due to changes in spine number and morphology can affect the activity of the circuit. The differential pattern of plasticity observed following chronic L-DOPA treatment may have a role in LID genesis. This is further supported by a study that found unusual and irregular glutamatergic corticostriatal synapse formation in rats with LID, suggesting aberrant excitatory input to the striatum (Zhang et al., 2013). Taken together, these data implicate that maladaptive synaptic plasticity occurs in the denervated striatum following L-DOPA treatment and

that this contributes to the development of LID. Identifying molecular markers that are involved in these plasticity changes is an important effort to better understand LID.

Nurr1: implications for Parkinson's and LID

Better clinical management of LID requires a stronger understanding of the molecular mechanisms behind the behavior. The omic-level studies discussed above have identified numerous important pathways and genes that may be involved in the maladaptive changes that lead to LID. One such gene is Nurr1, which has been identified as being upregulated in MSNs of dyskinetic mice (Heiman et al., 2014; Sodersten et al., 2014). Additional unpublished gene array data from our group corroborates these findings in rats, indicating that Nurr1 is a compelling target for further research in relation to LID.

I. Nurr1 biology and transcriptional regulation

Nurr1, or NR4A2, is a member of the NR4A nuclear receptor family and expressed in a number of brain regions (Law et al., 1992). Nurr1, like its family members NR4A1 (Nur77) and NR4A3 (NOR-1), is an IEG and its expression is rapidly induced by a variety of cellular signals, including ERK1/2 signaling (Sacchetti et al., 2006; Campos-Melo et al., 2013). Nurr1 mRNA is highly expressed in a range of structures in the developing mouse and rat brain (Zetterstrom et al., 1996; Hirokawa et al., 2008). Nurr1 expression persists into adulthood, with transcripts localized in the olfactory bulb, cortex, hippocampus, and SNc, among others (Xiao et al., 1996; Zetterstrom et al., 1996).

Nurr1 is an orphan nuclear receptor, meaning that it can regulate transcription independent of ligand binding (Giguere, 1999). In fact, analysis of the crystal structure of Nurr1 shows the ligand binding domain is filled with hydrophobic residues that take up the space for ligand binding seen in other nuclear receptors, further implying its orphan state (Wang et al., 2003). Still, Nurr1 activity can be regulated by its binding to fatty acids as well as introduction of synthetic compounds (Hammond et al., 2015; de Vera et al., 2016; de Vera et al., 2018). Nurr1 transcriptional activity has also been shown to change based on its expression levels (Johnson et al., 2011). For instance, lower levels of Nurr1 may inhibit inflammatory signaling, whereas high levels can induce it (Do, 2014). Nurr1-mediated gene transcription in some pathways are impacted similarly irrespective of Nurr1 levels, including the MAPK signaling cascade and DAergic synaptic proteins (Do, 2014).

Nurr1 activity is also dependent on its dimerization state. The transcription factor can act as a monomer or homodimer, as well as heterodimerizing with other NR4A family members (Maira et al., 1999; Hawk & Abel, 2011). Nurr1 can also heterodimerize with non-NR4A members, such as the Retinoid X receptor (RXR) receptor, to stimulate differential transcription (Zetterström et al., 1996; Volakakis et al., 2015). The dimerization state of the NR4A family affects what DNA sequences the proteins can bind to. As monomers, the binding specificity of NR4A transcription factors is the NGFI-B response element (NBRE) (Paulsen et al., 1995). As homodimers or heterodimers with other NR4A members, Nurr1 and its family members affect transcription by binding the Nur-responsive element (NurRE) (Philips et al., 1997; Maxwell & Muscat, 2006). Nurr1 again displays differential DNA binding when in heterodimers with RXR, where it

binds the DR5 motif to regulate retinoid signaling (Zetterström et al., 1996). Together, this shows that Nurr1 displays unique transcriptional activity based on its dimerization status and expression levels based on cellular activity.

II. Role of Nurr1 in dopaminergic neurons and Parkinson's disease

There are a number of reasons why Nurr1 is an interesting candidate to further study in regards to LID. One of the most well characterized roles of Nurr1 is in the development and long-term health of DA neurons of the SNc (Zetterstrom et al., 1997; Jiang et al., 2005; Kadkhodaei et al., 2009). As discussed above, the Nurr1 transcript is abundant in the SNc of both prenatal and postnatal rodents (Xiao et al., 1996; Zetterstrom et al., 1996). The importance of Nurr1 in development was shown in Nurr1 null mice, which do not develop DAergic neurons in the SNc and do not survive long after birth (Zetterstrom et al., 1997). DA levels in the striatum of these mice are reduced by 98% (Le et al., 1999). This is only observed in midbrain DA neurons, as smaller DAergic populations in the hypothalamus and olfactory bulbs are not susceptible in these mice (Le et al., 1999). This evidence shows that Nurr1 is a critical factor required for the proper development of the midbrain DA neurons that are susceptible in PD. The impact of Nurr1 goes beyond development, as heterozygous knockout Nurr1 mice begin to display DAergic dysfunction over time. The heterozygotes—which are viable—show motor deficits similar to parkinsonian models, decreased DA in the striatum, and a loss of SNc neurons (Jiang et al., 2005). Further, conditionally knocking down Nurr1 in the SNc of adult mice cause progressive SNc cell loss and motor deficits (Kadkhodaei et al., 2009). Nurr1 has been shown to regulate the transcription of many DA-specific genes, including *TH, AADC*, and *DAT* (Zetterstrom et al., 1997; Saucedo-Cardenas et al.,

1998; Sakurada et al., 1999; Decressac et al., 2013). Dysregulation of these genes in the knockout mouse models likely contributes to DA loss and cell death. Nurr1 also is involved in mitochondrial gene transcription, and its reduced expression may impact oxidative stress that promotes cell death (Decressac et al., 2013). These pivotal preclinical studies indicate that Nurr1 is both required for the development of SNc DA neurons and important for their health and maintenance in adulthood.

In humans, nigral Nurr1 expression decreases with aging—a major risk factor for PD (Chu et al., 2002). Accordingly, PD patients show a marked decrease in SNc Nurr1 expression that correlates with TH loss (Chu et al., 2006). There have been a number of Nurr1 polymorphisms identified that may be associated with familial PD, but it is unclear if these contribute to PD risk in the general population (Le et al., 2003; Zheng et al., 2003; Nichols et al., 2004; Liu et al., 2013).

An additional link to PD comes from research elucidating the interaction between Nurr1 and α-syn. Viral overexpression of α-syn in the rat SNc causes a subsequent down-regulation of Nurr1 (Decressac et al., 2012). Forcing the overexpression of Nurr1 in these neurons can reverse α-syn-mediated changes in gene expression (Volakakis et al., 2015). This evidence shows that Nurr1 is involved in the pathology and progression of PD at multiple levels. Because of its clear role in DAergic neuron health, a number of studies have worked to identify Nurr1 agonists as potential disease-altering treatments for PD (Kim et al., 2015; Smith et al., 2015).

III. Nurr1 in the dyskinetic striatum

While Nur77 and NOR-1 show endogenous expression in the striatum, Nurr1 does not (Xiao et al., 1996; Zetterstrom et al., 1996). It is expressed in a small population of TH-positive striatal interneurons, but not in MSNs (Cossette et al., 2004). This makes the upregulation of Nurr1 in the dyskinetic striatum highly unusual, and an interesting target for therapeutic approaches. Nothing is known about the role of Nurr1 in MSNs, as it has not been observed in these neurons until recently in LID-expressing animals. Thus, research elucidating how Nurr1 is acting in MSNs is appropriate to better understand LID.

A potential role for Nurr1 in LID could be through the induction of maladaptive plasticity. As discussed above, aberrant spine dynamics and neuronal activity are associated with LID development (Bastide et al., 2015). Previous work has shown that Nurr1 plays a substantial role in hippocampal synaptic plasticity required for learning and memory (Hawk & Abel, 2011). Nurr1 transcript is upregulated in rat brains after undergoing spatial learning tasks (Peña de Ortiz et al., 2000). Performance in memory task is impaired when Nurr1 is silenced in the rat hippocampus, further suggesting its importance in plasticity (Colon-Cesario et al., 2006). Knowing that Nurr1 retains these capabilities, it is reasonable to question if the transcription factor assumes a similar role when abnormally expressed in MSNs during LID.

Nurr1 expression in MSNs also opens the possibility of LID-driving transcription by heterodimerization with Nur77. Nur77 is endogenously expressed in the striatum, and is increased by L-DOPA treatment in denervated animals, primarily in dMSNs (St-Hilaire et al., 2003; Mahmoudi et al., 2009). In fact, it was recently shown that viral

overexpression of Nur77 in the striatum can exacerbate AIMs in rats, suggesting it plays a direct role in LID severity (Rouillard et al., 2018). Nur77 expression has additionally been shown to induce spine loss in hippocampal neurons, showing that other members of the NR4A family affect synaptic plasticity (Chen et al., 2014). It is possible that Nurr1 acts in a similar fashion to—or even in conjunction with—Nur77 to alter the striatum and promote LID. Taken together, the Nurr1 is an intriguing target to study in LID, especially considering potential Nurr1 agonist therapies for PD that could affect Nurr1 in the striatum.

Recombinant adeno-associated virus: using gene therapy as a research tool

I. rAAV biology

Major advancements in the current understanding of LID have been achieved by modulating gene expression in preclinical models using gene therapy techniques. One such tool used widely in neuroscience research employs recombinant adeno-associated virus, or rAAV. Wild type AAV is a non-enveloped parvovirus that does not cause any known disease or illness, with over 70% of the population having been infected at some time (Calcedo et al., 2009; Choudhury et al., 2017). The AAV genome was reengineered for research and clinical purposes as a vector delivery tool. The only remnants of the wild type genome in rAAV are the inverted terminal repeats (ITRs) which are required elements for packaging the genome into the viral capsid, as well as genome stabilization and transcription (Schultz & Chamberlain, 2008; Nonnenmacher & Weber, 2012).

The wildtype genes required for genome replication and capsid formation are removed in rAAV, leaving approximately 5 kilobases of space to insert genetic material of interest (Samulski & Muzyczka, 2014). The engineered cassette must include enhancer and promoter elements to either drive ubiquitous or cell-specific expression (Gray et al., 2011). Spatial and temporal expression can also be controlled using Cre-dependent genomes in transgenic animals, which can allow for conditional knockouts/knock-ins or cell-specific expression (Schnütgen et al., 2003). There are numerous approaches to control gene expression with rAAV depending on the expression cassette employed. A single gene of interest can be inserted into the vector to drive overexpression within cells. Numerous approaches for dual-expression using multicistronic genomes have been developed to allow for expression of multiple genes from one virus (Donnelly et al., 2001; Ngoi et al., 2004; Fagoe et al., 2013). Conversely, gene knockdown/knockout approaches are widely used, employing RNA interference (shRNAs, siRNAs, miRNAs, etc.) and gene editing (CRISPR). The versatility of rAAV makes it a robust research tool for research.

II. rAAV in neuroscience research

rAAV is a strong tool to use in the central nervous system (CNS) as it is able to transduce non-dividing cells (Choudhury et al., 2017). A number of rAAV capsid varieties—called pseudotypes—are capable of efficiently transducing neurons to achieve long-term, stable gene expression (Burger et al., 2004; McFarland et al., 2009). rAAV particles bind with extracellular receptors based on their pseudotype and are taken into the cell and trafficked into the nucleus, where the genome is released (Schultz & Chamberlain, 2008; Nonnenmacher & Weber, 2012). The viral DNA does not

integrate into the host genome, but instead is held episomally (Choudhury et al., 2017). Episomal DNA is not lost in non-dividing cells such as neurons, allowing for long-term, stable expression of the viral cassette in transduced cells. Stable rAAV-mediated gene expression has been observed two years following delivery in rat models, and 15 years in non-human primates (Klein et al., 2002; Aubert et al., 2005; Sehara et al., 2017).

rAAV has been used in many preclinical studies that have progressed our understanding of LID etiology. Virally-mediated expression of candidate genes of LID has helped elucidate genes that are involved in LID development and severity. For instance, rAAV-mediated overexpression of genes such as FosB, Nur77, and BDNF can exacerbate LID severity, indicating that these factors have direct involvement in dyskinesia (Berton et al., 2009; Cao et al., 2010; Cote et al., 2014; Tronci et al., 2017; Rouillard et al., 2018). Conversely, LID severity can be ameliorated in these models by virally silencing LID-driving genes, or overexpressing genes deficient in LID (Marongiu et al., 2016; Park et al., 2016). Thus, using rAAV is a strong tool for characterizing novel molecular markers in LID.

III. Clinical perspective on rAAV

rAAV is not only a powerful research tool, but useful for gene therapy in PD patients. rAAV vectors have been shown to be safe and well tolerated multiple clinical trials, as they do not generate a significant inflammatory response (Choudhury et al., 2017). Over the past few decades, a number of rAAV gene therapy clinical trials have been conducted to treat PD (Polinski, 2016). While these therapies have not shown efficacy above current therapeutics and research is ongoing, rAAV delivery has been found to

be clinically safe for use in these patients. This precedent for clinical rAAV use in PD patients paves the way for potential LID-managing gene therapies.

Overarching significance

The phenomenon of LID has plagued the most effective symptomatic PD treatment since its clinical introduction. The burden of these symptoms cannot be overstated, as they can dramatically affect the lives of patients and their caregivers, while physicians are left with few effective options for managing LID. While it is of grave importance that the field continues to study disease-altering treatments for PD, patients and their families still currently—and likely will continue to—rely on the symptomatic relief granted by L-DOPA. Research focused on understanding LID with the end-goal to eradicate these symptoms is an important aspect of the PD field. In this dissertation, I aimed to contribute to this field by characterizing LID etiology from both a presynaptic and postsynaptic perspective. Using rAAV as a research tool, I first aimed to close critical knowledge gaps in the presynaptic hypothesis of LID by supplementing 5-HT neurons with the DA autoreceptor, giving them the ability to regulate DA release. Additionally, I sought to understand molecular postsynaptic changes in LID by showing that the orphan nuclear receptor Nurr1 is a novel driver of LID. Lastly, this dissertation will present evidence showing that maladaptive Nurr1 expression can promote striatal changes that influence LID development. Together, the Specific Aims of my project add valuable insight to the etiology of LID, and suggest potential therapeutic strategies for LID management.

Aim 1: Regulation of dopamine neurotransmission from 5-HTergic neurons by ectopic expression of the dopamine D2 autoreceptor blocks levodopa-induced dyskinesia

Addressed in Chapter 2, in Aim 1, I virally expressed the DA autoreceptor, $D2R_S$ in serotonin neurons of the dorsal raphe nucleus of parkinsonian rats. I challenged these rats with escalating doses of L-DOPA and DA receptor agonists to determine if $D2R_S$ expression allowed for regulated release of DA from dorsal raphe neurons and, in turn, amelioration of LID. With this Aim, I sought to confirm the serotonin hypothesis of LID by showing that DA is abnormally released for dorsal raphe neurons, and that regulation of this release can mitigate LID.

Aim 2: Modulating levels of Nurr1 expression in the parkinsonian rat striatum impacts LID severity and development

Addressed in Chapter 3, in Aim 2 my goal was to characterize Nurr1 as a novel molecular factor in LID. I virally modulated Nurr1 levels in LID susceptible and resistant rats to determine if Nurr1 expression can exacerbate LID, and if its presence in MSNs is required for LID development. Additionally, I utilized specific D1 or D2 receptor agonists to determine if abnormal Nurr1 upregulation is induced by specific receptor stimulation, or if it requires activation of both the direct and indirect pathways.

Aim 3: Ectopic overexpression of Nurr1 induces an LID-like state in striatal medium spiny neurons

Addressed in Chapter 4, in Aim 3, I further determine the role of Nurr1 in LID development by showing how its ectopic overexpression alters striatal neurons. To

achieve this, we virally overexpressed Nurr1 in L-DOPA naïve animals and measured the electrophysiological properties of MSNs. I also determined if Nurr1 expression induces changes in the density and morphology of MSNs. This Aim allowed me to determine how Nurr1 influences the striatum in ways that may generate LID.

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Chapter 2: Regulation of dopamine neurotransmission from serotonergic neurons by ectopic expression of the dopamine D2 autoreceptor blocks levodopa-induced dyskinesia

Preface

In this chapter, *in vivo* microdialysis and HPLC experiments were carried out by Dr. Christopher Bishop at Binghamton University. Electrophysiology recordings were performed by Dr. Anthony West at Rosalind Franklin University. All other experiments and analysis were performed at Michigan State University by Rhyomi C. Sellnow.

Introduction

The hallmark motor symptoms in Parkinson's disease (PD) arise following substantial dopaminergic denervation within the striatum. Denervation results from the death of tyrosine hydroxylase (TH) expressing DA neurons of the substantia nigra pars compacta (SNc) as the disease progresses (Hoehn & Yahr, 1967; Kordower et al., 2013). The lack of proper DA signaling to the striatum creates an imbalance of the basal ganglia motor circuit, thus, causing bradykinesia, rigidity, tremor, and gait problems characteristic of PD (Goldman & Postuma, 2014). Current effective treatment strategies, while not affecting disease progression, are aimed at treating these primary motor symptoms. Since the late 1960s, L-3,4-dihydroxyphenylalanine (levodopa or L-DOPA) has been used as a catecholamine replacement therapy to alleviate motor symptoms (Cotzias et al., 1967). L-DOPA remains the gold-standard pharmacological treatment for PD.

While effective initially, the therapeutic window of L-DOPA narrows with the continuous loss of SNc neurons as the disease progresses, and higher doses are required to

maintain the anti-akinetic effects of L-DOPA. Moreover, chronic treatment with L-DOPA leads to the development of L-DOPA-induced dyskinesias (LID), a series of motor symptoms distinct and independent from the PD motor deficits being treated (reviewed in (Bastide et al., 2015)). These symptoms, comprised of painful and disrupting movements including hyperkinesia, dystonia, and chorea, occur in a majority of PD patients, developing in up to 50% of patients within 5 years of beginning treatment, and up to 90% of patients within 10 years (Ahlskog & Muenter, 2001; Manson et al., 2012).

Studies show that LID development is a multifaceted process. However, it is largely agreed upon that the intermittent oral dosing of L-DOPA results in large variations in extracellular DA. Ultimately, this pulsatile release of DA, together with the denervated state of the striatum, results in maladaptive molecular and structural changes in the DA-responsive neurons of the striatum, specifically medium spiny neurons (MSNs), leading to altered basal ganglia signaling (reviewed in (Cenci & Konradi, 2010)). Given the extreme nigrostriatal denervation at the time of diagnosis(Kordower et al., 2013) the actual source of striatal DA following L-DOPA administration has been debated over the past half century. The leading hypothesis is that uptake of L-DOPA and its subsequent dysregulated metabolism to DA, and release by 5-HTergic 5-hydroxytryptamine (5-HT) neurons in the dorsal raphe nucleus (DRN) may be linked to dyskinesogenesis (reviewed in (De Deurwaerdère et al., 2016)). These neurons express aromatic L-amino acid decarboxylase (AADC) and can therefore convert L-DOPA into DA. However, DRN neurons do not express the regulatory mechanisms to monitor and control DA synthesis and release into the synapse, allowing for the unregulated release of DA into a hypersensitized striatum (Maeda et al., 1999). Additionally, 5-HTergic innervation of the

striatum increases substantially following DA denervation, allowing the majority of L-DOPA to be metabolized and released as DA by 5-HTergic terminals (Maeda et al., 2003; Maeda et al., 2005; Yamada et al., 2007; Rylander et al., 2010; Roussakis et al., 2016)}. This overwhelming exposure of the DA-depleted striatal MSNs is hypothesized to be a large contributor to LID. In fact, studies in rats show that specifically lesioning the DRN (Carta et al., 2007; Eskow et al., 2009) or co-administering L-DOPA with 5-HT1 receptor agonists (Bezard et al., 2013; Politis et al., 2014; Ghiglieri et al., 2016; Meadows et al., 2017), effectively reduces or eliminates LID.

Normal regulation of DA signaling is mediated presynaptically primarily through the DA active transporter (DAT) and the DA autoreceptor. DAT directly regulates the levels of DA in the synapse by transporting synaptic DA back into the terminal. The dopamine autoreceptor ($D2R_S$) is an isoform of the D2 DA receptor ($D2R_L$) missing 29 amino acids from the third intracellular loop (Dal Toso et al., 1989). $D2R_S$ detects synaptic DA levels and regulates DA signaling in three ways, 1) by downregulating DA production through TH regulation, 2) regulation of reuptake through DAT, and 3) by directly inhibiting DA release (reviewed in (Ford, 2014)). Each of these modes of action are mediated through the inhibitory G_i alpha protein signaling pathways following $D2R_S$ activation.

These canonical G-protein-coupled receptor (GPCR) signaling pathways similarly inhibit 5-HTergic signaling in DRN neurons through 5-HT1 autoreceptor activation (Harrington et al., 1988; Okada et al., 1989). Previous studies using 5-HT1 agonists show promising reductions in LID. Unfortunately, these drugs can negate the anti-parkinsonian therapeutic benefits of L-DOPA animal models, and in some cases worsen PD

symptoms in clinical trials (Kannari et al., 2002; Olanow et al., 2004; Iravani et al., 2006; Cheshire & Williams, 2012).

While current evidence suggests a crucial role of 5-HTergic input and activity in LID, direct evidence of the abnormal dopaminergic neurotransmission and dysregulated DA release is lacking. In the present study, we sought to provide unequivocal evidence for the role of 5-HTergic DA neurotransmission in dyskinesogenesis and examine a novel therapeutic approach of modulating this non-physiological adaptation in the parkinsonian brain. To do this, we provided 5-HTergic neurons with DAergic regulatory mechanisms by ectopically expressing the $D2R_S$ autoreceptor in the DRN of parkinsonian 6-OHDA lesioned rats, and evaluated the effect of ectopic $D2R_S$ activity on L-DOPA efficacy, LID formation, response to DA agonists, and striatal DA release.

Methods

I. Adeno-associated virus production

The $D2R_S$ and GFP coding sequences were cloned into AAV genomes under the control of the chicken β-actin/cytomegalovirus (CBA/CMV) promoter for ubiquitous and robust expression. rAAV 2/9 was produced via triple-transfection of HEK 293T cells with the genome and helper plasmids. Virus was recovered from cells using freeze-thaw cycles, purified using an iodixanol gradient (Optiprep Density Gradient, Sigma-Aldrich, St. Louis, MO), followed by buffer exchange and concentration using concentrator columns (Orbital Biosciences, Topsfield, MA) as described previously (Benskey et al., 2016). The viral titer was determined using digital droplet PCR (ddPCR) and normalized to $1x10^{13}$ vector genomes (vg)/ml using Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO).

II. Animals and surgeries.

Studies were performed using adult male Fischer F344 rats (200-220g upon arrival; Charles River, Wilmington, MA) in accordance with the guidelines of Michigan State University (AUF MSU06/16-093-00), Binghamton University (AUF# 779-17), and Rosalind Franklin University (AUF# A3279-01) Institutional Animal Care & Use Committees. Rats were housed two per cage prior to behavioral testing, and then separated and individually housed with environment enrichment during behavior studies for the remainder of the experiments. The animals were housed in a light-controlled (12 hours light/dark cycle) and temperature-controlled (22±1 °C) room, and had free access to standard lab chow and water.

All 6-OHDA and vector surgeries were performed under 2% isoflurane. After being anesthetized, animals were placed in a stereotaxic frame and were injected using a glass capillary needle fitted to a Hamilton syringe (Hamilton, Reno, NV) (Benskey & Manfredsson, 2016). Three weeks following lesion surgery, animals were tested for spontaneous forepaw use (cylinder test) to estimate lesion efficacy. Vector treatment groups were normalized using forepaw deficits in order to ensure equal lesions between the treatment groups.

For lesion surgeries 5mg/ml 6-OHDA hydrobromide (Sigma-Aldrich, St. Louis, MO) was prepared in 0.2mg/ml ascorbic acid immediately prior to the injections. A subset of animals (n=7) destined for electrophysiological measures did not receive a 6-OHDA lesion. Animals received 2µl injections of 6-OHDA into the medial forebrain bundle (MFB) (from bregma: Anterior Posterior (AP) – 4.3mm, Medial Lateral (ML) +1.6mm, Dorsal Ventral (DV) -8.4mm from skull) and the SNc (from bregma: AP -4.8mm, ML

+1.7mm, DV -8.0mm from skull), for a total of 10µg 6-OHDA per site and 20µg per animal. The glass needle was lowered to the site and the injection started after 30 seconds. 6-OHDA was injected at a rate of 0.5µl/minute. The needle was removed two minutes after the injection was finished and cleaned between each injection.

Vector delivery was performed three weeks following the 6-OHDA lesion via stereotaxic delivery (Benskey & Manfredsson, 2016). Using the same procedure as described for the lesion surgeries, animals received a single 2µl injection of virus (AAV2/9-DR_S, $1x10^{13}$ vg/ml; AAV2/9-GFP, $1x10^{13}$ vg/ml) to the DRN (from bregma: AP -7.8, ML -3.1, DV -7.5 from skull). The stereotaxic arm was positioned in a 30° lateral angle in order to avoid the cerebral aqueduct.

Parkinsonian and vector-injected animals used for *in vivo* microdialysis were shipped to Binghamton University two weeks following the vector surgeries. Following quarantine, rats were acclimated to the colony room and habituated to handling for one week. Rats were then tested for baseline forepaw adjusting steps. Thereafter, microdialysis cannulation surgery was performed under 2-3% isoflurane in oxygen with the tooth bar set to 5 mm below the interaural line. Five minutes before surgery and 24 hours after surgery rats received an injection of Buprinex (0.03 mg/kg, i.p.). A unilateral dorsal striatal-directed cannula (CMA 12 Elite; Stockholm, Sweden) was implanted ipsilateral to lesion (from bregma AP: 1.2 mm; ML: -2.8 mm; DV: -3.7 mm). The cannula was fixed in place by four jeweler's screws, jet liquid, and dental acrylic (Lang Dental, Wheeling, IL). Two weeks following cannulation surgery, rats underwent behavioral testing.

Non-lesioned rats used for electrophysiological recordings of the DRN were shipped to Rosalind Franklin University two weeks following the vector surgeries and housed for an

additional 4-8 weeks prior to electrophysiological recordings. Burr holes (~1 mm in diameter) were drilled in the skull overlying the DRN. Prior to experimentation all animals were anesthetized with urethane (1.5 g/kg i.p.) and placed in a stereotaxic apparatus. Bipolar stimulating/recording electrodes were implanted in the frontal cortex and DRN on the right side using a micromanipulator (coordinates from Bregma: AP: 3.2 mm; ML: 0.8 mm lateral; DV: 4.4 mm ventral (frontal cortex) or AP: 7.8 mm; ML 3.1 mm; DV: 7.5 mm with the manipulator angled 30 degrees toward Bregma) as previously described (Chakroborty et al., 2017).

III. Abnormal involuntary movement (AIM) ratings and drug treatments.

Animals were allowed to recover for three weeks following vector injections, and to allow for peak expression of the viral transgene (Reimsnider et al., 2007). After this time, L-DOPA treatment and abnormal involuntary movement (AIM) scale ratings began. As has been described previously, the AIM rating scale can be used to evaluate the severity of LID and has been adapted for animal use (Lundblad et al., 2002; Steece-Collier et al., 2003). Briefly, AIMs were evaluated by scoring the level of dystonia of the limbs and body, hyperkinesia of the forelimbs, and orolingual movements. Each AIM is given two numerical scores—one indicating the intensity (0=absent, 1=mild, 2=moderate, or 3=severe) and frequency (0=absent, 1=intermittently present for <50% of the observation period, 2=intermittently present for >50% of the observation period, or 3=uninterruptable and present through the entire rating period) (Maries et al., 2006) Each AIM was given a severity score by multiplying the intensity and frequency, and the total AIM score is a sum of all the behaviors severities. An animal is considered non-dyskinetic with a score of ≤4, as non-dyskinetic parkinsonian rats can display low level

AIMs from exhibiting normal chewing behavior and a mild parkinsonian dystonic posture (Maries et al., 2006).

Animals received subcutaneous injections of L-DOPA/benserazide (Sigma-Aldrich, St. Louis, MO) three times per week and were rated using the AIM scale in 25-minute intervals post-injection until all AIM behavior had subsided. L-DOPA doses ranged between 2mg/kg-12mg/kg. Benserazide doses (12 mg/kg) remained constant for all L-DOPA injections. The same injection and rating paradigm was used for AIM evaluations with the non-selective DA agonist apomorphine (0.1mg/kg, R&D Systems, Minneapolis, MN), the D2/D3 receptor agonist quinpirole (0.2mg/kg, Sigma-Aldrich, St. Louis, MO) and the D1 receptor agonist SKF-81297 (0.8mg/kg, Sigma-Aldrich, St. Louis, MO). Peak AIM scores of DA agonists were determined based off the highest average AIM scores of control animals during the rating period.

IV. Parkinsonian motor evaluation.

To assess whether $D2R_s$ viral therapy affects the anti-parkinsonian properties of L-DOPA therapy, we evaluated parkinsonian motor behavior on and off L-DOPA using the cylinder task and the forepaw adjusting steps (FAS) test. Rats with significant lesions perform poorly on both these tests, with impairment to the forepaw contralateral to the lesion that is alleviated with L-DOPA treatment (Chang et al., 1999; Schallert, 2006). The cylinder task was conducted as previously reported (Manfredsson et al., 2007). Animals were placed in a clear Plexiglas cylinder on top of a light box for five to seven minutes while being recorded. Each animal was rated by counting ~20 weight-bearing forepaw placements on the cylinder (contralateral to the lesion, ipsilateral to the lesion, both) to determine the percentage use of the forepaw contralateral to the lesion, which

is derived by dividing the sum of contralateral touches and half of both forepaw touches by the total forepaw touches, and multiplying this number by 100. Trials were performed following the initial L-DOPA treatment (AIM evaluation) period, and tested either off L-DOPA or, on the following day, 50 minutes after receiving a 6mg/kg L-DOPA injection (12mg/kg benserazide).

The FAS test was performed as described previously (Meadows et al., 2017). Briefly, rats were restrained by an experimenter so that only one forepaw was free to touch the counter. Rats were then dragged laterally along a 90 cm distance over 10 sec while a trained rater blind to the experimental condition counted the number of steps. Data are represented as forehand percent intact, which are derived by taking the number of steps taken by the contralateral forehand and dividing it by the ipsilateral forehand, and then multiplying this number by 100. The test was performed over two days either off L-DOPA or 60 minutes following an 8 mg/kg or 12 mg/kg L-DOPA injection.

V. Tissue collection

Two hours following the final L-DOPA administration, animals from the AIM experimentation were sacrificed via sodium pentobarbital overdose and intracardially perfused with Tyrode's solution (137mM sodium chloride, 1.8mM calcium chloride dihydrate, .32mM sodium phosphate monobasic dihydrate, 5.5mM glucose, 11.9mM sodium bicarbonate, 2.7mM potassium chloride). Brains were rapidly removed and coronally hemisected, with the rostral portion of the left and right striatum dissected out and flash frozen in liquid nitrogen for biochemical analysis. The caudal portion of the brain was postfixed for 72 hours in 4% paraformaldehyde (PFA) in phosphate-buffered saline and then cryoprotected by saturation in 30% sucrose. Brains were frozen and

sectioned coronally at 40µm thickness using a sliding microtome into free floating sections and stored in cryoprotectant (30% ethylene glycol, 0.8mM sucrose in 0.5X tris-buffered saline) until further use.

VI. Immunohistochemistry

A 1:6 series of free-floating tissue was stained immunohistochemically for TH (MAB318, MilliporeSigma, Burlington, MA), D2R (AB5084P, MilliporeSigma, Burlington, MA), or GFP (AB290, Abcam, Cambridge, United Kingdom) using methods previously reported (Benskey et al., 2018). Sections were washed in 1x Tris-buffered saline (TBS) with .25% Triton x-100, incubated in 0.3% H_2O_2 for 30 minutes, and rinsed and blocked in 10% normal goat serum for 2 hours. Tissue was incubated in primary antibody (TH 1:4000, D2R 1:1000, GFP 1:20,000) overnight at room temperature. After washing, tissue was incubated in secondary antibody (biotinylated horse anti-mouse IgG 1:500, BA-2001; Vector Laboratories, Burlingame, CA; biotinylated goat anti-rabbit IgG 1:500, AP132B, Millipore-Sigma, Burlington, MA) followed by the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Tissue staining was developed with 0.5 mg/ml 3,3' diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) and 0.03% H_2O_2. Sections were mounted on glass slides, dehydrated, and coverslipped with Cytoseal (ThermoFisher, Waltham, MA).

Tissue for immunofluorescence dual labeling of $D2R_s$ or GFP with SERT (340-004, Synaptic Systems, Goettingen, Germany) were washed with 1x TBS with 0.25% Triton x-100, blocked in 10% normal goat serum for 2 hours, and probed with primary antibody overnight ($D2R_s$ 1:1000, GFP 1:20,000, SERT 1:300). Tissue was incubated with

secondary antibody (A11008 1:500, A11076 1:500; ThermoFischer, Waltham, MA) in the dark for two hours, and washed in TBS before being mounted and coverslipped with Vectashield Hardset Antifade Mounting Medium (Vector Laboratories, Burlingame, CA).

Images were taken on a Nikon Eclipse 90i microscope with a QICAM fast 1394 camera (fluorescence; QImaging, Surrey, British Columbia, Canada) or a Nikon D-1 camera (brightfield microscopy; Nikon, Tokyo, Japan). The figures were made using Photoshop 7.0 (Adobe, San Jose, CA) with the brightness, sharpness, and saturation adjusted only as needed to best represent the staining as it is viewed directly under the microscope.

VII. *In vivo* microdialysis.

As outlined above, a separate cohort of parkinsonian rats treated with GFP or $D2R_S$ were utilized for *in vivo* microdialysis. The night before the procedure, striatal probes (CMA 12 Elite; membrane length = 3 mm; 20,000 Dalton; Stockholm, Sweden) were inserted into the guide cannula so that they extended from bregma DV: -3.7 to -6.7 mm within the dorsal striatum. Rats underwent microdialysis at least two days following the last L-DOPA administration. During microdialysis, rats received intrastriatal infusion of filtered artificial cerebrospinal fluid (aCSF) (128mM NaCl, 2.5mM KCl, 1.3mM $CaCl_2$, 2.1mM $MgCl_2$, 0.9mM NaH_2PO_4, 2.0mM Na_2HPO_4, and 1.0mM glucose, pH 7.4). Dialysate samples were collected every 20 minutes. Briefly, rats were habituated to microdialysis for 1 hour. Fifty minutes into the procedure, rats received a subcutaneous injection of L-DOPA vehicle, which consisted of 0.9% NaCl, and 0.1 % ascorbate. Rats then underwent baseline testing for one hour to determine baseline levels of monoamines prior to L-DOPA treatment. After that a new collection tube was used and ten minutes later rats received an injection of L-DOPA (12 mg/kg + 12 mg/kg

Benserazide, s.c.). Samples were taken every 20 minutes for 3 hours. Following the procedure, rats were removed from the microdialysis bowl and striatal probes were replaced with a dummy probe. At least two days after microdialysis, rats were sacrificed via rapid decapitation, the anterior striatum was taken for verification of cannula placement, the posterior striatum was taken for HPLC, and the hindbrain was placed in 4% PFA for 3 days before being placed in 30% sucrose in phosphate-buffered saline (PBS). Brains were shipped on ice in a 50mL conical containing 30% sucrose in 0.1 M PBS to MSU.

VIII. High-performance liquid chromatography for monoamine tissue analysis

Striatal tissue and *in vivo* microdialysis samples were analyzed using HPLC. Reverse-phase HPLC was performed on striatal tissue samples as previously described (Kilpatrick et al., 1986; Meadows et al., 2017). Briefly, tissue samples were homogenized in ice-cold perchloric acid (0.1 M) with 1% ethanol and 0.02% ethylenediaminetetraacetic acid (EDTA). Homogenate was spun at 4°C for 45 min at 14,000 g. Supernatant was removed and, using an ESA solvent delivery system (Model 542; Chelmsford, MA, USA) ESA autoinjector (Model 582), analyzed for levels of norepinephrine, 3,4-dihydroxyphenylacetic acid (DOPAC), DA, 5-hydroxyindoleacetic acid (5-HIAA), and 5-HT. Monoamines and metabolites were detected as a generated current as a function of time by EZCHROM ELITE software via a Scientific Software, Inc (SS240x) Module. Data are displayed as peaks for monoamines and metabolites, which are compared to a standard curve made from monoamine and metabolite samples of known concentrations ranging from 1e-6 to 1e-9. Values were then normalized to tissue

weight and lesion deficits are reported as percent depletion, which is equal to 100 (1 – M Lesion/ *M* Intact).

Dialysate samples were analyzed via reverse-phase HPLC on an Eicom HTEC-500 System (Amuza Inc., San Diego, CA). Briefly, 10 µL of each dialysate sample was analyzed for NE, DA, and 5-HT using an Eicompak CAX column maintained at 35°C with a flow rate of 250 µL/min. Mobile phase (75mM Ammonium acetate, 9.36mM acetic acid, 1.33mM EDTA, 0.94mM Methanol, 50mM sodium sulfate). Samples were compared to known concentrations of monoamines (100, 10, 1, 0.1, and 0.05 ng/µL dissolved in a potassium phosphate buffer (0.1mM potassium phosphate monobasic, 0.1mM ethylenediaminetetraacetic acid, 0.02mM phosphoric acid), resulting in a final value of monoamine in ng/µL.

IX. Total enumeration of TH+ neurons to assess lesion severity

Lesion severity was determined using total enumeration of TH-positive neurons in three representative sections within the SNc identified by the presence and proximity to the medial terminal nucleus (MTN) of the accessory optic tract at levels equivalent to − 5.04 mm, − 5.28 mm and − 5.52 mm relative to bregma according to our previously validated method (Gombash et al., 2014) method. Briefly, the intact and lesion SNc were quantified for all TH immunoreactive cells using a 20X objective and MicroBrightfield StereoInvestigator software (MicroBrightfield Bioscience, Williston, VT). The total number of TH cells on the intact and lesioned hemispheres were averaged, and lesion efficacy was derived by dividing the lesioned hemisphere average by the intact hemisphere average and multiplying that value by 100.

X. Electrophysiology

Recording microelectrodes were manufactured from 2.0mm OD borosilicate glass capillary tubing and filled with sodium chloride (2M) solution. Electrode impedance was 5-15MΩ. The signal to noise ratio for all recordings was > 4:1. The level of urethane anesthesia was periodically verified via the hind limb compression reflex and maintained using supplemental administration as previously described (Sammut et al., 2010; Padovan-Neto et al., 2015). Temperature was monitored using a rectal probe and maintained at 37C° using a heating pad (Vl-20F, Fintronics Inc, Orange, CT). Electrical stimuli (duration= 500µs, intensity=1000 µA) were generated using a Grass stimulator and delivered in single pulses (0.5Hz) while searching for cells (Padovan-Neto et al., 2015). Once isolated, recordings consisted of basal (pre-drug), saline vehicle, and drug-treatment-(see below) induced changes in spike activity recorded in a series of 3-minute duration epochs.

All compounds and physiological 0.9% saline were prepared daily and administered intravenously (i.v.) through the lateral tail vein to enable rapid examination of potential acute effects of vehicle or drug on DRN neuronal activity. The selective 5-HT1A agonist 8-OH-DPAT (5µg/kg, i.v.), the selective 5-HT1A antagonist WAY100635 (100µg/kg, i.v.), and the D2R agonist Quinpirole (500µg/kg, i.v.) were dissolved in vehicle and administered systemically to either BFP or $D2R_S$ rats. DRN 5-HT neuron activity was recorded prior to and immediately following drug administration as described above.

XI. Statistical analysis

Statistical analysis was performed using Statview (version 5.0) or in SPSS version 23 with α set to 0.05. All graphs were created in GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA) or Excel (Microsoft, Redmond, WA). Lesion status was evaluated using unpaired, one-tailed t-tests. AIMs differences between vector groups were evaluated using a non-parametric Mann-Whitney U test, with $p \leq 0.05$ being considered statistically significant. Bonferroni post-hoc tests were employed when significant main effects were detected. Cylinder and FAS data for forehand and backhand stepping were submitted to a mixed model ANOVA with within-subjects factors of treatment (2: Baseline, L-DOPA) and between-subjects factors of vector (GFP, D2R). Overall percent intact values for FAS were determined by taking the overall number of right paw steps divided by the number of left paw steps and multiplying the quotient by 100. Similarly, overall percent intact values were analyzed via a repeated-measures ANOVA with within-subjects factor of treatment and between subjects factor of vector. Monoamine content (as determined by HPLC) was submitted to a mixed-model ANOVA with within-subjects factor of treatment (2: Vehicle, L-DOPA) and between-subjects factor of vector. Fisher's least significant difference (LSD) post-hocs and planned paired-samples t-tests were employed as appropriate to clarify significant effects. Additionally, independent-samples t-tests were employed to reveal effects of vector on the timing of DA, NE, and 5-HT efflux. HPLC values for striatal tissue were submitted to a mixed-model ANOVA with within-subjects factor of side and between-subjects factor of vector. Subsequently, since DA depletion did not vary as a function of vector, values for each monoamine for each side were collapsed across

treatments and compared via paired-samples t-tests. For electrophysiology experiments, the difference between the spontaneous and evoked electrophysiological activity of identified DRN-5-HT neurons across groups was determined and served as the dependent variable for our analyses. A two-way repeated measures ANOVA (GFP vs. gene therapy (ectopic expression of the DA D2 AR in 5-HT DR neurons)) x 2 (vehicle vs. drug treatment) with α set to 0.05 and all "n's" adequately powered for electrophysiological studies was conducted using Sigma Stat software (San Jose, CA), and the potential two-way interaction effect was examined to determine how treatment effects differ as a function of drug treatment or gene therapy (Padovan-Neto et al., 2015).

Results

I. Validation of lesion and transgene expression

In order to assess if exogenous expression of $D2R_S$ in the DRN could inhibit LID development or decrease LID severity, adult Fischer rats were rendered parkinsonian with 6-OHDA delivered to the SNc and MFB. Because LID is dependent on the severity of the lesion (Winkler et al., 2002) we validated *post mortem* that sufficient nigrostriatal denervation was achieved. Immunohistochemistry of the striatum (Figure 2.1B) and the SNc (Figure 2.1C) showed a near complete ablation of TH immunoreactivity with no difference in the number of SNc DA neurons between groups (Figure 2.1D; GFP=1.29%$\pm$0.29% remaining; $D2R_S$=1.45%$\pm$0.41% remaining; $t_{(9)}$=0.31, p>0.05). Similarly, HPLC analysis of DA and DOPAC levels from striatal tissue from rats employed in the microdialysis experiment confirmed that all animals displayed a near

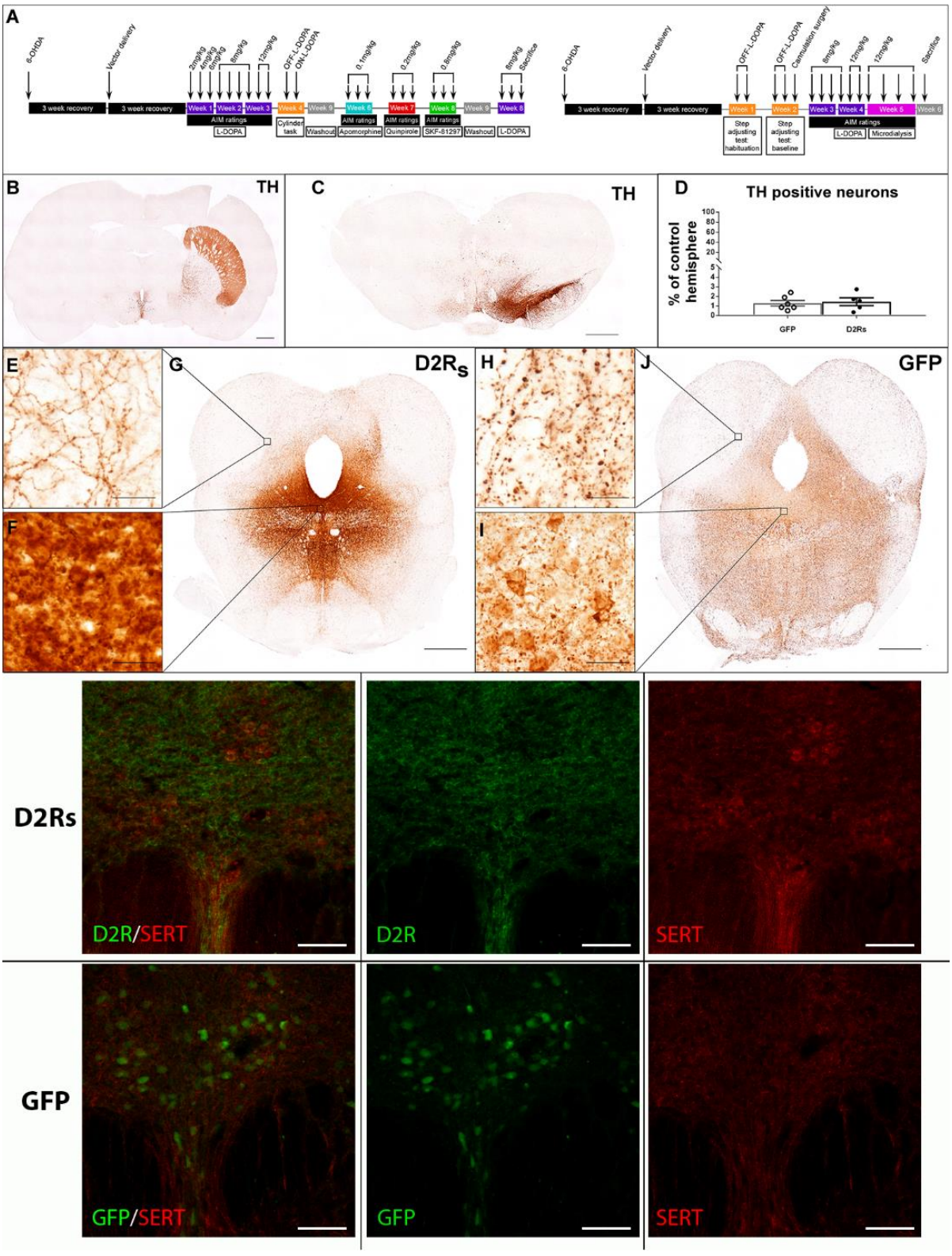

Figure 2.1 Experimental design and model validation

Figure 2.1 (cont'd)

(A) Experimental timeline showing LID-inducing L-DOPA paradigm, motor behavior evaluations, and DA agonist treatments. AIM score ratings were taken at each injection where indicated. (B and C) Representative TH immunoreactivity in the striatum (B) and substantia nigra (C) showing complete loss of TH-positive neurons and projections following 6-OHDA lesions (scale bar=1mm). (D) Total enumeration of remaining TH neurons in the substantia nigra. (E and F) IHC for the D2 receptor (E) or GFP (F) in the DRN, showing successful targeting of the structure and robust expression of the transgene (scale bar=1mm). Cell bodies were efficiently transduced in the DRN (E and F, bottom insets, scale bar=50um) and could be seen filling projection fibers in the peduncles (E and F, top insets, scale bar=50um). (K-P) Dual labeling transgene expression and SERT in rAAV-D2R_S (K-M) and rAAV-GFP (N-P) animals. Transgene expression was visualized with D2R_S (L) or GFP (O) staining, and serotonin fiber and cell integrity were confirmed by staining for SERT (M and P). No adverse effects on SERT fibers were observed following vector transduction with either construct (K and N) (K-P scale bar=100um).

complete reduction in striatal DA levels in the lesioned hemisphere as compared to the intact hemisphere (DOPAC=18.11±6.68% of intact hemisphere, DA=3.48± 1.36% of intact hemisphere) (Table 2.1). There was no difference in striatal DA depletion between groups (DOPAC $t_{(13)}$=0.73, p>0.05, DA $t_{(13)}$=17.21, p>0.05).

After a three-week recovery period, rAAV 2/9 expressing either $D2R_s$ or GFP was delivered by stereotaxic injection into the DRN. Following sacrifice, transduction was confirmed with immunohistochemistry (IHC) of $D2R_s$ or GFP (Figure 2.1E-J). Significant transgene expression was observed in the soma ($D2R_s$ Figure 2.1F; GFP Figure 2.1I) of the DRN as well as in DRN efferent projections ($D2R_s$ Figure 2.1E; GFP Figure 2.1H). The two transgenes exhibited a slightly different subcellular expression pattern where more GFP expression was seen in projections as compared to $D2R_s$ expression. It is unclear if this is due to increased 5-HT innervation in dyskinetic (i.e. GFP treated) animals (Maeda et al., 2003), or due to a different distribution pattern specific to the transgenes. The latter is to be expected as GFP is a soluble protein and typically fills the entire neuron. Vector transduction and transgene expression of both constructs did not adversely affect SERT expression in the DRN (Figure 2.1K-P). Four animals (rAAV-$D2R_s$: n=2, rAAV-GFP: n=2) that lacked sufficient vector expression in the DRN were removed from the analysis, leaving a total of n=15 rats included in the analysis (rAAV-$D2R_s$: n=7, rAAV-GFP: n=8).

II. $D2R_s$ delivery to the dorsal raphe eliminates LID

After a 4-week recovery period to allow for optimal transgene expression (Reimsnider et al., 2007), animals were treated with L-DOPA and rated for AIMs (see Figure 2.1A for

Monoamine	Lesioned Side	Intact Side	% Intact
NE	14.89 ±5.77*	41.93 ±2.19	35.53 ±13.33
DOPAC	311.39 ± 96.00*	2070.7± 207.05	18.107 ± 6.68
DA	378.18 ± 144.94*	12027 ±1175.20	3.48 ± 1.36
5-HIAA	532.68 ± 61.51	485.18 ±64.70	136.71 ± 26.74
5-HT	283.07 ± 45.73*	561.89 ± 105.76	64.46 ± 12.35

Table 2.1 Concentrations of monoamines in lesioned versus intact hemisphere

These data represent the picograms per microliter of monoamines and metabolites for striatal tissue taken from animals used in microdialysis experiments. Data verify successful DA lesion. There were no significant differences in monoamine concentrations in rAAV-GFP versus rAAV-D2R$_s$ rats. Thus, data are collapsed across groups. (*=$p<0.05$ lesioned vs. intact)

experimental timeline). With L-DOPA, rAAV-D2R$_S$ treated animals did not show significant LID at the typical peak-dose time point (75 minutes post-L-DOPA delivery) LID (defined as an AIM score ≥4) (Zhang et al., 2013) at any dose level (2 mg/kg AIMs=0±0, 4mg/kg AIMs=0.14±0.14, 6mg/kg AIMs=0±0, 8mg/kg day 8 AIMs=0.29±0.18, 8mg/kg day 10 AIMs=0.29±0.29, 8mg/kg day 12 AIMs=0.14±0.14, 8mg/kg day 15 AIMs=0.29±0.29, 12mg/kg day 17 AIMs=0.14±0.14, 12mg/kg day 19 AIMs=0.36±0.18) (Figure 2.2A, Figure A.2.1). rAAV-GFP controls began to show mild-to-moderate peak-dose AIMS with a moderate L-DOPA dose (6mg/kg peak dose AIMs=3±1.43), which increased to more significant levels of severity with higher doses of L-DOPA (8mg/kg peak dose AIMs: day 8=4.5±2.29, day 10=5.5±2.36, day 12=6.88±2.72, day 15=5.25±1.76; 12mg/kg peak dose AIMs: day 17=8.69±2.06, day 19=9.44±1.93) (Figure 2.2A, Figure A.2.1). When compared to rAAV-D2R$_S$ subjects, rAAV-GFP animals showed significantly higher total peak dose AIM scores per session starting with 8mg/kg doses (day 12 rAAV-D2R$_S$ (Md=0), rAAV-GFP (Md=4), $U=12$, $p<0.05$; day 15 rAAV-D2R$_S$ (Md=0), rAAV-GFP (Md=4.5), $U=12$, $p<0.05$; Mann-Whitney U test) (Figure 2.2B). This difference was maintained with the high dose of L-DOPA (12mg/kg day 17 rAAV-D2R$_S$ (Md=0), rAAV-GFP (Md=8.5), $U=0.5$, $p<0.001$; day 19 rAAV-D2R$_S$ (Md=0), rAAV-GFP (Md=9.25), $U=0$, $p<0.001$) (Figure 2.2C-F, Figure A.2.1). Taken together, these data show that D2R$_S$ expression in the DRN completely blocks the development of LID in parkinsonian rats, even with administration of high L-DOPA doses.

III. D2R$_S$ does not affect parkinsonian motor behavior

To assess if rAAV-D2R$_S$ treatment alters the anti-akinetic properties of L-DOPA, we examined motor behavior using the cylinder task (Figure 2.3A). There were no

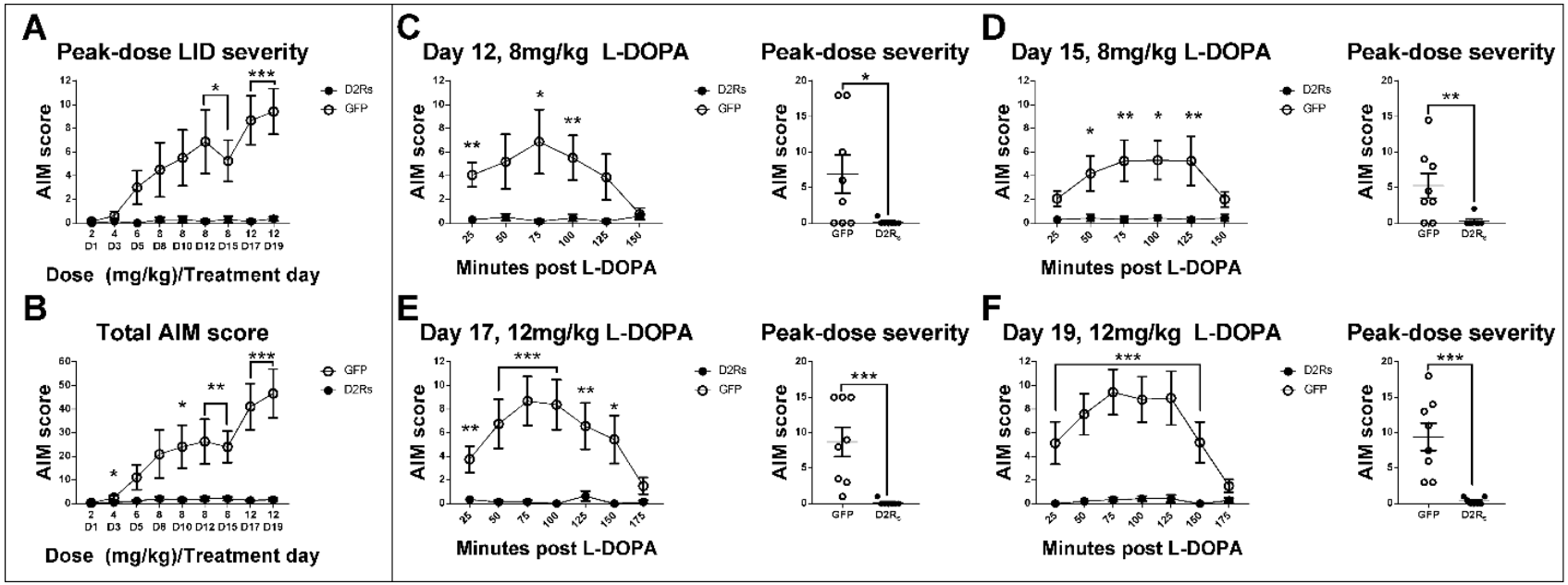

Figure 2.2 DRN D2R$_S$ expression blocks LID development

(A) rAAV-D2R$_S$-injected animals did not develop LID over the course of 19 days of treatment with increasing doses of L-DOPA, where rAAV-GFP controls developed AIMs. (B) The total AIM score for each rating session was significantly different between groups starting on treatment day 8 with 8m/kg L-DOPA. D2R$_S$ animals remained LID-. (C-F) AIM scores from days 12, 15, 17, and 19 showing LID severity in 25-minute intervals. GFP animals displayed a typical dyskinetic response to chronic L-DOPA treatment, with increasing AIM severity seen at higher doses. The peak-dose severity (AIM score at 75 minutes-post L-DOPA injection) was significantly higher in GFP animals than D2R$_S$ animals in the last four days of the L-DOPA paradigm. (*=p≤0.05, **=p≤0.01, ***=p≤0.001

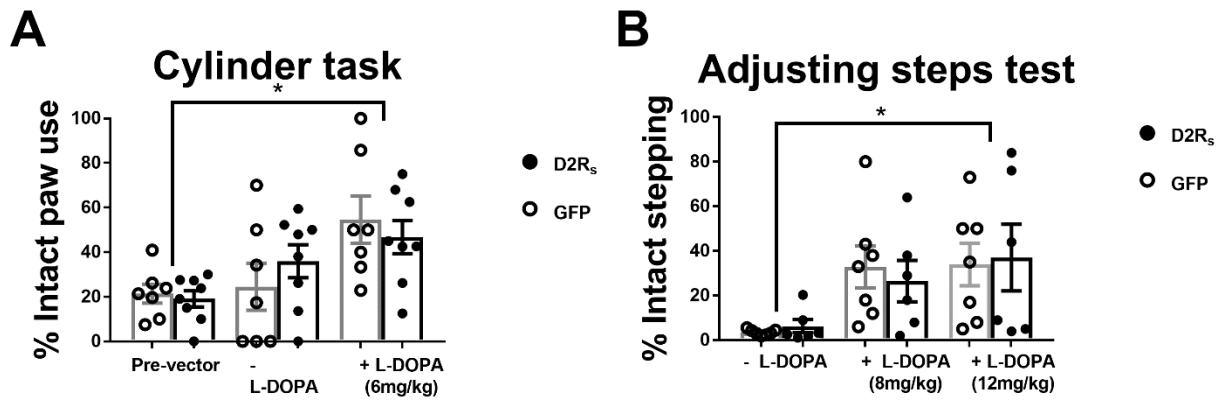

Figure 2.3 rAAV-D2R$_s$ does not impact L-DOPA efficacy

(A) Cylinder task was performed three weeks post-lesion (pre-vector), off L-DOPA (post-vector, post-L-DOPA paradigm) and on L-DOPA (6mg/kg, 50 minutes post injection). Both vector groups showed significant impairment following lesion and vector delivery, which was recovered with L-DOPA treatment. There were no significant differences between vector groups. (B) A second cohort received the same lesions and vector deliveries and motor function was evaluated using the adjusting steps tests. While all animals in both groups showed significant impairment on the test without L-DOPA, motor function was restored while on drug (8mg/kg and 12mg/kg). There were no differences between vector groups.

significant differences between the rAAV treatment groups without L-DOPA ($F_{(1,13)}$=0.008, p>0.05). Pre-vector scores for both groups and post-vector scores for rAAV-GFP showed a marked decrease from normal contralateral forepaw use, indicating significant impairment. rAAV-D2R$_s$ animals post-vector showed a trend towards more balanced forepaw use, but the differences were not significant. Both groups showed a significant increase from baseline increase towards balanced contralateral forepaw use while on L-DOPA (6mg/kg) ($F_{(2,26)}$=7.11, p<0.01). No significant differences were seen in impairment or improvement between vector treatment groups ($F_{(2,26)}$=0.72, p>0.05). A second separate cohort of animals underwent the adjusting steps test, both off and on (8-12mg/kg) L-DOPA. Both vector treatment groups showed significantly impaired adjusting steps without L-DOPA (GFP baseline=3.57%±0.49% intact stepping; D2R$_s$ baseline=6.33%±3.02% intact stepping; $t_{(11)}$=0.98, p>0.05), however, this deficit was rescued with the administration of both doses of L-DOPA (Figure 2.3B; $F_{(2,22)}$=9, p<0.01). As with the cylinder task, no differences in impairment nor improvement while on L-DOPA were seen between groups ($F_{(2,22)}$=0.24, p>0.05). Together, this suggests that ectopic D2R$_s$ expression in the DRN does not interfere with the anti-parkinsonian motor benefits of L-DOPA.

IV. Dopamine receptor agonists do not induce significant AIMs in L-DOPA-primed rAAV-D2R$_s$ rats

Next, we examined whether dopamine agonists could induce AIMs in the rAAV-D2R$_s$ treated rats that had remained resistant to LID after the L-DOPA dosing paradigm. Animals received three repeated doses each of a non-selective DA agonist (apomorphine, 0.1mg/kg), a D2/3-specific receptor agonist (quinpirole, 0.2mg/kg), and a

D1-specific receptor agonist (SKF-81297, 0.8mg/kg) and were evaluated for AIM severity (see timeline in Figure 2.1A). These DA agonists can induce AIMs in both L-DOPA-primed and unprimed parkinsonian animals (Boraud et al., 2001; Boyce et al., 2001; Chondrogiorgi et al., 2014). We hypothesized that directly activating the DA receptors with an agonist would bypass any protective effects of the rAAV-D2R$_S$ treatment in normalizing aberrant DA release, as these agonists do not require processing and release by DAergic or 5-HTergic terminals, and therefore would not be affected by presynaptic regulatory mechanisms. They also allowed us to compare DA receptor supersensitivity status between treatment groups. Interestingly, rAAV-D2R$_S$ animals challenged with both apomorphine and quinpirole did not show significant peak AIMs (rAAV-D2R$_S$ apomorphine third treatment 25-minute AIMS=1.86±1.32; quinpirole third treatment 25-minute AIMs=-1.57±0.66), while rAAV-GFP animals continued to express moderate-to-severe AIM behaviors (rAAV-GFP apomorphine third treatment 25-minute AIMS=10.75±2.10; quinpirole third treatment 25 minute AIMs=-11.81±2.45) (Figure 2.4A-F). rAAV-D2R$_S$ animals exhibited significantly lower peak-dose AIMs with both apomorphine and quinpirole treatment compared to rAAV-GFP animals (apomorphine third treatment 25-minute AIMs rAAV-D2R$_S$ (Md=0), rAAV-GFP (Md=12.75), U=4.5, $p<0.01$; quinpirole third treatment 25-minute AIMs rAAV-D2R$_S$ (Md=1.5), rAAV-GFP (Md=13), U=3.5, $p<0.01$). Treatment with SKF-81297 did induce mild-to-moderate AIM scores in rAAV-D2R$_S$ treated animals (third treatment 50-minute AIMs=3.92±0.73), but these scores remained significantly less severe than their control counterparts (third treatment 50-minute AIMs rAAV-D2R$_S$ (Md=3.5), rAAV-GFP (Md=13), U=2, $p<0.001$) (Figure 2.4G-I).

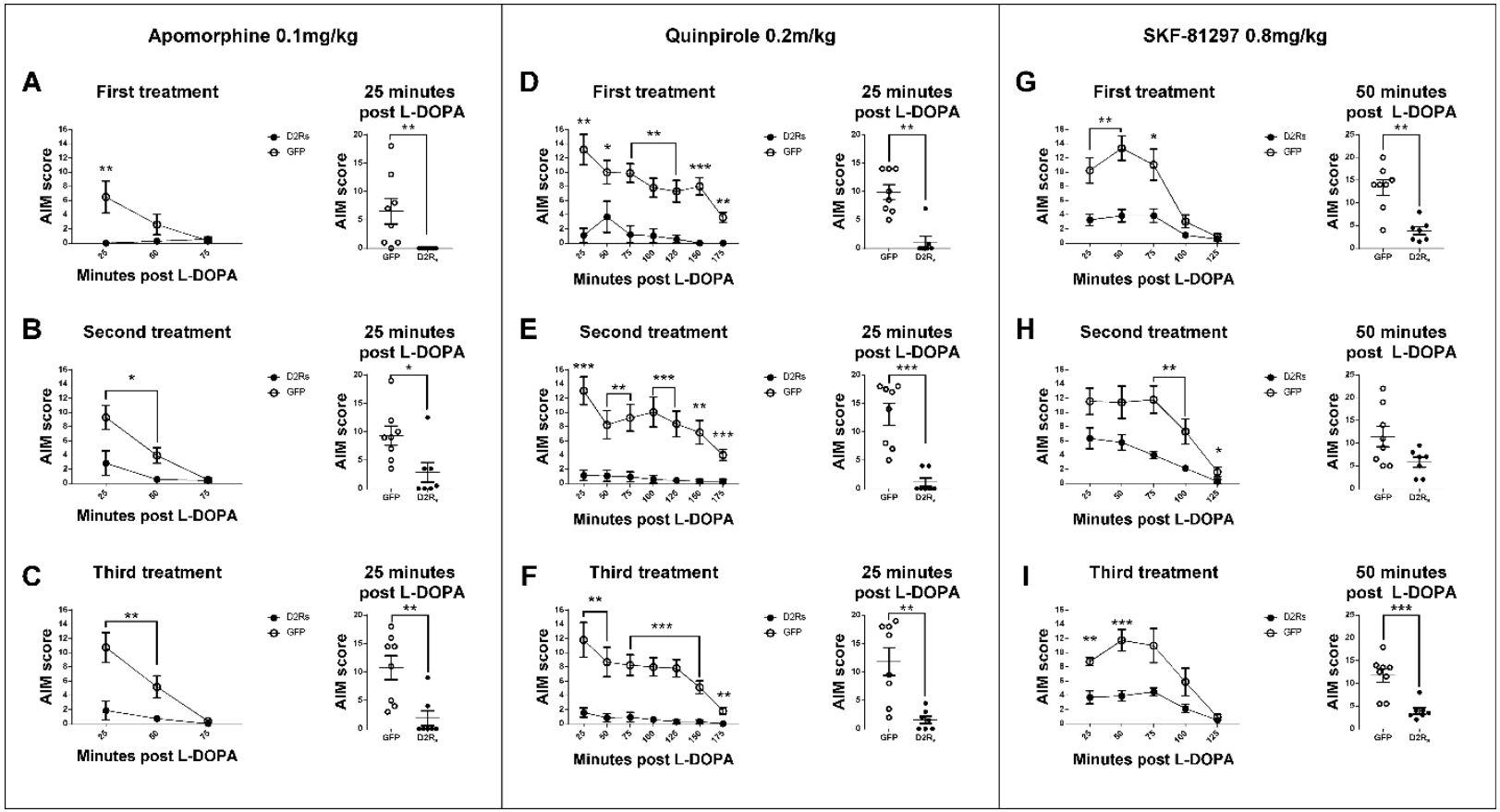

Figure 2.4 $D2R_S$-injected animals do not develop severe AIMs with DA agonist treatment

Animals were treated three times each with apomorphine (A-C) quinpirole (D-F) or SKF-81297 (G-I). (A-C) rAAV-$D2R_S$-injected animals remained AIM resistant with .1m/kg pan-DA agonist apomorphine treatment, while rAAV-GFP animals continued to exhibit dyskinetic behaviors. (D-F) .2mg/kg quinpirole (D2 agonist) did not elicit AIMs in rAAV-$D2R_S$ animals, where rAAV-GFP animals continued to exhibit moderate to severe AIMs. (G-I) rAAV-$D2R_S$ began to show mild-to-moderate AIMs with .8mg/kg of the D1 agonist SKF-81297 treatments, but remained significantly less severe than their rAAV-GFP counterparts. (*=p≤0.05, **=p≤.01, ***=p≤.001)

V. $D2R_s$ expression in the dorsal raphe reduces striatal dopamine efflux following L-DOPA delivery

In order to determine if ectopic $D2R_s$ expression in the DRN was inhibiting LID by moderating DA release from 5-HTergic neurons, we generated a second cohort of animals in order to perform in vivo microdialysis (rAAV-$D2R_s$ n=6, rAAV-GFP n=7). Animals were lesioned and received vector in an identical manner to the first cohort, and subsequently treated with L-DOPA to establish LID. In order to determine differences between vector groups in the absence of L-DOPA, striatal dialysate was analyzed via HPLC and data for monoamine content were examined using a 2 (vector) x 2 (treatment) mixed-model ANOVA. Overall, DA values were dependent upon treatment, $F_{(1,11)}=124.35$, $p<0.05$, and vector, $F_{(1,11)}=7.39$, $p<0.05$. Planned pairwise comparisons revealed that L-DOPA treatment increased striatal DA efflux in both groups. However, rats with rAAV-D_2R had lower levels of DA efflux than rats with the GFP vector ($p<0.05$) (Figure 2.5A). Finally, there was a vector-by-treatment interaction, $F_{(1,11)}=6.66$, $p<0.05$, such that rats with the D_2R vector had lower levels of DA efflux than rats with the GFP vector, but only after L-DOPA treatment. There was no effect of vector or treatment on striatal 5-HT efflux (Figure 2.5B; $F_{(11,121)}=0.867$, $p>.05$). DA values for each time point were also submitted to paired-samples t-tests in order to examine the effect of vector on DA efflux at each time point during microdialysis. There were significant differences between vector groups 60 ($t_{(5)}=3.42$, $p<0.05$), 80 ($t_{(5)}=2.77$, $p<0.05$), 100 ($t_{(5)}=4.68$, $p<0.01$), and 120 ($t_{(5)}=2.59$, $p<0.05$) minutes after L-DOPA administration, showing that rats with the GFP vector had elevated striatal DA efflux as compared to the rats with the D2R vector. This is the first direct evidence showing that

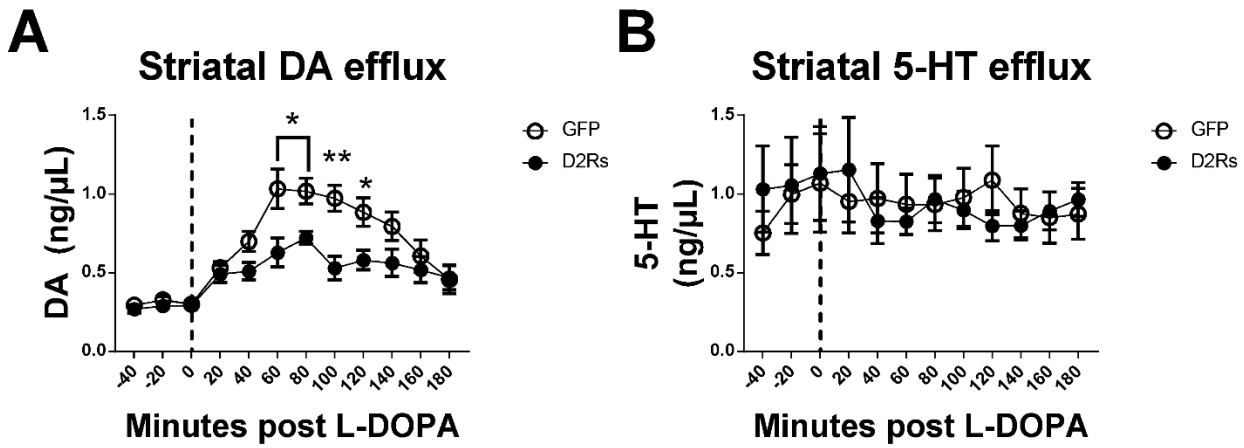

Figure 2.5 DRN D2R$_S$ reduces striatal efflux of DA

(A and B) *In vivo* microdialysis of rAAV-D2R$_S$ and rAAV-GFP animals in twenty-minute intervals following L-DOPA injection (12 mg/kg + 12 mg/kg benserazide). (A) rAAV-D2R$_S$ animals showed significantly decreased DA efflux in the striatum 60-120 minutes following injection. (B) No changes in serotonin efflux in the striatum between vector groups was observed following L-DOPA injection. (*=p≤0.05, **=p≤.01, ***=p≤.001)

mishandled DA by DRN neurons can be regulated exogenously, and this regulation reduces DA release in the striatum, thus suppressing LID.

VI. $D2R_S$ expression inhibits 5-HT neuron activity

In order to demonstrate that the ectopically expressed $D2R_S$ have the capacity to inhibit the activity of 5-HT neurons, we performed electrophysiological recordings on a separate cohort of non-parkinsonian, L-DOPA naïve animals. Animals received a stereotaxic delivery of either vector as described above, and 4-12 weeks later we performed *in vivo* single-unit extracellular recordings of DRN neurons. Putative 5-HT neurons were identified based initially on their firing characteristics (e.g., long-duration action potentials, regular firing pattern interrupted with burst activity). Next, neurons were identified as 5-HTergic based on well characterized responses to systemic administration (i.v.) of 5-HT1A receptor agonist (8-OH-DPAT) and reversal with an antagonist (WAY-100635) which restored 5-HT neuron firing to that of baseline (Figure 2.6B-D) (Hajos et al., 2007; Celada et al., 2013). Figure 2.6A shows typical traces of 5-HT and non-5HT DR neurons. Importantly, electrophysiologically identified 5-HT neurons recorded in the dorsal raphe of rats transduced with rAAV expressing BFP or $D2R_S$ responded similarly to systemic administration of vehicle, 5-HT1A receptor agonist, and reversal of 5-HT1A inhibition by 5-HT1A antagonism. Moreover, 5-HT cells recorded in AAV-$D2R_S$ injected rats administered the D2 agonist quinpirole exhibited clear inhibitory effects, whereas responses to quinpirole were variable and sometimes excitatory in BFP controls (Figure 2.6E-F). These data show that ectopic expression of $D2R_S$ in confirmed 5-HT neurons can act as a functional autoreceptor and inhibit impulse activity in 5-HTergic neurons.

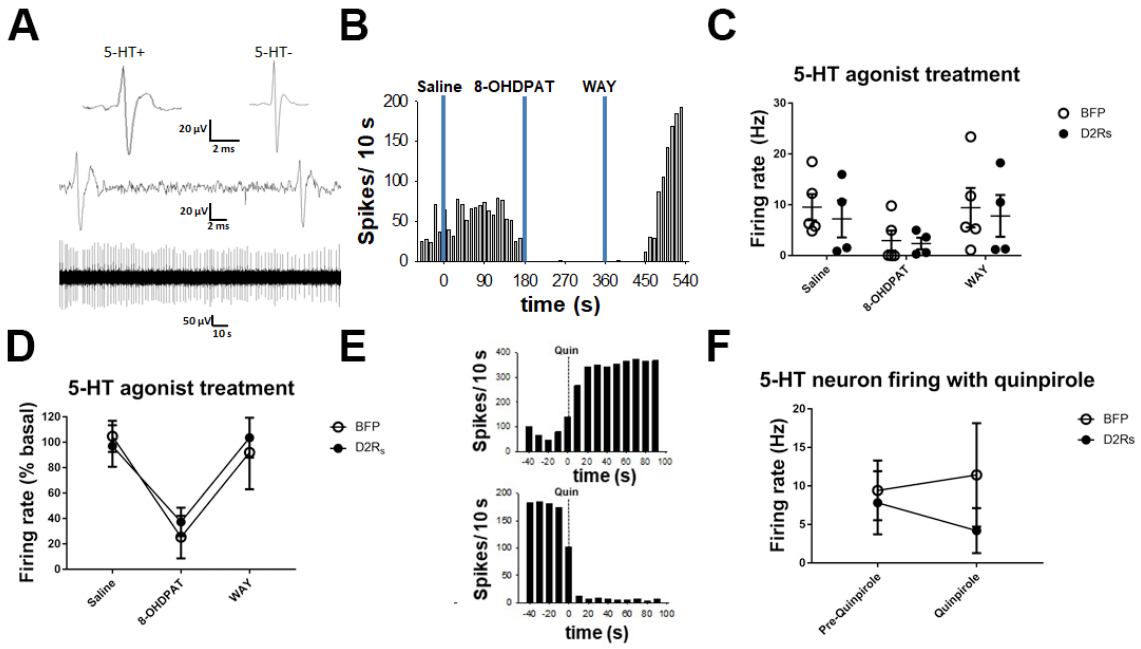

Figure 2.6 Ectopic DRN D2R$_S$ expression reduces 5-HT neuronal firing

A) Top: Traces show typical single-unit recordings of isolated DRN 5-HT neurons (5-HT+) (left) and non-5-HTergic (5-HT-) neurons (right). Middle/Bottom: 5-HT neurons often exhibit burst firing with short inter-spike intervals as well as regular spiking. B) Systemic administration of the selective 5-HT1A agonist 8-OH-DPAT (1 µg/kg, i.v.), but not saline (0.9%) vehicle, suppressed the spontaneous firing of a DRN neuron exhibiting spike characteristic of a 5-HT cell. A return to baseline firing was observed after the local application of WAY-100635 ((100 µg/kg, i.v.), vertical blue bars). C-D) firing rate distributions of DRN neurons recorded in BFP and D2R$_S$ expressing rats before and after the application of saline, 8-OH-DPAT and WAY-100635. Putative 5-HT neurons in both groups exhibited similar inhibitory responses to 5-HT1AR agonist and reversal of inhibition by 5-HT1AR antagonism. E) Firing rate histograms showing the effects of the D2R agonist quinpirole (500 µg/kg, i.v.) on 5-HT neurons recorded in BFP (top) or AAV-D2AR (bottom) injected rats. Control neurons that responded to Quin increased their firing activity to varying degrees, whereas the majority of 5-HT cells recorded in AAV-D2AR injected rats were inhibited. F) Cumulative electrophysiological data showing the mean ± S.E.M. firing rates of 5-HT DRN cells transfected with BFP or D2AR prior to, and after Quin administration.

Discussion

In this study, we used rAAV to ectopically express the dopamine autoreceptor ($D2R_S$) in order to equip DRN 5-HT neurons with a DA-mediated autoregulatory mechanism. Dysregulated DA release ("false neurotransmission") by 5-HT neurons has been heavily implicated as a key contributor to LID development (Maeda et al., 1999; Bibbiani et al., 2001; Maeda et al., 2003; Maeda et al., 2005; Carta et al., 2007; Eskow et al., 2009; Muñoz et al., 2009) . While a number of studies have supported this theory, to date, no direct evidence has been presented that shows that DRN neurons can release DA in the striatum in a way that ultimately effects LID. In the present study, our data demonstrate that providing DA-dependent autoregulation in 5-HT neurons can prevent LID formation by reducing striatal DA efflux, thus, providing unambiguous evidence that 5-HT neurons play a central role in DA-dependent symptomology.

A wealth of preclinical and clinical studies have shaped the serotonin hypothesis of LID, which suggest that DA processing and release from striatal 5-HT terminals is involved in AIM presentation. Studies ablating DRN neurons or dampening their activity with serotonin autoreceptor agonists are able to reduce or eliminate LID, the hypothesized reasoning being that reducing 5-HTergic neuronal activity leads to a reduction in DA release from DRN terminals (Iravani et al., 2006; Carta et al., 2007; Eskow et al., 2007). Although the mechanism by which 5-HT neurons process L-DOPA and release DA is not fully established, it is well established that the machinery to do so is present in 5-HTergic neurons (Arai et al., 1995; Tanaka et al., 1999; Gantz et al., 2015).

Dopamine autoregulation in the dorsal raphe blocks 5-HT neuron activity and LID development

In order to better delineate the role of DRN 5-HT neurons in dyskinesogenesis, we argued that expressing DA regulatory factors in 5-HT neurons would decrease LID severity. As 5-HT autoreceptors share a canonical signaling cascade with the D2-type DA autoreceptors—both are inhibitory GPCRs that reduce cellular cAMP to inhibit neuronal signaling (Harrington et al., 1988; Neve et al., 2004)—we hypothesized that ectopically expressing the DA autoreceptor $D2R_S$ in DRN neurons would serve such a function. In support of this theory, recent work demonstrated that expression of $D2R_S$ in the DRN of naïve mice results in a reduction of 5-HT-mediated currents (Gantz et al., 2015). Indeed, in this work we found that direct recordings of single 5-HT neurons in the DRN expressing the autoreceptor provide evidence that ectopic $D2R_S$ expression can provide an inhibitory neuromodulatory effect in 5-HT neurons, characterized by a strong decrease in spontaneous firing following systemic DA $D2R_S$ agonist administration. Accordingly, we utilized rAAV targeted to the DRN in hemiparkinsonian rats that subsequently received a LID-inducing dosing regimen of L-DOPA. As hypothesized, we found that DRN expression of $D2R_S$ provided complete protection against the development of LID, an effect that also persisted at high levels of L-DOPA. Importantly, there was no difference in the extent of nigrostriatal denervation between the groups, nor was there any demonstrable toxicity due to either treatment in the DRN. Thus, prevention of LID was explicitly due to expression of $D2R_S$ in the DRN.

Dopamine efflux into the striatum is reduced with dorsal raphe $D2R_s$ expression

Although there is a wealth of research supporting the 5-HTergic input in LID development (Nicholson & Brotchie, 2002; Scholtissen et al., 2006; Bastide et al., 2015), direct evidence showing that the contribution is due to an increase in DA release from these neurons is limited. Using *in vivo* microdialysis, we determined that L-DOPA-mediated DA efflux into the striatum can be significantly reduced by negatively regulating DRN serotonin neurons. We did not see a decrease in serotonin efflux in the striatum in rAAV-$D2R_s$ animals, suggesting serotonin release was not affected. However, one explanation for this observation may also be the lack of direct stimulation of the 5-HTergic system during our L-DOPA treatment, in which case one would not expect to see changes in 5-HT signaling. Our findings demonstrate that $D2R_s$ can induce DAergic regulation in 5-HT neurons, by 'hijacking' endogenous signaling cascades and reducing neuronal activity following L-DOPA administration. Our data indicate that exogenously provided $D2R_s$ can couple with $G_{\alpha i}$ subunits in DRN neurons, and induce the appropriate signaling cascades to reduce neuronal activity in the presence of DA. In conjunction with the LID studies utilizing serotonin agonists, our data confirm that reducing the activity of the serotonin system can dramatically inhibit LID. Additionally, this is the first evidence showing that 5-HTergic neurons can control DA release when supplied exogenously with a single DA-regulatory factor. Our study delivered rAAV-$D2R_s$ prior to beginning L-DOPA treatment. In future studies, showing that $D2R_s$ delivery post-L-DOPA and post-AIM establishment would be imperative to determine if the addition of regulation can reduce or reverse established LID.

Ectopic $D2R_S$ expression in the dorsal raphe blocks L-DOPA priming in the striatum

We hypothesized that the protective effects of $D2R_S$ treatment would be negated with DA-receptor agonist treatments which directly affect the postsynaptic target, striatal medium spiny neurons (MSNs). As the DA regulation of $D2R_S$ is a presynaptic mechanism, treatment with postsynaptic receptor agonists—which can induce dyskinesia in animal models and patients (Gomez-Mancilla & Bédard, 1992; Boraud et al., 2001; Boyce et al., 2001; Chondrogiorgi et al., 2014)—should induce AIMs in rAAV-$D2R_S$-treated animals resistant to LID. Interestingly, treatment with D1-, D2-specific, or pan-DA agonists did not induce severe AIMs in rAAV-$D2R_S$ animals, where only a rather modest dyskinetic response was seen with the D1 agonist SKF-81297. However, it is well established that LID development is preceded by a "priming-period" consisting of discontinuous striatal DA tone, resulting in morphological and molecular changes to the MSNs (Morelli et al., 1989; Pinna et al., 1997; Carta et al., 2003; Simola et al., 2009; Steece-Collier et al., 2009; Cenci & Konradi, 2010; Zhang et al., 2013). Our data therefore indicates that $D2R_S$-treated animals were blocked from the preceding L-DOPA priming, suggesting that $D2R_S$ in the DRN can counteract this discontinuous DA release to sufficiently block the LID-inducing priming of striatal MSNs. The fact that at the end of the treatment we began to observe a mild-to-moderate increase in AIM presentation in rAAV-$D2R_S$ animals with DA agonist treatment as compared to L-DOPA, suggest that these animals were in the early stages of priming, a phenomenon that is to be expected as direct MSN DA receptor activation would not be mitigated by DRN $D2R_S$ expression.

Inhibition of dorsal raphe 5-HTergic neurons does not mitigate the anti-parkinsonian benefits of L-DOPA

It was important to confirm that $D2R_S$ expression in the DRN does not negatively affect the therapeutic efficacy of L-DOPA in our PD model, as this has been an issue with 5-HT agonist-type therapies in clinical trials for LID (Kannari et al., 2002; Olanow et al., 2004; Cheshire & Williams, 2012), and an imperative problem to mitigate for potential future therapies. We saw no changes in motor improvement between control and $D2R_S$ animals of two separate cohorts using two different motor tests. Both tests demonstrated a significant improvement in motor function with the administration of L-DOPA, reflecting recovery back to a pre-lesion state. This shows that $D2R_S$ activity in 5-HTergic terminals of the striatum (or elsewhere) does not interfere with the pharmacological benefits of L-DOPA, and implicates $D2R_S$ therapy as a potential treatment option for LID. It is important to note that while many preclinical studies using 5-HT agonists did not show an effect on L-DOPA-induced motor improvement, these results have not translated clinically. While multiple trials have used a variety of 5-HT agonists and seen reductions in AIM scores, many of these compounds contribute to worsening of parkinsonian symptoms and OFF L-DOPA periods, or have been abandoned due to lack of efficacy (reviewed in (Cheshire & Williams, 2012)). The discrepancy between our $D2R_S$ approach and the use of agonists is unclear given that these two approaches conceivably evoke the same mechanism. Nevertheless, 5-HT1 compounds may produce their own side effects (Lindenbach et al., 2015). Second, their effects are dependent on an exogenously administered compound and dose as opposed to a gene therapy approach. Nevertheless, further studies would be required to

determine if $D2R_S$ expression in the raphe is successful in other preclinical models of LID.

Pharmacological manipulations of 5-HT neurons in the treatment of LID, although successful pre-clinically, have not been fully translated. The transient nature of the anti-dyskinetic effect of currently available 5-HT approaches may be due to pharmacologic limitations of these drugs, including lack of specificity and potency for the specific receptor. Moreover, timing and comparative pharmacodynamics with L-DOPA delivery may be preventative (Mazzucchi et al., 2015). Because of this, a genetic approach in the form of continuous 5-HT inhibition should bypass such pharmacological limitations and provide meaningful and lasting protection against LID. Moreover, the finding that $D2R_S$ gene therapy does not interfere with L-DOPA efficacy in our rat model provides promise for such an approach. Of course, the DR innervates a large part of the brain, providing many crucial functions, and the $D2R_S$ therapy undertaken here does not distinguish between various projections. Thus, understanding any off-target effects from DA-mediated regulation of 5-HT neurons remains one important caveat that requires further research. Further studies to better understand potential side effects and the effect on the 5-HTergic system may be warranted.

Our findings shed new light on previous work demonstrating changes in 5-HT innervation occurring concomitant with nigrostriatal denervation and PD. Both 5-HT hyperinnervation (Politis et al., 2010; Rylander et al., 2010; Bedard et al., 2011) as well as a decrease in 5-HT terminals (Scatton et al., 1983; Kim et al., 2003; Guttman et al., 2007; Kish et al., 2008) has been documented in human disease. Although the cause of these divergent findings is unknown, it is highly likely that 5-HT neurons play an

important role in PD symptomology and, as our findings would suggest, in LID. As nigrostriatal denervation in human PD is near complete at the time of diagnosis (Kordower et al., 2013) it is conceivable to speculate that changes in 5-HT innervation and function—and the capacity of these neurons to release DA—is a crucial component to dyskinesogenesis. To that end, understanding both the mechanisms of how 5-HT neurons process and release DA, and the underlying etiology of presynaptic 5-HT changes are important components as we begin to understand LID etiology and PD nonmotor symptoms, and represents a new therapeutic modality.

In conclusion, the current study shows that DA release from DRN 5-HT neurons can be regulated with ectopic expression of $D2R_S$, altering the release activity of these neurons. These data add important evidence to the current understanding of LID and serve as to confirm the serotonin hypothesis in LID, showing that directly regulating serotonin neurons activity can inhibit LID development.

APPENDIX

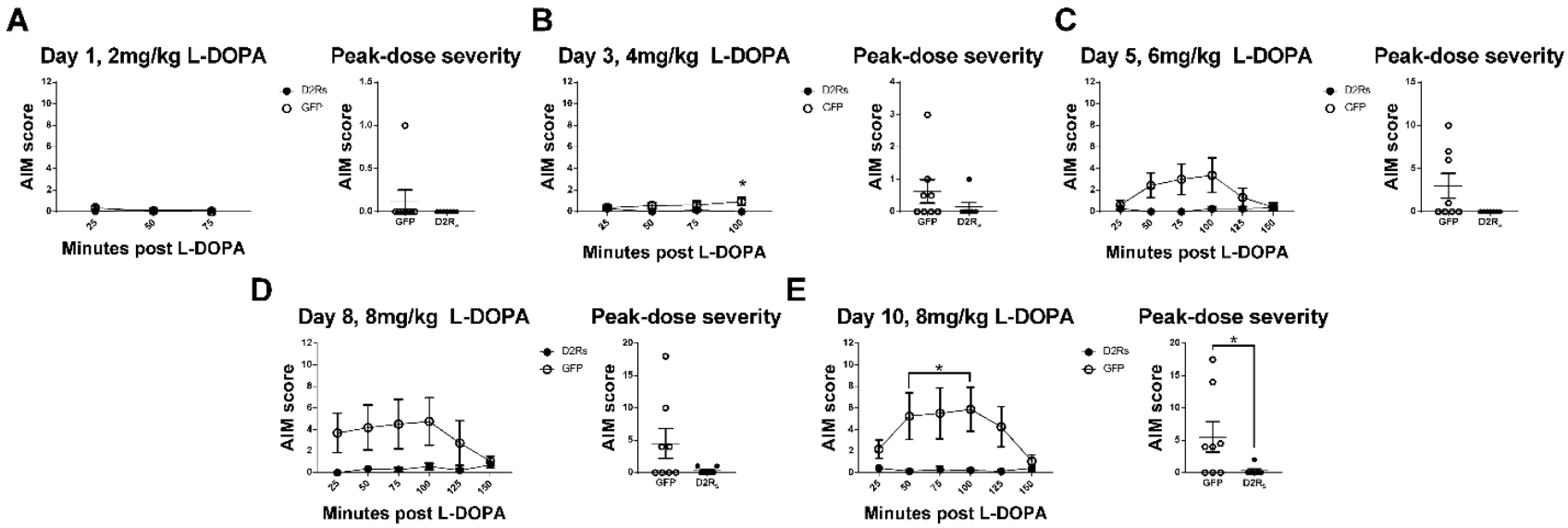

Figure A.2.1 AIM scores in L-DOPA dosing paradigm

AIM scores of days 1-10 in the L-DOPA dosing regimen, ranging from 2mg/kg-8mg/kg. Significantly more severe AIMs were observed in rAAV-GFP animals starting on day 10 with 8mg/kg. Peak-dose severity scores taken at 75 minutes post L-DOPA. (*=p≤0.05, **=p≤.01, ***=p≤.001)

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Chapter 3: Modulating levels of Nurr1 expression in the parkinsonian rat striatum impacts LID severity and development

Introduction

Levodopa (L-DOPA) is considered the gold-standard pharmacologic therapy for the motor symptoms in Parkinson's disease (PD). The precursor to dopamine (DA), L-DOPA therapy alleviates motor symptoms by restoring balance to basal ganglia signaling following a loss in DA signaling from degenerated substantia nigra pars compacta (SNc) neurons (Cotzias et al., 1967; Fox et al., 2011). Unfortunately, chronic treatment with L-DOPA leads to the inevitable development of drug-induced abnormal involuntary movements in the majority of patients (Ahlskog & Muenter, 2001; Manson et al., 2012). These symptoms, termed levodopa-induced dyskinesia (LID), consist of disruptive hyperkinetic movements and dystonic muscle tone associated with L-DOPA plasma levels.

Studies have focused on gene expression changes associated with LID to better understand their development and etiology. A previous study looking at gene expression differences between the direct and indirect pathway of the basal ganglia in dyskinetic rats showed a marked increase in expression of the transcription factor Nurr1 (Heiman et al., 2014). This has been corroborated by a study that observed Nurr1 transcript increase globally in the striatum of parkinsonian mice treated chronically with L-DOPA (Sodersten et al., 2014). Additionally, non-peer reviewed data from our group has shown LID-associated Nurr1 mRNA colocalized with markers of both the direct and indirect pathway, with a noted higher abundance in the direct pathway. Nurr1 is an orphan nuclear transcription factor and a member of the NR4A family (Giguere, 1999). It

has long been studied in PD as Nurr1 expression is crucial for DAergic neuronal development and long-term maintenance. Nurr1 knockout mice are not viable, and Nurr1+/- heterozygotes show DA dysfunction and loss of SNc neurons (Zetterstrom et al., 1997; Jiang et al., 2005; Kadkhodaei et al., 2009). Nurr1 expression in the SNc has been shown to decrease with age in humans, and in fact multiple Nurr1 isoforms have been found to be associated with some familial forms of PD (Dekker et al., 2003; Le et al., 2003). Because of this, Nurr1-based therapeutics are being researched as potential disease-modifying treatments for PD. The association of Nurr1 with LID, thus, begs for further characterization (Kim et al., 2015; Smith et al., 2015; Dong et al., 2016).

The upregulation of Nurr1 in the striatum of dyskinetic animals is especially of note as Nurr1 is not normally expressed in striatal medium spiny neurons (MSNs) (Xiao et al., 1996). This abnormal expression pattern associated with abnormal behavior suggests that Nurr1 plays a direct role in LID. However, this has not been shown directly. Nurr1 has been shown to be involved in synaptic plasticity required for learning and memory in the hippocampus (Peña de Ortiz et al., 2000; Colon-Cesario et al., 2006; Hawk & Abel, 2011; Hawk et al., 2012). As synaptic plasticity and spine dynamics have been previously implicated in LID formation (Fieblinger & Cenci, 2015), it is reasonable to question if Nurr1 plays a role in these changes. In the present study, we aim to show that Nurr1 is a key player in LID development and that it’s overexpression can induce LID.

Methods

I. Adeno-associated virus production

The human Nurr1 and GFP gene were cloned into the rAAV genome under control of the chicken β-actin/cytomegalovirus (CBA/CMV) promotor to allow for robust and ubiquitous expression in neurons. For shRNA experiments, a cassette expressing GFP as a transduction marker with either an shRNA targeted for the Nurr1 transcript (shRNA-Nurr1) or a scrambled control with no gene specificity (shRNA-SCR) was cloned under the same promoter element. rAAV pseudotype rAAV2/5 was produced via double transfection of HEK293 cells with rAAV genome and helper plasmids as previously described in this dissertation (Benskey et al., 2016). Virus was recovered using an iodixanol gradient and concentrated in concentration columns. Viral titer was ascertained by dot blot, and adjusted to a working titer of $1.0x10^{13}$ vg/mL.

II. Animals and surgeries

Adult male Fischer F344 and Lewis rats (200-220g on arrival, Charles River, Wilmington, MA) were used in the studies. Studies were conducted in accordance with the guidelines of Michigan State University (AUF MSU06/16-093-00). Rats were housed two per cage up until LID behavior testing began, when they were separated and singly housed. Animals were housed in a light-controlled (12 hours light/dark cycle) and temperature-controlled (22±1 °C) room, and had free access to standard lab chow and water.

All surgeries were performed as described previously in this dissertation. Animals were anesthetized with isoflurane and placed into a stereotaxic frame. All materials were

injected with a Hamilton syringe fitted with a glass capillary needle. Parkinsonian lesions were induced using 5mg/ml 6-hydroxydopamine (6-OHDA) hydrobromide mixed in 0.2mg/ml ascorbic acid immediately before injections. Animals received two 2µl injections of 6-OHDA (10µg per injection), one in the medial forebrain bundle (MFB, from bregma: Anterior Posterior (AP) – 4.3mm, Medial Lateral (ML) + 1.6mm, Dorsal Ventral (DV) - 8.4mm from skull) and one in the SNc (from bregma: AP - 4.8mm, ML + 1.7mm, DV - 8.0mm from skull). The needle was lowered to the site and the injection began after 30 seconds. The needle was removed two minutes after the injection was finished and cleaned between each injection. Lesion efficacy was estimated two and a half weeks following 6-OHDA injection with the cylinder task as previously described (Schallert, 2006; Manfredsson et al., 2007).

Viral delivery surgeries were performed similarly three weeks following 6-OHDA lesions. Animals in the overexpression studies received a single 2µl injection of either rAAV-Nurr1 or rAAV-GFP targeting lateral striatum (from bregma: AP + 0.5mm, ML + 3.7mm, DV – 5.3mm from skull). Animals in the knockdown studies received two 2µl injections of either rAAV-shRNA-Nurr1 or rAAV-shRNA-SCR. Injections targeted the striatum (1st injection from bregma: AP + 0.0mm, ML + 3.0mm, DV – 5.2mm from dura. 2nd injection from bregma: AP + 1.6mm, ML + 2.7mm, DV – 4.9mm from dura).

III. Abnormal involuntary movements ratings

Drug-induced dyskinesia severity was evaluated using the abnormal involuntary movement (AIM) scale described earlier in this dissertation. Animals received subcutaneous injections of either increasing doses of L-DOPA (2-8mg/kg) with benserazide (12mg/kg), or the DA receptor agonists SKF-81297 (0.8mg/kg) and

quinpirole (0.2mg/kg). Dosing occurred three days a week for three weeks for L-DOPA studies and one week for DA agonist studies. The AIM scale was used to rate drug-induced AIMs, as has been previously described (Steece-Collier et al., 2003; Maries et al., 2006). Briefly, AIM severity is evaluated by scoring the level of dystonia of the limbs and body, hyperkinesia of the forelimbs, and hyperoral movements. Each AIM is given two scores—one indicating the intensity (0=absent, 1=mild, 2=moderate, 3=severe) and frequency (0=absent, 1=intermittently present for <50% of the observation period, 2=intermittently present for >50% of the observation period, 3=uninterruptable and present through the entire rating period). Each AIM is given a severity score by multiplying the intensity and frequency, and the overall AIM score for each timepoint is a sum of all the behaviors severities. The sum of all AIM scores from each timepoint makes up the total AIM score. Peak-dose dyskinesia is considered to be 75 minutes post drug administration. An animal is considered non-dyskinetic with a score of ≤4 (Maries et al., 2006). Animals were observed in 25-minute increments following drug delivery until AIMs subsided.

IV. Tissue collection and processing

Animals received a final injection of either L-DOPA or DA agonists two hours prior to sacrifice. Rats were anesthetized deeply with a lethal dose of sodium pentobarbital, and intracardiacally perfused with Tyrode's solution (137mM sodium chloride, 1.8mM calcium chloride dihydrate, .32mM sodium phosphate monobasic dihydrate, 5.5mM glucose, 11.9mM sodium bicarbonate, 2.7mM potassium chloride) followed by 4% paraformaldehyde (PFA). Brains were removed rapidly and post-fixed for 72 hours in 4% PFA before being transferred into 30% sucrose. Brains were sectioned on a

freezing sliding microtome at 40µm and stored at -20°C in cryoprotectant (30% ethelyne glycol, 0.8mM sucrose in 0.5X tris-buffered saline).

Immunohistochemistry (IHC) was performed as previously reported (Benskey et al., 2018). A 1:6 series of free-floating tissue was stained for TH (MAB318, MilliporeSigma, Burlington, MA) Nurr1 (AF2156, R&D Systems, Minneapolis, MN) or GFP (AB290, Abcam, Cambridge, United Kingdom). Briefly, sections were washed in 1x TBS with .25% Triton x-100, incubated in 0.3% H_2O_2 for 30 minutes, and rinsed and blocked in 10% normal goat or donkey serum for 2 hours. Tissue was incubated in primary antibody (TH 1:4000, Nurr1 1.5ug/ml, GFP 1:20,000) overnight at room temperature. After washing, tissue was incubated in secondary antibody (Biotinylated horse anti-mouse IgG 1:500, BA-2001, Vector Laboratories, Burlingame, CA; biotinylated donkey anti-goat IgG 1:500, AP180B, Millipore-Sigma, Burlington, MA; biotinylated goat anti-rabbit IgG 1:500, AP132B, Millipore-Sigma, Burlington, MA) followed by the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Tissue staining was developed with 0.5 mg/ml 3,3’ diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) and 0.03% H_2O_2. Sections were mounted on slides, dehydrated, and coverslipped with Cytoseal (ThermoFisher, Waltham, MA).

V. *In situ* hybridization

In situ hybridization with IHC was performed using the RNAscope® 2.5 HD Duplex Assay according to the manufacturers’ protocol (Advanced Cell Diagnostics, Newark, CA). 40µm striatal sections were treated with the hydrogen peroxide solution for at least 10 minutes, or until active bubbling from the tissue subsided. Sections were washed in 1x TBS, mounted onto slides, and allowed to dry for at least 48 hours. Slides were then

boiled for 10 minutes in the Target Retrieval solution, followed by Protease Plus treatment. Tissue was hybridized with target probe for direct and indirect pathway markers (dynorphin (Dyn) or enkephalin (Enk), respectively) (Lu et al., 1998) for 2 hours at 40°C. Slides were rinsed in 1x RNAscope® Wash Buffer. Sequential amplification steps were then applied to the slides, with 1x Wash Buffer rinses between each amplification. After the sixth amplification, the probe was visualized using the Detect Red Signal solution for 10 minutes.

Immediately following *in situ* hybridization, the tissue was stained immunohistochemically for Nurr1. IHC was performed as described above, and Nurr1 protein was visualized using Vector SG Peroxidase kit (Vector Laboratories, Burlingame, CA).

VI. Stereology

Unbiased stereology was used to determine lesion status via TH loss as previously described (Polinski et al., 2015). Using Stereo Investigator software with the optical fractionator probe (MicroBrightfield Bioscience, Williston, VT), TH-positive neurons in every sixth section of the whole SNc were counted on the intact and lesioned hemisphere, giving an estimate of total TH-positive cells in the SNc.

VII. Statistical analysis

Statistical analysis was performed using Statview (version 5.0) or GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA. All graphs were created in GraphPad. Lesion status was evaluated using unpaired, one-tailed t-tests. AIMs were evaluated using a Mann-Whitney U test or the Kruskal-Wallis test. Differences between vector

groups were compared with p≤0.05 being considered statistically significant. Bonferroni post-hoc tests were employed when significant main effects were detected.

Results

I. Validation of lesion and transgene expression

Adult male Fisher and Lewis rats were lesioned with 6-OHDA injected into the medial forebrain bundle and substantia nigra. Lesion efficacy was validated post-mortem with TH staining and stereology. All animals included in analysis were sufficiently lesioned with ≥94% TH loss (Figure 3.1B-D). rAAV 2/5 expressing either Nurr1 (Fischer n=7, Lewis n=5) or GFP (Fischer n=7, Lewis n=5) was injected into the striatum of both strains of rats. Vector transduction and expression was confirmed with post-mortem IHC (Figure 3.1E-H). Vector delivery results in Nurr1 expression markedly higher than what is seen in rAAV-naïve, LID+ Fischer animals, where Nurr1 is abnormally upregulated (Figure A.3.1A). Lewis animals, however, express much lower level AIMs than Fischer rats, and do not express Nurr1 in the striatum (Figure A.3.1B).

II. Ectopic Nurr1 overexpression does not exacerbate AIMs in LID-susceptible rats

Three weeks were allowed after the virus delivery to ensure maximal transgene expression before animals were placed on a L-DOPA treatment paradigm (see Figure 3.1A for experimental timeline). AIMs were evaluated in 25-minute intervals post-L-DOPA delivery. Animals were first evaluated with vehicle (0mg/kg L-DOPA, 12mg/kg benserazide) to ensure that Nurr1 overexpression does not cause drug-independent AIMs. Indeed, both groups showed no AIMs with the vehicle administration (Figure

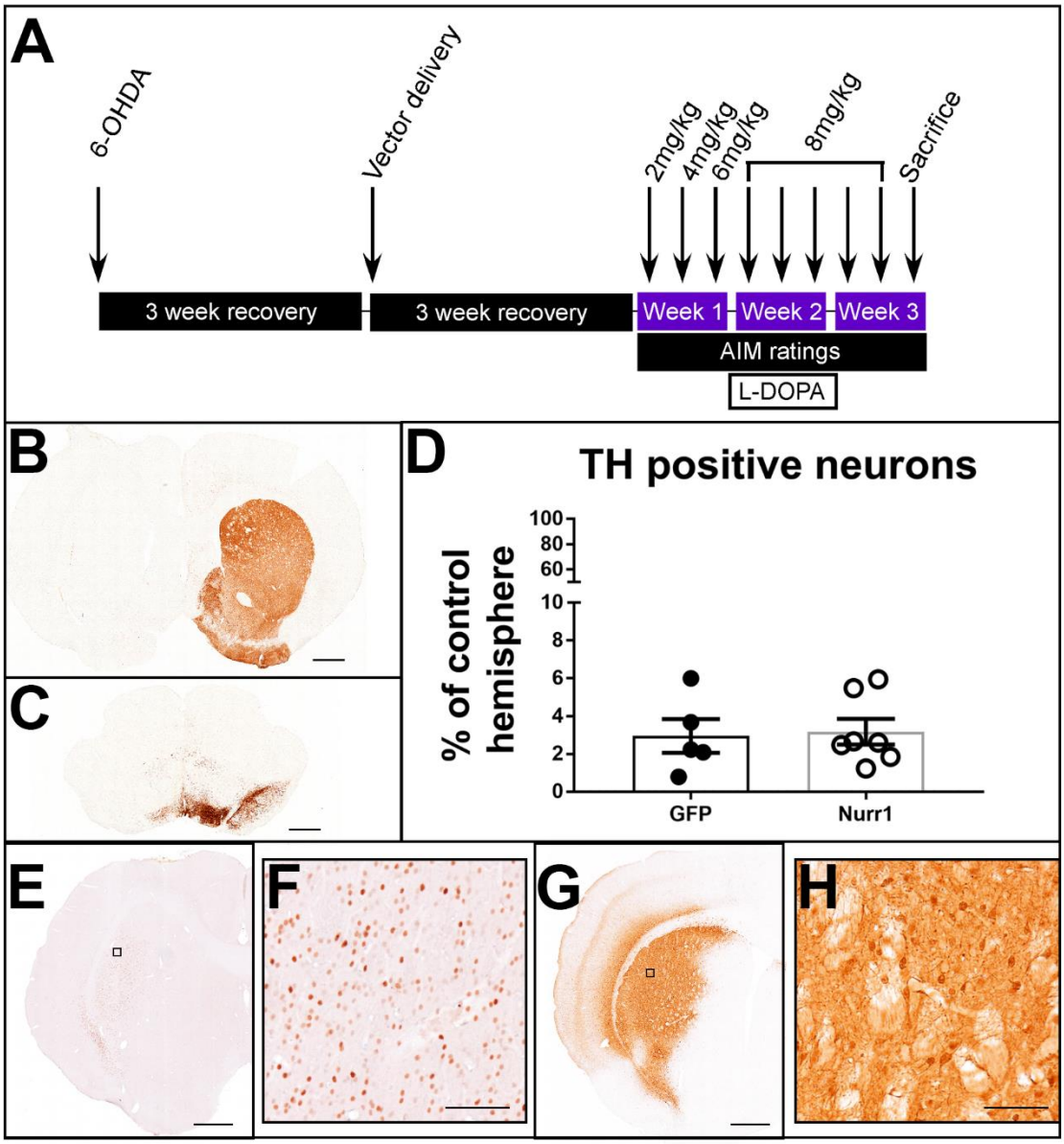

Figure 3.1 Experimental design and model validation

(A) Experimental timeline showing surgeries and LID induction timeline. AIM scores were evaluated at each injection where indicated. (B-D) 6-OHDA injections caused near-complete loss of TH fibers in the striatum (B) as well as TH-positive cells in the nigra (C). Stereology confirmed that all animals had greater than 94% TH cell loss in the nigra (D). (E-H) Nurr1 (E and F) and GFP (G and H) vector driven expression were confirmed in the striatum with IHC.
B, C, E, H scale bar=1mm
F, H scale bar=100µm

A.3.2). When treated with L-DOPA, there was no significant difference in total AIM scores at any rating timepoint in Fischer rats treated with rAAV-Nurr1 or GFP (Figure 3.2) (total AIM sum: D1 2mg/kg rAAV-Nurr1 (Md=0.5) rAAV-GFP (Md=1), U=17, $p>0.05$; D3 4mg/kg rAAV-Nurr1 (Md=8) rAAV-GFP (Md=6.5), U=10, $p>0.05$; D5 6mg/kg rAAV-Nurr1 (Md=39.5) rAAV-GFP (Md=29), U=14, $p>0.05$; D8 8mg/kg rAAV-Nurr1 (Md=55) rAAV-GFP (Md=42), U=12, $p>0.05$; D10 8mg/kg rAAV-Nurr1 (Md=61.5) rAAV-GFP (Md=52.5), U=11, $p>0.05$; D12 8mg/kg rAAV-Nurr1 (Md=57.5) rAAV-GFP (Md=48), U=14, $p>0.05$; D15 8mg/kg rAAV-Nurr1 (Md=55) rAAV-GFP (Md=63), U=15, $p>0.05$; D17 8mg/kg rAAV-Nurr1 (Md=62.5) rAAV-GFP (Md=54), U=13, $p>0.05$). In order to determine if Nurr1 affected specific AIM behaviors that may not be apparent in the overall AIM score, we separated the overall AIM score into individual axial, orolingual, and limb scores (AOL scores). Again, Nurr1 did not drive a difference in AIM severity in any individual LID behavior criteria (Figure 3.3). There was a difference observed on the final treatment (day 17) in forelimb AIM severity at 150 minutes post L-DOPA (rAAV-Nurr1 (Md=2) rAAV-GFP (Md=0), U=7, $p<0.05$). While statistically significant, this is likely not biologically relevant, as by this time point the AIMs of both groups were below our threshold for what is considered a dyskinetic score (AIM score≥4 to be LID+) (Maries et al., 2006). This data shows that Nurr1 did not have a specific effect on one particular set of AIM behaviors. We observed moderate and severe LID in both groups of Fischer rats, suggesting that ectopic Nurr1 expression does not exacerbate the AIMs of the LID-prone strain.

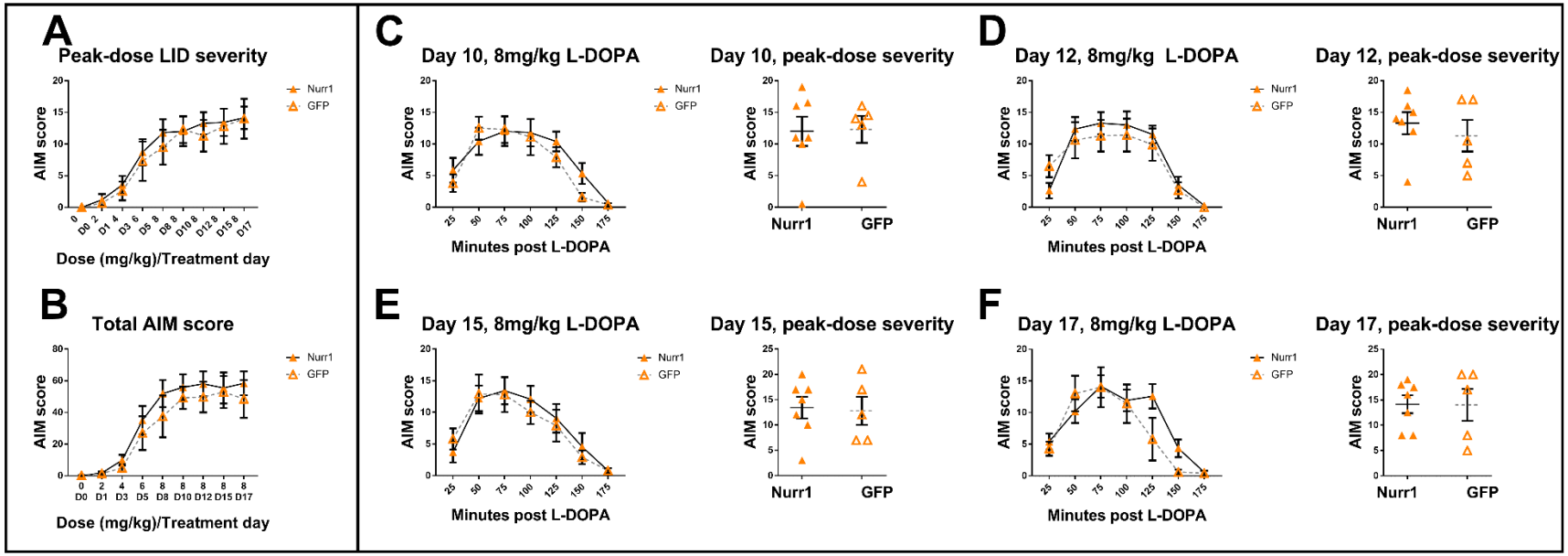

Figure 3.2 Ectopic Nurr1 does not exacerbate AIMs in LID-susceptible rats

(A) Peak-dose LID severity (75 minutes post injection) over each rating period. No differences in peak-dose severity between vector groups was observed in Fischer rats. (B) The total AIM score sum of each rating session showed no differences between rAAV-Nurr1 and rAAV-GFP animals. Both groups developed severe AIMs similarly over the treatment regimen. (C-F) Animals were treated with 8mg/kg L-DOPA on days 10-17. Both groups showed similar AIM expression over time (left panels) and peak-dose AIMs (right panels).

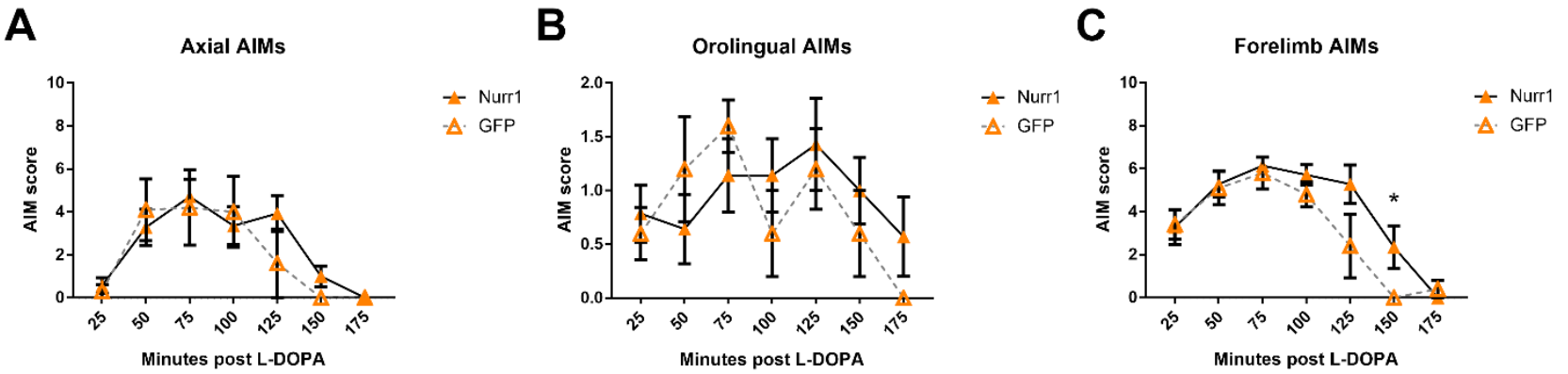

Figure 3.3 Nurr1 does not impact individual AOLs in LID-susceptible rats

(A) Axial AIMs—comprised of trunk and neck dystonia—are not different between rAAV-Nurr1 and rAAV-GFP Fischer rats. (B) Orolingual AIMs—comprised of chewing and tongue protrusions—are not different between vector groups. (C) Forelimb AIMs—comprised of forelimb hyperkinesia and dystonia—are not different at most time points between vector treatment groups. There was a significant difference between the groups at 150 minutes post injection. Rating period shown at day 17 with 8mg/kg L-DOPA dosing.

III. Ectopic Nurr1 overexpression severe induces AIMs in LID-resistant rats

Notably, when treated chronically with L-DOPA, rAAV-Nurr1 Lewis rats developed severe LID, where their rAAV-GFP control counterparts expressed low-level AIMs (Figure 3.4). This difference was first observed at the 6mg/kg treatment, in both total AIM score sum and peak-dose LID (total AIM sum 6mg/kg rAAV-Nurr1 (Md=34) rAAV-GFP (Md=3), $U=3$, $p<0.05$; peak-dose AIM 6mg/kg rAAV-Nurr1 (Md=13.5) rAAV-GFP (Md=4), $U=0$, $p<0.01$). rAAV-Nurr1 treated Lewis rats also displayed more severe AIMs than their rAAV-GFP counterparts on days 8, 10, and 19 with 8mg/kg L-DOPA (peak dose AIMs: day 8 rAAV-Nurr1 (Md=16) rAAV-GFP (Md=3), $U=2$, $p<0.05$; day 10 rAAV-Nurr1 (Md=16) rAAV-GFP (Md=6), $U=0$, $p<0.01$; day 17 rAAV-Nurr1 (Md=13.5) rAAV-GFP (Md=4), $U=0$, $p<0.01$; day 19 rAAV-Nurr1 (Md=16) rAAV-GFP (Md=3), $U=2$, $p<0.05$). The induction of severe AIMs with ectopic Nurr1 expression in an otherwise LID resistant strain shows that Nurr1 is directly involved in LID development.

When compared, rAAV-Nurr1 Lewis rats AIM scores were indistinguishable from rAAV-GFP Fischer rats (Figure 3.5). On day 17 of treatment, rAAV-GFP Lewis rats were significantly less dyskinetic than both rAAV-Nurr1 Lewis and Fischer treated animals (peak dose AIMs: rAAV-Nurr1 Lewis (Md=13.5) rAAV-GFP Lewis (Md=4), $U=0$, $p<0.01$; rAAV-Nurr1 Fischer (Md=16) rAAV-GFP Lewis (Md=4), $U=2$, $p<0.01$). Interestingly, rAAV-GFP Fischer animals did not express significantly more severe AIMs than rAAV-GFP Lewis rats (peak dose AIMs: rAAV-GFP Fischer (Md=17) rAAV-GFP Lewis (Md=4), $U=3$, $p>0.05$). Together, this data shows that the presence of Nurr1 is sufficient to overcome resistance to severe LID seen in Lewis animals.

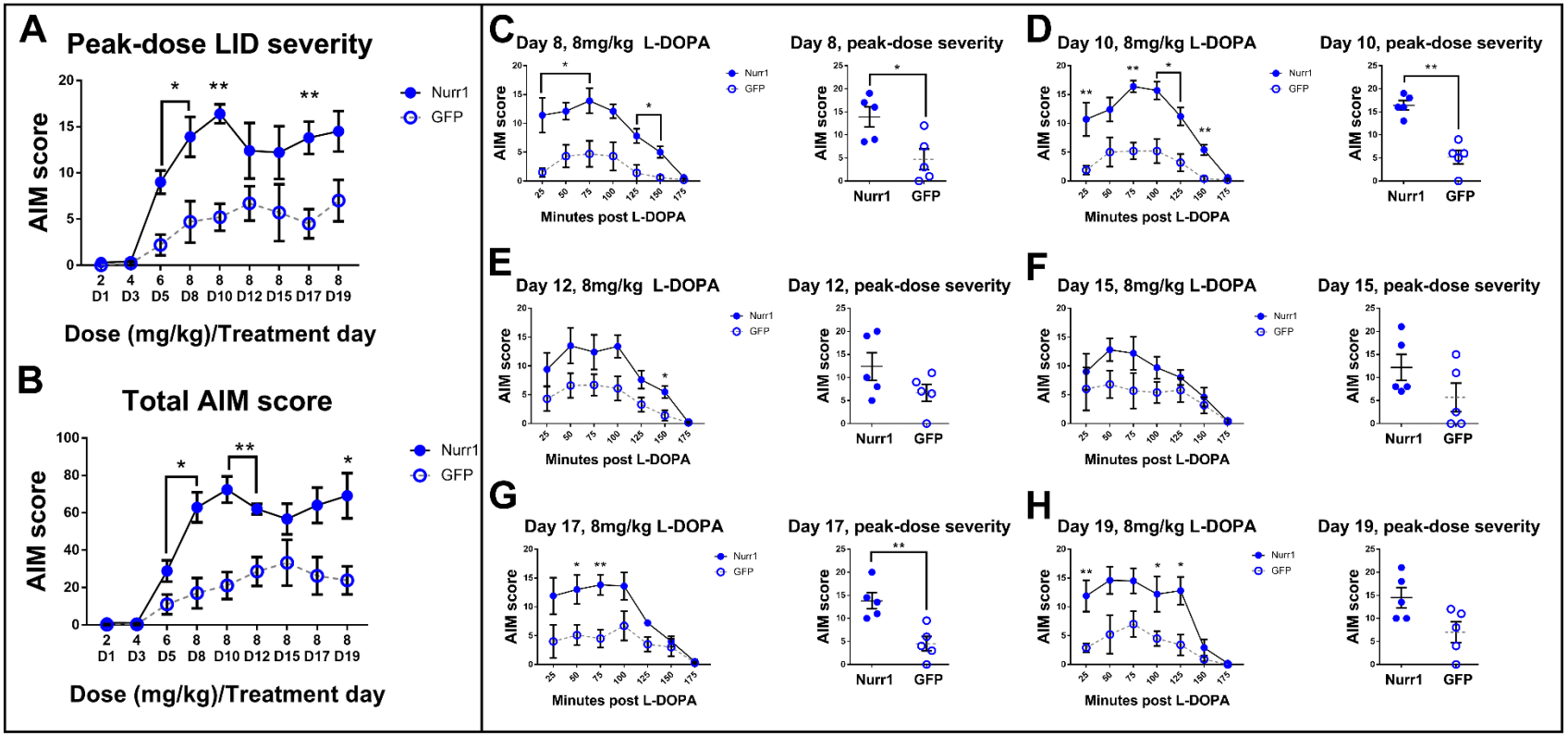

Figure 3.4 Ectopic Nurr1 expression induces severe AIMs in LID-resistant rats

(A) Peak-dose LID severity (75 minutes post injection) show rAAV-Nurr1 treated Lewis rats developing significantly more sever AIMs than rAAV-GFP animals at 6 and 8mg/kg doses. (B) Total AIM score sum showed exacerbated AIMs on days 5, 8, 10, 12, and 19 in rAAV-Nurr1 treated Lewis rats. (C-D) Individual rating periods on days 10-19 with 8mg/kg treatment. rAAV-Nurr1 animals developed AIMs more severe than rAAV-GFP animals at multiple timepoints during the observation period. (*=p≤0.05, **=p≤.01, ***=p≤.001)

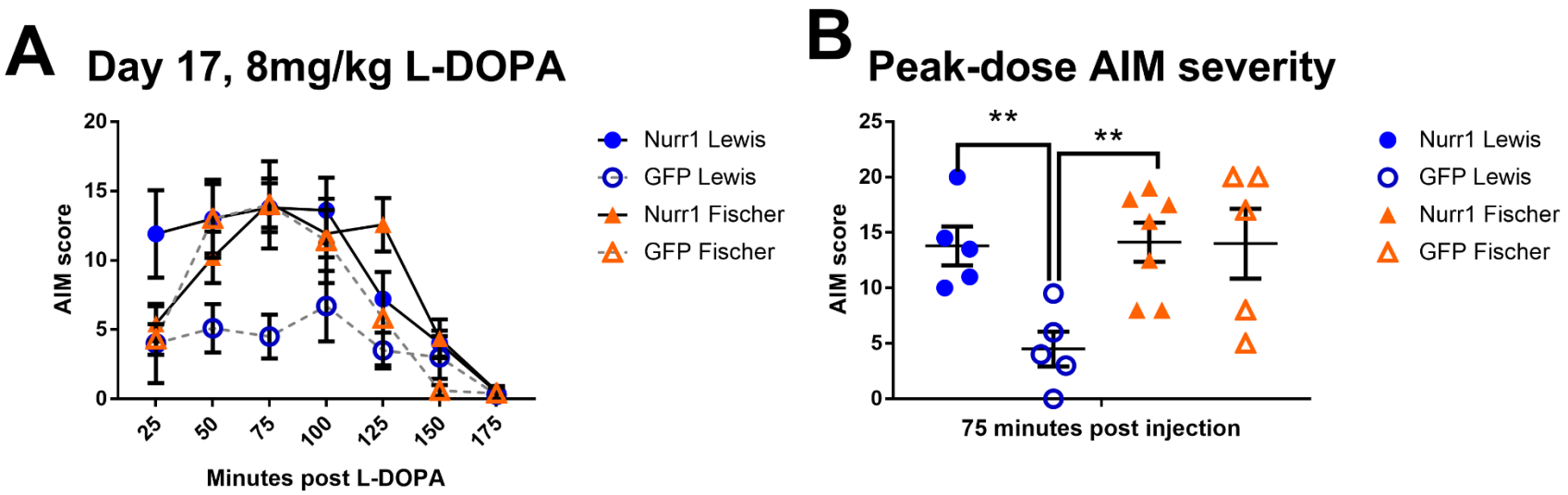

Figure 3.5 Ectopic Nurr1 induces AIMs in LID-resistant rats that are similar to LID-susceptible rats

(A) Day 17 AIM rating time course comparing rAAV treated LID-resistant Lewis rats to LID-susceptible Fischer rats. rAAV-Nurr1 Lewis animals showed indistinguishable AIMs compared to both groups of Fischer rats. (B) Peak dose (75 minutes post injection) AIM severity on day 17 of treatment. rAAV-GFP Lewis animals showed significantly lower LID severity than both rAAV-Nurr1 treated Lewis and Fischer rats. rAAV-GFP treated Fischer rats did not show significantly higher AIMs than rAAV-GFP treated Lewis rats. (*=p≤0.05, **=p≤.01, ***=p≤.001)

IV. Nurr1 expression is induced by direct pathway activation

We were next interested in characterizing Nurr1 induction in LID and if activation of either the direct or indirect pathway is sufficient for the abnormal expression to occur. Previous reports have found L-DOPA-induced Nurr1 expression in both direct and indirect pathway MSNs (dMSNs and iMSNs, respectively). To test this, we induced AIMs in parkinsonian Fischer rats with D1 agonist SKF-81297 or D2 agonist quinpirole (saline injection as a control). Animals were treated for one week and sacrificed 2 hours following the final dosing. Moderate and severe AIMs developed in animal treated with SKF-81297 (Figure 3.6). Quinpirole-treated rats expressed mild and moderate AIMs (Figure 3.6). No saline-treated animals developed AIMs. A Kruskal-Wallis test showed that drug-induced AIMs were significantly different between treatment groups (peak-dose AIMs: Kruskal-Wallis statistic=6.78, $p<0.05$). IHC showed Nurr1 expression in the lesioned striatum of SKF-81297 rats with AIMs, but not in those that did not develop AIMs, suggesting that Nurr1 is associated with AIM behavior and can be upregulated with direct pathway activation alone (Figure 3.6C-F). Notably, no staining for Nurr1 protein was observed in AIMs-expressing animals treated with quinpirole, despite moderate AIM scores (Figure 3.6G-J). This shows that indirect pathway activation alone does not induce maladaptive Nurr1 expression, and that direct pathway activation is required for this event.

In order to determine if direct pathway activation lead to Nurr1 expression only in D1 MSNs, we performed *in situ* hybridization with IHC to localize Nurr1 protein with mRNA of direct and indirect pathway markers (dynorphin (Dyn) and enkephalin (Enk), respectively) (Lu et al., 1998). We observed colocalization of Nurr1 protein and both

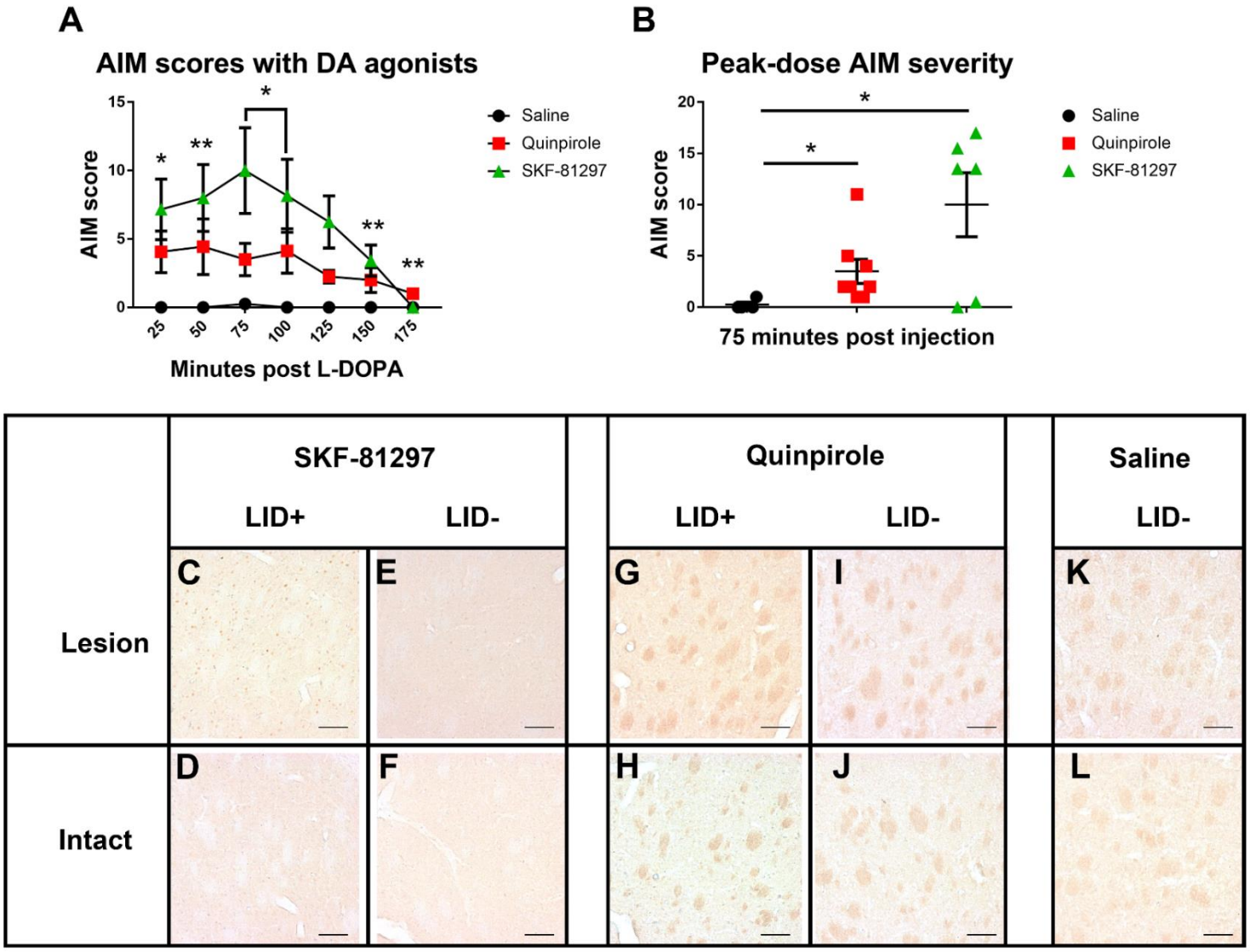

Figure 3.6 Abnormal striatal Nurr1 expression is induced by direct D1 receptor activation

Figure 3.6 (cont'd)

(A) AIM scores from the final treatment with either D1 agonist (SKF-81297), D2 agonist (quinpirole), or saline. D1-agonist treated rats displayed severe AIMs, while D2 agonist treated animals expressed moderate AIMs. No AIMs were observed in saline treated animals. (B) Peak-dose AIM severity from the final drug treatment. (C-L) Nurr1 IHC in animals treated with SKF-81297 (C-F), quinpirole (G-J) or saline (K-L). Nurr1 protein was only observed in the lesioned striatum of dyskinetic rats treated with SKF-81297 (C), but not in LID- animals with the same treatment (E) nor in the intact hemisphere (D and F). Nurr1 was not observed in the lesioned or intact striatum of LID+ rats treated with quinpirole (G and H), nor in LID- quinpirole treated animals (I-J). No Nurr1 was seen in saline treated animals (K and L). Scale bar=100μm

Dyn and Enk in SKF-81297 AIM-expressing animals (Figure 3.7). These findings suggest that Nurr1 expression in the indirect pathway is dependent on direct pathway activation. We also tested this in rats treated with quinpirole and saline, but no colocalization was observed as no Nurr1 protein was expressed in these animals (Figure A.3.3).

V. Viral knockdown of Nurr1 does not inhibit LID development

Our next objective was to determine if inhibiting the abnormal LID-associated Nurr1 expression in MSNs would block LID development. To achieve this, we used a vector cassette expressing an shRNA for Nurr1 (rAAV-shRNA-Nurr1) or a scrambled sequence with no gene specificity as a control (rAAV-shRNA-SCR). Animals were lesioned with 6-OHDA followed by stereotaxic vector delivery as described above. When challenged with L-DOPA, no significant reduction in AIMs was observed in rAAV-shRNA-Nurr1 animals (Figure 3.8). In fact, rAAV-shRNA-SCR animals expressed less severe AIMs than the rAAV-shRNA-Nurr1 animals (Total AIM sum, day 19: rAAV-shRNA-Nurr1 (Md=13.5) rAAV-shRNA-SCR (Md=2), $U=31$, $p<0.05$). This suggests that silencing Nurr1 prior to L-DOPA delivery is not sufficient to block LID in susceptible animals, indicating that there are likely redundant pathways to LID development that do not require Nurr1.

We noted that the animals treated with the rAAV-shRNA-SCR expressed notably less severe AIMs than expected for the strain. This could be due to inconsistent 6-OHDA lesions, or shRNA toxicity. To show that rAAV-shRNA-Nurr1 treatment did not exacerbate LID severity beyond what is expected for parkinsonian Fischer rats, we compared the rAAV-shRNA-Nurr1 treated animals to the control rAAV-GFP Fischer rats

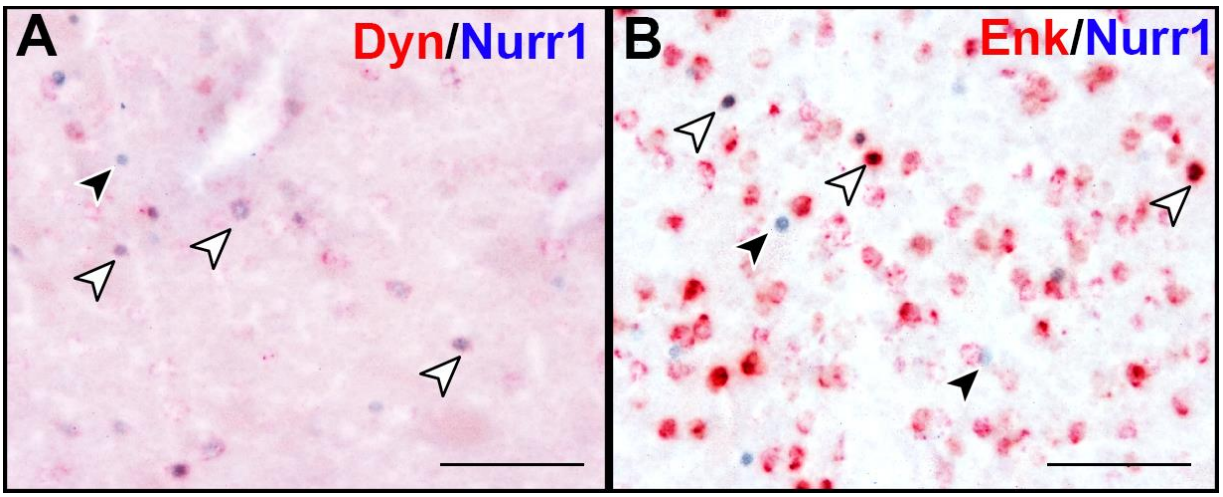

Figure 3.7 D1 stimulation induces Nurr1 expression in both indirect and direct pathway MSNs

(A) In situ hybridization for dynorphin (red) and IHC for Nurr1 protein (blue) in striatum of LID+ animal treated with D1 agonist SKF-81297. Dyn transcript and Nurr1 protein are colocalized (white arrows) in some neurons, but not others (black arrows). (B) *In situ* hybridization for enkephalin (red) and IHC for Nurr1 protein (blue) in the striatum of an LID+ animal treated with D1 agonist SKF-81297. The enk transcript is seen to colocalize with Nurr1 protein (white arrows). Some cells show Nurr1 expression with no enk transcript (black arrows) Scale bar=100µm

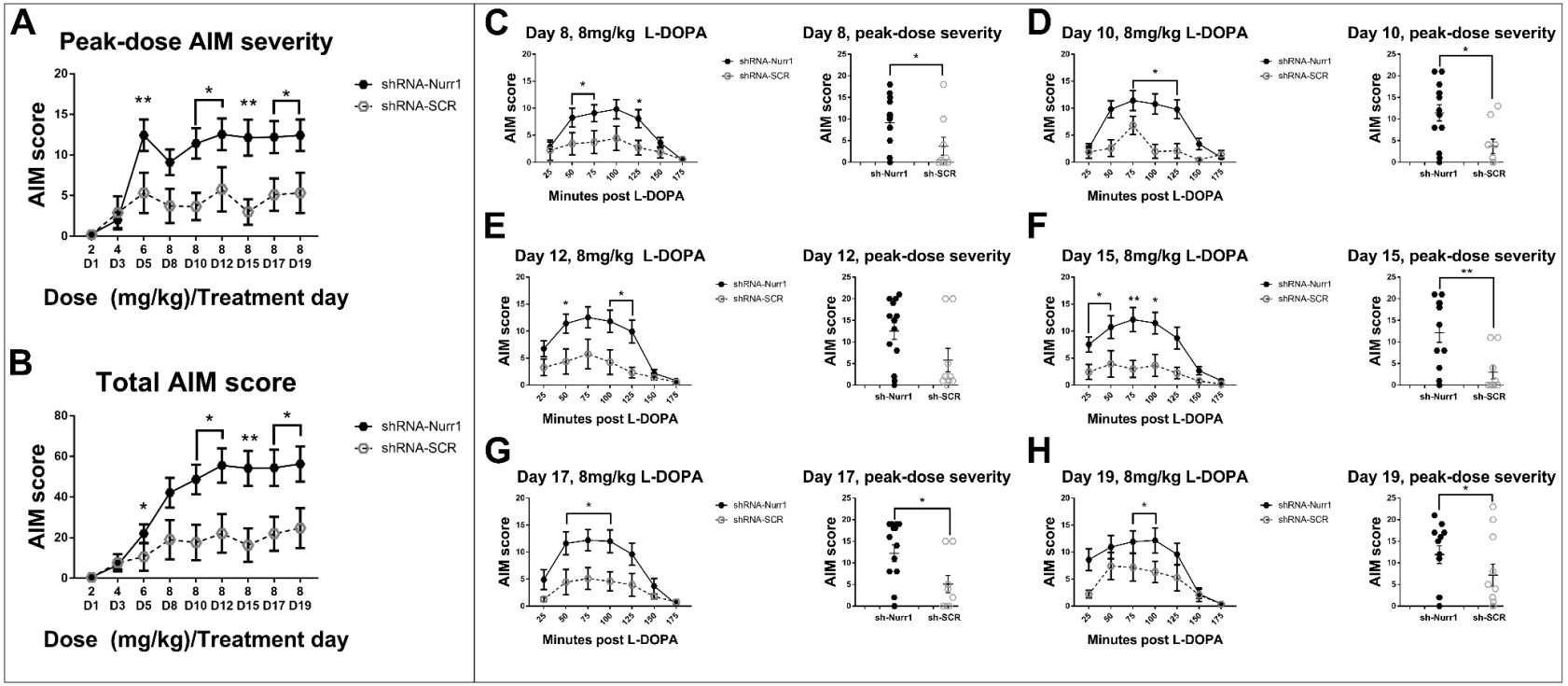

Figure 3.8 Nurr1 silencing does not inhibit LID development

(A) Peak dose (75 minutes post injection) AIM severity over the study duration. rAAV-shRNA-Nurr1 treated animals developed severe peak-dose AIMs over the study, while rAAV-shRNA-SCR animals developed moderate AIMs. (B) The sum AIM score from each rating period. shRNA-Nurr1 animals developed severe AIMs over time, more significant than shRNA-SCR animals. (C-H) Individual rating timepoints at 8mg/kg dosing. shRNA-Nurr1 did not inhibit sever AIMs from developing. rAAV-shRNA-SCR animals displayed moderate AIMs throughout the study.

from the overexpression study, as these animals developed severe AIMs more typical of the strain. rAAV-shRNA-Nurr1 animals developed AIMs similarly to rAAV-GFP animals (Figure 3.9) (rAAV-shRNA-Nurr1 day 17, peak dose AIMs=12.21±1.97, rAAV-GFP day 17, peak dose AIMs=14±3.15). This shows that the shRNA-Nurr1 delivery did not exacerbate LID, but also showed no benefit in reducing AIM severity.

Discussion

We aimed to test if Nurr1 impacts LID by inducing AIMs through altering the physiology of MSNs. Nurr1 has been previously associated with a dyskinetic state in rodent models, and its involvement is of note for multiple reasons (Heiman et al., 2014; Sodersten et al., 2014). Firstly, Nurr1 is known to be required for not only the development but also the long-term survival and health of DA neurons (Zetterstrom et al., 1997; Jiang et al., 2005; Kadkhodaei et al., 2009). This has prompted multiple groups to begin pursuing Nurr1-based therapies as PD-modifying treatments, including Nurr1 agonist compounds (Kim et al., 2015; Smith et al., 2015). How Nurr1 is involved in in LID must thus be well characterized and understood, as increasing its expression or activity could exacerbate LID. Second, Nurr1 is involved in learning-based plasticity in the hippocampus. This implies that Nurr1 may be influencing LID in the striatum by promoting spine plasticity that has been shown to be associated with LID. Finally, while other NR4A transcription factors are expressed endogenously in the striatum, Nurr1 is not (Xiao et al., 1996; Zetterstrom et al., 1996). This abnormal expression seen only with these adverse motor symptoms suggests the sole role of Nurr1 in MSNs is to promote these maladaptive changes.

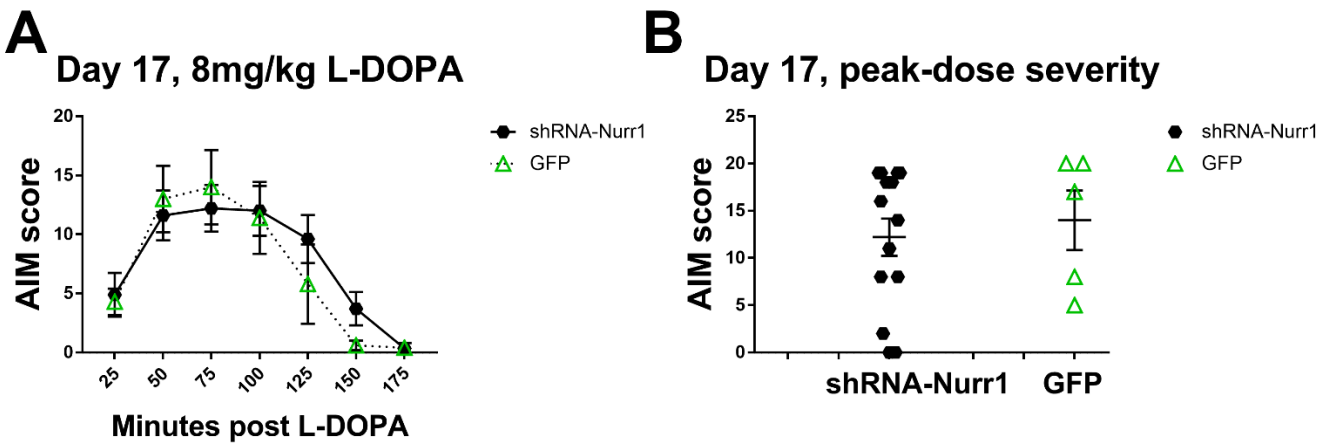

Figure 3.9 Nurr1 shRNA does not exacerbate LID

(A) Individual rating period comparing AIMs between rAAV-shRNA-Nurr1 and rAAV-GFP treated Fischer rats. Both groups show similar AIM expression patterns. (B) Peak dose (75 minutes post injection) AIMs show similar AIM severity in rAAV-shRNA-Nurr1 animals compared to rAAV-GFP animals.

Utilizing LID-prone Fischer rats and LID-resistant Lewis rats, we have shown that ectopic Nurr1 expression can induce severe LID in the Lewis animals. GFP-treated control Lewis rats displaying mild AIM scores do not show an endogenous upregulation of Nurr1 (see Figure A.3.1B), suggesting that the lack of Nurr1 contributes to the strain's resistance to severe LID. Interestingly, delivering rAAV-Nurr1 to Fischer rats did not induce more severe AIMs than control Fischer rats. It is likely that in strains that are prone to develop LID, Nurr1 cannot exacerbate the severe AIMs that would happen without intervention. This 'ceiling effect' suggests that Nurr1 is important for LID development and the appearance of severe AIMs, but increasing expression does not correlate with worsening AIMs. While vector delivery results in much higher Nurr1 expression than what is seen in an LID+ rAAV-naïve animal (see Figure 3.1E-F and Figure A.3.1A), our data suggests that the hyperexpression from viral delivery does not further LID severity. Likely, there is a critical threshold level of endogenous (or ectopic) Nurr1 expression must be reached to allow for LID induction, but higher expression levels do not exacerbate the behavior. This suggests that Nurr1 plays a role primarily in severe LID development. Additionally, it has been shown that Nurr1 displays differential transcriptional activity based on its expression level (Johnson et al., 2011; Do, 2014). It is possible that the higher expression levels achieved with viral expression promote gene transcription that is not related to LID escalation. However, the fact that LID can be induced in LID-resistant Lewis rats with viral Nurr1 expression suggests that this level of expression still allows for Nurr1-mediated transcription that influences LID. Our study did not evaluate the minimum level of Nurr1 expression or number of MSNs

expressing Nurr1 for LID to present, so further characterization of Nurr1 expression in LID is warranted.

Severe AIM expression in rAAV-Nurr1 treated Lewis animals suggests that Nurr1 is a key factor in LID development, as this strain of animals does not develop severe LID without Nurr1 ectopically being expressed. This additionally shows that Nurr1 is not a downstream byproduct of LID expression, and rather does play a role in their development. The ectopic expression of Nurr1 was sufficient to overcome LID resistance in these rats and induce AIMs as severe as those seen in LID-prone Fischer rats, suggesting that Nurr1 expression is a key difference in the two strains susceptibility. Identifying genetic or epigenetic differences between the strains that may explain their differential upregulation of Nurr1 in response to L-DOPA would be a valuable study to identify molecular traits involved in LID susceptibility.

Nurr1 has previously been observed in striatal MSNs in primarily the direct pathway (Heiman et al., 2014). We were curious as to whether direct DA receptor activation could induce Nurr1 expression in either or both the direct or indirect pathway. We observed Nurr1 induction in MSNs of AIM-expressing parkinsonian rats treated with a D1 receptor agonist, and confirmed that the expression was occurring in both MSNs of the direct and indirect pathway. This is notable because it indicates that Nurr1 induction is dependent on direct pathway activation, and additionally that D1 receptor activation is sufficient to induce Nurr1 expression in indirect pathway neurons. These data align with studies of other immediate early genes in LID, such as Homer-1a and FosB, which show that D1 priming is required for expression in D2 neurons (Pollack & Yates, 1999; Yamada et al., 2007; Pollack & Thomas, 2010).

Interestingly, no Nurr1 protein was observed in AIM-expressing animals treated with the D2 agonist quinpirole. This suggests that there are D2-dependent pathways to LID development that do not require Nurr1 expression, and further confirms that Nurr1 induction is reliant on direct pathway signaling. This could suggest a potential mechanism for low level LID development in Lewis rats that do not endogenously upregulate Nurr1 with AIMs. It is possible that differences in the DA receptors of Lewis rats promote more signaling through D2 receptors than D1, causing indirect pathway LID with no Nurr1 expression.

It is important to note that both Lewis rats treated with L-DOPA and Fischer rats treated with the D2 agonist quinpirole develop mild and moderate AIMs, much less severe than Fischer rats treated with L-DOPA or a D1 agonist. As both of these groups with moderate AIMs do not show abnormal Nurr1 upregulation, it is reasonable to hypothesize that Nurr1 is required for severe AIM development. Nurr1 may act as a ‘gate keeper’ of sorts that, when expressed, can induce striatal changes that lead to more severe dyskinesia.

In conclusion, this study has shown that Nurr1 is not only a marker of LID, but is directly involved in LID development and severity. This characterization of Nurr1 highlights its important role in LID, and suggests that Nurr1 agonists-based therapies for PD could be detrimental to patients by reducing the efficacious period of L-DOPA by promoting LID development.

APPENDIX

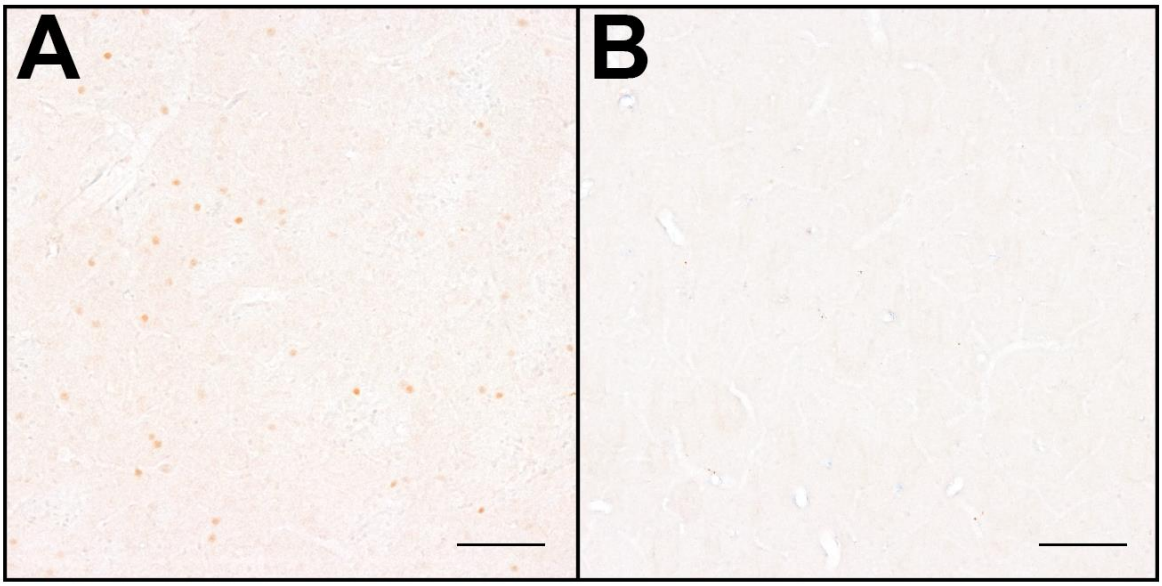

Figure A.3.1 Abnormal Nurr1 upregulation in dyskinetic rats

(A) Nurr1 IHC in the lesioned striatum of an LID+ Fischer rat. Abnormal Nurr1 expression can be seen throughout the lesioned striatum. (B) Nurr1 IHC in the lesioned striatum of an LID+ Lewis rat. No Nurr1 expression is seen in these animals. Scale bar=100µm.

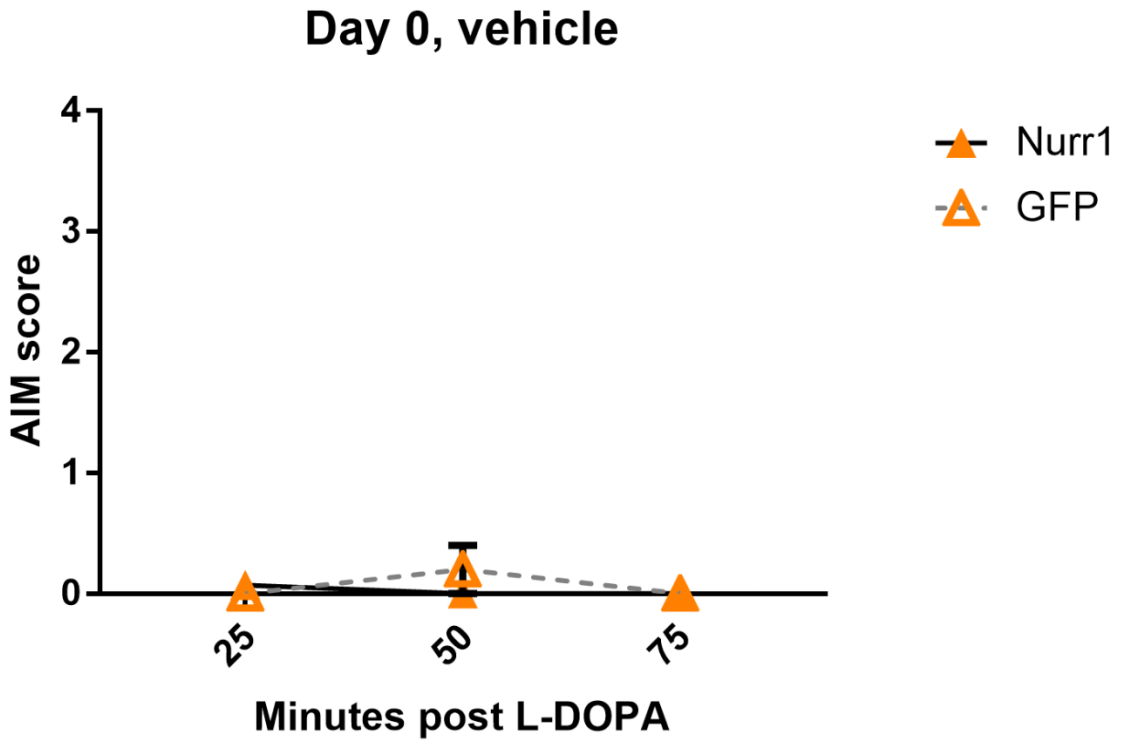

Figure A.3.2 Virally-expressed Nurr1 does not induce drug-independent AIMs

Fischer rats treated with rAAV-Nurr1 or rAAV-GFP do not express AIMs when treated with vehicle (0mg/kg L-DOPA, 12mg/kg benserazide) showing that Nurr1 overexpression does not promote AIMs without drug treatment.

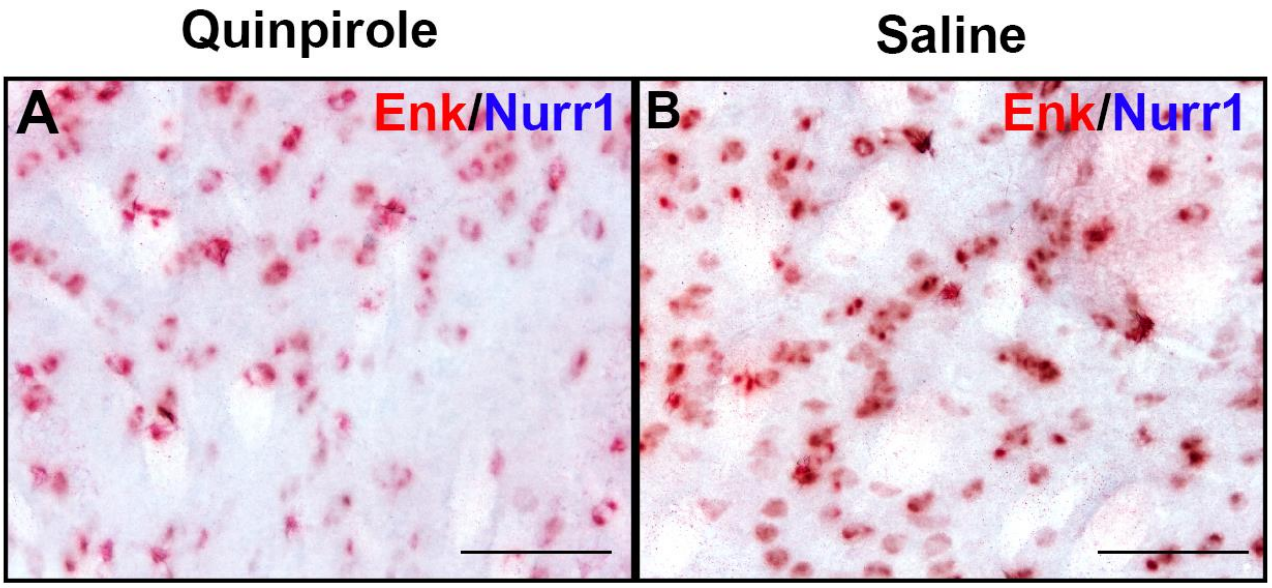

Figure A.3.3 Nurr1 colocalization with iMSNs in quinpirole and saline treated rats

(A) *In situ* hybridization for enkephalin (Enk) and with IHC for Nurr1 in a dyskinetic rat treated with quinpirole. No Nurr1 protein was observed in these animals. (B) *In situ* hybridization for Enk with IHC for Nurr1 in a saline treated animal show no abnormal Nurr1 induction in saline treated rats.

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Chapter 4: Ectopic overexpression of Nurr1 induces a LID-like state in striatal medium spiny neurons

Preface

In this chapter, electrophysiology experiments were performed by Dr. Anthony West and Dr. Kuei Tseng at Rosalind Franklin University. All other experiments and analysis were performed at Michigan State University by Rhyomi C. Sellnow.

Introduction

Levodopa (L-DOPA) induced dyskinesias (LID) are a debilitating side effect that occur in the majority of Parkinson's disease (PD) patients due to long term treatment with L-DOPA (Cotzias et al., 1967; Ahlskog & Muenter, 2001; Manson et al., 2012). LIDs are involuntary motor behaviors that include chorea, dystonia, and limb hyperkinesia that are distinct from parkinsonian motor behaviors. Though these symptoms can become debilitating and affect the daily living of patients, L-DOPA remains the gold-standard treatment for the motor symptoms in PD.

Much research has focused on LID-associated changes in the electrophysiology of the basal ganglia and its connecting structures. *In vivo* recordings of PD patients with deep brain stimulation (DBS) therapy have revealed impaired depotentiation in basal ganglia output structures associated with severe LID (Prescott et al., 2014). Indeed, this data reflects findings from animal studies which established a loss of bidirectional plasticity in LID (Picconi et al., 2003). Following DAergic denervation, both long-term potentiation (LTP) and long-term depression (LTD) are lost in the striatum. While L-DOPA treatment restores this corticostriatal plasticity, LTD is not present when LID develop (Picconi et al., 2003). Recent studies using optogenetically labeled neurons have shown dramatic

increase in activity in a specific population of direct pathway neurons, with simultaneous decreases in indirect pathway neuron activity (Ryan et al., 2018).This abnormal corticostriatal response and striatal neuron activity is a key factor in LID expression.

Some of these LID-associated changes in plasticity may be accounted for by changes in the dendritic spines on the majority population of neurons of the striatum—medium spiny neurons (MSNs). Multiple studies have shown that spine density and morphology changes dramatically in animal models following LID induction (Zhang et al., 2013; Fieblinger et al., 2014; Nishijima et al., 2014; Suárez et al., 2014). Dendritic spines cover MSN dendrites. These structures act as synaptic 'hot spots', forming connections with presynaptic terminals on the head and neck of the spines (Maiti et al., 2015). Spines are dynamic structures, and their strengthening and pruning back can occur due to normal neuronal processes such as memory and learning, or be indicative of diseased states (Bagetta et al., 2010; Spiga et al., 2014; Maiti et al., 2015). In animal models, dramatic spine loss is observed following DA depletion, followed by a reestablishment of spines in LID (Zhang et al., 2013; Fieblinger et al., 2014; Nishijima et al., 2014; Suárez et al., 2014). Some reports show differential changes in direct versus indirect pathway neurons, suggesting opposing mechanisms between pathways (Fieblinger et al., 2014; Suarez et al., 2016). Changes in spine morphology have also been observed, with increase in mature mushroom type spines being associated with LID (Zhang et al., 2013). While total spine density appears to return to a more basal level in LID, evidence suggests that the synapses being formed on these spines are maladaptive. There is a dramatic increase in corticostriatal synapses in dyskinetic animals, with increased multi-synaptic connections and synapses on dendrites (Zhang

et al., 2013). Together this research has shown that changes in spine density and morphology are an important event in LID development, but further research is needed to understand what factors influence these changes.

Nurr1 has been shown to be involved in synaptic plasticity required for learning and memory in the hippocampus (Peña de Ortiz et al., 2000; Colon-Cesario et al., 2006; Hawk & Abel, 2011; Hawk et al., 2012). As synaptic plasticity and spine dynamics have been previously implicated in LID formation (Fieblinger & Cenci, 2015), it is reasonable to question if Nurr1 plays a role in these changes. In the present study, we aim to show that Nurr1 influences LID by altering the physiology and morphology of MSNs.

Methods

I. Adeno-associated virus production

The same vector design and production methods were used as described in Chapter 3 of this dissertation. Briefly, the human Nurr1 and GFP gene were cloned into the AAV genome under control of a ubiquitous promoter. The genome was packaged into rAAV2/5 via double transfection of HEK293 cells with AAV genome and helper plasmids as previously described. Virus was recovered using an iodixanol gradient and concentrated in concentration columns. Viral titer was ascertained by dot blot, and adjusted to a working titer of $1.0x10^{13}$ vg/mL.

II. Animals and surgeries

Adult male Sprague Dawley or Fischer rats (200-220g on arrival, Charles River, Wilmington, MA) were used in the studies. Studies were conducted in accordance with the guidelines of Michigan State University (AUF MSU06/16-093-00) and Rosalind

Franklin University (AUF# A3279-01). Rats were housed two per cage up until LID behavior testing began, when they were separated and singly housed. Animals were housed in a light-controlled (12 hours light/dark cycle) and temperature-controlled (22±1 °C) room, and had free access to standard lab chow and water.

Surgeries were performed under similar conditions as described in Chapter 3 of this dissertation. Lesions were performed using 5mg/ml 6-hydroxydopamine (6-OHDA) hydrobromide mixed in 0.2mg/ml ascorbic acid immediately before injections. Sprague Dawley rats used for local field potential recordings and spine analysis were lesioned with two 2µL injections of 6-OHDA (10µg per injection) in the left striatum (1st injection from bregma: Anterior Posterior (AP) + 1.6mm, Medial Lateral (ML) 2.4mm, Dorsal Ventral (DV) – 4.2mm from skull; 2nd injection from 1st injection site: AP – 1.4mm, ML +0.2mm, DV – 2.8mm). Fischer rats used in antidromic cell recordings received two 2µl injections of 6-OHDA (10µg per injection), one in the medial forebrain bundle (MFB, from bregma: Anterior Posterior (AP) – 4.3mm, Medial Lateral (ML) + 1.6mm, Dorsal Ventral (DV) - 8.4mm from skull) and one in the SNc (from bregma: AP - 4.8mm, ML + 1.7mm, DV - 8.0mm from skull). The needle was lowered to the site and the injection began after 30 seconds. The needle was removed two minutes after the injection was finished and cleaned between each injection. Lesion efficacy was estimated two and a half weeks following 6-OHDA injection with the cylinder task as previously described (Schallert, 2006; Manfredsson et al., 2007).

Viral delivery surgeries were performed similarly three weeks following 6-OHDA lesions, as described previously (Benskey & Manfredsson, 2016). Animals received two 2µl injection of either rAAV-Nurr1 or rAAV-GFP targeting lateral striatum (1st injection from

bregma: AP + 1.0mm, ML + 3.0mm, DV – 4.0mm from dura; 2nd injection from bregma: AP – 1.6mm, ML + 3.8mm, DV – 5mm from dura).

III. Tissue collection and processing

Animals received a final injection of either L-DOPA or DA agonists two hours prior to sacrifice. Rats were anesthetized deeply with a lethal dose of sodium pentobarbital, and intracardiacally perfused with Tyrode's solution (137mM sodium chloride, 1.8mM calcium chloride dihydrate, .32mM sodium phosphate monobasic dihydrate, 5.5mM glucose, 11.9mM sodium bicarbonate, 2.7mM potassium chloride) followed by 4% paraformaldehyde (PFA). Brains were rapidly removed and post-fixed for 72 hours in 4% PFA before being transferred into 30% sucrose. Brains were sectioned on a freezing sliding microtome at 40µm and stored at -20°C in cryoprotectant (30% ethelyne glycol, 0.8mM sucrose in 0.5X tris-buffered saline).

Immunohistochemistry (IHC) was performed as previously reported. A 1:6 series of free-floating tissue was stained for TH (MAB318, MilliporeSigma, Burlington, MA) Nurr1 (AF2156, R&D Systems, Minneapolis, MN) or GFP (AB290, Abcam, Cambridge, United Kingdom). Briefly, sections were washed in 1x TBS with .25% Triton x-100, incubated in 0.3% H_2O_2 for 30 minutes, and rinsed and blocked in 10% normal goat or donkey serum for 2 hours. Tissue was incubated in primary antibody (TH 1:4000, Nurr1 1.5ug/ml, GFP 1:20,000) overnight at room temperature. After washing, tissue was incubated in secondary antibody (Biotinylated horse anti-mouse IgG 1:500, BA-2001, Vector Laboratories, Burlingame, CA; biotinylated donkey anti-goat IgG 1:500, AP180B, Millipore-Sigma, Burlington, MA; biotinylated goat anti-rabbit IgG 1:500, AP132B, Millipore-Sigma, Burlington, MA) followed by the Vectastain ABC kit (Vector

Laboratories, Burlingame, CA). Tissue staining was developed with 0.5 mg/ml 3,3' diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) and 0.03% H_2O_2. Sections were mounted on slides, dehydrated, and coverslipped with Cytoseal (ThermoFisher, Waltham, MA).

IV. Local field potential and single cell *in vivo* recordings

Striatal local field potential (LFP) response evoked by cortically stimulation were recorded as previously described (Thomases et al., 2013; Jayasinghe et al., 2015). Animals in both electrophysiology studies were shipped to Rosalind Franklin University three weeks following vector delivery. Animals were deeply anesthetized with 8% chloral hydrate. Electrodes were placed in the cortex for stimulation (from bregma: AP + 3.5mm, ML + 2.0mm, DV – 2.0mm from dura) and the striatum for recording (from bregma: AP + 0.7, ML + 3.4mm, DV – 4.5mm from dura). The cortex was stimulated with pulses delivered every 15 seconds (Thomases et al., 2013). LFP recordings were taken 30-120 minutes following injection of 5mg/kg L-DOPA with 12mg/kg benserazide.

In vivo single cell recordings of striatonigral projection neurons were measured in vector treated animals without L-DOPA treatment, or in established dyskinetic animals. Electrical stimulation and antidromic activation of striatonigral neurons was performed as previously described (Threlfell et al., 2009). Spikes evoked by stimulating the substantia nigra pars reticulata (SNr) were determined to be antidromically activated based on spike collision with orthodromic spikes occurring consistently over 10 trials (Threlfell et al., 2009).

V. Golgi-Cox impregnation and spine analysis

Animals for spine analysis were perfused as described above with Tyrode's solution followed by 4% PFA. Brains were removed and hemisected. The caudal portion of the brain was then postfixed in 4% PFA and used for lesion evaluation. The rostral portion was postfixed for 1 hour and then transferred to 0.2M phosphate buffe**r** until further processing. The rostral section was sectioned on a vibratome at 100µm. Sections were then processed for Golgi-Cox impregnation as described previously (Levine et al., 2013). Briefly, sections were sandwiched gently between two glass slides and placed into the Golgi-Cox solution (1% mercury chloride, 1% potassium chromate, 1% potassium dichromate) in the dark for 14 days. Sections were transferred into a 1% potassium dichromate solution for 24 hours. Sections were mounted on 4% gelatin-coated slides and the stain was developed with 28% ammonium hydroxide followed by 15% Kodak fixer. Slides were dehydrated in alcohol and xylene and coverslipped.

Spine density and morphology were quantified using Neurolucida (MicroBrightfield Bioscience, Williston, VT) as has been previously described (Zhang et al., 2013). Neurons of the dorsal striatum were selected for analysis. To be selected for quantification, a neuron needed at least four primary dendrites that projected radially, not bidirectionally, from the cell body. One dendrite per neuron was traced and the spines quantified and typified. Each spine was typified into one of four classes: thin, mushroom, stubby and branched (Maiti et al., 2015). Ten individual dendrites per hemisphere per animal were quantified. Spine quantity and phenotype were evaluated based on total dendrite length, or proximal (dendrite branch orders 1-2) or distal (branch orders 3-n) dendritic regions.

VI. Stereology

Unbiased stereology was used to determine lesion status via TH loss as previously described (Polinski et al., 2015). Using Stereo Investigator software with the optical fractionator probe, TH-positive neurons in every sixth section of the whole SNc were counted on the intact and lesioned hemisphere, giving an estimate of total TH-positive cells in the SNc.

VII. Statistical analysis

Statistical analysis was performed using Statview (version 5.0) or GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA). All graphs were created in GraphPad. Lesion status was evaluated using unpaired, one-tailed t-tests. Differences in spine quantity and morphology were evaluated using unpaired t-tests. Differences between vector groups at each recording timepoint within a single treatment were compared with t-tests, with $p \leq 0.05$ being considered statistically significant. Bonferroni post-hoc tests were employed when significant main effects were detected. A two-way repeated measures ANOVA (GFP vs. gene therapy (ectopic expression of the DA D2 AR in 5-HT DR neurons)) x 2 (vehicle vs. drug treatment) with α set to 0.05 and all “n’s” adequately powered for electrophysiological studies was conducted using Sigma Stat software (San Jose, CA), and the potential two-way interaction effect was examined to determine how treatment effects differ as a function of drug treatment or gene therapy (Padovan-Neto et al., 2015).

Results

I. Lesion validation and vector expression

Animals for all studies were rendered parkinsonian with 6-OHDA injections as described previously. TH immunoreactivity was markedly reduced in the injected hemisphere (Figure 4.1A-B). Animals included in the analysis showed ~75-80% cell loss in the injected hemisphere and there was no difference in lesion severity between vector groups (rAAV-Nurr1 % TH neurons remaining=21.48±3.12; rAAV-GFP % TH neurons remaining=27.87±5.26; $t_{13)}$=1.08, p>0.05). These lesions would be sufficient to induce striatal changes that occur following DA depletion. Transgene expression was confirmed with IHC, and all included animals showed robust transgene expression in the striatum (Figure 4.1.D-E). Animals were then used for electrophysiology (rAAV-Nurr1 n=5, rAAV-GFP n=5) or spine analysis (rAAV-Nurr1 n=4, rAAV-GFP n=3).

II. Striatal Nurr1 expression alone induces LID-like corticostriatal signaling

We were first interested in how Nurr1 could impact striatal medium spiny neurons on a physiological level. Rats were rendered parkinsonian and received striatal injections of either rAAV-Nurr1 (n=5) or rAAV-GFP (n=5). These animals were not treated chronically with L-DOPA—and thus non-dyskinetic—in order to evaluate the effect of Nurr1 on striatal MSN activity independent of L-DOPA. Local field potential (LFP) was measured in the striatum following cortical stimulation in 30 minutes intervals after receiving a therapeutic dose of L-DOPA (5mg/kg, 12mg/kg benserazide). Over the recording period, rAAV-GFP animals showed depotentiation in corticostriatal response compared to baseline. This response has been shown previously in parkinsonian rats (Mallet et al.,

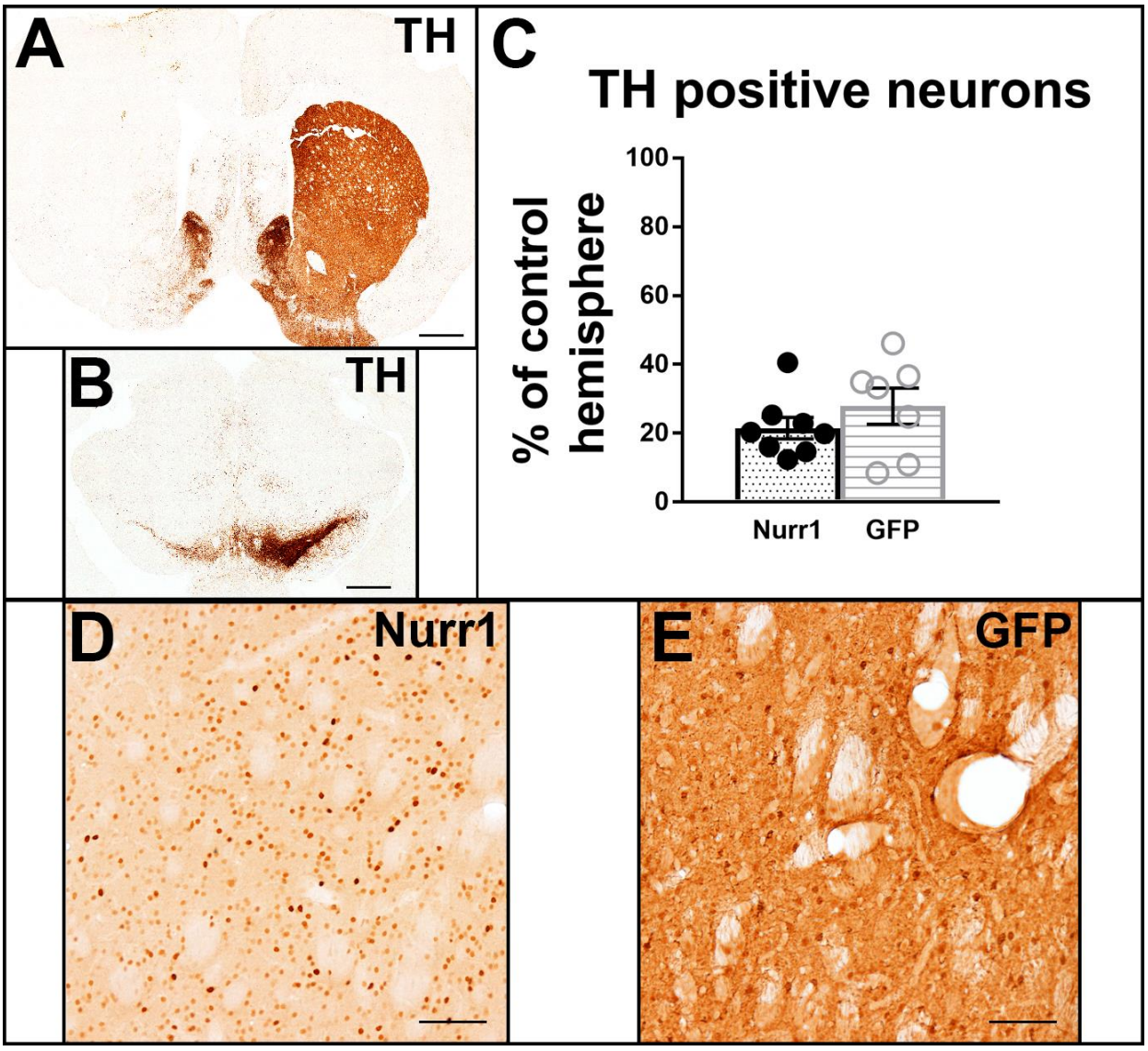

Figure 4.1 Lesion and vector confirmation

(A-C) Lesion status was confirmed with IHC for TH. TH immunoreactivity was dramatically reduced in the interjected hemisphere of the striatum (A) and substantia nigra (B). Stereological estimates of remaining TH-positive neurons show significant cell loss in both vector groups (C). (D-E) Transgene expression from viral vector delivery was confirmed in the striatum with IHC for Nurr1 (D) or GFP (E).
A and B scale bar=1mm
D and E scale bar=1µm

2006). Animals treated with rAAV-Nurr1, however, showed a potentiated response (Figure 4.2). This suggests that Nurr1 promotes increased neuronal activity and potentiation in DA-depleted MSNs. Importantly, the LFP profile of rAAV-Nurr1 non-dyskinetic animals was indistinguishable from that of rAAV-naïve animals with established LID. This indicates that Nurr1 expression induces LID-like activity in MSNs independent of L-DOPA.

To further understand how Nurr1 expression can impact MSN activity and signaling, we performed *in vivo* single cell recordings of direct pathway MSNs (dMSNs) of L-DOPA naïve parkinsonian rats injected with rAAV-Nurr1 or rAAV-GFP. Striatonigral dMSNs were identified by antidromically stimulating the SNr and recording from single neurons in the striatum. After a dMSN was identified, the cortex was stimulated to record cortically-evoked responses in the neurons (Figure 4.3A). Vector treated animals were compared with rAAV-naïve rats with established LID. dMSNs of rAAV-Nurr1 injected animals showed an increase in spike probability and average number of spikes compared to rAAV-GFP animals (Figure 4.3B-C). Importantly, rAAV-Nurr1 cortically-evoked spike response mimicked the spike profile of rAAV-naïve dyskinetic rats. This is similar to what we observed with LFP recordings. Together, this data suggests that dMSNs acquire LID-like activity with ectopic Nurr1 expression, and that these changes in firing rates are not dependent on DA signaling.

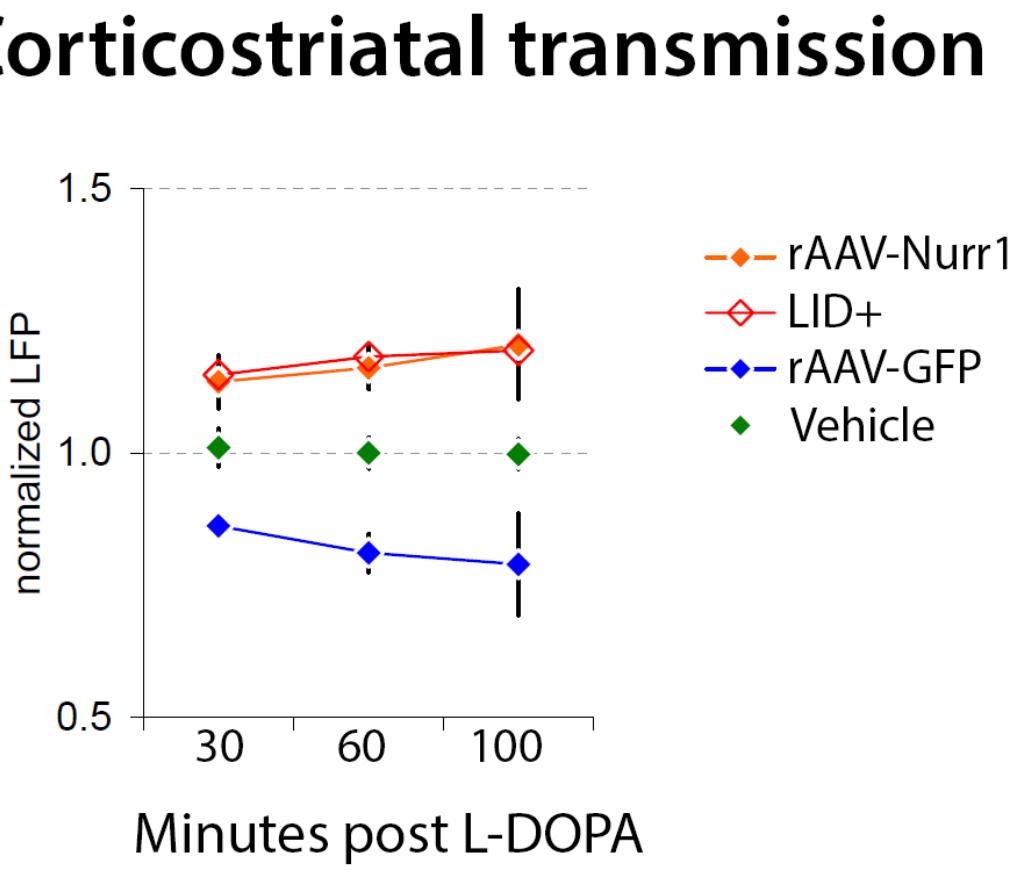

Figure 4.2 Striatal Nurr1 potentiates corticostriatal transmission

Local field potential (LFP) of the striatum in response to a therapeutic 5mg/kg dose of L-DOPA following cortical stimulation in animals injected with either rAAV-GFP (blue) or rAAV-Nurr1 (orange). A subset of animals did not receive a vector injection, but were instead rendered dyskinetic with L-DOPA priming (red). rAAV- GFP injected control subjects exhibited a suppression of the corticostriatal response, while rAAV-Nurr1 treated subjects exhibited a potentiation of corticostriatal transmission which was virtually identical to that of LID+ subjects. As expected, saline treatment (Vehicle) did not induce a response in rAAV-Nurr1 treated animals.

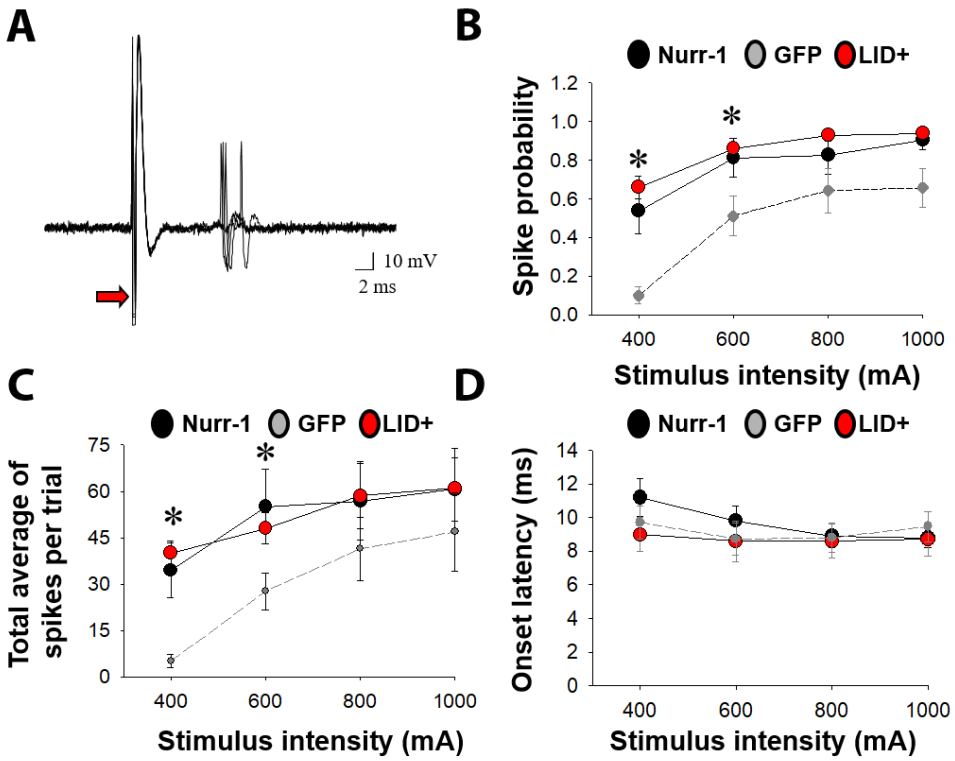

Figure 4.3 Cortically-evoked response of striatonigral MSNs to antidromic stimulation

Comparisons between cortically-evoked spike characteristics of antidromically-activated striatonigral projection neurons recorded from DA-depleted rAAV-naïve rats treated with chronic L-DOPA (LID+), rAAV-GFP injected L-DOPA naïve animals, or rAAV-Nurr1 injected L-DOPA naïve animals. (A) Traces of typical cortically-evoked responses from isolated striatonigral projection neurons. Ten consecutive overlaid responses are shown. (B-D) graphs compare the spike probability (B), average number of total spikes evoked during a stimulus trial at each current intensity tested (C), and onset latency of evoked spikes (D) during cortical stimulation. Stimulus intensity-dependent effects on cortically-evoked spike probability of striatonigral projection neurons were observed in both LID+ and Nurr-1 overexpressing rats as compared to vehicle treated DA-depleted GFP-expressing controls ($p < 0.001$). Post-hoc comparisons revealed a significant increase in the probability and number of evoked responses to cortical stimulation at the 400-600 µA current intensities in both LID+ and Nurr-1 overexpressing rats as compared to vehicle treated DA-depleted, GFP-expressing control rats (*$p < 0.05$). No significant differences in onset latency ($p > 0.05$) or SD of latency (data not shown) of cortically-evoked responses were observed.

III. MSN spine density and morphology changes are induced by Nurr1 expression

Dramatic changes in spine density and morphology in LID models has been previously shown (Fieblinger & Cenci, 2015). As Nurr1 has been shown to be involved in learning and memory-associated plasticity (Peña de Ortiz et al., 2000; Colon-Cesario et al., 2006), we sought to determine if Nurr1 expression can affect the spines of MSNs without exposure to L-DOPA. Parkinsonian rats were generated as in the electrophysiology experiment, and sacrificed three weeks following striatal vector delivery of rAAV-Nurr1 (n=4) or rAAV-GFP (n=3). These animals did not received L-DOPA at any time, allowing us to determine the effect of Nurr1 on DA-depleted MSNs independent of drug treatment. The striatum was processed using Golgi-Cox impregnation to visualize the spines. Spine analysis showed there was a significant decrease total spine density in rAAV-Nurr1 treated animals (rAAV-Nurr1 total spines/10µm=4.85±0.10, rAAV-GFP total spines/10µm=5.98±0.31; $t_{(5)}$=3.68, $p<0.05$) (Figure 4.4A). This difference was not specific to either the proximal or distal portions of the dendrite (rAAV-Nurr1 proximal spines/10µm=3.54±0.31, distal spines/10µm=5.58±0.41; rAAV-GFP proximal spines/10µm=4.24±1.45, distal spines/10µm=6.89±0.74). This indicates that Nurr1 can exert spine changes in MSNs and suggests its role in plasticity changes accompanying LID. Additionally, this effect is L-DOPA independent, showing that the capability of Nurr1 to induce spine changes does not require DA signaling.

We additionally compared spine morphology between rAAV-Nurr1 and rAAV-GFP animals. Spine morphology can be an indicator of synaptic strength and spine dynamics

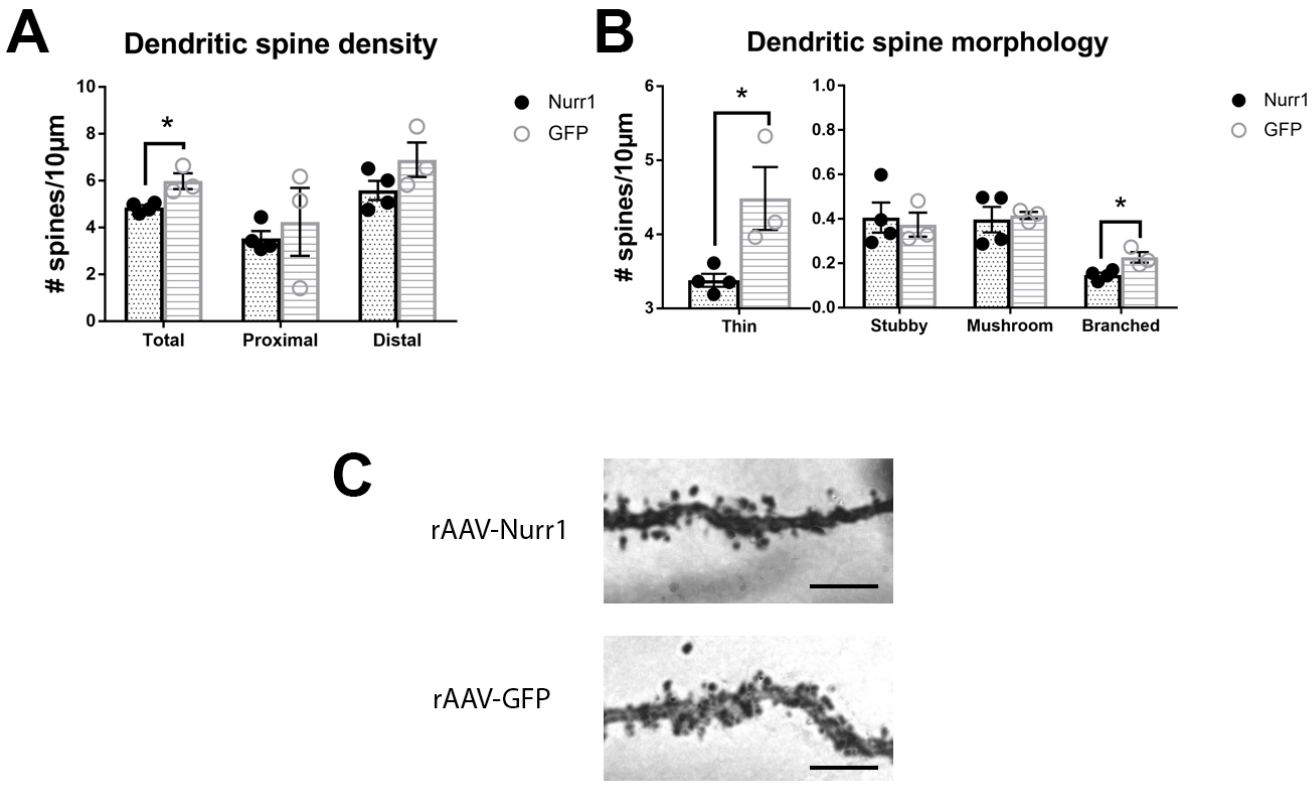

Figure 4.4 Nurr1-induced alterations in dendritic spine density and morphology

(A) Dendritic spine density of MSNs in the lesioned hemisphere of rats treated with rAAV-Nurr1 or rAAV-GFP. Nurr1 expression caused a decrease in total number of spines. (B) rAAV-Nurr1 expression induced a decrease in both thin and branched type spines compared to rAAV-GFP controls. (C) Representative image of dendritic spines on Golgi-Cox stained MSNs. Scale bar=50µm.

(Maiti et al., 2015). We found that ectopic Nurr1 expression lead to a change in two spine classes. rAAV-Nurr1 animals displayed significantly fewer thin type spines than controls (rAAV-Nurr1 thin spines/10µm=3.38±0.09, rAAV-GFP thin spines/10µm=4.48±0.43; $t_{(5)}$=2.98, $p<0.05$). Thin spines are typically transient and more likely to be formed and eliminated over time, unlike thicker spines that are more stable (Holtmaat et al., 2005). This suggests that Nurr1 could be influencing spine dynamics by altering turnover rates. We also observed fewer branched (also known as cupped or bifurcated) spines in rAAV-Nurr1 (branched spines/10µm=0.15±0.01) than in rAAV-GFP animals (branched spines/10µm=0.23±0.02; $t_{(5)}$=3.35, $p<0.05$) (Figure 4.4B). Branched spines are mature and much less dynamic than thin spines (Hering & Sheng, 2001). No difference between groups was observed with stubby or mushroom spines. The decrease in thin and branched spines with Nurr1 expression suggests that the transcription factor can independently alter spine dynamics and turnover. This implicates Nurr1 as a molecular regulator of the maladaptive spine plasticity that has been shown in animal models of LID.

Discussion

We studied the electrophysiology of the striatum with ectopic Nurr1 expression in order to understand how Nurr1 may be influencing LID. Our study shows that Nurr1 expression alone—independent of L-DOPA—can induce an LID-like signaling in the striatum as shown with LFP and single cell recordings. These animals were not chronically treated with L-DOPA, and thus the low dose given at the time of recording did not induce AIMs. This allowed us to determine that Nurr1 changes the activity of the striatum overall and in individual neurons independently, and its effect on LID

development is dependent on expression levels, not L-DOPA administration. The fact that rAAV-Nurr1 injected animals showed a near identical activity profile to rAAV-naïve, LID+ animals indicates Nurr1 has an active role in striatal changes associated with LID.

Additionally, we have shown that Nurr1 can independently induce changes in MSN architecture by affecting spine dynamics. Ectopic Nurr1 expression caused alterations in spine density and morphology, which have been shown previously to be linked with LID (Zhang et al., 2013; Fieblinger et al., 2014; Nishijima et al., 2014; Suárez et al., 2014). Interestingly, our study showed a decrease in total spine density in neurons ectopically overexpressing Nurr1. This is contrary to much of the literature investigating spine dynamics in LID, which have shown an overall increase in spine density associated with the behavior. However, some studies have shown that LID is associated with a decrease in spine density exclusively on dMSNs (Fieblinger et al., 2014; Nishijima et al., 2014; Suárez et al., 2014). As our study design did not allow for differentiation between dMSNs and indirect pathway MSNs (iMSNs), it is possible that through random sampling we selected primarily dMSNs. This would suggest that Nurr1 has an impact on the LID-associated spine loss in dMSNs. Alternatively, Nurr1 expression could lead to a differential effect on spine density between the two pathways that requires L-DOPA administration.

Our data implicate Nurr1 as a molecular factor involved in promoting these maladaptive changes. Nurr1 has previously been shown to be heavily involved in learning and memory-associated plasticity in the hippocampus (Peña de Ortiz et al., 2000; Colon-Cesario et al., 2006; Hawk et al., 2012). We have shown that Nurr1 can similarly affect plasticity when abnormally expressed in MSNs. Another NR4A family member Nur77

has been shown to induce spine loss in pyramidal neurons (Chen et al., 2014). Members of the NR4A family can form heterodimers to promote transcription (Maira et al., 1999). Since Nur77 is endogenously expressed in MSNs, it is possible that during abnormal Nurr1 induction in LID, an interaction between Nurr1 and Nur77 could promote transcriptional changes that induce the spine loss we observed in our experiments.

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Chapter 5: Discussion

Introduction

In this dissertation, we have addressed the hypothesis that LID is caused both by abnormal 5-HTergic DA release as well as maladaptive expression of Nurr1 in the striatum. First, I demonstrated the role of the DRN in LID development, and answered previously unexplored topics that provide compelling support for the 5-HT hypothesis of LID. I achieved this by virally expressing the DA autoreceptor in the DRN and observing changes in AIM behavior and DA signaling in the striatum. My second objective was to characterize postsynaptic molecular changes in MSNs that contribute to LID, specifically by determining that aberrant Nurr1 expression can induce LID. Finally, I demonstrated that Nurr1 may be able to influence LID by maladaptively altering striatal physiology through its activity alone, without L-DOPA. Together, the Aims of the present dissertation intend to close multiple knowledge gaps in the etiology of LID. There is an unmet clinical need for better management strategies for LID. Our hope with this dissertation is to shed light on integral aspects of LID development to broaden our understanding and lead to new therapeutic strategies.

Chapter 2: Inhibiting dorsal raphe-mediated dopamine release with AAV blocks LID development

I. Aim 1 findings, in brief

In Chapter 2, we showed LID development can be blocked by virally expressing the D2 autoreceptor, $D2R_S$, in 5-HTergic neurons of the DRN. Animals treated with rAAV-$D2R_S$ showed a complete inhibition of LID development without losing the anti-akinetic effects

of L-DOPA. $D2R_S$ expression was able to block the priming event required for LID development, as shown by the lack of AIMs expressed in these animals when treated with DA receptor specific agonists. Using *in vivo* microdialysis, we showed a marked decrease in striatal DA efflux in animals treated with rAAV-$D2R_S$. This is the first direct evidence showing that inhibition of DA release from 5-HT neurons is directly related to LID amelioration. We additionally showed that $D2R_S$ is functionally active in DRN neurons, as D2 agonist treatment caused reduced firing rates in transduced neurons. Together, this chapter confirms the 5-HT hypothesis of LID and shows that viral mediation of DRN neurons effectively blocks LID development.

II. Study implications and future directions

The 5-HT hypothesis of 'false neurotransmission' from DRN neurons has been a long-standing idea in the LID research community (Cheshire & Williams, 2012; De Deurwaerdère et al., 2016). There have been a number of strong studies that have supported this hypothesis and rationalized clinical trials modulating the 5-HT system in LID. The most compelling evidence supporting this hypothesis is that ablation of the DRN can eliminate LIDs completely (Carta et al., 2007; Eskow et al., 2009). Many studies using 5-HT receptor agonists in animal models have shown that pharmacological modulation of these neurons is also effective at reducing LID (Bezard et al., 2013; Politis et al., 2014; Ghiglieri et al., 2016; Meadows et al., 2017). However, until our study, no direct evidence has been offered that shows unequivocally modulating 5-HT neurons reduces abnormal DA release and effects LID severity. Our study has closed this knowledge gap by directly measuring a reduction in DA release with 5-HT modulation. Additionally, this is the first study to our knowledge utilizing

DAergic modulatory factors in 5-HT neurons in order to regulate their activity. While there have been a number of clinical trials aimed at modulating 5-HT activity using 5-HT receptor agonists, these studies have not shown dramatic improvements, and in some cases actually caused worsening PD symptoms (Kannari et al., 2002; Olanow et al., 2004; Iravani et al., 2006; Cheshire & Williams, 2012). The successful blockade of LID development in our model implicates $D2R_S$ gene therapy as a potential therapeutic strategy for modulating 5-HT neurons in a non-pharmacological way. Our study is the first of its kind, however, and further characterization would be necessary to determine if this type of therapy would be a viable clinical option, as many 5-HT agonists also showed highly promising preclinical results. Still, the precedent of AAV-based clinical trials in PD as well as the rationale and use of 5-HT agonists for LID treatment make $D2R_S$ an interesting candidate for therapeutic exploration.

There are a number of things that deservedly should be researched further with this approach. Firstly, a critical understand on how $D2R_S$ expression impacts 5-HTergic signaling will be essential moving forward. It has been shown previously that L-DOPA-derived DA displaces 5-HT in vesicles, suggesting that L-DOPA therapy itself downregulates global 5-HT signaling from DRN neurons (Waldmeier, 1985). This contributes further to LID as there is less activation of the 5-HT autoreceptors, causing over activity of these neurons and thus continual DA release. However, our treatment with $D2R_S$ reduces the activity and DA release from these neurons when treated with L-DOPA. While we did not observe changes in 5-HT efflux in the striatum, we were not directly activating 5-HTergic signaling during the recording period. Further analysis

looking at how $D2R_s$ expression impacts 5-HT release in different brain regions would be necessary to determine if $D2R_s$ negatively impacts the 5-HT system.

Our viral injections broadly targeted the entire DRN. However, not all DRN 5-HTergic neurons project to the striatum, the anatomical hub of LID. While detailed mapping of DRN projections is limited, it is clear that there are discreet cell populations that project to certain nuclei, including the striatum (Imai et al., 1986). A logical next step would therefore be to specifically target these neurons that project to the striatum, rather than the DRN as a whole. As DRN neurons release DA on other nuclei such as the substantia nigra, hippocampus, and prefrontal cortex (Navailles et al., 2010) it is important to determine if all DRN neurons or only those projecting to the striatum are involved in LID. There are a few ways this could be achieved. One approach would be to utilize a split rAAV vector and two-site delivery (Hirsch et al., 2016). Split vectors have been developed to circumvent the small packaging capacity of AAV. A transgene can be split between two vectors and packaged separately. When a cell is transduced with both vectors, the genomes naturally join via homologous recombination (Hirsch et al., 2016). For this application, one vector would be delivered to the DRN and the other to the striatum. If the striatal virus delivered was an rAAV pseudotype designed for efficient retrograde transport, this approach would result in the full transgene being expressed only in DRN cells that project to the striatum. The $D2R_s$ gene could be reengineered into a split vector and used in this way to determine if the strength of its anti-LID effect remains when expressed specifically in these neurons. This technique could also be used with a split designer receptor exclusively activated by designer drugs (DREADD). Further, this design would allow for specific ablation of striatal-projecting

DRN neurons by introducing vectors expressing split forms of apoptotic-promoting proteins, such as Caspase. As full ablation of the DRN has been shown to block LID (Carta et al., 2007), this specific lesion would definitively determine if only striatal-projecting DRN neurons are involved in LID.

We showed that $D2R_S$ DRN expression can effectively block L-DOPA priming by promoting more physiologic DA release on MSNs. This is evident in the fact that L-DOPA-primed rAAV-$D2R_S$ treated with direct DA agonists do not develop AIMs. As the protective mechanism of $D2R_S$ expression is a presynaptic mechanism, future experiments should challenge $D2R_S$ expressing animals that have not been treated with L-DOPA with DA agonists. I would hypothesize that these rats would develop severe AIMs similar to control animals, as the DRN signaling is not involved in DA receptor activation mediated by direct agonists. Additionally, as DA receptor agonists are known to cause dyskinesia in parkinsonian models and patients (Kuno, 1997; Rascol et al., 2001), it is likely that if we had extended DA agonist treatments in our behavioral cohort, severe AIMs would have eventually developed in the $D2R_S$—expressing animals as they became primed.

In this study, we delivered rAAV prior to treating with L-DOPA and establishing LID and successfully blocked LID development. This brings out the natural question: can delivering rAAV-$D2R_S$ to an animal with established dyskinesia revert the phenotype? Answering this question would have obvious clinical implications, as a patient receiving therapy would have been exposed to L-DOPA and developed LID. Thus, this reversal experiment would be very informative to determine if this approach could translate clinically. This study could be designed nearly identically to the study we performed,

only changing the order of treatments. Lesioned animals would be treated with L-DOPA to establish LID. Only after this would we delivery rAAV. If $D2R_S$ is capable of reversing LID, we would expect AIM scores to drop when these animals were reintroduced to L-DOPA.

Chapter 3: Nurr1 is a molecular driver of LID development

I. Aim 2 findings, in brief

In Chapter 3, we tested the hypothesis that Nurr1 plays an active role in LID development. We showed this by inducing severe AIMs in rats that are typically resistant to LID with viral overexpression of Nurr1 in the striatum. While Nurr1 overexpression did not further exacerbate LID in Fischer rats that are susceptible to sever LID development, the overexpression did induce severe AIMs in LID-resistant Lewis rats. These rats exhibited LID as severe as the Fischer rats. We next utilized direct DA receptor agonists to determine how pathway-specific activation affected Nurr1 expression. We found that Nurr1 expression is dependent on direct pathway signaling, and that D1 activation is sufficient to induce abnormal Nurr1 expression in both dMSNs and iMSNs. Finally, we found that an shRNA against Nurr1 was not sufficient to inhibit LID development in susceptible rats. Overall, our study provides evidence that Nurr1 is a novel molecular driver of LID.

II. Study implications and future directions

LID development is a complex, multifaceted process involving thousands of molecular changes. Gene expression studies have shown drastic expression changes in thousands of genes in dyskinetic models, highlighting a range of cellular pathways that

influence LID (Konradi et al., 2004; Heiman et al., 2014; Sodersten et al., 2014; Charbonnier-Beaupel et al., 2015). Nurr1 has been previously identified as being upregulated in MSNs of dyskinetic mice (Heiman et al., 2014; Sodersten et al., 2014). Our study has showed that Nurr1 is a critical factor in LID development, and not a molecular byproduct with no active role in the behavior. This identification and characterization of a novel molecular driver of LID is an important addition to the current understanding of how LID develop. Identification of key genes involved in LID etiology allows for better understanding of their development, which is necessary for developing novel treatment strategies. Additionally, we believe that our study demands a critical reappraisal of new Nurr1-based therapeutics intended to deliver neuroprotection PD. Pharmacological strategies to promote Nurr1 activity in the SNc could concurrently promote maladaptive activity in the striatum. It is possible that this could lead to earlier development or more severe LID in patients. Thus, caution moving forward with these strategies is warranted. Studies interrogating the effect of Nurr1 agonists on LID development in animal models would describe the risk associated with these therapies.

There is convincing evidence in the literature that can explain how this abnormal Nurr1 expression occurs in LID. The MAPK signaling pathway through and activation of ERK1/2 is well established as being hyperactive in LID (Fiorentini et al., 2013). *In vitro* studies have found that ERK1/2 signaling is required for the transcription of NR4A receptors including Nurr1 (Darragh et al., 2005). Nurr1 expression is regulated by a number of MAPK downstream effectors, including CREB and MSK-1 (Saucedo-Cardenas et al., 1997; McEvoy et al., 2002). In fact, inhibition of the MAPK cascade completely blocks activity-induced Nurr1 expression (Darragh et al., 2005). The

persistent MAPK signaling and ERK1/2 activation that occurs in LID is thus sufficient to promote Nurr1 expression in MSNs.

The MAPK cascade is, of course, not specific to a dyskinetic state and activated under normal physiological conditions in MSNs (Girault, 2012; Hutton et al., 2017). This physiological signaling does not, however, lead to Nurr1 expression in MSNs. However, the understanding of molecular changes in LID and Nurr1 regulation suggest that histone modifications may be involved in this abnormal allowance of Nurr1 expression. MSK-1 induces histone phosphorylation—specifically phosphorylation of histone H3—following its activation by extracellular stimuli (Kim et al., 2008). Notably, one study found dramatic increases in H3 phosphorylation mice treated with L-DOPA (Sodersten et al., 2014). Importantly, the researchers found that Nurr1 gene regulation was associated with specific H3 phosphorylation, and that Nurr1 transcript drastically increased in the striatum of dyskinetic mice (Sodersten et al., 2014). Together, this evidence suggests that histone modification by MSK-1 is essential in LID-associated gene expression, including Nurr1.

Our study using shRNA to block Nurr1 upregulation failed to inhibit LID development. This would suggest that Nurr1 is not required for LID development and that there are functionally redundant pathways. This is contradictory to our study showing that Nurr1 is required to induce LID in resistant Lewis rats. A possible explanation is that limitations with our shRNA approach did not achieve sufficient knockdown of Nurr1. While we achieved strong transduction with our vectors, it is near impossible to transduce 100% of neurons. Indeed, while our shRNA successfully blocked Nurr1 expression in transduced cells, we observed LID-associated Nurr1 upregulation in non-transduced

neurons (Figure 5.1). Because of this, it is likely that the critical threshold of Nurr1 expression to induce LID was achieved in these animals. This would reflect in the behavior data, as we saw severe LID development even with shRNA treatment. Currently, we do not know what the minimum amount of Nurr1 required for LID development is. Until this is characterized further, we can assume that the number of non-transduced neurons able to upregulate Nurr1 was sufficient to induce LID in our study. An shRNA approach may not be the strongest way to address the hypothesis that Nurr1 silencing can attenuate LID, and thus further studies using more robust knockdown approaches would be useful. Full genetic silencing of Nurr1 in the striatum using a CRISPR approach would ensure complete knockdown of the gene. However, this approach would require viral delivery of the CRISPR cassette, and would result in the same limitation as the shRNA if not enough cells are transduced. A conditional knockout model may be most viable, which would allow specific ablation of Nurr1 by using a tamoxifen-inducible Cre driven by MSN-specific regulatory elements. A similar approach has previously been used to selectively knockout Nurr1 in DAergic neurons (Kadkhodaei et al., 2013). This approach would ensure Nurr1 silencing in the entire striatum and allow us to determine if LID can be blocked in susceptible animals by disallowing Nurr1 expression. This model additionally would allow us to determine if silencing Nurr1 in established dyskinetic animals can revert AIM behavior.

Our study found that Nurr1 expression in both the direct and indirect pathway is dependent on D1 signaling. While dyskinesia-induced Nurr1 expression occurs in both classes of MSNs, we did not characterize the role of Nurr1 in dMSNs versus iMSNs. In order to determine if Nurr1 expression has a differential effect on LID in dMSNs or

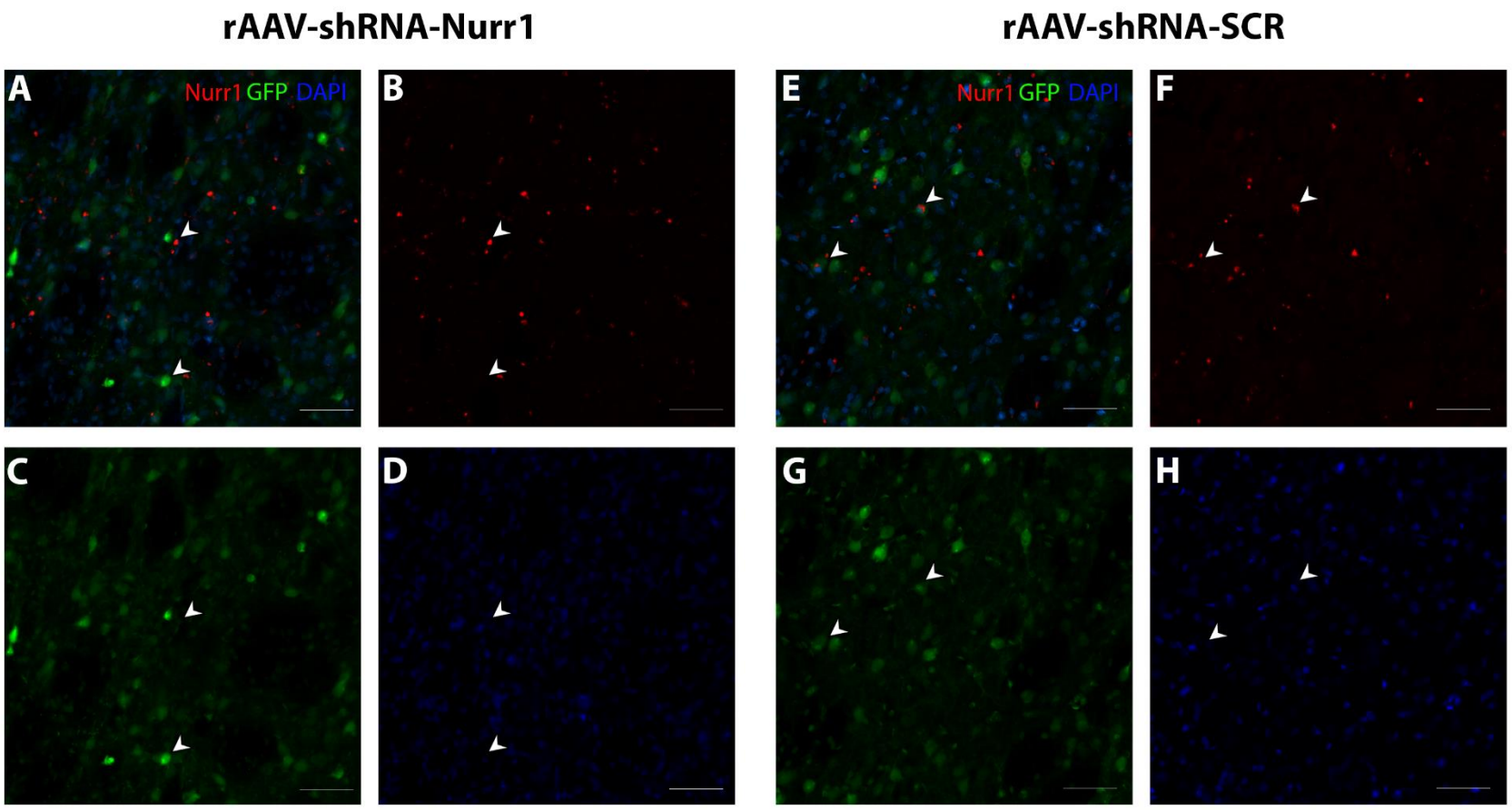

Figure 5.1 Nurr1 expression in shRNA treated animals

(A-D) IHC for Nurr1 (red, B), transduction marker GFP (green, C) and dapi (blue, D) in the striatum of an animal treated with rAAV-shRNA-Nurr1. No Nurr1 was observed to colocalize with GFP, and Nurr1 was only observed in neurons that were not transduced (arrows). (E-H) IHC for Nurr1 (red, E), transduction marker GFP (green, G), or dapi (blue, H) in the striatum of animals treated with the control vector rAAV-shRNA-SCR. Nurr1 can be seen colocalizing with GFP (arrows). Scale bar=50µm.

iMSNs, a Cre-dependent genetic vector could be used to drive expression specifically in D1 or D2 expressing neurons. Cre-dependent FLEx vectors can be used to achieve expression of the viral transgene only in cells expressing Cre (Schnütgen et al., 2003). These vectors could be packaged into rAAV and delivered to the striatum of transgenic rats with Cre expression driven by the promoter of either the D1 receptor or D2 receptor. Animals would then be treated with L-DOPA to monitor changes in AIMs due to specific Nurr1 expression. This would address the hypothesis that Nurr1 expression primarily effects LID through the direct pathway.

Chapter 4: Nurr1 expression induces an LID-like striatum

I. Aim 3 findings, in brief

In Chapter 3, we tested the hypothesis that Nurr1 expression induces abnormal changes in the firing patterns and ultrastructure of MSNs. We characterized the effect of Nurr1 on MSNs independent of L-DOPA. Using rAAV-mediated gene modulation, we showed altered corticostriatal activity in the striatum transduced with rAAV-Nurr1. These animals exhibited a potentiation in response to continuous stimuli, where control animals showed a depotentiation over time. Additionally, we found that the firing of rate individual striatonigral neurons was increased in rAAV-Nurr1 treated animals. In both of these studies, the activity profile of L-DOPA naïve rAAV-Nurr1 treated rats mimicked the behavior of neurons from a dyskinetic rat. Finally, we found that ectopic Nurr1 expression reduced dendritic spine density and lowered the number of thin and branched type spines. Taken together, this aim shows that Nurr1 can induce activity and morphology changes in MSNs that promote LID.

II. Study implications and future directions

A number of previous studies have found a strong link between altered MSN activity and morphology and LID (Picconi et al., 2003; Bastide et al., 2015; Fieblinger & Cenci, 2015). Our study provides evidence that Nurr1 is a molecular instigator of these alterations. Importantly, Nurr1 is able to induce these changes independent of L-DOPA, suggesting that Nurr1-regulated activity and transcription in MSNs does not require DAergic signaling. This evidence shows that once Nurr1 expression is induced in MSNs, it can promote these changes through its transcriptional activity.

We showed that individual dMSNs display increased firing rates with ectopic Nurr1 overexpression. Antidromic labeling cannot reliably identify iMSNs, as stimulus of the GPe would lead to inadvertent activation of dMSN fibers passing through the structure (Threlfell et al., 2009). As the antidromic labeling technique could only identify dMSNs, future studies should evaluate activity changes in iMSNs as well. This has been done recently in parkinsonian and dyskinetic mice using optogenetic identification of D1 or D2 expressing neurons in the striatum (Ryan et al., 2018). A similar approach could be employed in animals virally overexpressing Nurr1. An alternative approach would be to drive DREADD expression in dMSNs or iMSNs. Neurons could then be recorded from and identified by applying a designer drug to determine the neuron class. This or optogenetic identification would allow us to determine how Nurr1 alters the activity of both pathways, with or without L-DOPA. iMSNs show a decrease in firing potential in dyskinetic mice (Ryan et al., 2018), thus we hypothesize that iMSNs transduced with Nurr1 would show reduced firing rates independent of L-DOPA.

We saw a marked decrease in total spine density as well as thin and branched class spines in animals with rAAV-Nurr1 delivered to the striatum. These results indicate that Nurr1 can impact dendritic spine dynamics in MSNs, independent of L-DOPA. This is the first characterization of the impact of Nurr1 on dendritic spines of MSNs. Previous work has found that NR4A family member Nur77 can reduce spine density in hippocampal neurons, a similar effect we observed in MSNs with Nurr1 overexpression (Chen et al., 2014). As it is known that NR4A family members can heterodimerize to regulate transcription (Maira et al., 1999) Nurr1 could be impacting spine dynamics through its interaction with Nur77 in these neurons. Nur77 expression additionally has been shown to impact LID development in parkinsonian rats (Rouillard et al., 2018). As Nur77 and Nurr1 are not endogenously coexpressed in MSNs, their presence together in LID suggests that they coregulate maladaptive gene expression that drives LID. Further studies to determine if a Nurr1/Nur77 interaction occurs in MSNs and its effect on LID-associated gene transcription would add compelling evidence to support that NR4A nuclear orphan receptors are molecular drivers of LID.

LID-associated alterations in MSNs, from gene expression to spine dynamics to activity, are not consistent between the direct and indirect pathway. The use of Golgi-Cox impregnation of neurons does not allow for differentiation between the two neuron classes, and while we did observe Nurr1-induced changes in spine density and morphology, our study does not inform how these changes are reflected in each pathway. Previous studies have used retrograde labeling using neuron-filling dye injected into the SNr to identify dMSNs or the GPe to identify iMSNs (Nishijima et al., 2014). Other groups have used transgenic mouse models with fluorescent proteins

expressed under the D1 or D2 promoter to differentiate between pathways (Fieblinger et al., 2014; Suárez et al., 2014). Fluorescent neurons can be identified in slices and injected with dyes to fill the neuron and spines. Using the same experimental design from our study, employing one of these approaches would allow us to determine the effect of Nurr1 expression on each specific class of MSNs. We hypothesize that Nurr1 induces LID-associated spine loss in dMSNs and spine increases on iMSNs, and that this effect is independent of L-DOPA. In the present study, we saw global decreases in spine numbers when not differentiation between neurons. Assuming that we sampled dMSNs and iMSNs equally, our current data would suggest that Nurr1 reduces spines in both neuron classes. This could be confirmed with these approaches for differentiating dMSNs and iMSNs.

In the present dissertation, we studied the downstream effects on behavior and striatal physiology that are caused by Nurr1 overexpression. However, as Nurr1 is a transcription factor, it is likely not the direct effector of these changes, rather a regulator of genes that have direct impact. While our study characterized behavioral and physiological changes associated with Nurr1, we did not explore the downstream effectors regulated by Nurr1 and their effect on LID. Further studies identifying the Nurr1-regulated molecular pathway affecting LID are warranted. The data from this dissertation would suggest that the effector genes modulate synaptic plasticity through spine dynamics and signaling activity. Indeed, multiple genes regulated by Nurr1, including BDNF, the glutamate receptor GluR2, and kinesin family member KIF5C (Volpicelli et al., 2007; Do, 2014) can effect dendritic spines and synaptic transmission (Passafaro et al., 2003; Kellner et al., 2014; Willemsen et al., 2014). Pathway analysis

has shown that Nurr1 affects a wide array of cellular pathways, including MAPK signaling, cellular response to stimuli and stress, and cell growth and architecture (Do, 2014). Further characterization of genes regulated by Nurr1 in LID is necessary to better understand how its expression promotes LID.

Final remarks

This dissertation offers new evidence exhibiting the critical role of both the 5-HT system and Nurr1 in LID development. Dysregulated DA release from 5-HT neurons of the DRN promote a host of changes in MSNs, including the expression of the newly identified driver of LID, Nurr1. There are many networks and pathways involved in LID, and this dissertation sheds new light on how both presynaptic input and abnormal induction of one gene can influence the behavior. This dissertation supports a multifaceted mechanism for LID where abnormal DA release from 5-HT neurons induces Nurr1 expression in MSNs, which changes striatal physiology in a manner that promotes LID (Figure 5.2). Additionally, our studies provide compelling rational for the clinical development of novel LID management strategies targeting DRN neurons. Further characterization of LID-associated pathways, including aberrant Nurr1 expression, will be important as the field moves closer towards effective therapies for both PD and LID. The results presented in this dissertation have closed multiple gaps in knowledge in LID etiology, highlighting the impact of 5-HT regulation in LID and the role of Nurr1 expression in LID-associated plasticity.

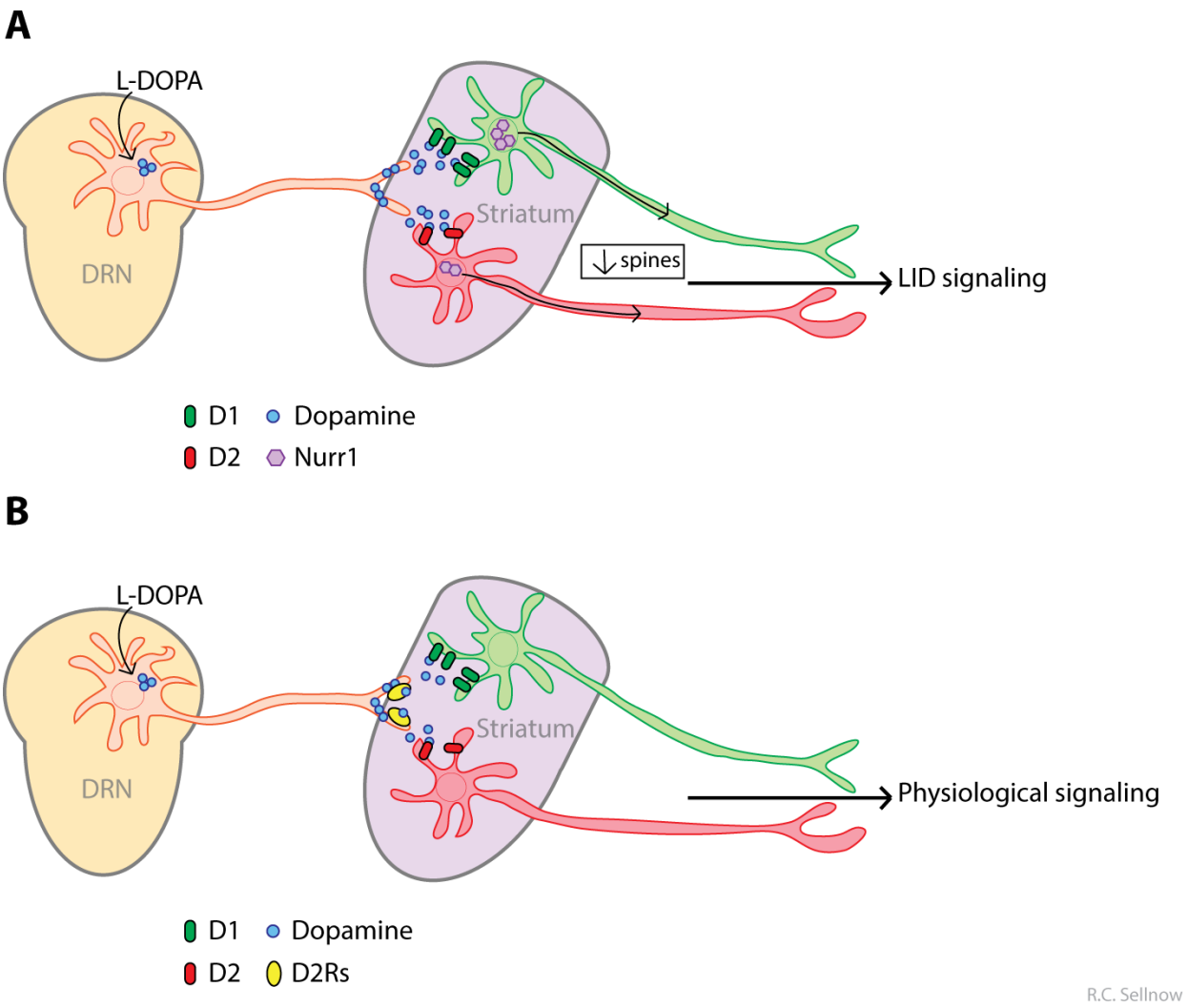

Figure 5.2 Proposed mechanism of LID development

(A) This schematic shows the proposed mechanism of LID development supported by the findings of this dissertation. Serotonin neurons of the dorsal raphe nucleus (DRN) convert L-DOPA to DA and indiscriminately release the neurotransmitter onto striatal MSNs. Hypersensitive MSNs respond to DAergic stimulation by maladaptively upregulating the transcription factor Nurr1, which induces changes in dendritic spines and MSN signaling activity. These aberrant changes promote LID. (B) LID development can be blocked by regulating DA release from DRN neurons. Ectopic expression of the DA autoreceptor $D2R_S$ in the DRN regulates striatal DA efflux, disallowing priming and maladaptive expression and synaptic plasticity required for LID development.

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