EXTRASTRIATAL $GABA_A$ RECEPTORS AS A NONDOPAMINERGIC TARGET IN THE TREATMENT OF MOTOR SYMPTOMS OF PARKINSON'S DISEASE AND LEVODOPA-INDUCED DYSKINESIA

by

ROBERT ASSINI

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written under the direction of Professor Elizabeth D. Abercrombie and approved by

_____

Collin J. Lobb, PhD

_____

James M. Tepper, PhD

_____

Pierre-Olivier Polack, PhD

_____

Tibor Koos, PhD

_____

Elizabeth D. Abercrombie, PhD

_____

Juan Mena-Segovia, PhD

Newark, New Jersey

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ABSTRACT OF THE DISSERTATION

Extrastriatal $GABA_A$ receptors as a nondopaminergic target in the treatment of motor symptoms of Parkinson's disease and levodopa-induced dyskinesia

By Robert Assini

Dissertation Director: Prof. Elizabeth D. Abercrombie

Parkinson's disease is a progressive neurodegenerative disorder resulting from the death of the dopaminergic nigrostriatal projection to the basal ganglia. Extrastriatal nuclei within this circuit have been shown to exhibit synchronous oscillatory activity entrained to excessive cortical beta oscillations following dopamine depletion. Zolpidem binds to $GABA_A$ receptors at the benzodiazepine site, potentiating inhibitory postsynaptic currents with selectivity for receptors expressing the α_1 subunit. Coincidentally, the nuclei expressing the α_1 subunit within the BG are also those that have been shown to have increased synchronous bursting activity in a dopamine-depleted state. We hypothesize that this differential expression of the α_1 subunit indicates zolpidem-sensitive $GABA_A$ receptors may constitute a potential non-dopaminergic therapeutic intervention in the treatment of PD motor symptoms. The work described in this dissertation explores this possibility using behavioral neuropharmacology and analytical chemistry.

Acknowledgments

This dissertation is dedicated to my grandparents, Dolores and Eugene Billeci, who passed away during the completion of this work. I would also like to thank my family, friends, and colleagues for their support throughout.

Preface

A version of the work described in Chapter III has been published (Assini and Abercrombie, 2018, *European Journal of Neuroscience*). Currently, Chapters IV and V are in preparation.

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List of Abbreviations

5-HIAA:	5-hydroxyindoleacetic
5-HT:	5-hydroxytryptamine (serotonin)
6-OHDA:	6-hydroxydopamine
ACSF:	artificial cerebrospinal fluid
BG:	basal ganglia
DA:	dopamine
DOPAC:	3,4-dihydroxyphenylacetic acid
GABA:	γ-aminobutiric acid
GPe:	globus pallidus external segment
GPi:	globus pallidus internal segment
HPLC:	high performance liquid chromatography
IPSC:	inhibitory postsynaptic current
L-DOPA:	L-3,4-dihydroxyphenylalanine
LID:	levodopa-induced dyskinesia
PAM:	positive allosteric modulator
PD:	Parkinson’s disease
SNc:	substantia nigra pars compacta
SNr:	substantia nigra pars reticulata
STN:	subthalamic nucleus
UPDRS:	Unified Parkinson’s Disease Rating Scale
Zol:	zolpidem

CHAPTER I

INTRODUCTION

1.1 Significance

It has been over 200 years since Dr. James Parkinson described the "shaking palsy" in his now-famous essay (Parkinson, 1817; 2002; Drew, 2016). It would be over 140 years from that formal characterization of the cardinal motor symptoms until they would become linked to the loss of dopamine (DA), which would quickly spur a pharmacological strategy to mitigate them (Ehringer & Hornykiewicz, 1960; Birkmayer & Hornykiewicz; 1961). This success would be short-lived, however, as shortly thereafter DA replacement therapies would be shown to induce debilitating side effects in a large percentage of patients. From then, Parkinson's disease (PD) would come to be recognized as the 2nd most common neurodegenerative disorder worldwide (Tanner and Goldman, 1996).

Despite decades of research, DA replacement therapies have remained the gold standard in the pharmacological management of PD motor symptoms for over 50 years. There have been countless attempts at investigating potential non-dopaminergic therapeutic targets, but few have yielded sufficient symptomatic relief absent deleterious side effects (Fox, 2013). Given the increased relative incidence of PD and the lack of progress in investigations of pharmacological alternatives/adjuncts to DA replacement, it follows that continued investigation of potential non-dopaminergic targets is critical.

It is not uncommon for clinical neurologists to investigate whether a novel pharmacological intervention for a given neurological disorder could address symptoms of another, especially in cases of overlap in symptoms or pathophysiology. Such was the

case when post-encephalitic patients rendered in a minimally conscious state were given levodopa (L-DOPA), as documented in *Awakenings* by Dr. Oliver Sacks and later immortalized in a 1990 film by the same title. The patient response was remarkable, but also short-lived, as the hallmark side effects of L-DOPA soon emerged as in PD patients.

Interestingly, the hypotheses that serve as the foundation of the following doctoral dissertation were inspired in a similar fashion. It was recently found that patients reduced to a minimally conscious state following traumatic brain injury respond to zolpidem in a paradoxical fashion. Zolpidem, a hypnotic with known sedative properties that is typically prescribed for insomnia, produced a robust increase both in cognitive and motor function in these patients (Schiff and Posner, 2007). These findings were attributed to the unique selective binding affinity of zolpidem within the basal ganglia (BG), which is also the principal neural circuitry affected by PD. Remarkably, there is a direct overlap in the natural distribution of zolpidem-sensitive receptors and the pathophysiology of PD motor symptoms, as well as L-DOPA-induced dyskinesia.

Given this overlap, the studies described in this dissertation aimed to investigate the utility of the zolpidem-sensitive $GABA_A$ receptor as a non-dopaminergic target in the treatment of PD motor symptoms and L-DOPA-induced dyskinesia. These experiments were designed to provide a preclinical proof of concept in a valid translational model of PD, using behavioral neuropharmacology and analytical chemistry. Further, this work was aimed at elucidating the interactions between amino acid neurotransmitters and DA in the BG circuit, and the impact of this interaction on the manifestation of DA-mediated behaviors.

1.2 Overview of the Introduction

In Section 1.3, I will review the neural correlates of PD motor symptoms and describe what is known of the role of the BG in volitional movement. Next, I will go over the consequences of chronic DA depletion within the BG, and how they have been elucidated using lesion-based translational models. Finally, I will describe the concordance of findings in PD translational models with those found in the PD patient population.

In Section 1.4, I describe levodopa-induced dyskinesia (LID). First, its prevalence in the PD population followed by the mechanism of action of levodopa. Subsequently, I will provide an exploration of the literature investigating potential neural mechanisms of levodopa-induced dyskinesia. This will cover both receptor-based insights and the neurophysiological correlates. To wrap up Section 1.4, I discuss the caveats current behavioral assays for rodent models of LID and propose the necessity for a more quantitative measure.

In Section 1.5, I describe the ionotropic $GABA_A$ receptor. In this Section, a review of receptor structure and its relationship to selective sensitivity to drugs will be provided. In addition, PD-related changes in GABAergic neurotransmission with in the BG are discussed. Lastly, this section will provide a hypothetical framework by which exploitation of $GABA_A$ receptor distribution within the BG could yield insight into potential treatment of PD motor symptoms.

Section 1.6 provides a review of the selectivity of zolpidem, its known comparative pharmacokinetics across species, and the neurophysiological consequences

of that selectivity within the BG circuit. This Section will conclude with a review of the promise of zolpidem in the treatment of neurological disorders, including a review of case studies in PD patients.

Finally, Section 1.7 briefly introduces the 3 data chapters (III-V).

1.3 Parkinson's disease: Motor symptoms, pathophysiology, and translational models

1.3.1 Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disorder, second to Alzheimer's disease in prevalence (Tanner and Goldman, 1996). PD is primarily characterized by a selective degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc) and their projections to the basal ganglia (BG) via the nigrostriatal pathway. Neurodegeneration has also been observed in other regions; including the locus coeruleus, basal forebrain, pedunculopontine nucleus, raphe nuclei, and hypothalamus (Hirsch *et al*, 1987; German *et al*, 1992; Dickson, 2012; reviewed in Giguere *et al*, 2018). With the exception of the pedunculopontine nucleus, however, postmortem reports investigating neuronal loss within these regions have been contradictory (Cheshire *et al*, 2015). Further, neurodegeneration within these regions have not been found to correlate with disease severity or duration. For these reasons, as well as the plethora of concordance of pathophysiological evidence observed in the PD patient population with translational models utilizing a selective DA lesion, the focus of this Introduction will remain on the consequences of DA depletion within the BG circuit.

Cardinal motor symptoms of PD include bradykinesia, tremor, rigidity, and inability to volitionally initiate/suppress motor output. Indeed, progression of nigral degeneration has been directly correlated to the severity and progression of motor symptoms of PD, particularly bradykinesia and rigidity (Greffard *et al*, 2006; Beach *et al*, 2009; reviewed in Dickson *et al*, 2009). The onset of motor symptoms, however, is typically preceded by a pre-motor/prodromal period lasting up to 20 years (Pont-Sunyer *et al*, 2015; Kalia & Lang, 2015). An array of nonmotor symptoms have been described during this period; including gastrointestinal issues, sleep disorders, and cognitive/mood deficits. In fact, motor symptoms may not manifest—resulting in clinical diagnosis of PD—until nearly 80% of the nigrostriatal projection has been lost (Bernmeier *et al*, 1973; Abercrombie *et al*, 1990; Hornykiewicz, 1993; Kirik *et al*, 1998; Dauer and Przedborski, 2003). This delayed onset of PD motor symptoms highlights the remarkable—in this case, tragic—adaptive capacity of the central nervous system. Knowing this, it is not unreasonable to consider PD motor symptoms to be a consequence of not only depletion of dopaminergic tone, but the intrinsic compensatory responses to DA depletion across the BG circuit.

There is currently no intervention capable of reversing the neurodegeneration that is known to result in PD motor symptoms. As a result, much focus has also been spent on the pharmacological mitigation of motor abnormalities. Current pharmacological treatments for motor symptoms of PD include dopamine (DA) replacement therapies, which require the administration of the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) and/or DA agonists. As the disease progresses, however, DA replacement therapies become less effective. In fact, these therapies induce debilitating side effects

such as choreic dyskinesia (discussed in Section 1.4). Given this, novel alternatives and/or supplements to DA replacement therapies that can address motor symptoms—and/or the complications accompanying DA replacement—are still needed.

1.3.2 Pathophysiology of Parkinson's disease in the basal ganglia

Execution and initiation of volitional movement requires an intact and functioning nigrostriatal DA system and BG. Under these circumstances, striatal afferents depolarize spiny projection neurons (SPNs) from a quiescent DOWN state to a depolarized UP state. Upon receiving enough convergent excitatory input, GABAergic striatonigral SPNs of the so-called "direct" pathway disinhibit neurons of the motor thalamus (VA/VL) via BG output nuclei: internal globus pallidus/substantia nigra pars reticulata/entopeduncular nucleus (GPi/SNr/EPN). In turn, thalamocortical projections disfacilitate the cerebral cortex, which facilitates movement via descending corticospinal neurons. Striatopallidal SPNs of the so-called "indirect" pathway also receive excitatory inputs from motor cortex, but send GABAergic efferents to the external globus pallidus (GPe), disinhibiting glutamatergic projections from subthalamic nucleus (STN) to BG output nuclei. The net result of increased inhibitory drive from GPi/SNr/EPN to thalamus suppresses movement and/or competing motor programs. Similarly, cortico-subthalamic projections of the so-called "hyperdirect" pathway also depolarize glutamatergic STN neurons (Magill *et al*, 2000). STN efferents, in turn, depolarize BG output nuclei as well as GPe neurons, some of which in turn send GABAergic projections back to striatum acting as a fast braking mechanism in the inhibition of movement (Tepper *et al*, 2007; Mallet *et al*, 2012; Iwamuro *et al*, 2017).

It is now hypothesized that competition between these 3 pathways underlies the execution or cancellation of movement and regulation of this "race" is likely mediated by DA. Schmidt *et al* (2013) showed that "stop" cues in a go/no-go task produced an increase of STN neural activity, but also that successful stops occurred only when BG output nuclei responded to this excitatory signal with an increase in activity. In other words, cumulative "go" signals from inhibitory striatonigral/pallidonigral inputs must be outweighed by excitation from STN in BG output nuclei in order for successful cessation/inhibition of a motor program (Dunovan *et al*, 2015). Similarly, cortico-subthalamic depolarization of BG output nuclei is critical for response inhibition, and can serve as a means of "increasing the decision threshold" during conflict (Baunez and Robbins, 1997; Frank, 2006; Frank *et al*, 2007; Eagle *et al*, 2008; Cavanaugh *et al*, 2011; Zavala *et al*, 2014).

Local infusion of DA agonists has elucidated the potential contribution of DA to the execution/inhibition of a movement. Hassani & Féger (1999) found that infusion of apomorphine into STN of intact rats reliably reduced the firing rate of the neurons therein, indicating a role for DA in the suppression of the cortico-subthalamic "fast braking" mechanism and, thus facilitation of movement. Similarly, infusion of DA into the GPe of intact rats induces an increase in single-unit activity and subsequent reduction of activity in both STN and SNr (Mamad *et al*, 2015). When DA is chronically depleted, the responsiveness of STN neurons to cortical activation increases, entraining the GPe-STN microcircuit to cortical beta oscillations across species (Nini *et al*, 1995; Magill *et al*, 2001; Cassidy *et al*, 2002; Sharrot *et al*, 2005; Canessa *et al*, 2016). This is evidently

mediated by “deranged” glutamatergic activation of NMDA receptors via the cortico-subthalamic pathway (Pan *et al*, 2014).

This evidence indicates that DA is critical in mitigating aberrant depolarization of STN, acting as a counterbalance to the potent glutamatergic drive of cortico-subthalamic afferents. Additional DA-mediated inhibition “tips the scale” in favor of inhibition of BG output nuclei from the striatonigral/pallidonigral pathways *versus* the combined disinhibitory/excitatory drive of striatopallidal and cortico-subthalamic pathways. According to this literature a chronic loss of DA will likely increase excitation along the cortico-subthalamic pathway, increasing depolarization of BG output nuclei, resulting in reduced disinhibition of thalamocortical projection neurons and an akinetic/bradykinetic motor phenotype.

To better illustrate this, consider performing a biceps curl. According to current hypotheses concerning the role of these 3 pathways, the striatonigral pathway would encourage flexion of the biceps muscle while the striatopallidal pathway would relax the triceps (Mink and Thach, 1991; Desmurget and Turner, 2007). To reverse the course of movement, or to abruptly inhibit it, the somatotopically organized cortico-subthalamic pathway would act as a sort of “emergency brake” (Baunez and Robbins, 1997; Frank *et al*, 2007; Eagle *et al*, 2008; Iwamuro *et al*, 2017). If the functional role of DA is as proposed above, a loss of DA would result in the absence of a potent source of inhibition of the brake. This would provide an opposing force to both the initiation and competent execution of a movement by encouraging co-flexion of opposing muscle groups (Hore and Vilis, 1980). Put simply, the motor symptoms of PD could be likened to driving with the emergency brake on. This makes sense, considering PD patients have been shown to

exhibit increased muscle tone, which is reversed by L-DOPA (Burleigh *et al*, 1995). Further, both parkinsonian rats and PD patients have been shown to exhibit trains of rhythmic muscle contractions at rest (Caviness *et al*, 2003; Metz *et al*, 2005).

1.3.3 Neurotoxin lesion models of Parkinson's disease

Prior to the emergence of advantageous study of neurosurgical patients with PD, the majority of knowledge pertaining to neural correlates of PD motor symptoms was gained from the use of translational animal models. Of these, the most commonly utilized translational models of PD require the administration of catecholamine-selective neurotoxins (Tieu, 2011). Decades of research using these models has exposed the neural correlates of PD motor symptoms, the network dynamics of the BG circuit, and the neurochemical dependency of BG function on DA. Much of this evidence has later been corroborated in the neurosurgical patient population. Given the need for a valid translational model for both basic research and clinical trials exploring potential interventions, the following section is dedicated to a brief explanation of these models, with particular emphasis on the validity of that utilized in this dissertation: the 6-hydroxydopamine (6-OHDA) rodent model of PD.

SNc DA neurons are extremely sensitive to acute environmental insult. As a result, parkinsonian motor deficits can manifest from a myriad of factors including viral encephalitis, thyroid issues, and traumatic brain injury (Dauer and Przedborski, 2003). Of these, one of the most notable instances of environmentally-induced parkinsonism occurred when intravenous drug users unknowingly self-administered 1-methyl-4-

phenyl-1,2,3,6-tetrahydropyridine (MPTP; Davis *et al*, 1979; Langston *et al*, 1983; 1999). Since, systemic administration of MPTP in non-human primates has become the gold standard in the dissection of PD pathophysiology and potential treatment for PD motor symptoms. MPTP-treated primates mimic both the symptoms and underlying BG pathphysiology observed in PD patients (Porras *et al*, 2012; described in Section 1.3.5). This selective neurotoxin can also be administered to rodents, representing a more cost-effective and statistically powerful approach to modeling PD. However, there is a considerable lack of toxicity in rats relative to mice (Giovanni *et al*, 1994). Parkinsonism can also be induced by systemic administration of herbicide/pesticide toxins such as paraquat and rotenone, but these methods tend to be avoided due to nonspecificity and variability of phenotypic outcome (Ferrante *et al*, 1997; Thiruchelvam *et al*, 2000). For these reasons, the most commonly studied rodent-based approach to the study of PD remains the unilateral acute intracerebral 6-OHDA infusion model.

Interestingly, use of 6-OHDA as an experimental manipulation predates its use in animal models of PD. First identified in 1959, it was originally used in experiments exploring the effects of noradrenergic depletion on cardiac function as well as the effects of selective destruction of sympathetic nerve terminals (Senoh and Witkop; 1959; Porter *et al*, 1963; Tranzer and Thoenen, 1968). Following the discovery that loss of striatal DA underlies the motor symptoms of PD, Ungerstedt (1968) found that intracerebral injection of 6-OHDA results in a selective destruction of central monoaminergic systems. Combined with systemic pre-treatment with a noradrenergic reuptake inhibitor, however, intracerebral infusion of 6-OHDA results in a reproducible and selective destruction of dopaminergic neurons (Waddington, 1980).

The mechanism by which 6-OHDA results in cell death has been investigated, but is not yet completely understood. Once introduced into the extracellular space, 6-OHDA gains access to monoaminergic neurons via dopamine or noradrenergic transporters (Luthman *et al*, 1989). Intracellularly, 6-OHDA has been shown to inhibit oxidative phosphorylation complexes in the mitochondrial electron transport chain (Glinka and Youdim, 1995; reviewed in Glinka *et al*, 1997). A more conventional explanation of 6-OHDA toxicity is related to its proclivity toward rapid auto-oxidation, resulting in formation of reactive oxygen species and subsequent cytotoxicity (Saner and Thoenen, 1971; Cohen and Heikkila, 1974; Graham, 1978; reviewed in Zigmond *et al*, 1992; Requejo-Aguilar and Bolaños, 2016). There is also evidence that 6-OHDA-induced toxicity is related to the regulation of intracellular Ca^{2+} release, as activation of the σ_1 receptor—a chaperone protein located at mitochondria-associated endoplasmic reticulum membranes—has been shown to protect against 6-OHDA toxicity (Hayashi and Su, 2007; Francado *et al*, 2014).

To induce a parkinsonian motor phenotype in rats, 6-OHDA can be infused into several locations: neostriatum, SNc, or the medial forebrain bundle (MFB; Deumens *et al*, 2002). Infusion of 6-OHDA into any of these locations can produce motor deficits that can be interpreted as a parkinsonian motor phenotype. However, these methods differ in the degree of striatal DA depletion produced, and thus are considered models of different stages of neurodegeneration. For example, intrastriatal 6-OHDA results in partial lesion of the nigrostriatal pathway, and can be considered a model of earlier disease stages (Yuan *et al*, 2005). Given the volume and functional complexity of the neostriatum, however, different sites of injection produce differing locomotor phenotypes

(Carli *et al*, 1985; Sabol *et al*, 1985; Cousins and Salamone, 1996). In addition, production of the requisite 80% striatal DA depletion requires several infusion sites across the rostrocaudal axis (Kirik *et al*, 1998; Chang *et al*, 1999).

Lesions of the MFB and SNc, on the other hand, typically result in near complete destruction of the nigrostriatal pathway, making this approach more of a late-stage PD model. Between these, MFB lesions appear to be more advantageous for the study of robust parkinsonian motor deficit, evident by a more replicable locomotor phenotype and drug-induced rotational behavior (Ungerstedt, 1971b; Casteneda *et al*, 1990; Hudson *et al*, 1993). It should be noted that MFB lesions can be produced bilaterally, and that this would certainly mimic late stage PD to a more accurate degree (Ungerstedt, 1971c). But animals subjected to bilateral 6-OHDA lesions of the MFB express profound deficits, including adipsia and aphagia. Additionally, unilateral lesions contribute an advantage such that the created imbalance in interhemispheric DA allows for additional motor assay. For example, unilaterally lesioned rats can be tested for disparate forelimb use, as well as drug-induced behavioral asymmetry (Schwarting and Huston, 1996a; Tillerson *et al*, 2001; Deumens *et al*, 2002).

Considering that the disease state to be studied is that of late stage, when DA replacement therapies have lost efficacy, the experiments described herein utilized unilateral 6-OHDA infusion into the MFB. This choice was made considering the disease state, replicability of lesion/motor deficit, as well as the dependency on near-total lesion of rodent locomotor assays (Tillerson *et al*, 2001). A discussion of the validity of this model is provided in Chapter VI.

1.3.4 Concordant electrophysiological evidence of extrastriatal hyperexcitation across species

DA depletion, as occurs in PD, alters BG network activity inducing oscillatory entrainment of downstream BG nuclei to cortical beta frequencies (15-30 Hz; Fillon, 1979; Magill *et al*, 2001; Mallet *et al*., 2008; Avila *et al*., 2010). This has also been observed in the form of altered neuronal activity patterns. Changes have been observed from a predominantly tonic pattern to dramatically increased burst firing within the GPe-STN microcircuit, but also in the circuit's output nuclei (GPi and SNr), and even ventrolateral thalamus (Magnin *et al*, 2000; Lee & Tepper, 2007; Tachibana *et al*., 2011; Lobb & Jaeger, 2015; reviewed in Lobb, 2014). DA depletion-induced alterations in to increasingly bursty activity patterns have been observed in 6-OHDA-lesioned rodents (Sanderson *et al*, 1986; MacLeod *et al*, 1990; Magill *et al*, 2001; Walters *et al*, 2007; Zold *et al*, 2007; Mallet *et al*, 2008; 2012), MPTP-treated non-human primates (Fillon & Tremblay, 1991; Boraud *et al*, 1998; Soares *et al*, 2004; Wichmann and Soares, 2006) and PD patients (Starr *et al*, 2005; Gale *et al*, 2009). Furthermore, the severity of PD motor symptoms has repeatedly been found to be positively correlated with oscillatory activity in STN at beta frequencies (Sharrot *et al*, 2014; 2018; van Wijk *et al*, 2016; Neumann *et al* 2016; 2017).

Recent evidence also suggests that DA depletion induces a switch in the responsiveness of BG output nuclei from striatonigral/striatopallidal input to that from the hyperdirect, such that activity of downstream BG nuclei correlates more strongly with activity in the STN than with striatum (Deffains *et al*, 2016). This is likely due to a perturbation of the inhibitory/excitatory balance within these BG nodes, and indicates an

increase in the physiological salience of the cortico-subthalamic pathway (Pan *et al*, 2014). Accordingly, direct electrical depolarization of STN in intact rats was found to be sufficient to induce a parkinsonian behavioral phenotype, as well as an increasingly bursty firing pattern therein (Tai *et al*, 2012). In addition, constant hyperpolarizing currents applied to STN were sufficient to improve parkinsonian symptoms in 6-OHDA-lesioned rats and abolish burst patterns of neural activity.

1.3.5 Insights from rodent rotational behavior

Additional insight into PD pathophysiology may be gained from a review of literature investigating pharmacological manipulations within the BG. It is well-documented that interhemispheric imbalances in BG circuit output generate overall behavioral asymmetries in rodents. It follows that unilateral infusion of substances with known effects on overall inhibition/excitation within a BG output nucleus, and its relationship to behavioral asymmetry, would certainly aid in the deduction of general governing principles of this phenomenon. In turn, these can be compared to the behavioral asymmetries observed in a PD translational model with a unilateral toxin-induced pathology.

TABLE 1.1. Behavioral asymmetries following unilateral intranigral (pars reticulata) infusion of GABAergic drugs. Adapted and compiled from Pycock, 1980. ACHC: Cis-3-aminocyclohexane carboxylic acid, GHB: γ-hydroxybutyric acid.

Source(s)	Drug	SNr Activity	Behavioral Asymmetry
Olpe et al, 1977; Scheel-Kruger et al, 1977; Martin et al, 1978, Arnt and Scheel-Kruger, 1979	GABA	↓	Contraversive
Oberlander et al, 1977; Olpe et al, 1977; Scheel-Kruger et al, 1977; Martin and Haubrich, 1978; Martin et al, 1978; Olianas et al, 1978b; Waddington, 1977; 1978a, b; Reavill et al, 1979; Waddington and Cross; 1978; 1979; Arnt et al, 1979; Arnt and Scheel-Kruger; 1979	Muscimol	↓	Contraversive
Olpe et al, 1977; Scheel-Kruger et al, 1977; James and Starr, 1978; Olianas et al, 1978b; Reavill et al 1979	Picrotoxin	↑	Ipsiversive
Scheel-Kruger et al, 1977; James and Starr, 1978; Olianas et al, 1978a	Bicuculline	↑	Ipsiversive
Waddington, 1977; 1978a	Flurazepam	↓	Contraversive
James and Starr, 1978	ACHC	↓	Contraversive
Olpe et al, 1977; Kelly and Moore, 1978	GHB	↓	Contraversive

The evidence outlined in Table 1.1, describing the behavioral correlates of SNr pharmacological manipulation, appears to converge on a general rule governing rotational behavior. With respect to the contribution of SNr: unilateral excitation produces an ipsiversive bias, inhibition contralateral. Put simply, an ipsiversive locomotor bias indicates increased excitatory drive within the ipsilateral BG output nuclei. Interestingly, undrugged unilaterally 6-OHDA-lesioned rats display ipsiversive rotational/locomotor bias both spontaneously and in response to external sensory stimuli (Ungerstedt, 1971a). When combined with the neurophysiological evidence outlined in Section 1.3.2 and 1.3.3, this overlap may be interpreted as such: In the event of chronic DA depletion, extrastriatal BG nuclei become prone to hyperexcitation via the cortico-subthalamic pathway. Excessive influence of glutamatergic afferents to STN are propagated to BG output nuclei (SNr in rats), resulting in a net excitation relative to the contralateral hemisphere and an ipsiversive locomotor bias. It follows that potentiation of GABAergic inputs to these nuclei could potentially oppose this pathophysiology.

1.3.6 SUMMARY

Initiation, execution, and suppression of volitional movement requires an intact BG circuit and nigrostriatal DA pathway. Chronic disruption of nigrostriatal DA, in both PD patients and translational models, alters the excitatory/inhibitory balance across extrastriatal BG nuclei. The literature outlined above indicates that loss of dopaminergic tone likely leaves extrastriatal BG nuclei prone to increased excitatory influence from glutamatergic afferents via the cortico-subthalamic pathway. This is supported by anatomical, neurophysiological, pharmacological, and behavioral evidence. We

hypothesize that a unifying characteristic among these nuclei could yield insight into a potential pharmacological target that directly addresses the pathophysiological alterations induced by DA depletion across species.

1.4 Levodopa-induced dyskinesia

1.4.1 Dopamine replacement as a short-lived therapeutic intervention

Currently, the most effective pharmacological intervention for PD is DA replacement therapy, which requires administration of DA agonists, such as apomorphine or ropinirole, or the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA). This intervention is effective at first, but debilitating side effects such as L-DOPA-induced dyskinesia (LID) soon develop in response to both DA replacement as well as DA agonists. Early studies by Duvoisin (1974) found that nearly half of PD patients develop LID within 6 months of treatment, and more recent longitudinal studies have found that up to 80% of PD patients develop LID within 5 years of treatment (Rascol *et al*, 2000).

Typically, LID is observed when DA or agonist levels exceed a concentration that is sufficient to activate postsynaptic DA receptors within the BG ("peak-dose dyskinesia"), but can also occur when DA level is too low ("early morning dystonia"), and even when DA levels rise and fall following L-DOPA treatment ("biphasic dyskinesia"; Cotzias, *et al*, 1969, Melamed *et al*, 1979, Muenter *et al*, 1977). Once these side effects begin to manifest, treatment options include further pharmacological intervention as well as surgical procedures. For the purposes of this dissertation, LID will be used to refer to peak-dose dyskinesia.

1.4.2 Levodopa: Mechanism of action

L-DOPA is typically administered orally to PD patients, concomitant with a peripheral AADC inhibitor (benserazide/carbidopa) which allows for passage through the blood-brain barrier into the CNS. Under normal circumstances, L-DOPA is enzymatically decarboxylated to DA within dopaminergic terminals by AADC. In PD, however, DA afferents to the BG have degenerated, and thus cannot be the sole source of conversion to DA (Hefti *et al*, 1981). It was once proposed that tyrosine hydroxylase containing striatal interneurons were the source of DA conversion following degeneration of nigrostriatal terminals, but it was subsequently found that these neurons lack AADC and thus cannot synthesize DA (Melamed *et al*, 1981; Xenias *et al*, 2015).

It is now generally accepted that L-DOPA is converted to DA by serotonergic afferents to the BG, which is supported by immunohistochemical assays, behavioral analyses, and *in vivo* neurochemical manipulations (Arai *et al*, 1994; Bishop *et al*, 2009; Iderberg, *et al*, 2015; Maeda *et al*, 2005; McCreary *et al*, 2016; Tison *et al*, 1991). Following release from 5-HT terminals, the now-converted DA is degraded by enzymatic activity of endogenous monoamine oxidase (Arai *et al*, 1996). Given that 5-HT neurons lack intrinsic mechanisms for DA level stabilization engendered by SNc DA neurons, this "anarchic" production/release of DA will inevitably lead to the hallmark complications of DA replacement therapies for a large cohort of PD patients (Bezard *et al*, 2001; de la Fuente Fernandez *et al*, 2004). This indicates that treatments for LID should focus upon ameliorating effects of exogenous L-DOPA at postsynaptic targets within the BG circuit rather than pharmacological regulation of DA levels, as the latter approach could lead to unintended dystonic and further dyskinetic effects resulting from DA level fluctuation.

1.4.3 Dopamine receptor supersensitivity

It is well established that DA depletion (as in PD) induces compensatory responses in postsynaptic DA receptor expression within the BG. This, in turn, leads to a subsequent behavioral supersensitivity to DA agonists (Ungerstedt, 1971b; Zigmond & Stricker, 1980). For example, unilaterally 6-OHDA-lesioned rodents are known to exhibit rotational behavior in response to the nonselective DA agonist apomorphine as well as DA synthesized from exogenous L-DOPA (Abercrombie *et al*, 1990). Indeed, receptor binding studies have illustrated significant increases in striatal D_2 receptors in both 6-OHDA-lesioned rodents and post mortem studies of untreated PD patients (Creese *et al*, 1977; Lee *et al*, 1978). In contrast, striatal D_1 receptor expression has been shown to be unchanged or even reduced (Lee *et al*, 1978). Furthermore, Lee *et al* (1978) showed that L-DOPA therapy may in fact reverse the upregulation of D_2 receptors within the striatum.

Given the lack of consensus in the literature describing sustained DA receptor upregulation following L-DOPA, Gerfen (1995) proposed that it is necessary to look beyond these changes in explaining the pathogenesis of LID as well as potential treatments. It is therefore reasonable to posit that while DA receptor supersensitivity may induce neuroplastic alterations underlying LID, evidence that L-DOPA can reverse elevated DA receptor expression indicates that receptor expression alone cannot explain the continued phenotypic manifestation of LID. Therefore, pharmacological intervention should focus upon mitigating neurophysiological correlates of LID within the BG rather than reversal of DA receptor supersensitivity.

1.4.4 Neurophysiological correlates of levodopa-induced dyskinesia

While studies of L-DOPA-induced DA release and compensatory responses in DA receptor expression level have focused primarily on the neostriatum, numerous investigations of the neurophysiological correlates of LID have elucidated aberrations in neural activity within downstream nodes of the BG circuit. Seminal studies by Fillon and colleagues showed that DA agonists induce reductions in firing rate of GPi in MPTP-treated primates, which was also observed in later primate studies (Fillon, 1979; Fillon *et al*, 1991; Papa *et al*, 1999). Similar results have been obtained in numerous studies of parkinsonian patients undergoing neurosurgery (Hutchinson *et al*, 1997; Stefani *et al*, 1997; Lozano *et al*, 2000). In contrast, metabolic studies have found that ventral anterior (VA) and ventrolateral (VL) regions of the thalamus—which receive dense GABAergic projections from GPi—show marked reductions in 2-deoxyglucose (2-DG) activity in MPTP-treated primates that exhibited LID-like behaviors versus those that did not (Mitchell *et al*, 1992). Lack of consensus between observations of firing rate of GPi and metabolic activity of its thalamic targets indicates that a shift in firing pattern, and not necessarily firing rate, may underlie the relationship between neural activity of BG output nuclei and motor impairment.

Indeed, neurophysiological recordings from both GPi and GPe in MPTP-treated primates and subsequent analyses of firing pattern have shown that L-DOPA or apomorphine administration provokes a shift to irregular and burst patterns of firing (Boraud *et al*, 1998; Boraud *et al*, 2001). Furthermore, alterations in firing pattern of GPi neurons were found to be correlated with the onset of LID-like symptoms. This shift in firing pattern, despite a decrease in rate, may be indicative of excessive excitation within

the BG circuitry. In agreement with this hypothesis, Trugman (1995) found that systemic administration of L-DOPA induced increases in 2-DG levels in EPN, SNr, as well as STN in unilaterally 6-OHDA-lesioned rats. Similarly, a study using *in vivo* microdialysis found that acute systemic L-DOPA administration elicited significant increases in extracellular glutamate levels in the SNr of unilaterally 6-OHDA-lesioned rats (Robelet, 2004). Furthermore, ibotenic acid-induced ablation of STN ipsilateral to the lesion was found to reduce L-DOPA-induced abnormal involuntary movements in 6-OHDA-lesioned rats (Aristieta *et al*, 2012).

The aforementioned evidence, when combined with hypotheses proposed by Crossman (1990) and Bezard *et al* (2001) regarding the importance of an overactive GPe in the pathophysiology of LID, indicate that potential pharmacological interventions should focus on counterbalancing excessive excitation in downstream nodes of the BG circuit (GPe-STN) as well as the output nuclei (SNr, GPi/EPN). Interestingly, optogenetic and pharmacological inactivation of STN has recently been shown to reduce DA agonist/precursor related behavioral asymmetries in unilaterally 6-OHDA-lesioned rodents (Petri *et al*, 2013; Yoon *et al*, 2016). Furthermore, a shared feature of all of these BG regions is the expression of α_1 containing $GABA_A$ receptors on principal projection neurons. In theory, a drug with selectivity for this receptor subtype—such as zolpidem—could selectively target these regions simultaneously and result in counteracting hyperexcitation of these nuclei evident in the literature.

1.4.5 Measuring levodopa-induced dyskinesia in rodent models of Parkinson's disease

Quantification of L-DOPA-induced behavioral asymmetry in translational rodent models is typically achieved using a variation of the Abnormal Involuntary Movements (AIMs) scoring system (Cenci *et al*, 1998; Lundblad *et al*, 2002; Steece-Collier *et al*, 2003; Cenci & Lundblad, 2007; Breger *et al*, 2013). Following systemic administration of L-DOPA, unilaterally lesioned rodents are observed and scored for specific behavioral stereotypies: axial contortion of the trunk and neck contralateral to the lesion (axial AIMs), myoclonic movements of the forelimb contralateral to the lesion (limb AIMs), repetitive movements of the lower mandible and tongue protrusion (orolingual AIMs), contralateral rotational behavior (ambulatory AIMs). These behaviors are scored on a scale of 0-4, representing the following criteria: 0→ absence of the specific behavior over the rating period, 1→ present for less than half of the rating period, 2→ present for longer than half of the rating period, 3→ present for the entire rating period, but suppressed by external stimuli (noise or cage movement), 4→ present for entire rating period, but not suppressed by external stimuli. Scores for each specific AIM are added together, the sum of which yields a total AIM score (out of 16) for that specific time point. In some studies, a Global AIM score is calculated by adding all scores across epochs analyzed per testing session.

Although these methods are efficient, there are confounds. These methods are ostensibly quantitative, but implementation of likert-type scales of behavioral rating results in a more categorical measure of behavioral asymmetry. As a result, it becomes difficult to detect less obvious changes in behavior. Further, treatment of a categorical variable as continuous introduces confounds when analyzed with conventional statistics.

Second, scoring is typically performed live with the experimenter present during the rating period, which can induce stress-related effects on the manifestation of behavioral asymmetry (Ungerstedt, 1971a). Additionally, these methods could be susceptible to inter-rater variability based upon the expertise of the experimental rater, which can lead to lack of reliability and reproducibility between laboratories. Third, these rating scales appear to be sensitive to doses lower than 4 mg/kg with all higher doses resulting in similar scores (Putterman *et al*, 2007). Fourth, studies have failed to correlate AIMs scores with striatal DA efflux in the lesioned hemisphere, which indicates that this measurement is not sufficiently sensitive to changes in extracellular DA levels (Lindgren *et al*, 2010).

The ability to provide a preclinical framework for future study of treatments for LID is dependent on a sufficiently sensitive assay. Given the pitfalls of AIMS-like systems, the necessity emerges for the development of an assay that can adequately detect changes in L-DOPA-induced behavioral asymmetry. This assay should be quantitative in nature, and correlated with striatal DA, as LID-like behaviors are DA-dependent. As a result, the experiments described in Chapter V sought to establish such a measure.

1.4.6 SUMMARY

In the pharmacological management of PD motor symptoms, the utility of DA replacement therapies is short-lived. The emergence of LID among a large proportion of the PD population engenders a need for investigations into potential alternative antiparkinsonian compounds, or those that can be utilized to combat LID itself. In the

assessment of potential drug candidates, the evidence outlined above indicates that pharmacological manipulation of L-DOPA-derived DA levels or management of DA receptor systems is not likely to be the most effective approach. Evidence gathered from neurophysiological study of LID correlates within the BG, however, have elucidated that extrastriatal BG nuclei are likely involved. We hypothesize that a compound with selective affinity across these BG nuclei could potentially impact the manifestation of LID.

1.5 $GABA_A$ receptors as a potential therapeutic target

1.5.1 Interventions beyond the dopamine system

The hallmark motor symptoms of PD—tremor, rigidity, and bradykinetic movement—are indeed directly related to DA depletion as a consequence of the progressive death of SNc dopaminergic neurons (Section 1.3). However, findings that non-dopaminergic drugs may be of use in the treatment of PD motor symptoms indicate that the consequences of DA depletion within the BG alter neurotransmitter/neuromodulatory systems beyond the nigrostriatal DA system. Pharmacological agents that target noradrenergic (fipamezole: α_2-antagonist; propranolol: β-adrenergic antagonist), serotonergic (clozapine: $5\text{-}HT_{2A/2C}$ antagonist), cholinergic (benztropine: M_4 muscarinic antagonist), glutamatergic (amantadine: NMDA antagonist; $mGluR_5$ antagonists), GABAergic (progabide: GABA analog/antiepiletic), and purinergic systems (instradefylline, preladenant: A_{2A} antagonists) have shown promise, likely due to their action within the BG circuit (Fox, 2013). Investigations into

most of these potential pharmacological interventions for PD showed a reduction in motor symptoms as well as L-DOPA-induced abnormal involuntary movements, but also off-target side effects that were deemed to outweigh the benefit of motor symptom reduction.

1.5.2 Role of subunit composition in selective affinity to drugs

GABA is the principal inhibitory neurotransmitter in the vertebrate central nervous system (Bloom and Iversen, 1971; Sivilotti and Nistri, 1991). This amino acid neurotransmitter acts via three subtypes of ligand-gated receptor: $GABA_A$, $GABA_B$, and $GABA_C$. Among these subtypes, the ionotropic $GABA_A$ receptor is the most widely distributed. The $GABA_A$ receptor is a pentameric receptor complex, whose subunits join to form a pore permeable to Cl^- (Sakkman *et al*, 1983; Bormann *et al*, 1987). Several variants of $GABA_A$ receptor subunits have been identified, including six different α-subunits ($\alpha_1 – \alpha_6$), three β-subunits ($\beta_1 – \beta_3$), three γ-subunits ($\gamma_1 – \gamma_3$), three ρ-subunits (ρ_1-ρ_3), as well as one δ- and ε-subunit (Hevers and Ludens, 1998). Despite the potential for robust diversity, the most commonly observed $GABA_A$ receptor in the central nervous system contains two α-, two β-, and one γ-subunit ($\alpha_x\beta_y\gamma_z$; Ernst *et al*, 2003).

Not only does the combination of subunits determine electrophysiological properties, but subvariants of the $GABA_A$ receptor can also be further distinguished pharmacologically (Sieghart, 1995; Sieghart *et al*, 1999; reviewed in Hevers and Ludens, 1998). For example, differential expression of α-subunit variants confers differential sensitivity to drugs that bind at the benzodiazepine site (Barnard *et al*, 1998; Olsen and

Sieghart, 2008). Receptors expressing the α_1 subunit, for example, are sensitive to both benzodiazepines and zolpidem while receptors expressing the α_2 subunit are sensitive only to benzodiazepines (Arbilla *et al*., 1986). From this, the possibility arises that identification of differences in the localized expression of specific $GABA_A$ receptor isoforms could be exploited to target the pathophysiology of neurological disorders, in a region- or even cell type-specific manner.

1.5.3 GABAergic receptor distribution in the basal ganglia: Insights for therapeutic targeting?

Inhibitory neurotransmission in the BG is primarily mediated by ionotropic $GABA_A$ receptors. Within the BG, the α_2 subunit is exclusively expressed in the caudate/putamen (striatum in rodents), while the α_1 subunit is expressed postsynaptically within the external globus pallidus (GPe), subthalamic nucleus (STN), internal globus pallidus/entopeduncular nucleus (GPi/EPN), and substantia nigra pars reticulata (SNr; Waldovogel *et al,* 1999; Pirker *et al*, 2000; Boyes and Bolam, 2007; Goetz *et al*, 2007; Gross *et al*, 2011; Fig 1.1). Interestingly, the nuclei expressing the α_1 subunit within the BG are also those that have been shown to exhibit oscillatory bursting activity in a DA-depleted state (Section 1.3.2). We hypothesize that this unifying characteristic among extrastriatal nuclei could yield insight into a potential pharmacological therapeutic intervention that directly addresses the pathophysiological alterations induced by DA depletion across species.

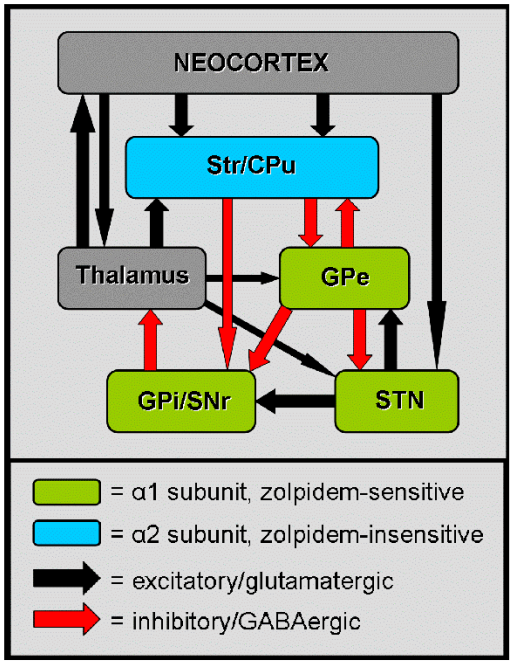


FIGURE 1.1: **Schematic representation of zolpidem sensitivity within the basal ganglia.** Autoradiography and in-situ hybridization studies have elucidated the differential distribution of $GABA_A$ receptor alpha-subunit variants within this circuit. The α_1-subunit is expressed in the GPe, STN, GPi/EPN, and SNr; making these nuclei zolpidem-sensitive (green). The α_2 subunit is expressed within the Str/CPu, making projection neurons within this region insensitive to zolpidem (blue). Black arrows represent excitatory/glutamatergic projections. Red arrows represent inhibitory/GABAergic projections.

1.5.4 GABAergic changes in Parkinson's disease

If a non-dopaminergic intervention for PD motor symptoms is to be effective, it should likely target regions implicated in the pathophysiology of these symptoms. Insights into potential systems/neural regions to be targeted should be informed by studies investigating the compensatory reactions to DA depletion within the brains of PD patients/translational models, within regions directly implicated in clinical manifestation of symptoms. The GABAergic system has emerged as a system of interest in this case, as the disinhibitory nature of the BG circuit is predicated upon reliable neurotransmission via ionotropic $GABA_A$ receptors.

Interestingly, GABAergic neurotransmission appears to be altered as a result of DA depletion within this circuit. Early post-mortem tissue analysis showed that GABA binding appears to be altered in human PD patients (Lloyd *et al*, 1979). Investigations using *in vivo* microdialysis have shown that levels of ambient GABA are increased 2-fold in the BG as a consequence of 6-OHDA lesion in rats (Bianchi *et al*, 2003). $GABA_A$ receptor subunit-specific mRNA binding studies have also indicated alterations in GABAergic systems as a consequence of DA depletion. For example, Pan *et al* (1985) showed that $GABA_A$ receptor binding is reduced in striatum and GPe and increased in BG output nuclei (EPN/GPi, SNr) 5 months after unilateral 6-OHDA lesion in rats. Furthermore, $GABA_A$ receptor α_1-subunit mRNA has been shown to be reduced in GPe but upregulated in STN, EPN, and SNr 3-5 weeks following unilateral 6-OHDA lesion (Chadha *et al*, 2000; Yu *et al*, 2001; Table 1.1). Combined with the overlap of zolpidem's selectivity, PD pathophysiology within the BG, as well as the evident efficacy of GABA analogs in the treatment of PD motor symptoms, this evidence indicates that a

positive allosteric modulator (PAM) acting upon the $GABA_A$ receptor system within the BG—such as zolpidem—could yield promising results (Ziegler *et al*, 1987; Daniele *et al*, 2016).

TABLE 1.2. Changes in $GABA_A$ receptor expression in the rodent basal ganglia as a result of unilateral 6-OHDA lesion. *Adapted from: 1) Pan *et al* (1985), 2) Chadha *et al*, 2000, 3) Yu *et al*, 2001. BZ = benzodiazepine.

#*	Subjects	Manipulation	Measure	Time post-lesion	Str	GP	STN	EPN/GPi	SNr
1	SD rats	Unilateral 6-OHDA in MFB	$GABA_A$ receptors	1 week	-1%	-49%	N/A	+41%	+11%
				4 weeks	-19%	-54%	N/A	+32%	+32%
				5 months	-18%	-32%	N/A	+40%	+60%
			BZ receptors	1 week	-18%	-6%	N/A	+7%	-6%
				5 weeks	-16%	-51%	N/A	+15%	+24%
				5 months	-18%	-33%	N/A	+13%	+39%
2	SD rats	Unilateral 6-OHDA in MFB	α_1-subunit mRNA	3 weeks	0	-18%	0	0	+11%
			α_2-subunit mRNA		0	0	0	0	0
			α_3-subunit mRNA		0	0	0	0	0
			α_4-subunit mRNA		+10%	0	0	0	0
3	Wistar Rats	Unilateral 6-OHDA in MFB	$GABA_A$ receptor	5 weeks	-21%	-14%	+20%	+40%	+29%
				10 weeks	-19%	-18%	+23%	+33%	+30%
			α_1-subunit mRNA	5 weeks	N/A	-12%	+10%	+15%	+14%
				10 weeks	N/A	-14%	+9%	+13%	+15%
			α_2-subunit mRNA	5 weeks	-7%	N/A	N/A	N/A	N/A
				10 weeks	-8%	N/A	N/A	N/A	N/A
			$mGluR_5$ mRNA	5 weeks	-7%	N/A	N/A	N/A	N/A
				10 weeks	-9%	N/A	N/A	N/A	N/A

1.5.5 Enhancing inhibition to combat extrastriatal hyperexcitation?

If excessive excitation is responsible for the alteration of neuronal firing patterns from tonic to bursting within the BG, it is reasonable to posit that enhancing inhibitory neurotransmission may mitigate this pathophysiology. Indeed, pharmacological manipulations of $GABA_A$ receptors have been shown to reduce neurophysiological and behavioral manifestations of DA depletion using translational models. Tachibana and colleagues (2011) observed that pathological oscillations in BG output nuclei were attenuated in MPTP primates by direct infusion of $GABA_A$ agonists into either GPe or STN. Similarly, amphetamine- and L-DOPA induced behavioral asymmetries were shown to be reduced as a result of muscimol infusion into the STN ipsilateral to the lesion in unilaterally 6-OHDA-lesioned rats (Petri *et al*, 2013). Concordantly, direct infusion of gabazine into the GPe of 6-OHDA-lesioned rats—presumably disinhibiting STN—resulted in contralateral rotational behavior akin to that observed following L-DOPA or apomorphine (Xue *et al*, 2010). This evidence suggests that manipulations of $GABA_A$ receptors within extrastriatal BG nuclei directly influence parkinsonian motor symptoms. Thus, their targeting in the treatment of PD motor symptoms and L-DOPA induced dyskinesia could prove fruitful.

1.5.6 SUMMARY

The truncated window for the clinical benefits DA replacement therapies has sparked investigations into alternative therapeutic targets. The GABAergic neurotransmitter system has emerged as a target of interest in this case, as the BG circuit

relies on ionotropic $GABA_A$ receptors. Similarly, nigrostriatal DA depletion has been shown to result in robust changes in $GABA_A$ receptor expression. Differential expression of $GABA_A$ receptor subunits, and thus differential sensitivity to compounds within this circuit, could yield insights into methods of selective therapeutic targeting. As discussed above, it has been shown that extrastriatal BG nuclei—which have been shown to exhibit hyperexcitation in a DA depleted state—exclusively express the α_1-subunit. From this, it can be hypothesized that a compound with selective affinity for these nuclei, capable of potentiating GABAergic inhibition, could yield therapeutic benefits.

1.6 Zolpidem

1.6.1 Selectivity

Zolpidem is an imidazopyridine (nonbenzodiazepine) hypnotic, commonly prescribed for insomnia under the trade name Ambien® (Sanofi-Aventis). The effects of zolpidem are mediated $GABA_A$ receptors, via interaction with the benzodiazepine binding site (Squires and Braestrup, 1977; Mohler and Okada, 1977). Despite a similar binding site, zolpidem has been shown to differ from classical benzodiazepines in its selective affinity. Benzodiazepines are known to have affinity for $GABA_A$ receptors containing α_1-, α_2- , α_3-, or α_5-subunit (Barnard *et al*, 1998; Mohler *et al*, 2002). Zolpidem, on the other hand, has been repeatedly demonstrated (across species) to have a selective affinity for receptors expressing the α_1-subunit (Arbilla *et al*, 1985; Benavides *et al*, 1988; Dennis *et al*, 1988; Langtry and Benfield, 1990). In fact, the selective affinity for the α_1-subunit ranges from 10x-30x the affinity for α_2-, α_3-, or α_5-subunits (Pritchett

and Seeburg; 1990; Hadingham *et al*, 1993; Damgen and Luddens, 1999). From these studies, it can be deduced that systemic administration of zolpidem would result in preferential modulation of neurons expressing α_1-subunit containing $GABA_A$ receptors.

1.6.2 Actions within the basal ganglia

As discussed above (Section 1.5.3, Fig 1.1), zolpidem-sensitive receptors are expressed postsynaptically on projection neurons in extrastriatal BG nuclei (GPe, EPN/GPi, STN, SNr). The effects of zolpidem on IPSCs within these nuclei has been directly investigated *ex vivo*. Within GPe, zolpidem (100 nM) was shown to increase decay time but not amplitude of both mIPSCs and sIPSCs (Chen *et al*, 2004). Conversely, Gross and colleagues (2011) found that zolpidem (100 nM) increased both the amplitude and duration of IPSCs evoked by striatal stimulation. Within STN, zolpidem (100nM) increased both the decay and rise time of mIPSCs but also increased mIPSC amplitude at a higher concentration (1 μM; Chen *et al*, 2007). Similar to GPe, zolpidem (100 nM) increased decay time but not amplitude of both mIPSCs and sIPSCs within SNr (Zhang *et al*, 2008).

Similarly, *in vivo* single-unit recording studies report that the presence of zolpidem reliably decreases the firing rate of zolpidem-sensitive cells in response to GABA, but not alone (Duncan *et al*., 1995). In other words, the zolpidem-induced reduction in unit activity was GABA-dependent. *In vivo* single-unit recordings within SNr also revealed that intravenous administration of zolpidem dose-dependently silenced

BG output neurons (Mereu *et al*, 1990). In this experiment, doses ranging from 0.03-10 mg/kg resulted in firing rate reductions that ranged from ~25-100%, respectively.

Zolpidem has also been selectively infused unilaterally into sensitive BG nuclei, showing nucleus-specific effects on rotational behavior. When infused into GPe, zolpidem (1 mM) produced ipsilateral rotational behavior, suggesting disinhibition of subthalamonigral projections via potentiation of striatopallidal afferents (Chen *et al*, 2004). In STN, infusion of zolpidem (1mM) resulted in contralateral rotation (Chen *et al*, 2007). This implies that a potentiation of GABAergic pallido-subthalamic afferents is sufficient to bias the responsiveness of ipsilateral BG output nuclei toward striatopallidal afferents to a degree capable of producing behavioral asymmetry. Similarly, infusion of zolpidem (1 mM, 0.2 µL) into SNr produced contralateral rotational behavior, suggesting a potentiation of striatopallidal afferents (Zhang *et al*, 2008). The results of these experiments show that zolpidem possesses potent effects on locomotor behavior, opposite to that observed in unilaterally 6-OHDA-lesioned rodents.

1.6.3 Comparative pharmacokinetics

Prior to the prosecution of the efficacy of a drug candidate, it is best to inform the investigation using available pharmacokinetic/pharmacodynamic data in the species to be studied (if such data are available). Fortunately, the prevalence of zolpidem in the clinic is rooted in an in-depth knowledge of these parameters across species. Zolpidem is frequently hailed for an advantageous pharmacokinetic profile, as it is rapidly adsorbed and eliminated (Langtry and Benfield, 1990; Salvo and Costa, 1995; Rush, 1998).

Indeed, maximal drug concentrations (C_{max}) in both blood and brain repeatedly occurred within the first sampling period in rats, regardless of route of administration (Garrigou-Gadenne *et al*, 1988; Durand *et al*, 1992; Trenque *et al*, 1994, Table 1.3). It should be noted that the sampling intervals differed between these studies, with the shortest time from administration measured being 15 min. Although it cannot be assumed that t_{max} does not occur earlier, the experiments described in this dissertation were designed with a t_{max} of 15 min in mind due to a lack of published data to the contrary. A summary of the pharmacokinetics of zolpidem following an acute dose can be found in Table 1.3.

Notably, there appears to exist a wide gap in bioavailability (F) of zolpidem between rats and humans when given orally. Bioavailability can be defined as the percentage of unchanged drug that reaches systemic circulation, and can be used as a means of estimating first-pass effects metabolism. Since intravenous administration grants unmolested access to the circulatory system, the calculation of bioavailability can be considered the dose-adjusted fraction of drug observed following an alternate route of administration divided by that observed intravenously:

$$F = \frac{AUC_{other} * Dose_{IV}}{AUC_{IV} * Dose_{other}}$$

In this equation: AUC is the area under the curve observed over the course of the experiment for either intravenous (IV) or the other route, Dose is the amount of drug administered for each route. Knowing this, it is possible to calculate the estimated bioavailability for the intended route of administration in this dissertation: intraperitoneal (i.p.). Using the values in Table 1.3 for i.v. or i.p. administration, the result is $F_{\text{i.p.}}$ = 37.8%.

An additional reason zolpidem has garnered clinical favor is due to its rapid and near total elimination from the body via the urine and feces. Zolpidem is rapidly metabolized hepatically, and is undetectable in the brain within 3 h in rats (Garrigou-Gadenne *et al*, 1988; Durand *et al*, 1992). Of the 7 major metabolites, only one has been shown ability to penetrate the central nervous system. This metabolite, a carboxylic acid derivative, has not been shown to be neuropharmacologically active (Drover, 2004). It should be noted, however, that cis-1,3-aminocyclohexane carboxylic acid is a known GABA reuptake inhibitor that has been shown to be active within SNr (Neal and Bowery, 1977; James and Starr, 1978).

Table 1.3. Comparative pharmacokinetics of zolpidem across species and routes of administration. Values have been compiled from indicated studies. Abbreviations are as follows; i.v.: intravenous, p.o.: oral, i.p.: intraperitoneal, t_{max}: time of max concentration, C_{max}: maximal concentration, AUC: area under the curve, $t_{1/2}$: biological half-life, *F*: bioavailability, n/r: not reported.

Study	*Garrigou-Gadenne et al, 1988*				*Durand et al, 1992*						*Trenque et al, 1994*
Species	**Rat**				**Rat**		**Monkey**		**Human**		**Rat**
Dose	2.63 mg/kg				2.63 mg/kg		2.63 mg/kg		10 mg		5 mg/kg
Route	i.v.		p.o.		i.v.	p.o.	i.v.	p.o.	i.v.	p.o.	i.p.
Sample	plasma	brain	plasma	brain	plasma		plasma		plasma		plasma
t_{max} (h)	0.25	0.25	0.25	0.25	0.25	0.25	0.5		1.03		0.5 (1st sample)
C_{max} (ng/mL)	2685	998	862	27	862		29-42		139		2341 ± 540
AUC (ng/mL*h)	n/r		n/r		3020	805	2673-2952	22-54	483	362	1734 ± 489
$t_{1/2}\alpha$ (h)	0.2-0.3	0.3	n/r		0.2-0.3		0.2-0.4		n/r		0.51 ± 0.06
$t_{1/2}\beta$ (h)	1.3-1.5	n/r	n/r		1.3-1.5		0.7-2.0		1.9 ± 0.3		n/r
F (%)	27%				27%		1-2%		66.6%		n/r
Excretion	n/r				Urine, feces		n/r		n/r		n/r
Elimination (%)	n/r				99.3	98.4	100	100	n/r	92.3	n/r

1.6.4 Zolpidem in the treatment of movement disorders

While this work is the first to utilize translational models to investigate whether zolpidem may be effective in the treatment of PD motor symptoms, other research has demonstrated similar effects in treatment of motor dysfunction. Miyazaki *et al* (2012) report that zolpidem improved symptoms in patients with medication-resistant dystonia, attributing this efficacy to zolpidem's actions within the BG. Similarly, studies have shown that administration of zolpidem to patients in an injury-induced minimally cognitive state induces recovery of motor responsiveness, ambulatory ability, and recovery of spoken language (Brefel-Courbon *et al*, 2007; Shames & Ring, 2008; Cohen & Duong, 2008).

Schiff and Posner (2007) proposed that the mechanism underlying this paradoxical response to zolpidem lies within the BG. It is hypothesized that zolpidem's action within the GPi may compensate for striatal dysfunction in minimally conscious patients by restoring disinhibition of thalamocortical projections, subsequently promoting disfacilitation of cortical pyramidal neurons (Schiff, 2009). It should be noted, however, that zolpidem's binding affinity within the BG is not restricted to the GPi, but also includes the GPe-STN microcircuit as well as the SNr. Further, coordinated activity of both "direct" and "indirect" pathways is critical to properly facilitate movement via thalamocortical projections (Cui et al, 2013). We propose that zolpidem may act to amplify inhibitory drive throughout the BG in these cases, restoring the physiological salience of striatal afferents along both BG pathways and promoting disinhibition of downstream thalamic nuclei.

1.6.5 Case studies of efficacy in Parkinson's disease

As described above, the efficacy of zolpidem in the treatment of PD motor symptoms has not been investigated in translational models. It has, however, been discussed in case studies of PD patients:

- Daniele and colleagues (1997) describe the case of a 61-year-old woman with advanced PD (25 year disease duration) who received zolpidem for symptoms of insomnia. Administration of 10 mg of zolpidem produced antiparkinsonian effects without producing drowsiness. Daniele *et al* went on to conduct a small placebo-controlled study using a single oral 10 mg dose of zolpidem in 10 PD patients, which produced a significant improvement of UPDRS-III motor scores (mean improvement = 18.4%).

- Ruzicka and colleagues (2000) describe the case study of a 45 year-old woman with advanced PD (12-year disease course) who had developed DA-induced choreic dyskinesia as a result of levodopa treatment. 30 minutes following administration of 2.5 to 5 mg of zolpidem, antidyskinetic and antiparkinsonian effects were reported. The woman subsequently maintained a treatment of 30 mg of zolpidem daily (5 mg doses, 6 times daily), and reported antidyskinetic effects "ON" levodopa and improved motor symptoms in the "OFF" medication state in the absence of drowsiness. This case study did not include quantitative analysis of motor symptom improvement.

- Chen *et al* (2008) describe the case study of a 53-year-old man (23-year disease course) who had remained severely disabled following pallidotomy and bilateral DBS implant procedures. After administration of 10 mg of zolpidem to treat insomnia, he "was able to speak well and his dystonia resolved. He was also able to get up from the bed, and to walk with minimal aid, as well as open his mouth, chew and swallow. His dyskinesia also improved." Treatment with 10 mg zolpidem produced a 27% improvement in UPDRS-III motor scores, and also produced a 26% improvement on a subsequent evaluation 6 months later (in the absence of drowsiness). The man was maintained on a regimen of 5mg doses, three times per day, which improved dystonic and dyskinetic symptoms. Chen and colleagues report that a 10 mg dose was required for improvement of motor symptoms.

1.6.6 SUMMARY

Zolpidem is a rapidly absorbed sedative hypnotic with selective affinity for $GABA_A$ receptors expressing the α_1-subunit. Acting at the benzodiazepine binding site, zolpidem increases the duration of IPSCs in the GPe, STN, and BG output nuclei. Given the overlap between the pathophysiology of various neurological disorders within the BG, and the selective affinity of zolpidem within this circuit, this drug has repeatedly been reported to have beneficial effects. This is true for PD, as several case studies have noted both antiparkinsonian and antidyskinetic effects. A controlled preclinical study of this phenomenon in a valid translational model, however, is absent in the literature.

1.7 Introducing the Data Chapters

The neuropathophysiology within the BG and related behavioral consequences of DA depletion are well understood based on decades of research into the clinical manifestation of PD motor symptoms. Aside from DA replacement therapies, however, investigations into alternative pharmacological therapeutic interventions absent of debilitating side effects have not been fruitful. Given the overlap between the neurophysiological alterations induced by DA depletion within the BG as well as those observed concurrent with LID, with the expression of the $GABA_A$ receptor α_1 subunit, investigation into the efficacy of zolpidem as a non-dopaminergic alternative is warranted.

Using behavioral neuropharmacology and analytical chemistry, this doctoral dissertation sought to provide a behavioral proof of concept in rats sustaining unilateral nigrostriatal DA depletion, thus providing evidence for this circuit-based hypothesis. First, this dissertation sought to establish whether systemic administration of zolpidem resulted in an anti-parkinsonian effect. In Chapter III, we first identify the threshold for deleterious effects on locomotor behavior and coordination in intact rats using the rotarod balance beam test. Using this as a guide for dosage in 6-OHDA-lesioned animals, we then investigated the efficacy of zolpidem using behavioral assays known to be sensitive to unilateral nigrostriatal lesion.

As discussed above, there is also evidence that zolpidem may possess antidyskinetic properties. However, current behavioral assays in 6-OHDA-lesioned

rodents lack a quantitative basis, and are also loosely related to L-DOPA-induced striatal DA efflux. As a result, we sought to establish a novel quantitative, dopamine-correlated assay of L-DOPA-induced behavioral asymmetry. The experiments in Chapter IV were designed to explore the pharmacokinetics/pharmacodynamics of L-DOPA-induced behaviors using a dose-response design. From that dose-response experiment, we designed our assay: The Behavioral Asymmetry Scoring System (BASS). Next, we tested the ability of this assay to discriminate between degrees of severity of LID-like behaviors as a function of dose. Finally, we investigated the relationship between scores obtained from BASS with L-DOPA-induced DA efflux within the lesioned hemisphere, using *in vivo* microdialysis.

Finally, we sought to investigate the antidyskinesia potential of zolpidem using BASS. In Chapter V, the documented experiments explore whether zolpidem reduces L-DOPA-induced behavioral asymmetry. Next, we sought to determine whether any observed antidyskinetic properties of zolpidem can be attributed to altered striatal DA efflux in the lesioned hemisphere using *in vivo* microdialysis. Last, we assessed whether zolpidem exerted an effect on the impact of striatal DA efflux on LID-like stereotypy and behavioral asymmetry.

CHAPTER II

GENERAL METHODS

2.1 Animals

All procedures were approved by the Rutgers University—Newark Institutional Animal Care and Use Committee (IACUC), in compliance with the Guide for the Care and Use of Laboratory Animals as adopted by the National Institute of Health (NIH).

Adult Male Sprague-Dawley rats (Charles River Laboratories) were used for all experiments described in this dissertation. Animals were housed individually under conditions of constant temperature (21ºC) and humidity (40%) and maintained on a 12/12 light dark cycle (0700 on, 1900 off). Animals were exposed to handling daily for 1 week prior to behavioral training/testing or lesion procedures.

2.2 Stereotaxic surgery

2.2.1 Unilateral 6-hydroxydopamine treatment

In some animals, DA lesion was carried out by local infusion of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA). Animals received desipramine (25 mg/kg in 0.9% NaCl, i.p., Sigma Chemicals) 20 min prior to surgery to prevent destruction of noradrenergic nerve terminals (Waddington, 1980), then were anesthetized with chloral hydrate (400 mg/kg, i.p.) or sodium pentobarbital (40 mg/kg, i.p.). Once anesthesia was confirmed, rats were placed into a stereotaxic frame (David Kopf Instruments) with skull flat between lambda and bregma. The scalp was shaved,

sterilized, and infiltrated with bupivacaine HCl (0.5% w/v; Hospira Inc.) to provide local anesthesia. Body temperature (37ºC) was maintained with a heating pad (Gaymar Industries) throughout the procedure. A small burr hole was made over the site of injection, and a 30g cannula was lowered at a rate of 1 mm/min to the medial forebrain bundle (MFB) at the following coordinates (in mm): AP: -3.2 (bregma), ML: -1.5, and DV: -7.2 from dura (Paxinos and Watson, 1982). After 5 min, 2 µL of 6-OHDA (3 µg/µL in 0.02% w/v ascorbate, freebase wt.) was infused over 4 min. Following infusion, the cannula was kept in place for 5 min in order to prevent redux of solution. The cannula was then retracted at a rate of 1 mm/min, and the incision was closed with wound clips. Post-operative analgesia was provided using meloxicam (2 mg/kg, s.c.; Henry Schein).

For experiments described in Chapter III, animals recovered for 14 days and then were assessed for rotational behavior in response to the DA agonist apomorphine (0.05 mg/kg, s.c.). 4-10 d after apomorphine-induced rotation testing, experiments on these animals began. For experiments described in Chapters IV, animals recovered for 14-28 days before behavioral testing. For those described in Chapter V, animals recovered for 14-35 days prior to microdialysis probe implant procedures and behavioral testing (Sections 2.2.2 & 2.4, respectively).

2.2.2 Microdialysis implant procedure

Animals were anesthetized with ketamine (90 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.), and placed into a stereotaxic frame (David Kopf Instruments, Tujunga, CA,

USA). The scalp was shaved and infiltrated with bupivacaine HCl (0.5% w/v; Hospira Inc.) to provide local anesthesia. Body temperature was maintained with a heating pad (Gaymar Industries) throughout the procedure. Anesthesia was supplemented, as needed, using an additional i.p. injection of ketamine (30 mg/kg). Following establishment of flat skull position, a small burr hole was drilled and the microdialysis probe was implanted into the right dorsal striatum at the following coordinates: AP: +0.5 mm (bregma), ML: -2.5 mm (midline), DV: -6.0 mm (dura; Paxinos & Watson, 1982). The probe was lowered at a rate of 1 mm/min. Additional (2) burr holes were drilled to accommodate small screws to further anchor the probe. Once in place, the screws and probe were cemented into place with dental cement. Post-operative analgesia was provided using meloxicam (2 mg/kg, s.c.; Henry Schein) once following completion of the procedure.

2.3 Behavioral Testing

2.3.1 Rotarod Balance Beam Test

Animals were tested for overall motor coordination using a rotarod apparatus (Economex, Columbia Instruments), following procedures outlined by Carter *et al* (2001). Briefly, animals were allowed to habituate to the testing room for ≥ 60 min prior to training and testing sessions. Training consisted of 4 trials at a fixed speed of 10 rpm, repeated over 3 consecutive days. Latency to fall was recorded and a 60 s trial termination cutoff was used. The inter-trial interval was 5 min. 5 days after the initiation of training (48 h after last training session), animals were tested at a fixed speed of 20 rpm, which served as the pre-lesion/undrugged measure. Subjects were given 2

trials, and both trials were averaged together for each animal in order to perform statistical analysis.

2.3.1.1 *Rotarod performance in intact rats*

In order to determine non-sedative doses of zolpidem for further examination of effects on motor deficits in DA-lesioned animals, a group of intact rats (n = 14) received an i.p. injection of zolpidem (0.5, 1, 2.5, 5, 10 mg/kg; i.p.) or vehicle 15 minutes before being placed onto the rotarod apparatus. This delay was chosen to ensure testing was conducted coincident with the reported t_{max} for zolpidem in the rat (Durand *et al*, 1992). In order to allow for drug washout, at least 48 h separated testing sessions.

2.3.1.2 *Rotarod performance in unilaterally dopamine-depleted rats*

Following pre-lesion rotarod training and testing, unilateral 6-OHDA lesion procedure was conducted in some animals (n = 26). Approximately 1 mo after lesion surgery, animals with confirmed lesions—based on the prior apomorphine challenge data—were tested for post-lesion performance in the rotarod task. Approximately 1 week later, these animals also were tested 15 min after receiving an i.p. injection of zolpidem (0.1, 0.25, 0.5 mg/kg) or vehicle. All animals received each dose following the Latin Square method, with at least 48 hours in between testing sessions to allow for washout of the drug and its metabolites. Animals that remained on the apparatus > 45 s in the undrugged post-lesion condition were dropped from analysis. Similarly, animals that were shown to have < 80% DA depletion in the lesioned hemisphere (relative to unlesioned hemisphere) were dropped from analysis.

2.3.2 Cylinder/Paw preference test

Forelimb use symmetry was assessed using the cylinder/paw preference test, using a modified version of a previously described paradigm (Schallert and Tillerson, 2000). Briefly, animals (n = 13) were placed individually in a plastic cylinder (diameter = 30 cm) lined with fresh bedding (BETA chips) in a dimly lit room for 25 min. No habituation to the cylinder was allowed. Once the animal was in the cylinder, the experimenter left the room and exploratory activity was video recorded from above. 15 min of each video was analyzed, beginning at the onset of first paw contact with the side of the cylinder. Number of weight-bearing contacts made by each forepaw was recorded, and a ratio of contacts made by the forepaw contralateral to the lesion to total contacts was calculated:

$$\frac{contralateral}{ipsilateral + contralateral}$$

Animals were tested pre-lesion (32 d prior to surgery), post-lesion (undrugged; 20 d post-surgery), and 15 minutes following i.p. injection of zolpidem (0.1 mg/kg) or vehicle (51-58 d post-surgery). Testing was temporally spread as such in an effort to reduce habituation to the cylinder, thus ensuring the testing arena remained novel. Animals that did not execute ≥ 10 total forepaw contacts in each experimental condition were excluded from analysis. Similarly, animals that were shown to have < 80% DA depletion in the lesioned hemisphere (relative to unlesioned hemisphere) were dropped from analysis.

2.3.3 Levodopa-induced behavioral asymmetry

2.3.3.1 *Levodopa dose-response*

Following lesion surgery (19-24 days), animals (n = 41) were assessed for rotational behavior and abnormal involuntary movements in response to an acute dose of L-DOPA methyl ester (Sigma Chemicals) or saline in a dimly lit room. Animals habituated to the testing room for 60 min, were pretreated with the peripheral aromatic amino acid decarboxylase (AADC) inhibitor Ro4-4602 (15 mg/kg, i.p.; freebase wt.; Sigma Chemicals), then placed directly into a plastic cylinder (diameter = 30 cm) containing fresh cage bedding, and allowed to habituate for 30 minutes. Animals were then removed from the cylinder, given an i.p. injection of L-DOPA (2.5, 5, 10, 25, 50 mg/kg, i.p.; freebase wt.) or 0.9% NaCl, and placed back into the cylinder. Behavior was video recorded from above, beginning immediately after Ro4-4602 pretreatment and ending 3 h after L-DOPA administration.

The recorded behavior was analyzed in 5 min blocks at 10 min intervals (i.e., 0-5 min, 10-15 min, etc. post-DOPA). Net number of contraversive rotations and amount of time spent displaying unilateral stereotypy was recorded. Only full 360° rotations were counted. Unilateral stereotypies included axial twisting of the trunk and myoclonic movements of the forepaw contralateral to the lesioned hemisphere. The relationship between rotational behavior and time spent displaying unilateral stereotypy was assessed using Spearman's correlation ($\alpha = 0.05$).

2.3.3.2 *Effects of zolpidem on levodopa-induced behavioral asymmetry*

The effects of zolpidem on L-DOPA-induced behavioral asymmetry was assessed in unilaterally 6-OHDA-lesioned rats. Animals habituated to the dimly-lit testing room for 60 min, were pretreated with the peripheral aromatic amino acid decarboxylase (AADC) inhibitor Ro4-4602 (15 mg/kg, i.p.; freebase wt.; Sigma Chemicals), then placed directly into a plastic cylinder (diameter = 30 cm) containing fresh cage bedding, and allowed to habituate for 30 minutes. Animals were then removed from the cylinder, given an i.p. injection of L-DOPA (10 mg/kg, i.p.; freebase wt.) or 0.9% NaCl, and placed back into the cylinder. Animals assigned to zolpidem or vehicle groups were once again removed from the cylinder 40 min post-DOPA, and given an i.p. injection of zolpidem (0.1, 0.5 mg/kg) or vehicle. Behavior was video recorded from above, beginning immediately after Ro4-4602 pretreatment and ending 3 h after L-DOPA administration.

The recorded behavior was analyzed in 5 min blocks at 10 min intervals (i.e., 0-5 min, 10-15 min, etc. post-DOPA). Net number of contraversive rotations and amount of time spent displaying unilateral stereotypy was recorded. Only full 360° rotations were counted. Unilateral stereotypies included axial twisting of the trunk and myoclonic movements of the forepaw contralateral to the lesioned hemisphere.

2.3.3.3 *Behavioral asymmetry scoring system (BASS)*

In order to gain a more quantitative insight into L-DOPA-induced behavioral asymmetry, we devised a novel assessment method. As described above, behavior was scored by counting the number of net contraversive rotations and the time spent

displaying unilateral stereotypy within a 5 min time block. The obtained values were plugged into the following formula, generating an Asymmetry Score (A-Score) between 0 and 1:

$$\text{A-Score} = \frac{\text{rotations}}{\text{total time (s)} - \text{time dys (s)}} + \frac{\text{time dys (s)}}{\text{total time (s)}}$$

It should be noted that "rotations" represents the number of net contraversive rotations such that full 360° ipsiversive rotations were subtracted from the number of contraversive rotations. "Time dys" refers to the amount of time spent by the animal displaying unilateral stereotypy, which were considered dyskinesia-like behaviors. All times are entered into the formula in s. Since each analyzed time block lasted 5 min, the "total time" variable in this formula was 300 s.

2.4 *In vivo* microdialysis

2.4.1 Microdialysis probe construction and calibration

Microdialysis probes were designed to be vertical and concentric, as previously reported by our laboratory (Cobb and Abercrombie, 2002). The probe inlet consisted of PE-10 tubing (Clay Adams; Parsippany, NJ, USA), with a length of fused silica capillary tubing (I.D. 75 μm, O.D. 150 μm; Polymicro Technologies, Phoenix, AZ, USA) serving as the outlet. A semi-permeable microdialysis membrane (MW cut-off = 13 kDa; O.D. 216 μm; Spectrum Laboratories, Rancho Dominguez, CA, USA) was placed over the end of the silica tubing, glued to the PE-10 tubing, and coated with a thin layer of epoxy. The active exchange area was limited to a length of 2 mm at the end of the probe. Probes were

continuously perfused with artificial cerebrospinal fluid (aCSF; NaCl 147 m*M*, KCl 2.5 m*M*, CaCl2 1.3 m*M*, MgCl2 0.9 m*M*, pH = 7.4) using a microliter infusion pump (Harvard Apparatus, Holliston, MA, USA) and a single-channel fluid swivel (Instech Laboratories, Plymouth Meeting, PA, USA) at a rate of 1.5 μL/min. Prior to implantation procedures, each probe was calibrated *in vitro* to determine the relative recovery rate. Only probes showing recovery rates between 10-15% were used in experiments. Prior to implantation procedures, the probe inlet and outlet tubing were fed through a metal spring and cemented into place using dental cement.

2.4.2 *In vivo* microdialysis procedure

Experiments commenced following an 18-24 h recovery period. Animals were assessed to ensure that they had resumed normal activity such as eating, drinking, and grooming; and that there was no bleeding from probe implant site. Prior to initiation of pharmacological manipulations, 15 min baseline dialysate samples were collected over the course of 1 h to ensure a stable baseline. Animals that produced baseline samples containing detectable levels of DA were not considered to be lesioned, and were not tested further.

2.4.3 Pharmacological/Behavioral procedures – *in vivo* microdialysis

Following collection of baseline dialysate samples, animals (n = 13) were carefully removed from the testing cylinder and pretreated i.p. with Ro4-4602 (15 mg/kg; freebase wt.; Sigma Chemicals), placed back into the enclosure, and allowed to habituate

for 30 minutes. Animals were again removed from the cylinder, given an i.p. injection of L-DOPA (10 mg/kg; freebase wt.), and placed back into the cylinder. A subset of animals (n = 6) also received an i.p. injection of zolpidem (0.5 mg/kg), concomitant with L-DOPA.

Dialysate samples were collected every 15 min. Behavior was video recorded from above, beginning immediately after Ro4-4602 pretreatment and ending 3 h after L-DOPA administration. The recorded behavior was analyzed in 5 min blocks at 15 min intervals, coinciding with the central 5 min of dialysate sample collection (i.e., 5-10 min, 20-25 min, etc. post-DOPA; see below). Video recordings of behavior were assessed for contralateral rotations, time spent displaying dyskinesia-like behaviors, as described above.

2.5 High performance liquid chromatography (HPLC)

2.5.1 Analysis of dialysate samples

Dialysate samples were collected over 15 min, and 20 µL of the dialysate was immediately assayed for DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxyindole-3-acetic acid (5-HIAA) using high performance liquid chromatography with electrochemical detection (HPLC-ED), according to previously described methods (Callahan & Abercrombie, 2011). Briefly, the HPLC-ED system contained a Rheodyne injector (Rheodyne, Cotati, CA, USA), a Velosep RP-18 column (100 x 3.2 mm; PerkinElmer, Waltham, MA, USA), and a Shimadzu LC-10AD VP solvent delivery pump (Shimadzu Scientific Intruments Inc., Columbia, MD, USA). Mobile phase (pH =

4.20) was delivered at a flow rate of 0.7 mL/min, which was composed of 0.1 *M* sodium acetate buffer, 0.1 m*M* EDTA, 1.2 m*M* sodium octyl sulfate, and 8% methanol (v/v). A Coulochem II electrochemical detector (ESA Inc., Chelmsford, MA, USA) with a flow cell electrode delivering an applied potential of +260 mV was used, the output of which was coupled to a PowerChrom data acquisition system (PowerChrom, Denistone East, NSW, Australia). Elements of interest (DA, DOPAC, 5-HIAA) were identified by retention time, and quantified based on the height of the oxidation peak relative to the peak of a 10^{-8} *M* standard prepared in 0.1 N perchloric acid. Standard was injected every hour and prepared fresh prior to injection.

2.5.2 Analysis of tissue dopamine content

After completion of the experiments, neurochemical analysis of DA loss was performed in all 6-OHDA-treated animals from which data were obtained. Rats were given chloral hydrate (400 mg/kg, i.p.), rapidly decapitated, and the striatum of each hemisphere was dissected and frozen on dry ice. The dissected striata were stored at -80° C until homogenization and centrifugation. Upon thawing, each striatum was weighed then homogenized in 0.1 M perchloric acid and 100 µM EDTA (20µl/mg wet tissue). Homogenates were centrifuged at 14,000 rpm for 20 min. Samples of resulting supernatant (sample vol. = 20 µl) were assayed for DA content using high performance liquid chromatography coupled with electrochemical detection (HPLC-ED). DA was separated on a Velosep RP-18 column (100×3.2 mm; Applied Biosystems, Inc., Foster City, CA, USA) and quantified by measuring oxidative current at a glassy carbon wall-jet electrode (model ECD-700; EICOM Corporation) set at +0.350 V *versus* an Ag/AgCl

electrode and coupled to a potentiostat (ECD-700; EICOM Corporation) and a PowerChrom data acquisition system (PowerChrom, Denistone East, NSW, Australia). The mobile phase consisted of a 0.1 M sodium acetate buffer, pH 4.2, 0.1 mM EDTA, 1.2 mM sodium octyl sulfate, 8% (v/v) methanol. A solvent delivery pump (model EP-700; EICOM Corporation) delivered the mobile phase at 0.5 ml/min. Retention time was used to identify DA, which was quantified on the basis of the peak height of oxidative current. The detection limit of the assay was 0.7 pg DA per sample. The extent of striatal DA depletion produced by 6-OHDA lesion was determined as the percent decrease in the concentration of DA in striatal tissue in the lesioned *versus* intact hemisphere.

2.6 Drugs

Chloral hydrate (trichloroacetaldehyde hydrate), sodium pentobarbital, desipramine hydrochloride, xylazine, benserazide (Ro4-4602), and L-DOPA methyl ester were dissolved in sterile 0.9% NaCl and made fresh before use. Chloral hydrate, sodium pentobarbital, and desipramine were mixed according to pre-defined concentrations (8%, , and 12.5 mg/ml, respectively), and doses were administered according to weight (mg/kg). Ro4-4602 and L-DOPA methyl ester were dissolved according to the dose administered, and were normalized so that the amount of solution injected was proportional to weight (1 mL/kg). Ketamine was purchased as 100 mg/mL stock, and administered according to weight (mg/kg). Zolpidem was first dissolved in 0.1 *M* glacial acetic acid, and brought up to volume with sterile 0.9% NaCl according to the doses being administered (final concentration of vehicle = 1% acetic acid). Zolpidem and vehicle were pH adjusted with NaOH (final pH = 5.0). Drug concentrations for each of

the reported doses were normalized so that the amount of solution injected was proportional to weight of the animal (1 mL/kg). Vehicle for control experiments contained 1% acetic acid dissolved in 0.9% NaCl, and was injected at a volume proportional to weight of the animal (1 mL/kg).

2.7 Data Analysis

2.7.1 General considerations

With the exception of rotarod, all behavioral assays were video recorded and analyzed offline for target behaviors. All data analysis and statistical testing was conducted using either MATLAB (2016a) or GraphPad Prism 7 software. All figures were made using Prism 7. Specific analyses used are described below. Unless otherwise stated, statistical testing was considered statistically significant when $\alpha < 0.05$.

2.7.2 Analyses for experiments in Chapter III

For Chapter III, behavioral testing is described in Sections 2.3.1 and 2.3.2. Differences between doses/conditions for the zolpidem dose-response experiment (2.3.1.1) were investigated using the Wilcoxon Rank Sum test. Error correction methods were not used in this specific analysis, as the purpose of this experiment was to find the lowest possible dose that may cause any significant motor impairment. Data from experiments described in Section 2.3.1.2, statistical analysis was performed within subjects using Friedman's nonparametric ANOVA for Repeated Measures, a significant

main effect was further investigated between treatment conditions using the Wilcoxon Sign Rank test using the Bonferroni correction method for family-wise error rate for the following comparisons: intact vs. lesion, lesion vs. vehicle/0.1/0.25/0.5 Zol, vehicle vs. 0.1/0.25/0.5 Zol (8 comparisons, $\alpha_{adj} = 0.00625$). For the cylinder/paw preference test (Section 2.3.2), differences between treatment conditions were explored within subjects using One-Way Repeated Measures ANOVA with a multiple comparison *post hoc* investigation using the Bonferroni correction for all possible interactions (6 comparisons, $\alpha_{adj} = 0.0083$).

2.7.3 Analyses for experiments in Chapter IV

In Chapter IV, L-DOPA dose-response data was analyzed several ways. First, each dose was analyzed independently for time bins containing statistically significant increases in rotational behavior and time spent displaying dyskinesia-like behavior, using a Friedman's Test. A significant main effect was further analyzed with Dunn's multiple comparisons tests comparing each post-DOPA time point to a baseline time bin (10-15 min post-DOPA; 17 comparisons, $\alpha_{adj} = 0.003$). The relationship between rotational behavior and time spent displaying unilateral stereotypy was assessed using Spearman's correlation ($\alpha = 0.05$), both by dose and with all doses pooled. To avoid bias in correlational analyses, matched pairs occurring prior to the time of onset of rotational behavior (defined below) as well as those following behavioral offset were excluded. Matched pairs occurring after the behavioral maximum containing 0 net contraversive rotations AND < 30 s displaying dyskinesia-like stereotypy were classified as behavioral

offset, and thus excluded. However, any matched pairs following the behavioral maximum that violated the offset exclusion criteria were included.

Behavioral footage was also reviewed for latency to onset of both component behaviors (rotations/dyskinesia). For rotations, this was the timestamp (s) of the first completed contraversive rotation. For dyskinesia, this was the timestamp (s) of the onset of the first bout of unilateral stereotypy lasting ≥ 3 sec. Within each dose, latency to onset for rotations was compared to that of dyskinesia using Wilcoxon Sign Rank tests ($\alpha = 0.05$). Each component behavior was also assessed for linearity across doses ($\alpha = 0.05$)

To test the sensitivity of rotations, time spent displaying dyskinesia, or A-Scores (Section 2.3.3.3) to dosage, each variable was analyzed using a Two-Way ANOVA. Significant main effect of dose was further deconstructed using Bonferroni-corrected multiple comparisons at time points 20, 40, 60, 80, 100, 120, 140, and 160 min post-DOPA injection. All dose interactions were considered and included in the correction (15 comparisons per time point; $\alpha_{adj} = 0.0033$). Mean net contraversive rotations, time spent displaying dyskinesia-like stereotypy, and A-Scores was also compared across doses using One-Way ANOVAs followed by Bonferroni-corrected multiple comparisons ($\alpha_{adj} = 0.0033$).

Dose-response data from time points described above, as well as mean behavioral measures, were fit using a general four-parameter Hill equation according to the "sigmoid E_{max} model" (Holford and Sheiner, 1981a, b; reviewed in Goutelle *et al*, 2008):

$$E = E_0 + \frac{E_{max} D^{\alpha}}{{ED_{50}}^{\alpha} + D^{\alpha}}$$

In this equation: E is the effect of drug, E_0 is the effect at a dose of 0 (data from saline condition, in this case), E_{max} is the maximal effect, ED_{50} is the dose at which 50% of the maximal effect is obtained, D is the dose, and α is the Hill coefficient.

Striatal DA, DOPAC, and 5-HIAA data obtained from *in vivo* microdialysis experiments (Sections 2.4 and 2.5.1) were first analyzed for statistically significant increases following L-DOPA using a Geisser-Greenhouse-corrected One-Way Repeated Measures ANOVAs. A significant main effect was further investigated using Bonferroni-corrected multiple comparisons, comparing each post-DOPA time bin to the mean of the baseline dialysate samples (14 comparisons, $\alpha_{adj} = 0.0036$). Striatal DA and DOPAC samples were then time-matched to corresponding behavioral observations (rotations, dyskinesia, A-Score), the relationship between matched pairs was tested using Pearson's correlation ($\alpha = 0.05$).

2.7.4 Analyses for experiments in Chapter V

In Chapter V, the behavioral data was analyzed in a similar manner to that described in Section 2.7.2. First, rotational and dyskinesia-like behavior data was analyzed independently for effects within condition as a function of time using a Friedman's Test for each condition. A significant main effect was further analyzed with Dunn's multiple comparisons tests comparing each post-DOPA time point to a baseline time bin (10-15 min post-DOPA; 17 comparisons, $\alpha_{adj} = 0.003$).

To test the effects of zolpidem/vehicle, a Two-Way Repeated Measures ANOVA was used comparing rotational, dyskinesia, and A-Score data obtained from each

condition 40-90 min post-DOPA. A significant main effect of drug was further using corrected (Holm-Sidak) multiple comparisons at 40, 50, 60, 70, 80, and 90 min post-DOPA (5 comparisons: DOPA vs DOPA/VEH, DOPA vs DOPA/0.1 ZOL, DOPA vs. DOPA/0.5 ZOL, DOPA/VEH vs. DOPA/0.1 ZOL, DOPA/VEH vs. DOPA/0.5 ZOL; $\alpha = 0.05$). Mean rotations, time spent displaying dyskinesia-like stereotypy, and A-Scores over the course of the analysis window (40-90 min post-DOPA) were also compared across conditions using a One-Way ANOVA. A significant main effect was further deconstructed using Bonferroni-corrected multiple comparisons ($\alpha_{adj} = 0.01$).

Striatal DA, DOPAC, and 5-HIAA data obtained from *in vivo* microdialysis experiments were first analyzed as described above using Friedmans's Tests followed by Dunn's multiple comparisons. Neurotransmitter and metabolite content data were compared between DOPA and DOPA/ZOL conditions using a Two-Way Repeated Measures ANOVA, with a significant main effect of condition further investigated using Bonferroni-corrected multiple comparisons (18 comparisons; $\alpha_{adj} = 0.0028$). As described above, microdialysis data was time-matched to behavioral observations and the relationship was explored using Pearson's correlation ($\alpha = 0.05$).

Behavioral data from *in vivo* microdialysis experiments was compared between DOPA and DOPA/ZOL conditions using a Two-Way Repeated Measures ANOVA, for which a statistically significant main effect of treatment was investigated using Bonferroni-corrected multiple comparisons (12 comparisons; $\alpha_{adj} = 0.0042$). Mean contraversive rotations, time spent displaying dyskinesia-like behavior, and A-Scores were also compared across conditions using Mann-Whitney U tests ($\alpha = 0.05$).

Finally, the relationships between L-DOPA-induced striatal DA efflux and A-Scores was compared between the two treatment conditions (DOPA vs. DOPA/ZOL) using an Analysis of Covariance (ANCOVA). Slopes as well as elevations/y-intercepts were compared ($\alpha = 0.05$ for each comparison).

CHAPTER III

ZOLPIDEM AMELIORATES MOTOR IMPAIRMENTS IN THE UNILATERALLY 6-HYDROXYDOPAMINE-LESIONED RAT

3.1 Rationale

Inhibitory neurotransmission in the basal ganglia (BG) is primarily mediated by ionotropic $GABA_A$ receptors, which can be distinguished pharmacologically according to α-subunit expression. Within the BG, the α_2-subunit is exclusively expressed on spiny projection neurons in the striatum. The α_1-subunit is expressed postsynaptically within the external globus pallidus (GPe), subthalamic nucleus (STN), internal globus pallidus (GPi), and substantia nigra pars reticulata (SNr, Arbilla *et al*, 1986; Waldovogel *et al*, 1999; Boyes and Bolam, 2007; **Fig 1.1**).

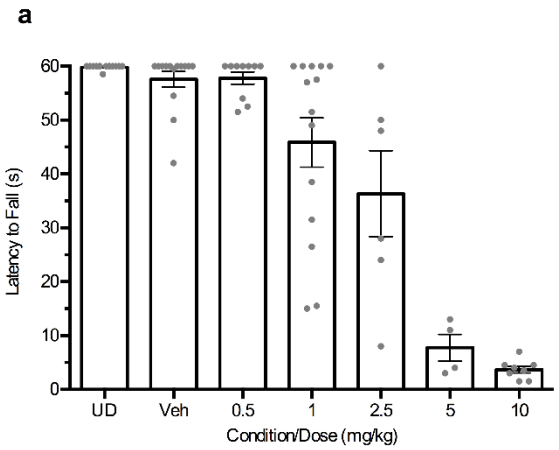
Zolpidem (trade name Ambien®) is an imidazopyridine that binds to $GABA_A$ receptors at the benzodiazepine site, acting as a positive allosteric modulator with selective affinity for receptors expressing the α_1-subunit. *Ex vivo* studies have shown that application of zolpidem results in an increase in decay time constant of Cl^- channel opening and potentiation of iPSCs in zolpidem-sensitive nuclei within the BG (Chen *et al*, 2004; Chen *et al*, 2007; Zhang *et al*, 2008). Similarly, zolpidem potentiates GABA-induced decreases in the firing rate of zolpidem-sensitive cells (Duncan *et al*, 1995; Mereu *et al*, 1990). Furthermore, zolpidem-sensitive $GABA_A$ receptors have been hypothesized to mediate the ataxic and myorelaxant properties of benzodiazepines, due to high expression levels of the α_1-subunit within extrastriatal BG nuclei (Milic *et al*, 2012).

Interestingly, the BG nuclei expressing the α_1-subunit have also been shown to exhibit oscillatory entrainment to cortical activity in a DA-depleted state (Magill *et al*, 2001; Mallet *et al*, 2008; Avila *et al*, 2010; Deffains *et al*, 2016). This oscillatory entrainment, and associated alterations in firing pattern, have been observed in PD models (Sanderson *et al*, 1986; MacLeod *et al*, 1990; Soares *et al*, 2004; Wichmann and Soares, 2006; Walters *et al*, 2007; Zold *et al*, 2007; Lobb and Jaeger, 2015; reviewed in Lobb, 2014), and are associated with severity of motor symptoms in PD patients (Sharrott *et al*, 2014). Combined with the observed efficacy of the GABA analog progabide in the treatment of PD motor symptoms, available evidence indicates that a compound with selectivity for extrastriatal $GABA_A$ receptors—such as zolpidem—could yield promising results (Ziegler *et al*, 1987). Investigations of this concept utilizing translational models, however, have not been reported.

3.2 Overview of Methods

We examined whether zolpidem-sensitive $GABA_A$ receptors constitute a potential therapeutic target in the treatment of PD motor symptoms using a translational model. The presented experiments tested whether zolpidem ameliorates motor deficits in unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rats, utilizing assays sensitive to DA depletion (Iancu *et al*, 2005). First, we performed a dose-response investigation in intact rats to determine the threshold dose at which zolpidem impairs motor coordination using rotarod. Second, we investigated whether an acute subthreshold dose of zolpidem, as determined in the dose-response experiment, improved motor performance and

forelimb use symmetry in unilaterally 6-OHDA-lesioned rats using both rotarod and the paw preference/cylinder test.



b

	UD	Veh	0.5	1	2.5	5	10
UD		N.S.	N.S.	**	**	***	****
Veh	N.S.		N.S.	*	**	**	****
0.5	N.S.	N.S.		N.S.	N.S.	**	***
1	**	*	N.S.		N.S.	**	***
2.5	**	**	N.S.	N.S.		*	**
5	***	**	**	**	*		N.S.
10	****	****	***	***	**	N.S.	

FIGURE 3.1: **Zolpidem induces a dose-dependent impairment on rotarod in intact rats.** Animals (n = 14) were tested 15 min following an i.p. injection of zolpidem at the indicated dose. Following analysis using Wilcoxon Rank Sum tests, it was found that all doses > 0.5 mg/kg induced a significant impairment compared to the undrugged (UD) condition. **(a)** Data are expressed as mean ± SEM, dots represent each animal. **(b)** Matrix indicating significant interactions between conditions. Number of symbols indicates p-values as follows: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), **** ($p < 0.0001$).

3.3 Results

3.3.1 Zolpidem induces a dose-dependent impairment on rotarod in intact rats

In order to empirically ascertain a dose of zolpidem that would not interfere with motor coordination, a dose-response experiment was conducted assessing rotarod performance in intact rats (n = 14). Doses of 0.5, 1.0, 2.5, 5.0, and 10.0 mg/kg zolpidem (i.p.) were tested, as well as vehicle. Mean (± SEM) latency to fall (in s) and number of animals given each dose were as follows (**Fig. 3.1a**): 59.21 ± 0.96 (undrugged, n = 14), 57.57 ± 2.09 (vehicle, n = 14), 57.8 ± 1.72 (0.5 mg/kg, n = 10), 45.86 ± 4.68 (1 mg/kg, n = 14), 36.33 ± 9.22 (2.5 mg/kg, n = 6), 7.75 ± 4.17 (5 mg/kg, n = 4), 3.69 ± 0.96 (10 mg/kg, n = 8).

Dose interactions pertinent to subsequent experiments were as follows (**Fig 3.1b**): Doses ≥ 1.0 mg/kg produced a statistically significant impairment compared to the undrugged condition (1.0 mg/kg: $p = 0.0045$; 2.5 mg/kg: $p = 0.0012$; 5.0 mg/kg: $p = 5.05 \times 10^{-4}$; 10.0 mg/kg: $p = 3.48 \times 10^{-5}$). A dose of 0.5 mg/kg did not produce a significant impairment compared to the undrugged ($p = 0.3534$), nor vehicle conditions ($p = 0.9709$). Doses ≥ 1.0 mg/kg produced a significant impairment compared to vehicle (1.0 mg/kg: $p = 0.0298$; 2.5 mg/kg: $p = 0.0067$; 5 mg/kg: $p = 0.0032$, 10 mg/kg: $p = 6.88 \times 10^{-5}$). As a result, the maximal dose utilized in subsequent experiments was 0.5 mg/kg.

		Hemisphere		
Group/Test	*n*	*Intact (ng/g)*	*Lesion (ng/g)*	*Percent DA Loss (%)*
Rotarod	26			
included	10	6576.22 ± 1192.65	74.45 ± 8.92	96.72 ± 1.84
excluded (low deficit)	13	9000.01± 1385.06	126.70 ± 36.93	98.17 ± 0.43
excluded (low lesion)	3	11055.65 ± 1339.75	9511.64 ± 1390.04	14.38 ± 5.38
Cylinder	13			
included	10	6456.26 ± 1320.85	95.04 ± 14.78	96.99 ± 0.93
excluded	3	7605.5 ± 5058.67	258.79 ± 137.53	95.65 ± 0.78

TABLE 3.1: Dopamine depletion as a result of unilateral 6-OHDA infusion**.**

3.3.2 Behavioral and neurochemical effects of unilateral 6-OHDA lesion

The effect of unilateral 6-OHDA lesion was quantified by comparing pre-lesion (intact) to post-lesion performance on rotarod and cylinder test using the Wilcoxon Sign Rank test. A significant effect of lesion was found in both rotarod and cylinder tests. 6-OHDA lesion significantly impaired animals (n = 10) that met inclusion criteria in the rotarod experiment (p_{adj} = 0.0156; **Fig 3.2b**), and induced a significant unilateral bias in forelimb use in the cylinder test (n = 10, $p_{adj} = 8.78 \times 10^{-7}$; **Fig 3.3b**).

The extent of 6-OHDA-induced lesion was assessed via HPLC-ED (see Methods). In animals that met the inclusion criteria for the rotarod experiment (see Methods), mean (± SEM) tissue DA depletion was 96.72 ± 1.84%. In animals tested in the cylinder/paw preference experiment, mean (± SEM) DA depletion was 96.99 ± 0.93% (**Table 1**).

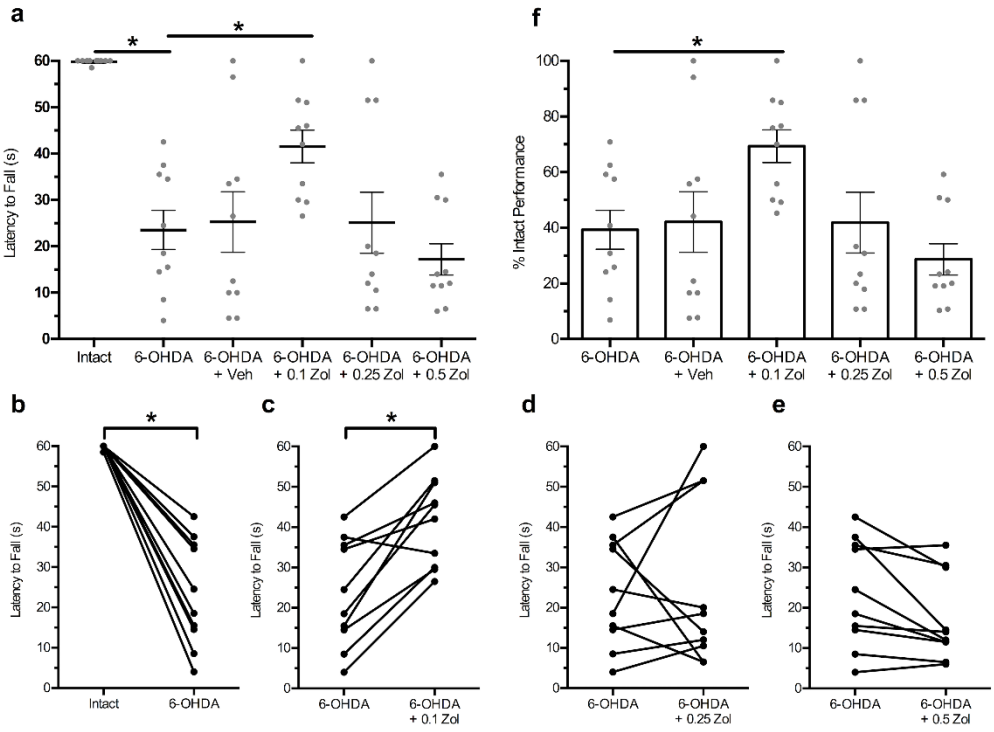


FIGURE 3.2: **Zolpidem improves rotarod performance in unilaterally 6-OHDA-lesioned rats.** Animals (n = 10) were tested 15 min following i.p. injection of zolpidem or vehicle. Following analysis using Friedman's nonparametric ANOVA for Repeated Measures and Wilcoxon Sign Rank tests, it was found that 0.1 mg/kg zolpidem significantly improved rotarod performance compared to the lesion condition. **(a)** Performance of each animal for each experimental condition. Data are expressed as mean ± SEM, dots represent each animal. **(b-e)** Comparisons of experimental conditions: **(b)** intact (pre-lesion) *vs*. 6-OHDA (post-lesion), **(c)** 6-OHDA *vs*. 6-OHDA + 0.1 Zol, **(d)** 6-OHDA *vs*. 6-OHDA + 0.25 Zol, **(e)** 6-OHDA *vs*. 6-OHDA + 0.5 Zol. **(f)** Rotarod performance of each animal (in each condition) was compared to its performance in the intact condition, and a % intact performance value was calculated. It was found that 0.1 Zol significantly improved this measure

compared to the lesion condition. Data are expressed as mean ± SEM, dots represent each animal. Top lines indicate significant interactions. * denotes significance ($p < 0.05$).

3.3.3 Zolpidem improves rotarod performance in unilaterally 6-OHDA-lesioned rats

The efficacy of zolpidem in the treatment of PD motor symptoms was assessed using the rotarod balance beam test. In order to ensure that the animals in this study showed a significant impairment, the criteria for inclusion in this experiment were as follows: (1) post-lesion rotarod fall latency ≤ 45 sec, (2) verification of DA depletion ≥ 80% compared to the intact hemisphere. Of the animals tested (n = 26), only 10 met both of these criteria. Among the animals that were excluded, the predominant reason for exclusion from this study was a lack of adequate impairment on rotarod performance despite > 80% DA depletion in the lesioned hemisphere (n = 13, mean latency to fall = 56.62 sec, mean DA depletion = 98.15%). In a few cases, animals that showed sufficient impairment on rotarod did not meet the inclusion criteria based on DA depletion (n = 3, mean latency to fall = 28.67, mean DA depletion = 14.37%).

Animals that met both criteria (n = 10) were placed on the rotarod apparatus 15 min following i.p. injection of zolpidem (0.1, 0.25, 0.5 mg/kg) or vehicle. Testing was conducted at a constant velocity of 20 rpm, and a 60 s cutoff was used for each trial. Mean (± SEM) latency to fall (in sec.) for each condition was as follows: 59.85 ± 0.15 (intact), 23.55 ± 4.21 (lesion), 25.25 ± 6.52 (vehicle), 41.55 ± 3.55 (0.1 mg/kg), 25.10 ± 6.57 (0.25 mg/kg), 17.20 ± 3.37 (0.5 mg/kg).

A statistical analysis using Friedman's nonparametric ANOVA for Repeated Measures yielded a significant main effect ($\chi^2 = 22.37$, $p = 3.08 \times 10^{-5}$). Post-hoc investigations of effects between treatments were conducted using Wilcoxon Sign Rank tests with a family-wise error rate adjusted using the Bonferroni method for the following comparisons: intact vs. lesion, lesion vs. vehicle/0.1/0.25/0.5 Zol, vehicle vs. 0.1/0.25/0.5 Zol (8 comparisons, $\alpha_{adj} = 0.00625$). It was found that zolpidem improved rotarod performance in a dose-dependent manner (**Fig 3.2a**). An i.p. injection of 0.1 mg/kg zolpidem significantly improved rotarod performance when compared to undrugged post-lesion performance ($p_{adj} = 0.0312$, **Fig 3.2b**). Some animals (6/10) improved slightly following a dose of 0.25 mg/kg, but this difference was not significant when compared to post-lesion performance ($p_{adj} = 6.9375$, **Fig 3.2d**). An i.p. injection of 0.5 mg/kg zolpidem did not significantly alter rotarod performance, but in most cases (8/10) resulted in shorter latency to fall than the post-lesion condition ($p_{adj} = 0.1250$, **Fig 3.2e**). Rotarod performance following 0.1 mg/kg did not differ significantly from vehicle ($p_{adj} = 0.3125$). Performance following 0.25 mg/kg also did not significantly differ from vehicle ($p_{adj} = 4.8281$), nor did 0.5 mg/kg ($p_{adj} = 3.8125$). Post-lesion (undrugged) performance did not significantly differ from performance following vehicle ($p_{adj} = 7.8438$). These data suggest that 0.1 mg/kg zolpidem improves motor performance in unilaterally 6-OHDA-lesioned rats as measured by rotarod.

To directly compare performance in each experimental condition to the intact/undrugged state, a "% intact performance" value was calculated by dividing the latency to fall (in s) of each animal (in each experimental condition) by its performance in the intact condition. Mean (± SEM) percentage values obtained for each condition

were as follows: 39.27 ± 7.01% (lesion), 42.10 ± 10.86% (vehicle), 69.36 ± 5.86% (0.1 mg/kg), 41.88 ± 10.93% (0.25 mg/kg), 28.69 ± 5.61% (0.5 mg/kg). An analysis of this data using Friedman's nonparametric ANOVA for Repeated Measures yielded a significant main effect ($\chi^2 = 12.2$, $p = 0.0159$; **Fig 3.2f**). Post-hoc investigations of effects between experimental conditions were conducted using Wilcoxon Sign Rank tests with a family-wise error rate adjusted using the Bonferroni method for the following comparisons: lesion vs. vehicle/0.1/0.25/0.5 Zol (4 comparisons, $\alpha_{adj} = 0.0125$). Interestingly, we found that an injection of 0.1 mg/kg zolpidem resulted in a significantly higher % intact performance value when compared to the lesion condition ($p_{adj} = 0.0156$). All other comparisons were nonsignificant. Of note, however, was a nonsignficant trend indicating a reduction in % intact performance following a dose of 0.5 mg/kg compared to the lesion condition ($p_{adj} = 0.0781$). This analysis indicates that a dose of 0.1 mg/kg significantly improved rotarod performance, and that a dose of 0.5 mg/kg potentially further exacerbated motor impairment in unilaterally 6-OHDA-lesioned rats.

The data presented here indicate that zolpidem improved motor deficits in a translational model of PD. However, there are some potential caveats in the rotarod component of this study. First, there was no significant difference between vehicle and 0.1 mg/kg zolpidem conditions. This can potentially be explained by practice effects, as an analysis of the relationship between the number of trials prior to the vehicle condition and rotarod performance indicated a positive relationship ($r = 0.2805$; data not shown). A similar analysis of the 0.1 mg/kg zolpidem condition showed the opposite ($r = -0.5535$; data not shown), indicating that prior trials did not affect performance in this condition. Second, nearly half of the animals tested using rotarod did not meet the inclusion criteria

such that they did not exhibit sufficient deficit in the post-lesion condition despite > 80% DA depletion in the lesioned hemisphere. Interestingly, an analysis of apomorphine-induced rotational behavior using a Student's t-test suggests that there was no difference between the included/excluded animals (120.10 ± 18.31 *vs*. 140.31 ± 23.51; $p = 0.7240$). This can likely be attributed to the stringent nature of the rotarod inclusion criteria, as well as the 60 s trial duration used in this study, which was intended to ensure that the included animals were significantly impaired following 6-OHDA treatment.

Rotarod is one of the most commonly utilized assays intended to assess motor behavior, and has indeed been shown to be sensitive to manipulations of the nigrostriatal DA system in rodents. This assay, however, utilizes the escape contingency and can also be considered a test of forced locomotion. Furthermore, this test has been shown to be sensitive to manipulations of other brain regions such as the cerebellum (Dixon *et al*, 2005; Lekic *et al*, 2011). Thus, it is possible that regions beyond the BG can compensate for damage to the nigrostriatal DA system in this test (Wang *et al*, 2015). Since the primary deficit in PD is difficulty in the volitional initiation of movement, we therefore sought to test the successful dose (0.1 mg/kg) utilizing an assay that avoids the escape contingency, and encapsulates a more volitional aspect of movement initiation: the cylinder/paw preference test.

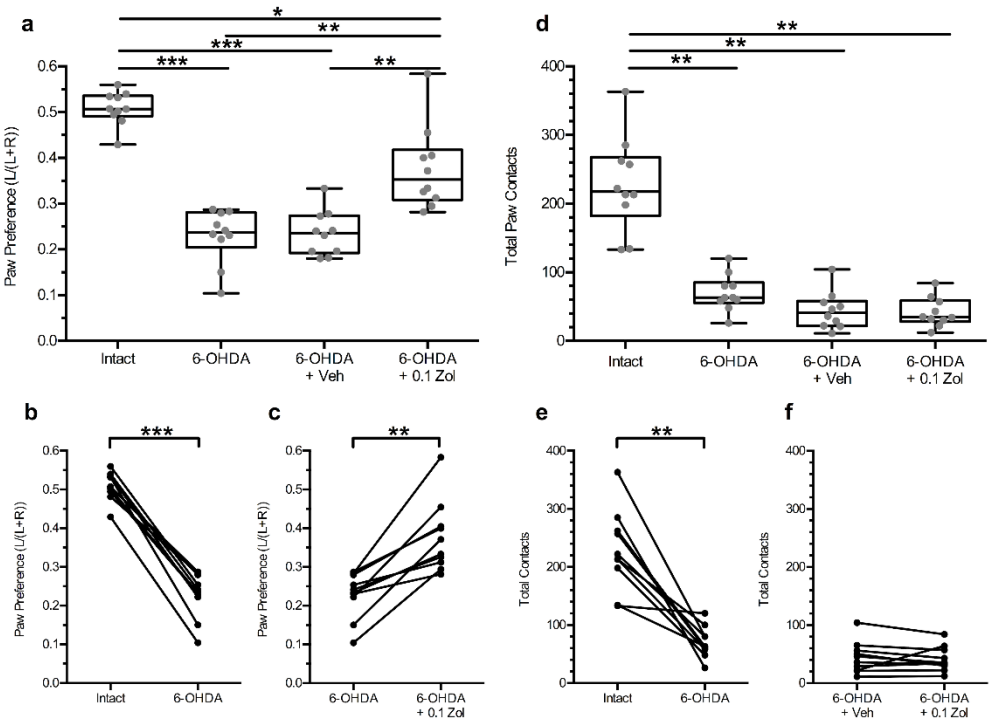


FIGURE 3.3: **Zolpidem reduces forelimb use asymmetry in unilaterally 6-OHDA-lesioned rats in the cylinder/paw preference test.** Animals (n = 10) were tested for volitional forelimb use pre-lesion (intact), post-lesion (6-OHDA), and 15 min following i.p. injection of vehicle (6-OHDA + Veh) or 0.1 mg/kg zolpidem (6-OHDA + 0.1 Zol). Analysis using a One-Way Repeated Measures ANOVA and multiple comparisons *post hoc* testing indicated that zolpidem significantly improved use of the forelimb contralateral to the lesion. **(a)** Box plot illustrating results of the cylinder/paw preference test for each experimental condition, dots represent each animal **(b)** Unilateral 6-OHDA lesion induced a significant forelimb use bias. **(c)** A dose of 0.1 mg/kg zolpidem significantly reduced forelimb use bias. **(d)** Box plot illustrating total forepaw contacts in each experimental condition. **(e)** Unilateral 6-OHDA lesion induced a significant reduction in total forepaw contacts. **(f)** There was no significant

difference in total forepaw contacts between vehicle and 0.1 Zol conditions. Dots represent each animal. Top lines indicate significant interactions. * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.00001$.

3.3.4 Zolpidem improves forelimb use symmetry in unilaterally 6-OHDA-lesioned rats

Unilateral 6-OHDA lesion induces an asymmetry in forelimb use that can be measured by placing an animal in a cylindrical enclosure, and counting total weight-bearing forepaw contacts with the walls of the enclosure by each forelimb during exploratory activity. Typically, a unilaterally 6-OHDA-lesioned rodent will show a forelimb use bias, such that use of the forepaw contralateral to the lesion is significantly reduced compared to the ipsilateral forepaw. As a result, we tested whether zolpidem decreased forelimb use asymmetry in unilaterally 6-OHDA-lesioned rats. Inclusion in statistical analysis for this experiment required: (1) ≥ 10 total forepaw contacts in each experimental condition, (2) verification of DA depletion ≥ 80% compared to the intact hemisphere. Of the animals tested (n = 13), 10 satisfied both of these criteria, due to a subset of animals (n = 3) not recording 10 total forepaw contacts in every experimental condition.

Animals that met the inclusion criteria (n = 10) were tested for forelimb use symmetry pre-lesion (intact), post-lesion (lesion), and 15 min following i.p. injection of 0.1 mg/kg zolpidem or vehicle. The number of weight-bearing forepaw contacts with the walls of the enclosure during rearing activity was counted for each limb, and a paw preference ratio was calculated (see Methods). Mean (± SEM) paw preference ratio for

each experimental condition was as follows: 0.5073 ± 0.01(intact), 0.2273 ± 0.02 (lesion), 0.2401 ± 0.02 (vehicle), 0.3797 ± 0.03 (0.1 mg/kg).

Statistical analysis using One-Way Repeated Measures ANOVA found a significant main effect ($F_{3,27} = 55.82$, $p < 0.0001$; **Fig 3.3a**). Multiple comparisons *post-hoc* analyses revealed that zolpidem significantly reduced forelimb use bias compared to post-lesion ($p_{adj} = 0.0029$; **Fig 3.3c**), as well as vehicle ($p_{adj} = 0.0045$) conditions. Post-lesion forelimb use did not differ significantly from vehicle ($p_{adj} = 3.97$), whereas vehicle performance was found to significantly differ from pre-lesion (intact) measures ($p_{adj} = 7.98 \times 10^{-6}$). Forelimb use following 0.1 mg/kg zolpidem was significantly different from intact performance ($p_{adj} = 0.0128$).

It is also possible that the effects of zolpidem observed in this experiment could be attributed to a reduction in total forepaw contacts, likely due to habituation to the enclosure with increased experimental trials. This could lead each contact of the target forepaw (contralateral to the lesion) to carry a greater weight, thus having a greater influence on the paw preference ratio and generating a false positive result. In order to test this possibility, we compared the total number of forepaw contacts across experimental conditions. Mean (± SEM) total forepaw contacts per experimental condition were as follows: 218.1 ± 22.07 (intact), 61.5 ± 5.34 (lesion), 42.5 ± 7.96 (vehicle), 40.5 ± 6.82 (0.1 mg/kg). Statistical analysis using a One-Way Repeated Measures ANOVA yielded a significant main effect ($F_{3,27} = 48.89$, $p < 0.0001$; **Fig 3.3d**).

Differences between experimental conditions were further investigated using multiple comparisons *post hoc* analyses, adjusted using the Bonferroni correction method

(6 comparisons, $\alpha_{adj} = 0.0083$). It was found that 6-OHDA lesion significantly reduced total forepaw contacts compared to the intact condition ($p_{adj} = 4.50 \times 10^{-4}$; **Fig 3.3e**). Similarly, total contacts in the intact condition differed significantly compared to zolpidem ($p_{adj} = 3.04 \times 10^{-4}$) as well as vehicle ($p_{adj} = 1.42 \times 10^{-4}$) conditions. I.p. injection of 0.1 mg/kg zolpidem did not significantly alter total forepaw contacts compared to undrugged post-lesion ($p_{adj} = 0.1734$) nor vehicle ($p_{adj} = 4.2847$; **Fig 3.3f**) conditions. There was also no statistically significant difference between lesion and vehicle conditions ($p_{adj} = 0.2545$). These analyses indicate that zolpidem reduced forelimb use bias in the absence of a reduction in total forepaw contacts compared to the undrugged post-lesion state (nor vehicle), and thus improved volitional use of the affected forelimb in unilaterally 6-OHDA-lesioned rats.

3.4 Summary of Results

The presented experiments investigated whether zolpidem-sensitive $GABA_A$ receptors may serve as a potential novel target in the treatment of motor symptoms of PD. First, we explored the dose-response relationship between zolpidem and motor coordination using rotarod in order to determine the threshold dose at which zolpidem encumbers motor behavior in intact rats. Next, we tested the hypothesis that zolpidem may improve motor deficits in unilaterally 6-OHDA-lesioned rats using rotarod and cylinder/paw preference tests. Our data indicate that zolpidem induced a dose-dependent impairment on rotarod performance in intact rats, and that a low dose of zolpidem (0.1 mg/kg) selectively improved rotarod performance as well as forelimb use symmetry. Notably, the overall number of forepaw contacts were similar in both zolpidem and

vehicle conditions, indicating that the observed improvement was not due to increased "weight" of each individual contact in the zolpidem condition.

CHAPTER IV

A QUANTITATIVE, DOPAMINE-CORRELATED MEASURE OF LEVODOPA-INDUCED BEHAVIORAL ASYMMETRY IN THE UNILATERALLY 6-HYDROXYDOPAMINE-LESIONED RODENT

4.1 Rationale

As discussed in Chapter I, the most effective pharmacological intervention for Parkinson's disease (PD) is dopamine (DA) replacement therapy, which requires administration of DA agonists, such as apomorphine or ropinirole, or the DA precursor L-DOPA. This intervention is effective at first, but debilitating side effects such as L-DOPA-induced dyskinesia (LID) soon develop in response to DA replacement therapies in a majority of PD patients (Cotzias *et al*, 1969; Rascol *et al*, 2000). Investigations into the underlying neural mechanisms as well as potential pharmacological interventions for LID are quite numerous in both translational and clinical disciplines. However, in-depth study of neural correlates has been limited by available methodology in the measure and interpretation of LID-like behaviors in translational rodent models.

Quantification of L-DOPA-induced behavioral asymmetry in translational rodent models is typically achieved using a variation of the Abnormal Involuntary Movements (AIMs) scoring system (Cenci *et al*, 1998; Lundblad *et al*, 2002; Steece-Collier *et al*, 2003; Cenci & Lundblad, 2007; Breger *et al*, 2013). Although these methods are efficient, there are confounds. These methods are ostensibly quantitative, but implementation of likert-type scales of behavioral rating results in a more categorical measure of behavioral asymmetry. As a result, it becomes difficult to detect less obvious

changes in behavior. Further, treatment of a categorical variable as continuous introduces confounds when analyzed with conventional statistics. Second, scoring is typically performed live with the experimenter present during the rating period, which can induce stress-related effects on the manifestation of behavioral asymmetry (Ungerstedt, 1968; 1971a). Additionally, these methods could be susceptible to inter-rater variability based upon the expertise of the experimental rater, which can lead to lack of reliability and reproducibility between laboratories. Third, these rating scales appear to be sensitive to doses lower than 4 mg/kg with all higher doses resulting in similar scores (Putterman *et al*, 2007). Fourth, studies have failed to correlate AIMs scores with striatal DA efflux in the lesioned hemisphere, which indicates that this measurement is not sufficiently sensitive to changes in extracellular DA levels (Lindgren *et al*, 2010).

As a result, we sought to establish a more quantitative approach to LID-like behavioral assessment in the unilateral 6-hydroxydopamine (6-OHDA) lesion model of PD. First, we investigated the relationship between contraversive rotational behavior and amount of time spent displaying LID-like stereotypy. Next, we devised a formula for quantifying overall levodopa-induced behavioral asymmetry that may account for this relationship: the Behavioral Asymmetry Scoring System (BASS). Third, we sought to validate this method and to test dose-sensitivity of this assay using a dose-response paradigm. Finally, we investigated the relationship between BASS and DA efflux in the lesioned striatum using *in vivo* microdialysis.

4.2 Overview of Methods

4.2.1 Levodopa dose-response

The overall goal of this study was to establish a more quantitative approach to LID-like behavioral assessment in the unilateral 6-hydroxydopamine (6-OHDA) lesion model of PD. To best inform and validate our approach, we conducted a dose-response experiment. Briefly, unilaterally 6-OHDA-lesioned rats (n = 41) were pretreated with benserazide (15 mg/kg, i.p.) 30 min prior to receiving an i.p. injection of L-DOPA (2.5, 5, 10, 25, or 50 mg/kg) or saline (0 mg/kg). Each animal was tested only once. Videos were scored for rotational behavior and time spent displaying unilateral stereotypy within 5 min time blocks, every 10 min.

This experiment served several purposes: 1) assess the relationship between rotational behavior and dyskinesia-like behavior, 2) devise a formula (BASS) to account for this relationship (see Section 2.3.3.3), 3) test BASS for sensitivity to escalating doses of L-DOPA, 4) determine whether BASS is superior to measuring rotational behavior or unilateral stereotypy alone.

4.2.2 Behavioral asymmetry scoring system (BASS)

In order to gain a more quantitative insight into L-DOPA-induced behavioral asymmetry, we devised a novel assessment method. As described above, behavior was scored by counting the number of net contraversive rotations and the time spent displaying unilateral stereotypy within a 5 min time block. The obtained values were

plugged into the following formula, generating an Asymmetry Score (A-Score) between 0 and 1:

$$\text{A-Score} = \frac{\text{rotations}}{\text{total time (s)} - \text{time dys (s)}} + \frac{\text{time dys (s)}}{\text{total time (s)}}$$

It should be noted that "rotations" represents the number of net contraversive rotations such that full 360° ipsiversive rotations were subtracted from the number of contraversive rotations. "Time dys" refers to the amount of time spent by the animal displaying unilateral stereotypy, which were considered dyskinesia-like behaviors. All times are entered into the formula in s. Since each analyzed time block lasted 5 min, the "total time" variable in this formula was 300 s.

4.2.3 *In vivo* microdialysis

We also sought to investigate the relationship between A-Score and striatal DA efflux using *in vivo* microdialysis. Additionally, we investigated the relationship between striatal DA efflux and both component behaviors (rotations, time spent displaying dyskinesia-like behavior).

Briefly, unilaterally 6-OHDA-lesioned rats (n = 7) were implanted with a microdialysis probe in dorsolateral striatum (see Sections 2.2.2 and 2.4). On test day, baseline dialysate samples were collected over the course of 1 h. Animals were then pretreated with benserazide (as above) and given 10 mg/kg L-DOPA 30 min later. Dialysate samples were collected every 15 min, and immediately analyzed for DA, DOPAC, and 5-HIAA content (see Section 2.5.1). Behavior was video recorded from

above, and was analyzed offline. The recorded behavior was analyzed rotational and time spent displaying stereotypy behaviors in 5 min blocks at 15 min intervals, coinciding with the central 5 min of dialysate sample collection (i.e., 5-10 min, 20-25 min, etc. post-DOPA; see below).

4.3 Results

4.3.1 Behavioral effects of levodopa administration

All doses ≥ 5 mg/kg resulted in robust rotational and dyskinesia-like behavior (**Fig 4.1a**). Analysis of rotational behavior using Friedman's test revealed a significant main effect in the 5 ($\chi^2 = 86.23$, $p = 2.95 \times 10^{-11}$), 10 ($\chi^2 = 107.2$, $p = 4 \times 10^{-15}$), 25 ($\chi^2 = 37.43$, $p = 2.89 \times 10^{-3}$), and 50 ($\chi^2 = 62.74$, $p = 3.68 \times 10^{-7}$) mg/kg conditions. This was also the case for dyskinesia-like behavior (5: $\chi^2 = 94.36$, $p = 9.76 \times 10^{-13}$; 10: $\chi^2 = 109.8$, $p = 10^{-15}$; 25: $\chi^2 = 35.38$, $p = 0.0056$; 50: $\chi^2 = 39.89$, $p = 0.0013$). Pharmacodynamics were further explored using *post hoc* Dunn's tests, comparing each time point to a behavioral baseline (10-15 min pre-DOPA).

A dose of 5 mg/kg produced a statistically significant increase in rotational behavior from 40-60 min post-DOPA ($p_{40} = 0.009$, $p_{50} = 0.032$, $p_{60} = 0.03$), and time spent displaying dyskinesia-like behavior 50-80 min post-DOPA ($p_{50} = 0.006$, $p_{60} = 0.001$, $p_{70} = 0.001$, $p_{80} = 0.008$). In the 10 mg/kg condition, we observed a statistically significant increase in rotational behavior from 20-70 min post-DOPA ($p_{20} = 3.11 \times 10^{-4}$, $p_{30} = 0.002$, $p_{40} = 0.008$, $p_{50} = 0.001$, $p_{60} = 0.005$, $p_{70} = 0.014$) and dyskinesia-like

behavior 30-100 min post-DOPA (p_{30} = 0.0098, p_{40} = 0.001, p_{50} = 9.59 x 10^{-4}, p_{60} = 1.47 x 10^{-4}, p_{70} = 0.001, p_{80} = 6.76 x 10^{-5}, p_{90} = 1.82 x 10^{-4}, p_{100} = 5.79 x 10^{-4}).

Though the behavioral responses to doses of 25 and 50 mg/kg were more severe and of more prolonged duration, the time bins in which the behavioral response reached statistically significant levels fluctuated throughout the testing period. The increased rotational response in the 25 mg/kg condition, for example, reached statistically significant levels at 30 (p_{30} = 0.02), 80-90 (p_{80} = 0.01, p_{90} = 0.02), and 110-120 (p_{110} = 3.3 x 10^{-4}, p_{120} = 0.007) min post-DOPA. The dyskinesia response reached statistical significance 60-70 (p_{60} = 0.002, p_{70} = 0.015) and 100-130 (p_{100} = 0.001, p_{110} = 0.035, p_{120} = 0.006, p_{130} = 5.99 x 10^{-4}) min post-DOPA. A dose of 50 mg/kg resulted in an immediate statistically significant increase in rotational behavior at 10 min (p_{10} = 0.003), but also 30-70 min (p_{30} = 3.72 x 10^{-4}, p_{40} = 1.08 x 10^{-4}, p_{50} = 0.001, p_{60} = 0.003, p_{70} = 0.018) post-DOPA. Additionally, 50 mg/kg induced a statistically significant increase in dyskinesia-like behavior 80-120 min (p_{80} = 5.32 x 10^{-4}, p_{90} = 8.5 x 10^{-4}, p_{100} = 0.002, p_{110} = 0.014, p_{120} = 0.012) post-DOPA.

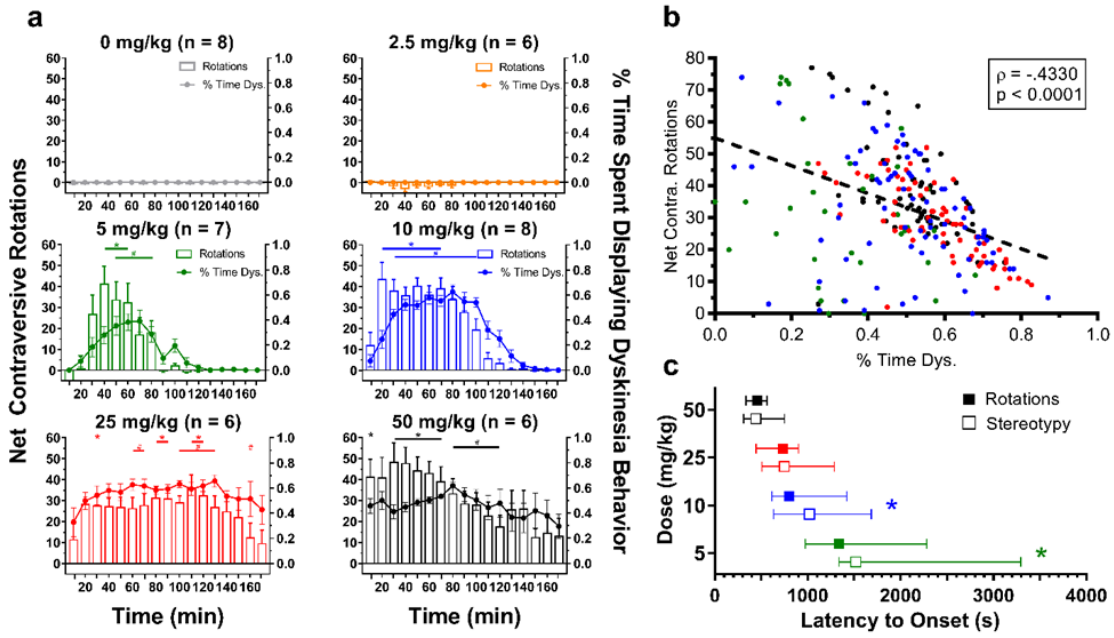


FIGURE 4.1. **L-DOPA-induced rotational behavior and time spent displaying dyskinesia-like behavior are negatively correlated.** Animals (n = 41) were pretreated with benserazide (15 mg/kg), then given L-DOPA (2.5, 5, 10, 25, or 50 mg/kg) or saline 30 min later. **(a)** Net contraversive rotations and % time spent displaying dyskinesia behavior by dose, over course of experiment (180 min). All doses ≥ 5 mg/kg produced statistically significant increases in both rotational and dyskinesia-like behavior. Bars represent mean ± *SEM* net contraversive rotations, dot/lines represent % time displaying dyskinesia behavior (% Time Dys). Top symbols/lines denote time bins with statistically significant difference compared to baseline: * denotes significance for rotations, # for dyskinesia behavior ($p < 0.05$). **(b)** Relationship between number of net contraversive rotations and % time spent displaying dyskinesia behavior (5-50 mg/kg pooled). Color of dot indicates dose. **(c)** Latency to the onset (s) of rotational behavior and dyskinesia-like stereotypy by dose.

Data represented as median ± range. * denotes statistically significant difference within dose ($p < 0.05$).

4.3.2 Levodopa-induced rotations and unilateral behavioral stereotypy are inversely correlated

We first sought to investigate the relationship between L-DOPA-induced rotational behavior and unilateral behavioral stereotypy. Surprisingly, we observed a statistically significant negative correlation between number of net contraversive rotations and amount of time spent displaying dyskinesia-like stereotypy (Spearman's $\rho = -.4330$, $p < 0.$ 10-13; **Fig 4.1b**). When each dose was analyzed independently, doses ≥ 10 mg/kg each produced statistically significant negative correlations (10 mg/kg: $\rho = -0.3760$, $p = 5.87 \times 10^{-4}$; 25 mg/kg: $\rho = -0.7564$, $p = 3.7 \times 10^{-14}$; 50 mg/kg: $\rho = -0.4749$, $p = 1.26 \times 10^{-4}$). These data suggest that rotations and dyskinesia-like stereotypy are distinct behaviors that are unlikely to occur simultaneously. Further, a unified behavioral scale for LID-like behavior should account for this in some way.

4.3.3 Rotational behavior precedes stereotypy

We then sought to determine whether there was a temporal relationship between the respective latencies to onset of both rotational and stereotypy behavior, as well as whether this changed as a function of dose. Therefore, we compared the latency to the first contraversive rotation to the emergence of dyskinesia-like stereotypy using Wilcoxon sign rank tests. Indeed, the latency to onset of rotational behavior was

significantly shorter than stereotypy in both 5 ($p = 0.0156$) and 10 ($p = 0.0078$; **Fig 4.1c**) mg/kg conditions. Both 25 and 50 mg/kg conditions were nonsignificant, suggesting that the levels of DA receptor stimulation necessary to produce a shift from rotational behavior alone to unilateral stereotypy were reached more rapidly at higher doses. Interestingly, we also found a linear relationship between dose and latency to onset for both rotational behavior ($R^2 = 0.6147$, $p = 6.53 \times 10^{-7}$) and stereotypy ($R^2 = 0.584$, $p = 3.1 \times 10^{-6}$). Taken with the findings above, these data suggest that although these behaviors are distinct, they may also represent a progression of DA-mediated behaviors. Presumably, this progression occurs as a function of DA receptor stimulation.

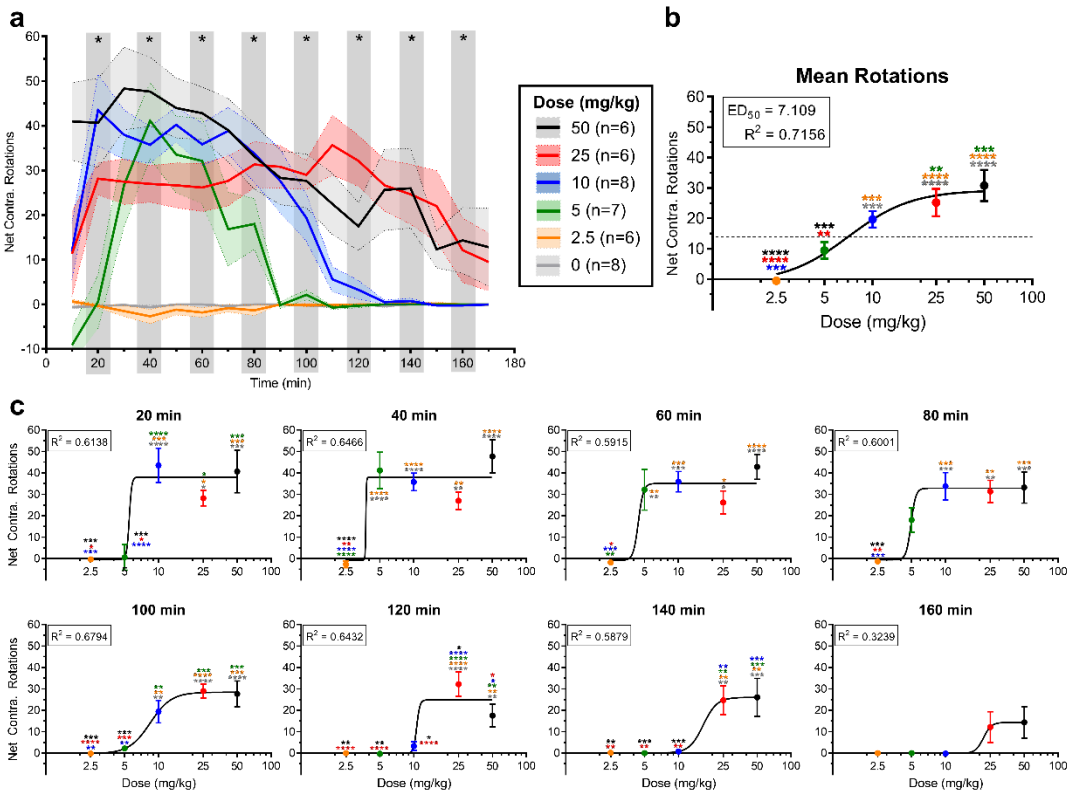


FIGURE 4.2. **Rotational behavior poorly discriminates between doses. (a)** Net contraversive rotations by dose. Lines represent mean rotations at given time point, shaded regions represent *SEM*. Top symbols denote statistically significant main effect at that time point (ANOVA, $p < 0.05$). **(b)** Mean overall net contraversive rotations by dose. **(c)** Comparison of rotational behavior at each time point analyzed. **(b-c)** Dots and error bars represent mean ± *SEM*. Symbols indicate significant interactions: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$. Color of * represents comparator dose. Solid line illustrates best fit line using a four-parameter Hill formula, dotted line **(b)** shows estimated ED_{50} based on that fit.

4.3.4 Rotational behavior poorly discriminates between doses

Rotational behavior in response to DA receptor agonists is a primary indicator of lesion size in unilaterally 6-OHDA rodents (Ungerstedt, 1971a, b). As such, it has also been utilized as an indicator of levodopa response in this translational model (Abercrombie *et al*, 1990). However, the utility of this behavior in isolation as a dependent measure in evaluating the severity of LID-like behaviors remains questionable. Thus, quantification of rotational behavior in response to escalating doses of L-DOPA should yield additional insight.

Analysis of rotational behavior using a Two-Way ANOVA yielded a significant main effect of both time ($F_{16, 560} = 23.41$, $p < 10^{-15}$) as well as dose ($F_{5, 35} = 18.76$, $p = 4.9 \times 10^{-9}$). Dose interactions were explored further using multiple comparisons *post hoc* testing in 20 min increments from 20 to160 min post-DOPA (Holm-Sidak; gray bars in **Fig 4.2a**).

Dose interactions are outlined in **Fig 4.2c**. Briefly, 5 mg/kg produced significantly more rotations compared to saline ($p_{40} = 1.07 \times 10^{-5}$, $p_{60} = 0.001$) and 2.5 mg/kg ($p_{40} = 1.53 \times 10^{-5}$, $p_{60} = 0.001$) 40 and 60 min post-DOPA. A dose of 10 mg/kg resulted in a statistically significant increase in rotations compared to saline ($p_{20} = 3.52 \times 10^{-5}$, $p_{40} = 5.41 \times 10^{-5}$, $p_{60} = 1.7 \times 10^{-4}$, $p_{80} = 1.73 \times 10^{-4}$, $p_{100} = 0.0014$) and 2.5 mg/kg ($p_{20} = 1.1 \times 10^{-4}$, $p_{40} = 7.07 \times 10^{-5}$, $p_{60} = 2.36 \times 10^{-4}$, $p_{80} = 2.88 \times 10^{-4}$, $p_{100} = 0.0033$) from 20 to 100 min post-DOPA, before returning to levels indistinguishable from those conditions. Comparisons with 5 mg/kg yielded statistically significant differences at 20 ($p_{20} = 8.38 \times 10^{-5}$) and 100 min ($p_{100} = 0.007$). Both 25 and 50 mg/kg conditions produced statistically significant increases in rotational behavior from 20 to 140 min

post-DOPA, compared to both saline and 2.5 mg/kg conditions. In contrast, these doses were only distinguishable from 5 mg/kg 20 min and 100-160 min post-DOPA. Interestingly, a difference between 25 and 50 mg/kg emerged at 120 min (p_{120} = 0.0165), indicating that a dose of 25 mg/kg resulted in a more sustained rotational response.

We also sought to investigate whether doses could be distinguished by overall mean rotational response. For this analysis, mean number of net contraversive rotations served as the dependent variable. Mean (± *SEM*) net contraversive rotations for each dose were as follows: -0.15 ± 0.08 (saline), -0.53 ± 0.47 (2.5 mg/kg), 9.46 ± 2.77 (5 mg/kg), 19.68 ± 2.70 (10 mg/kg), 25.21 ± 4.50 (25 mg/kg), 30.81 ± 5.14 (50 mg/kg). A One-Way ANOVA revealed a statistically significant main effect ($F_{5, 35}$ = 18.73, p = 5.01 x 10^{-10}; **Fig 4.2b**). Differences between doses were further explored using Bonferroni-corrected multiple comparisons *post hoc* tests. Compared to saline, we observed a statistically significant increase in mean rotational behavior following doses of 10 (p = 1.4 x 10^{-4}), 25 (p = 9.69 x 10^{-6}), and 50 (p = 2.09 x 10^{-7}) mg/kg. This was also the case compared to 2.5 mg/kg (p = 2.72 x 10^{-4}, p = 2.25 x 10^{-5}, p = 6.19 x 10^{-7}, respectively). Doses of 25 (p = 0.0073) and 50 (p = 0.0002) mg/kg also produced statistically significant increases in mean rotational behavior compared to 5 mg/kg. When fit using a four-parameter Hill equation, using rotational behavior alone as a means of quantifying L-DOPA-induced behavioral asymmetry yielded an estimated ED_{50} of 7.11 mg/kg (R^2 = 0.7156).

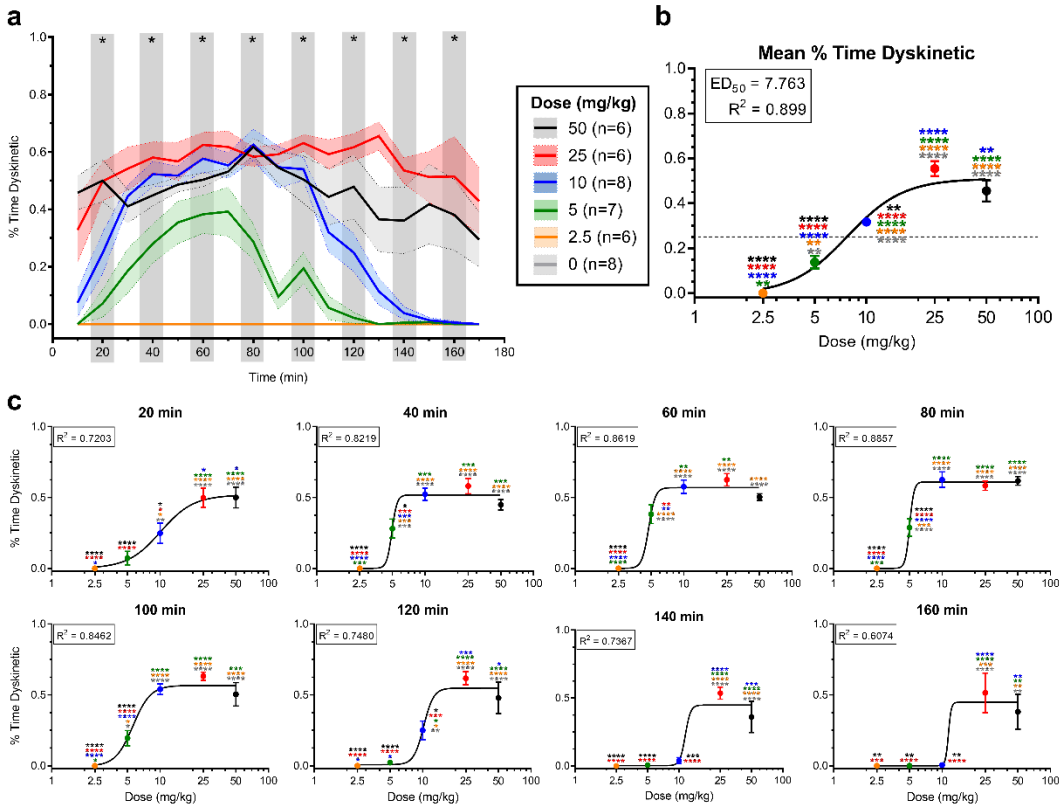


FIGURE 4.3. **% Time spent displaying dyskinesia-like behavior can be used to discriminate between doses. (a)** % Time spent displaying dyskinesia-like behavior (% Time Dys.) by dose. Lines represent mean % time dyskinetic. at given time point, shaded regions represent *SEM*. Top symbols denote statistically significant main effect at that time point (ANOVA, $p < 0.05$). **(b)** Mean % Time Dys. by dose. **(c)** Comparison of % Time Dys. at each time point analyzed. **(b-c)** Dots and error bars represent mean ± *SEM*. Symbols indicate significant interactions: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$. Color of * represents comparator dose. Solid line illustrates best fit line using a four-parameter Hill formula, dotted line **(b)** shows estimated ED_{50} based on that fit.

4.3.5 Time spent displaying dyskinesia-like behavior is superior to rotational behavior in distinguishing between doses

Asymmetrical behavioral stereotypy, as characterized by the AIMs scoring system, constitutes the most readily translatable component of the L-DOPA response in unilaterally DA-lesioned rodents. Traditionally, this dyskinesia-like behavior has been chunked into 3 distinct behaviors: Axial twisting of the trunk and neck, myoclonic movements of the forepaw contralateral to the lesion, and excessive orolingual movements. Though more commonly-used assays score these behaviors separately, they have been found to be highly correlated (Putterman *et al*, 2007). As a result, we sought to ascertain whether the expression of both axial and limb-based stereotypy (scored together) was sufficient to determine the severity of LID-like behaviors in a dose-response paradigm.

Analysis of time spent displaying dyskinesia-like behaviors (axial or limb-based) using a Two-Way ANOVA yielded a significant main effect as a function of both time ($F_{16, 560} = 19.45$, $p < 10^{-15}$) as well as dose ($F_{5, 35} = 85.86$, $p < 10^{-15}$; **Fig 4.3a**). Dose interactions were explored further using Holm-Sidak's multiple comparisons *post hoc* testing, as described above (**Fig 4.3c**). Treatment with 5 mg/kg L-DOPA resulted in statistically significant increases in behavioral stereotypy compared to both saline ($p_{40} = 1.91 \times 10^{-4}$, $p_{60} = 2.89 \times 10^{-7}$, $p_{80} = 5.4 \times 10^{-5}$, $p_{100} = 0.0107$) and 2.5 mg/kg ($p_{40} = 4.14 \times 10^{-4}$, $p_{60} = 1.12 \times 10^{-6}$, $p_{80} = 1.17 \times 10^{-4}$, $p_{100} = 0.0168$) from 40 to 100 min post-DOPA. A dose of 10 mg/kg L-DOPA resulted in a statistically significant increase in dyskinesia-like behavior from 20 to 120 min, compared to saline ($p_{20} = 0.0099$, $p_{40} = 4.93 \times 10^{-10}$, $p_{60} = 9.15 \times 10^{-12}$, $p_{80} = 1.42 \times 10^{-12}$, $p_{100} = 2.44 \times 10^{-10}$, $p_{120} = 0.0093$), and 2.5 mg/kg ($p_{20} =$

0.0152, p_{40} = 2.88 x 10^{-9}, p_{60} = 6.23 x 10^{-11}, p_{80} = 1.12 x 10^{-11}, p_{100} = 1.57 x 10^{-9}, p_{120} = 0.0166). Further, there was a statistically significant difference between 5 and 10 mg/kg from 40 to 120 min post-DOPA (p_{40} = 9.45 x 10^{-4}, p_{60} = 0.0063, p_{80} = 4.32 x 10^{-6}, p_{100} = 6.35 x 10^{-6}, p_{120} = 0.0216), which indicates that this behavior is more sensitive to dose than rotational behavior alone.

Both 25 and 50 mg/kg resulted in a statistically significant increase in unilateral behavioral stereotypy compared to saline, 2.5 mg/kg, as well as 5 mg/kg, for the duration of the experiment. The sole exception was a lack of statistical significance between 5 and 50 mg/kg at 60 min post-DOPA. Additionally, there was a statistically significant difference between these doses and 10 mg/kg at 20 min (25: p_{20} = 0.015, 50: p_{20} = 0.015) and 120 to 160 min (25: p_{120} = 2.51 x 10^{-4}, p_{140} = 4.51 x 10^{-8}, p_{160} = 5.41 x 10^{-5}; 50: p_{120} = 0.0239, p_{140} = 1.12 x 10^{-4}, p_{160} = 0.0028) post-DOPA.

We also compared the mean overall dyskinesia response between doses. Mean (± *SEM*) % time spent dyskinetic for each dose were as follows: 0 ± 0 (saline), 0 ± 0 (2.5 mg/kg), 0.1374 ± 0.03 (5 mg/kg), 0.3172 ± 0.01 (10 mg/kg), 0.5541 ± 0.03 (25 mg/kg), 0.4556 ± 0.05 (50 mg/kg). Analysis using a One-Way ANOVA revealed a statistically significant main effect ($F_{5, 35}$ = 85.86, $p < 10^{-15}$; **Fig 4.3c**). Multiple comparisons *post hoc* testing (Bonferroni) also showed statistically significant differences between doses. All doses ≥ 5 mg/kg resulted in increased mean dyskinesia-like behavior compared to both saline (5: p = 0.0035, 10: p = 2.12 x 10^{-11}, 25: $p < 10^{-15}$, 50: p = 8 x 10^{-15}) and 2.5 (5: p = 0.0079, 10: p = 1.5 x 10^{-9}, 25: $p < 10^{-15}$, 50: p = 5.53 x 10^{-13}) mg/kg conditions. Compared to 5 mg/kg, there was a statistically significant increase in dyskinesia-like behavior following doses of 10 (p = 7.84 x 10^{-5}), 25 (p = 2.41 x 10^{-12}), and 50 (p = 2.87 x

10^{-9}) mg/kg. Doses of 25 (p = 1.1 x 10^{-6}) and 50 (p = 0.0052) mg/kg also produced significantly more dyskinesia than 10 mg/kg. There was no difference between 25 and 50 mg/kg. When fit using a four-parameter Hill equation, using dyskinesia-like stereotypy as a means of quantifying L-DOPA-induced behavioral asymmetry yielded an estimated ED_{50} of 7.76 mg/kg (R^2 = 0.899; **Fig 4.3b**).

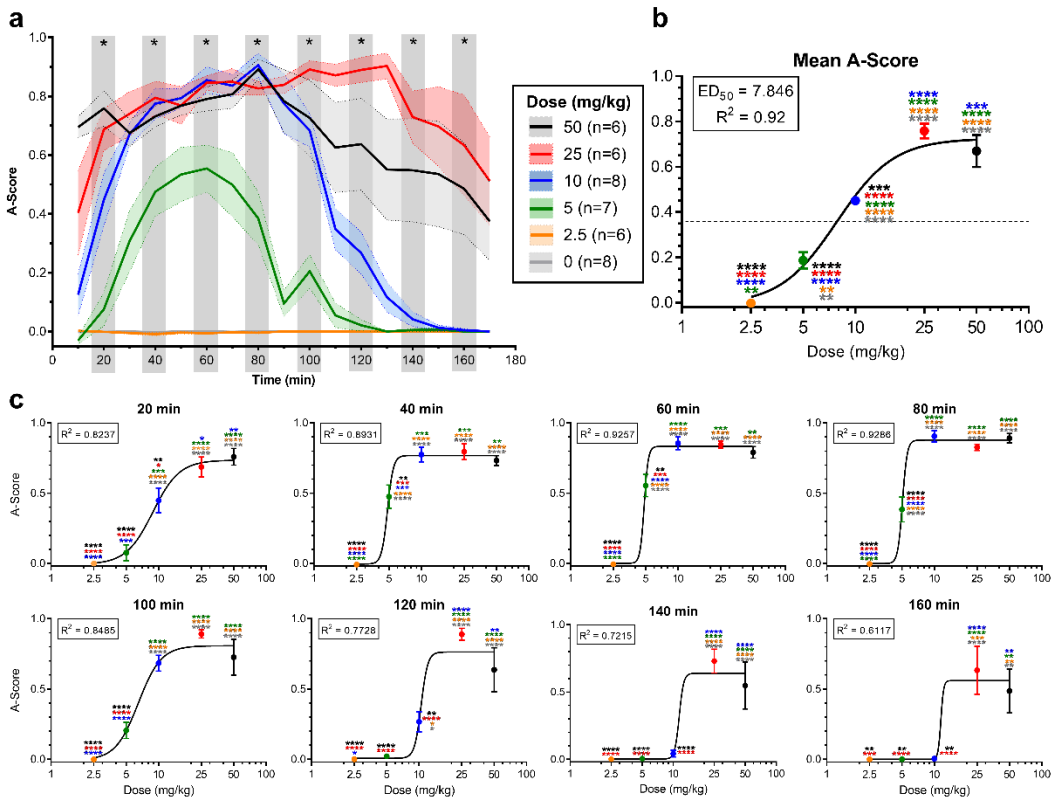


FIGURE 4.4. **BASS can be used to assess severity of LID-like behaviors as a function of dose.** **(a)** Asymmetry Scores (A-Scores) by dose. Lines represent mean A-Score at given time point, shaded regions represent *SEM*. Top symbols denote statistically significant main effect at that time point (ANOVA, $p < 0.05$). **(b)** Mean Overall A-Score by dose. **(c)** Comparison of A-Scores at each time point analyzed. **(b-c)** Dots and error bars represent mean A-Score ± *SEM*. Symbols indicate significant interactions: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$. Color of * represents comparator dose. Solid line illustrates best fit line using a four-parameter Hill formula, dotted line **(b)** shows estimated ED_{50} based on that fit.

4.3.6 BASS can be used to assess severity of LID-like behaviors as a function of dose

In seeking to establish a quantitative measure of LID-like behaviors, we sought to combine both rotational behavior and unilateral dyskinesia-like stereotypy into a unified behavioral scale. We arrived at a formula that can be described as the rotational rate added to the percentage of time spent displaying unilateral stereotypy in a given epoch. However, our data indicate a need to account for the inverse relationship between these behavioral markers (see **Fig 4.1**). As a result, we attempted to account for this in our BASS formula by isolating the amount of time available for rotational behavior. When the corresponding measures are entered into the BASS formula, an Asymmetry Score (A-Score) between 0 and 1 is obtained (see Section 2.3.3.3). This value was utilized in the following analyses, as well as in the neurochemical experiments described below.

Analysis of A-Scores using a Two-Way ANOVA yielded a significant main effect as a function of both time ($F_{16, 560} = 29.68$, $p < 10^{-15}$) as well as dose ($F_{5, 35} = 99.05$, $p < 10^{-15}$; **Fig 4.4a**). Dose interactions were explored by multiple comparisons *post hoc* testing (Holm-Sidak method), as described above, and are outlined in **Fig 4.4c**. A dose of 5 mg/kg L-DOPA resulted in statistically significant increases in A-Score compared to both saline ($p_{40} = 1.98 \times 10^{-7}$, $p_{60} = 3.06 \times 10^{-10}$, $p_{80} = 1.46 \times 10^{-6}$) and 2.5 mg/kg ($p_{40} = 5.88 \times 10^{-7}$, $p_{60} = 1.26 \times 10^{-9}$, $p_{80} = 3.81 \times 10^{-6}$) from 40 to 80 min post-DOPA. A dose of 10 mg/kg L-DOPA resulted in a statistically significant increase in A-Score from 20 to 120 min, compared to saline ($p_{20} = 1.22 \times 10^{-5}$, $p_{40} = 6.86 \times 10^{-13}$, $p_{60} < 10^{-15}$, $p_{80} < 10^{-15}$, $p_{100} = 2.34 \times 10^{-9}$, $p_{120} = 0.0319$), and 2.5 mg/kg ($p_{20} = 4.05 \times 10^{-5}$, $p_{40} = 4.05 \times 10^{-13}$, $p_{60} = 6 \times 10^{-15}$, $p_{80} = 3 \times 10^{-15}$, $p_{100} = 1.27 \times 10^{-8}$, $p_{120} = 0.0483$). Comparisons with 5 mg/kg yielded a statistically significant increase in A-Score from 20 to 100 min ($p_{20} = 3.15 \times 10^{-}$

^{4}, p_{40} = 4.92 x 10^{-4}, p_{60} = 7.42 x 10^{-5}, p_{80} = 2.98 x 10^{-9}, p_{100} = 5.53 x 10^{-6}). Both 25 and 50 mg/kg resulted in a statistically significant increase in A-Score compared to saline, 2.5 mg/kg, as well as 5 mg/kg, for the duration of the experiment.

As with rotational behavior and time spent displaying dyskinesia-like behavior, we also compared mean overall A-Score between doses. Mean (± *SEM*) A-Scores for each dose were as follows: -0.0007 ± 0 (saline), -0.0018 ± 0 (2.5 mg/kg), 0.1874 ± 0.04 (5 mg/kg), 0.4505 ± 0.02 (10 mg/kg), 0.7578 ± 0.03 (25 mg/kg), 0.6697 ± 0.07 (50 mg/kg). A One-Way ANOVA yielded a statistically significant main effect ($F_{5, 35}$ = 99.05, $p < 10^{-15}$; **Fig 4.4b**), which was investigated further using Bonferroni-corrected multiple comparisons. All doses ≥ 5 mg/kg resulted in increased mean A-Score compared to both saline (5: p = 0.0021, 10: p = 2.67 x 10^{-11}, 25: $p < 10^{-15}$, 50: p = 3 x 10^{-15}) and 2.5 (5: p = 0.0047 , 10: p = 1.92 x 10^{-10}, 25: $p < 10^{-15}$, 50: p = 2 x 10^{-15}) mg/kg conditions. Compared to 5 mg/kg, there was a statistically significant increase in A-Score following doses of 10 (p = 1.25 x 10^{-5}), 25 (p = 7.76 x 10^{-13}), and 50 (p = 7.75 x 10^{-11}) mg/kg. Doses of 25 (p = 1.45 x 10^{-6}) and 50 (p = 4.79 x 10^{-4}) mg/kg also produced significantly higher A-Scores than 10 mg/kg. There was no difference in mean A-Score between 25 and 50 mg/kg. When fit using a four-parameter Hill equation, using BASS as a means of quantifying L-DOPA-induced behavioral asymmetry yielded an estimated ED_{50} of 7.85 mg/kg (R^2 = 0.92; **Fig 4.4b**).

4.3.7 BASS is superior to either rotational behavior and dyskinesia-like stereotypy alone

Finally, we sought to utilize the dose-response data to compare the 3 approaches to assessing LID-like behaviors outlined above. To do so, each time point was fit using a four-parameter Hill formula for rotations (**Fig 4.2c**), dyskinesia-like stereotypy (**Fig 4.3c**), and A-Scores (**Fig 4.4c**), as described above. We hypothesized that a superior fit using the Hill formula for a given parameter could signify a higher degree of accuracy in predicting the dose-response relationship. Therefore, we used the goodness of fit obtained from each timepoint for each parameter as the dependent variable. Given that both stereotypy and A-Scores were superior to rotational behavior alone in discriminating doses, rotational behavior was excluded from this analysis. A direct comparison between the goodness of fit values obtained from A-Score and dyskinesia-like stereotypy using a Wilcoxon sign rank test yielded a statistically significant difference ($p = 0.039$). This analysis provides additional evidence that the BASS formula is superior to each individual behavior parameters in assessing the severity of LID-like behaviors.

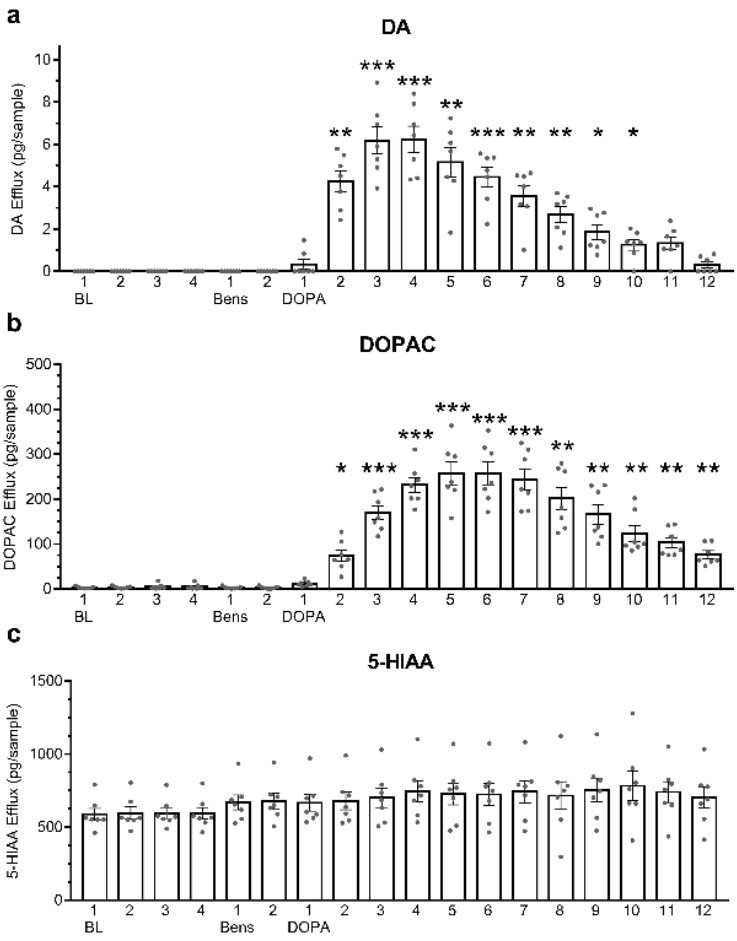


FIGURE 4.5. **L-DOPA-induced DA, DOPAC, and 5-HIAA efflux.** Animals (n = 7) were implanted with microdialysis probes in dorsolateral striatum. Following 1 h baseline sample collection (BL1 - 4), animals were pretreated (Bens 1 – 2) and given L-DOPA (10 mg/kg, i.p.). Samples were collected every 15 min over 3 h post-DOPA (DOPA 1 – 12). Repeated Measures ANOVAs found statistically significant increases in DA **(a)** and DOPAC **(b)** efflux, but not 5-HIAA **(c)**. Symbols indicate statistically significant interactions (compared to baseline): * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

4.3.8 Levodopa-induced dopamine, 3,4-dihydroxyphenylacetic acid, and 5-hydroxyindoleacetic acid efflux

Systemic administration of L-DOPA has been shown to produce robust increases in DA within the lesioned striatum of unilaterally 6-OHDA-lesioned rodents (Abercrombie *et al*, 1990; Miller & Abercrombie, 1999; reviewed in Zigmond *et al*, 1992). However, establishment of a direct relationship between DA efflux in the lesioned striatum and a measure of L-DOPA-induced behavioral asymmetry has been difficult (Lindgren *et al*, 2010). Since BASS has been shown to discriminate between escalating doses of L-DOPA, we sought to investigate the relationship between A-Score and striatal DA efflux using *in vivo* microdialysis. In addition, we investigated the relationship between striatal DA efflux and both component behaviors (rotations, time spent displaying dyskinesia-like behavior).

As expected, L-DOPA produced robust increases in striatal DA efflux. Analysis of DA efflux over the course of the experiment using a One-Way Repeated Measures ANOVA (Geisser-Greenhouse correction) yielded a statistically significant main effect ($F_{2.185,\ 13.11} = 55.74$, $p = 2.87 \times 10^{-7}$; **Fig 4.5a**). We then compared each time point to mean baseline DA efflux using multiple comparisons *post hoc* tests (Bonferroni). It was found that DA efflux remained increased at a statistically significant level from 15 to 150 min ($p_{15\text{-}30} = 0.002$, $p_{30\text{-}45} = 9.64 \times 10^{-4}$, $p_{45\text{-}60} = 7.3 \times 10^{-4}$, $p_{60\text{-}75} = 0.004$, $p_{75\text{-}90} = 9.96 \times 10^{-4}$, $p_{90\text{-}105} = 0.005$, $p_{105\text{-}120} = 0.005$, $p_{120\text{-}135} = 0.023$, $p_{135\text{-}150} = 0.034$) post-DOPA.

Similar results were found for DOPAC, a primary DA metabolite. Analysis of DOPAC efflux over the course of the experiment using a corrected One-Way Repeated Measures ANOVA yielded a statistically significant main effect ($F_{2.134,\ 12.81} = 62.45$, $p =$

2×10^{-7}; **Fig 4.5b**). We then compared each time point to mean baseline DOPAC efflux using Bonferroni-corrected multiple comparisons *post hoc* tests. It was found that DOPAC efflux remained increased at statistically significant levels from 15 to 180 min ($p_{15\text{-}30} = 0.02$, $p_{30\text{-}45} = 0.001$, $p_{45\text{-}60} = 10^{-4}$, $p_{60\text{-}75} = 8.14 \times 10^{-4}$, $p_{75\text{-}90} = 7.69 \times x10^{-4}$, $p_{90\text{-}105} = 8.09 \times 10^{-4}$, $p_{105\text{-}120} = 0.003$, $p_{120\text{-}135} = 0.004$, $p_{135\text{-}150} = 0.008$, $p_{150\text{-}165} = 0.002$, $p_{165\text{-}180} = 0.003$) post-DOPA.

Analysis of 5-HIAA efflux over the course of the experiment yielded a statistically significant main effect ($F_{2.087,\ 12.522} = 5.13$, $p = 0.0226$; **Fig 4.5c**). However, Bonferroni-corrected multiple comparisons *post hoc* tests did not reveal any statistically significant differences between any post-DOPA samples and baseline samples. As a result, the relationship between 5-HIAA efflux and L-DOPA-induced behavioral asymmetry was not investigated further.

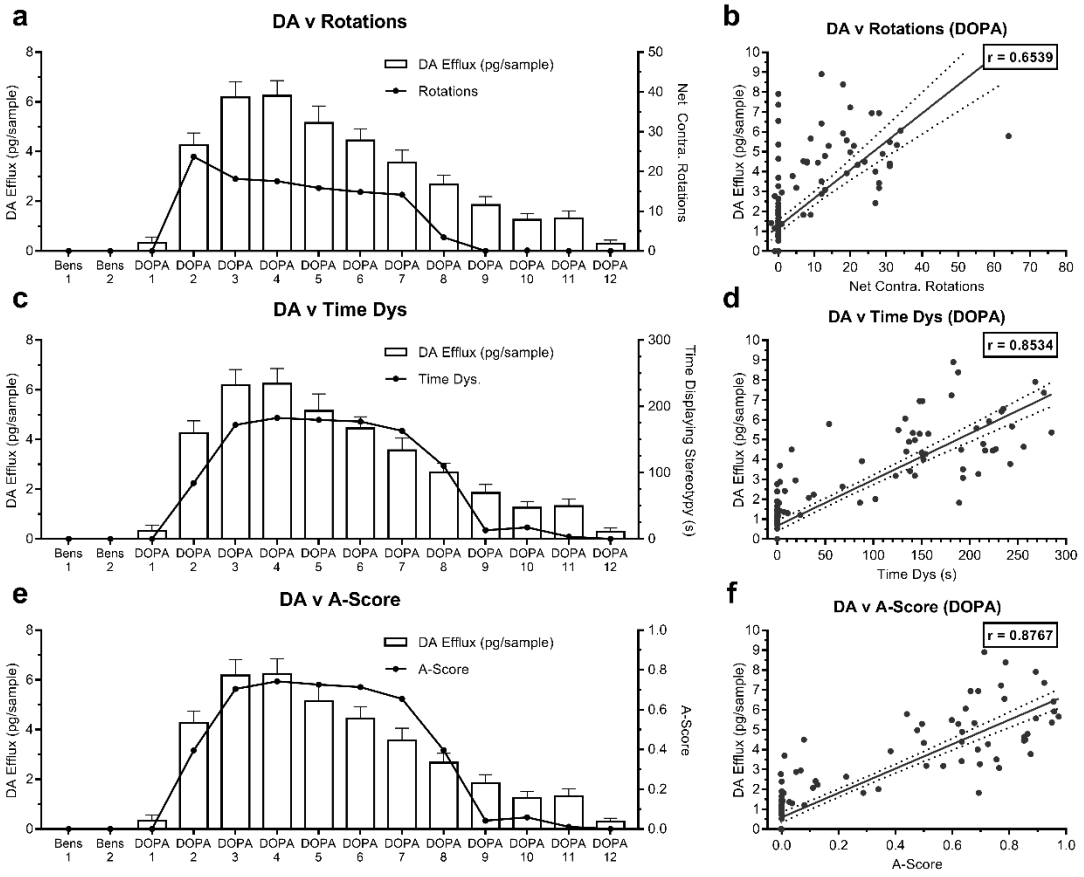


FIGURE 4.6. **Relationship between DA efflux and L-DOPA-induced behavioral asymmetry. (a, c, e)** DA efflux versus rotational behavior **(a)**, dyskinesia behavior **(c)**, and A-Score **(e)** over course of experiment. **(b, d, f)** Correlational analysis of the relationship between L-DOPA-induced DA efflux and net contraversive rotations **(b)**, time spent displaying dyskinesia-like behavior **(d)**, and A-Score **(e)**. All correlations were statistically significant ($p < 0.05$). Lines represent best fit for the relationship; dotted lines denote 95% confidence interval for the fit line.

4.3.9 Relationship between dopamine efflux and levodopa-induced behavioral asymmetry

As in the dose response experiment (**Figs 4.1-4.5**), 10 mg/kg L-DOPA produced an increase in rotational behavior, the peak of which preceded peak dyskinesia-like behavior (**Fig 4.6**). Interestingly, peak rotational behavior also preceded t_{max} of striatal DA efflux (**Fig 4.6a**). An analysis using Pearson's correlation indicated a moderate positive relationship ($r = 0.6539$, $p < 10^{-15}$, **Fig 4.6b**) between rotational behavior and striatal DA efflux. When observational pairs containing 0 rotations were removed, this relationship decreased ($r = 0.5236$, $p = 2 \times 10^{-6}$, data not shown)

In addition to rotational behavior, 10 mg/kg L-DOPA resulted in a robust dyskinesia-like behavioral response. Notably, this behavior reached its peak coincidentally with peak striatal DA efflux (**Fig 4.6c**). We found that there was a strong relationship between time spent displaying dyskinesia-like behavior and striatal DA efflux ($r = 0.8534$, $p < 10^{-15}$, **Fig 4.6d**). When observational pairs in which there was no time spent displaying these behaviors were removed, this strength of this relationship decreased ($r = 0.7681$, $p = 2 \times 10^{-15}$, data not shown). Importantly, this mirrored our findings that time spent displaying dyskinesia-like behavior was better able to discriminate between doses of L-DOPA, compared to rotational behavior (see above). Thus, a unified behavioral rating scale should take this into account by emphasizing asymmetrical stereotypy.

4.3.10 BASS is highly correlated with dopamine efflux

We then sought to discern whether our BASS assay was directly correlated with striatal DA efflux. The kinetics of both A-Score and striatal DA efflux were remarkably similar, with t_{max} for both measures coinciding 45-60 min post-DOPA (**Fig 4.6e**). Further, we observed a very strong correlation ($r = 0.8767$, $p < 10^{-15}$; **Fig 4.6f**) between A-Scores and striatal DA efflux. When observational pairs containing a 0 value for A-Score were removed, this relationship also decreased ($r = 0.8029$, $p < 10^{-15}$, data not shown). Of note, this correlation was stronger than either net contraversive rotations or time spent displaying dyskinesia-like behavior alone.

4.4 Summary of Results

We have devised a novel method in quantifying L-DOPA-induced behavioral asymmetry in unilaterally 6-OHDA-lesioned rats: the BASS. In the studies outlined above, we have shown that this assay can discriminate the severity of behavioral asymmetry as a function of dose. Additionally, our BASS method was found to be strongly correlated with L-DOPA-induced striatal DA efflux. Both % time displaying dyskinesia-like behavior and BASS were superior to rotational behavior alone in discriminating between doses. We also found that BASS was superior to dyskinesia-like behavior in predicting this dose-response relationship. This finding was also reflected in our investigation of the relationships between these each measures and L-DOPA-induced increases in striatal DA efflux. In sum, we have shown that BASS can be used as a

quantitative and DA-correlated means of measuring L-DOPA-induced behavioral asymmetry in unilaterally 6-OHDA-lesioned rodents.

CHAPTER V

EFFECTS OF ZOLPIDEM ON LEVODOPA-INDUCED BEHAVIORAL ASYMMETRY IN THE UNILATERALLY 6-HYDROXYDOPAMINE-LESIONED RAT

5.1 Rationale

As discussed above (Chapters I & IV), DA replacement therapies are currently the most effective pharmacological intervention for PD motor symptoms, but this treatment usually leads to debilitating side effects such as choreic dyskinesia and dystonia. This is likely due to a combination of compensatory responses to DA depletion within the BG, fluctuations in DA levels, and the resulting long-lasting synaptic potentiation within BG nuclei when DAergic tone is restored (Cotzias, *et al*, 1969; Melamed *et al*, 1979; Muenter *et al*, 1977; Ungerstedt, 1971; Zigmound & Stricker, 1980; Creese *et al*, 1977; Lee *et al*, 1978; reviewed in Gerfen, 1995). Metabolic and neurochemical studies as well as *in vivo* neurophysiological recordings in PD translational models have indicated that the BG circuit may indeed exhibit hyperexcitation in response to DA agonists (Mitchell *et al*, 1992; Trugman, 1995; Robelet *et al*, 2004). This is evident in findings demonstrating that a shift in firing pattern from regular to irregular/bursting emerges within the GPe-STN microcircuit and BG output nuclei as a result of L-DOPA and DA agonist administration (Boraud *et al*, 1998; Boraud *et al*, 2001). It is hypothesized that a potential pharmacological intervention for these side effects should be selective for BG nuclei that exhibit hyperexcitation, acting as a counterbalance to restore inhibitory drive to these nuclei.

Several studies have shown that interventions focusing upon downstream BG nuclei, notably GPe-STN and output nuclei (GPi/EPN, SNr), are effective in the reduction of L-DOPA-induced involuntary movements in PD translational models. Neurochemical lesion as well as optogenetic and pharmacological inactivation of STN have been shown to reduce L-DOPA-induced behavioral asymmetry in unilaterally 6-OHDA-lesioned rodents (Aristieta *et al*, 2012; Petri *et al*, 2013; Yoon *et al*, 2016). In addition, direct brain stimulation of STN or GPi has been shown to reduce LID in PD patients. Given the overlap between the sites of these specific interventions and the expression profile of the α_1 subunit within the BG, it is possible that the zolpidem-sensitive $GABA_A$ receptor may serve as a novel pharmacological target in the treatment of L-DOPA-induced abnormal involuntary movements.

In the proposed experiments of this specific aim, the effects of zolpidem on L-DOPA-induced behavioral asymmetries were evaluated in unilaterally 6-OHDA-lesioned rats. As outlined in Chapter IV, we have devised and validated a novel and DA-correlated quantitative analysis of LID-like behavior in a translational rodent model of PD (BASS). In the studies presented within this Chapter, we utilized BASS to assess the effects of zolpidem on the manifestation of LID-like behaviors in unilaterally 6-OHDA-lesioned rats. Next, we assessed whether any potential effects of zolpidem on L-DOPA-induced behavioral asymmetry can be attributed to alterations in the production and release of DA in the lesioned striatum using *in vivo* microdialysis. Finally, we examined whether the presence of zolpidem potentially altered the impact of DA on behavioral asymmetry as measured by BASS.

5.2 Overview of Methods

5.2.1 Behavioral effects of zolpidem on L-DOPA-induced behavioral asymmetry

The overall goal of this experiment was to determine whether zolpidem exerted an anti-dyskinetic effect, and whether this effect depended on dose. Adult, male Sprague-Dawley rats (n = 25) were first subjected to unilateral 6-OHDA lesion procedures (see Section 2.2.1). Behavioral testing commenced approximately 3-6 weeks following lesion. Briefly, animals were pretreated with benserazide (15 mg/kg, i.p.) 30 min prior to receiving an i.p. injection of L-DOPA (10 mg/kg). Animals assigned to zolpidem/vehicle groups also received an i.p. injection of zolpidem (0.1, 0.5 mg/kg) or vehicle (1% acetic acid) 40 min post-DOPA. The reason for this delay was to ensure that t_{max} for both L-DOPA-derived DA efflux and zolpidem were coincident (Durand *et al*, 1992). Each animal was tested only once. Videos were scored for rotational behavior and time spent displaying unilateral stereotypy within 5 min time blocks, every 10 min. In order to ensure that variability in L-DOPA offset is not falsely attributed to zolpidem, we analyzed a time window lasting 60 min from zolpidem administration (40-90 min post-DOPA).

5.2.2 Neurochemical effects of zolpidem on L-DOPA-induced DA efflux

As outlined below, there is possibility that zolpidem may exert anti-dyskinesia effects via a reduction in L-DOPA-derived striatal DA efflux. We therefore aimed to test this possibility using *in vivo* microdialysis. Animals (n = 13) were rendered parkinsonian via unilateral infusion of 6-OHDA. Approximately 3-8 weeks following lesion

procedures, animals were implanted with a microdialysis probe into the lesioned dorsal striatum. Testing commenced following a 24 h recovery period.

On testing day, animals habituated to the dimly-lit test setting for ≥ 60 min. Then, baseline dialysate samples were collected over the course of 1 h (15 min samples, n = 4 samples). Animals that produced samples with detectible levels of DA during the baseline period were considered to be insufficiently lesioned, and were not tested further. Animals with confirmed lesions were then pretreated with benserazide (15 mg/kg, i.p.) and given 10 mg/kg L-DOPA 30 min later. Those assigned to the zolpidem group also received an additional i.p. injection of 0.5 mg/kg zolpidem. This timing was chosen in order to test whether zolpidem affected pharmacokinetics/pharmacodynamics of L-DOPA. Dialysate samples were collected every 15 min over 3 h post-DOPA (n = 12 samples), and immediately analyzed for DA, DOPAC, and 5-HIAA content (see Section 2.4). Animals were tested once. Behavior was video recorded from above, and was analyzed offline. The recorded behavior was analyzed rotational and time spent displaying stereotypy behaviors in 5 min blocks at 15 min intervals, coinciding with the central 5 min of dialysate sample collection (i.e., 5-10 min, 20-25 min, etc. post-DOPA; see below). These behavioral parameters were inserted into the BASS formula, and resulting A-Scores were also compared between groups.

5.3 Results

5.3.1 Behavioral effects of levodopa/zolpidem administration

All experimental conditions resulted in robust rotational and dyskinesia-like behavior (**Fig 5.1a**). Analysis of rotational behavior using Friedman's nonparametric ANOVA for repeated measures revealed a significant main effect in the L-DOPA (χ^2 = 107.2, $p = 4 \times 10^{-15}$), L-DOPA + Veh (χ^2 = 75.05, $p = 2.87 \times 10^{-9}$), L-DOPA + 0.1 ZOL (χ^2 = 74.66, $p = 3.36 \times 10^{-9}$), and L-DOPA + 0.5 ZOL (χ^2 = 87.4, $p = 1.81 \times 10^{-11}$) conditions. This was also the case for dyskinesia-like behavior (L-DOPA: χ^2 = 109.8, $p = 10^{-15}$; L-DOPA + Veh: χ^2 = 73.5, $p = 5.34 \times 10^{-9}$; L-DOPA + 0.1 ZOL: χ^2 = 89.36, $p = 7.97 \times 10^{-13}$; L-DOPA + 0.5 ZOL: χ^2 = 91.33, $p = 3.49 \times 10^{-12}$). Pharmacodynamics were further explored using *post hoc* Dunn's tests, comparing each time point to a behavioral baseline (10-15 min pre-DOPA).

In the L-DOPA condition, we observed a statistically significant increase in rotational behavior from 20-70 min post-DOPA ($p_{20} = 3.11 \times 10^{-4}$, p_{30} = 0.002, p_{40} = 0.008, p_{50} = 0.001, p_{60} = 0.005, p_{70} = 0.014) and dyskinesia-like behavior 30-100 min post-DOPA (p_{30} = 0.0098, p_{40} = 0.001, $p_{50} = 9.59 \times 10^{-4}$, $p_{60} = 1.47 \times 10^{-4}$, p_{70} = 0.001, $p_{80} = 6.76 \times 10^{-5}$, $p_{90} = 1.82 \times 10^{-4}$, $p_{100} = 5.79 \times 10^{-4}$). The L-DOPA/Veh condition yielded statistically significant increases in rotational behavior 30-70 min post-DOPA (p_{30} = 0.0318, p_{40} = 0.0046, p_{60} = 0.0191, p_{70} = 0.0191), with the exception of the 50 min bin (p_{50} = 0.0572). Increases in dyskinesia-like stereotypy reached statistical significance 50 through 100 min post-DOPA (p_{50} = 0.0172, p_{60} = 0.0112, p_{70} = 0.0081, p_{80} = 0.0018, p_{90} = 0.009, p_{100} = 0.0428).

In the DOPA/0.1 ZOL condition, we also observed a statistically significant increase in rotational behavior 30-70 min (p_{30} = 0.0418, p_{40} = 0.0382, p_{60} = 0.0291, p_{70} = 0.0136), and stereotypy 40 through 100 min (p_{40} = 0.0009, p_{50} = 0.0013, p_{60} = 0.0061, p_{70} = 0.0136, p_{80} = 0.005, p_{90} = 0.0092, p_{100} = 0.0136) post-DOPA. In the DOPA/0.5 ZOL condition, there was a statistically significant increase in rotational behavior 30 min (p_{30} = 0.0012) as well as 70 to 100 min post-DOPA (p_{70} = 0.0075, p_{80} = 0.0023, p_{90} = 0.0136, p_{100} = 0.0457). Analysis of dyskinesia-like stereotypy within this condition also yielded a statistically significant increase 40-110 min (p_{40} = 0.0005, p_{50} = 4.93×10^{-5}, p_{60} = 0.0015, p_{70} = 0.005, p_{80} = 0.0075, p_{90} = 0.0457, p_{100} = 0.0242, p_{110} = 0.0242) post-DOPA.

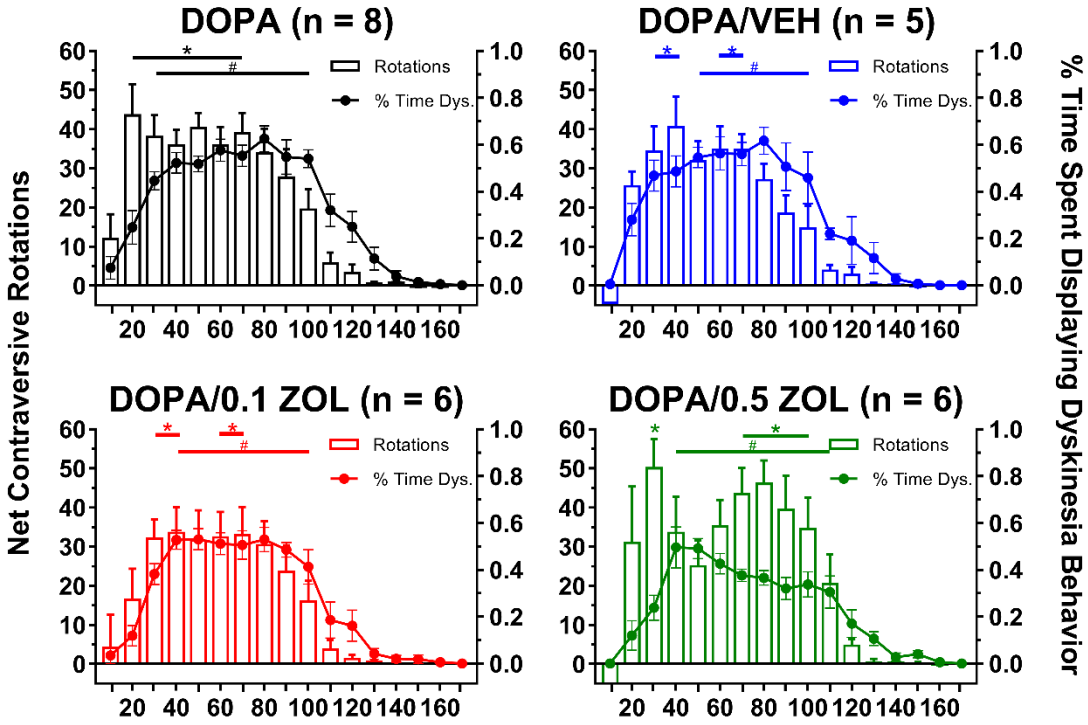


FIGURE 5.1. **L-DOPA-induced rotational behavior and time spent displaying dyskinesia-like behavior by experimental condition**. Animals (n = 25) were pretreated with benserazide (15 mg/kg, i.p.) 30 min prior to receiving an i.p. injection of L-DOPA (10 mg/kg). Animals assigned to zolpidem/vehicle groups also received an i.p. injection of zolpidem (0.1, 0.5 mg/kg) or vehicle (1% acetic acid) 40 min post-DOPA. Plots show net contraversive rotations and % time spent displaying dyskinesia behavior by treatment, over course of experiment (180 min). All conditions yielded statistically significant increases in both rotational and dyskinesia-like behavior. Bars represent mean ± *SEM* net contraversive rotations, dot/lines represent % time displaying dyskinesia behavior (% Time Dys). Top symbols/lines denote time bins with statistically significant difference compared to baseline: * denotes significance for rotations, # for dyskinesia behavior ($p < 0.05$).

5.3.2 Effects of zolpidem on rotational behavior

We investigated whether zolpidem influenced rotational behavior, illustrated in **Fig 5.2**. Analysis of net contraversive rotations using a Repeated Measures Two-Way ANOVA revealed a statistically significant main effect of time ($F_{5, 105} = 4$, $p = 0.0023$), but not dose ($F_{3, 21} = 0.3096$, $p = 0.8182$). As a result, dose interactions within the analysis window (40-90 min post-DOPA) were not analyzed further.

We also analyzed mean rotational response within the analysis window. A One-Way ANOVA failed to find a statistically significant main effect of condition ($F_{3, 21} = 0.2692$, $p = 0.8469$).

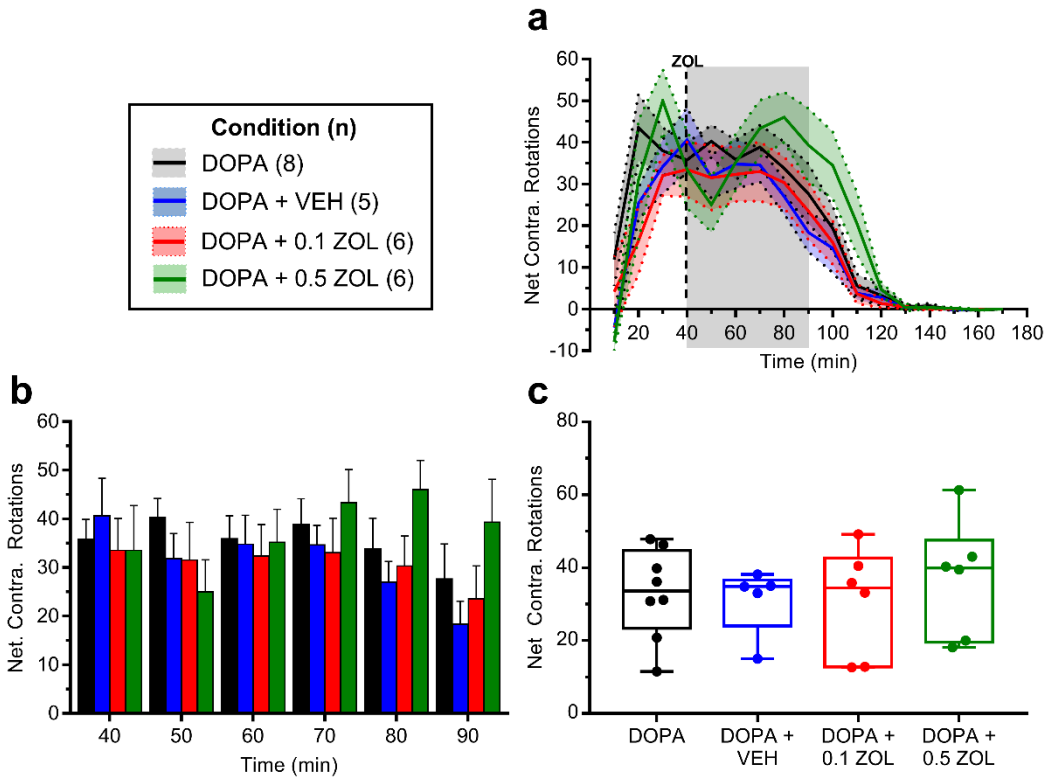


FIGURE 5.2. **Effects of zolpidem on net contraversive rotations. (a)** Net contraversive rotations by treatment. Lines represent mean rotations at given time point, shaded regions represent *SEM*. Gray box indicates analysis window (40-90 min post-DOPA). **(b)** Comparison of rotational behavior at each time point in analysis window. Bars represent mean ± *SEM*. **(c)** Mean overall net contraversive rotations by treatment. Box plot illustrates minimum to maximum mean rotations for each condition. Dots represent each animal.

5.3.3 Zolpidem reduced dyskinesia-like stereotypy

We then sought to determine whether zolpidem had an effect on amount of time spent displaying dyskinesia-like stereotypy, shown in **Fig 5.3a**. Analysis using a Repeated Measures Two-Way ANOVA revealed a statistically significant main effect of dose ($F_{3, 21} = 3.779$, $p = 0.0259$), but not time ($F_{5, 105} = 1.423$, $p = 0.222$). Further dose interactions were explored using Bonferroni-corrected multiple comparisons.

Interactions between experimental conditions are displayed in **Fig 5.3b**. As expected, we found no difference between DOPA and DOPA/Veh conditions. There was also no statistically significant difference between the 0.1 ZOL condition and DOPA or DOPA/Veh conditions. We did observe a statistically significant difference between the 0.5 ZOL and DOPA conditions 70-90 min ($p_{70} = 0.0497$, $p_{80} = 0.0015$, $p_{90} = 0.0067$) post-DOPA. However, there was only a significant difference between 0.5 ZOL and DOPA/Veh conditions 80 min post-DOPA ($p_{70} = 0.0779$, $p_{80} = 0.0076$, $p_{90} = 0.073$).

We also sought to determine whether there was a difference in mean time spent displaying dyskinesia-like stereotypy during the analysis window. A One-Way ANOVA failed to find a statistically significant main effect of drug treatment on this parameter ($F_{3, 21} = 2.803$, $p = 0.0648$; **Fig 5.3c**).

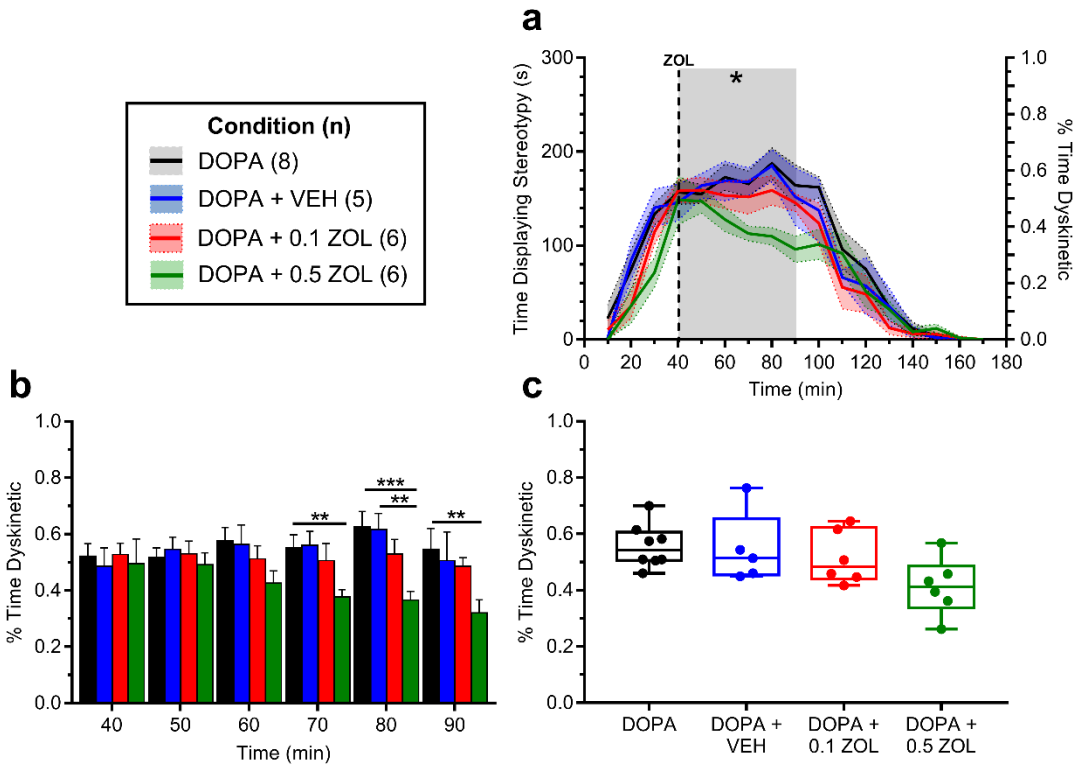


FIGURE 5.3. **Effects of zolpidem on time spent displaying dyskinesia-like behavior. (a)** Time spent displaying dyskinesia-like behavior by treatment. Lines represent mean time dyskinetic at given time point, shaded regions represent *SEM*. Gray box indicates analysis window (40-90 min post-DOPA). **(b)** Comparison of % time spent displaying dyskinesia-like behavior at each time point in analysis window. Bars represent mean ± *SEM*. Above lines and symbols indicate statistically significant interactions. **(c)** Mean % time dyskinetic by treatment. Box plot illustrates minimum to maximum mean % time dyskinetic for each condition. Dots represent each animal. Symbols indicate significant interactions: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$. Color of * represents comparator condition **(c)**.

5.3.4 Zolpidem reduced overall behavioral asymmetry

As explored and described in Chapter IV of this dissertation, we have established a novel quantitative analysis method: the BASS. Our BASS method is a single unified scale that incorporates both rotational behavior and dyskinesia-like stereotypy. Other benefits to using this method include a tight correlation with striatal DA efflux, and a higher degree of sensitivity in discriminating severity of L-DOPA-induced behavioral asymmetry compared to rotational behavior or time spent displaying stereotypy alone.

As a result, we used this method to examine the effects of zolpidem on this behavior. Overall A-Scores for each condition are shown in **Fig 5.4a**. Analysis of A-Scores using a Repeated Measure Two-Way ANOVA yielded statistically significant main effects for both time ($F_{5, 105} = 3.459$, $p = 0.0062$), and dose ($F_{3, 21} = 6.148$, $p = 0.0036$). Further dose interactions were explored using multiple comparisons *post hoc* testing (Bonferroni).

Again, we found no difference between the DOPA and DOPA/Veh conditions at any time point. This was also the case when the 0.1 ZOL condition was compared to both DOPA and DOPA/Veh conditions. A dose of 0.5 mg/kg zolpidem resulted in a statistically significant reduction in A-Scores compared to L-DOPA alone 60-90 min post-DOPA ($p_{60} = 0.0093$, $p_{70} = 0.0094$, $p_{80} = 0.0004$, $p_{90} = 0.0034$). However, a statistically significant reduction in A-Score was restricted to 70-80 min post-DOPA when compared to the vehicle condition ($p_{60} = 0.0614$, $p_{70} = 0.0364$, $p_{80} = 0.0154$, $p_{90} = 0.3256$).

As with rotational behavior and dyskinesia-like stereotypy, we also compared the mean A-Scores within the analysis window across experimental conditions. Mean (± *SEM*) A-Scores in each condition were as follows: 0.8233 ± 0.028 (DOPA), 0.7793 ± 0.056 (DOPA/Veh), 0.7168 ± 0.031 (DOPA/0.1 ZOL), 0.617 ± 0.039 (DOPA/0.5 ZOL). A One-Way ANOVA of mean A-Scores yielded a statistically significant main effect ($F_{3, 21} = 6.148$, $p = 0.0036$). Bonferroni-corrected multiple comparisons *post hoc* testing revealed a statistically significant reduction in mean A-Scores in the 0.5 ZOL condition, compared to both DOPA ($p = 0.0023$) and vehicle ($p = 0.0425$) conditions. There were no other statistically significant comparisons. In sum, these data suggest that a dose of 0.5 mg/kg zolpidem reduced overall L-DOPA-induced behavioral asymmetry.

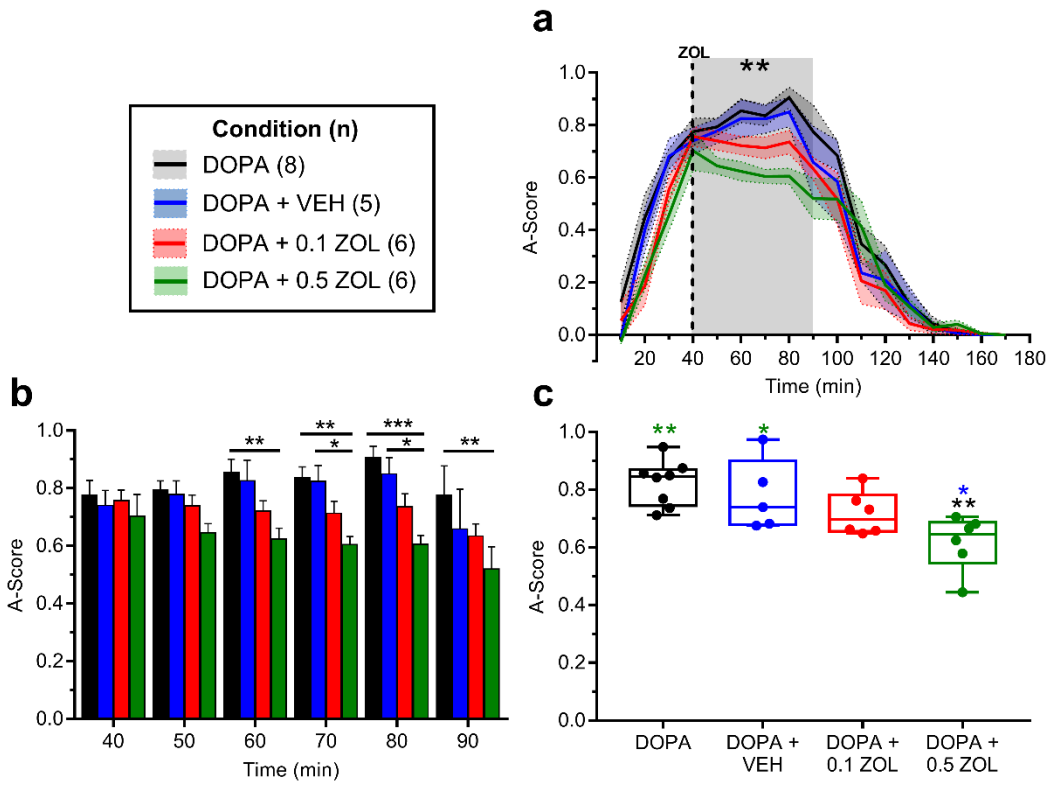


FIGURE 5.4. **Effects of zolpidem on A-Scores. (a)** A-Scores by treatment. Lines represent mean A-Scores at given time point, shaded regions represent *SEM*. Gray box indicates analysis window (40-90 min post-DOPA). **(b)** Comparison of A-Scores at each time point in analysis window. Bars represent mean ± *SEM*. Above lines and symbols indicate statistically significant interactions. **(c)** Mean A-Score by treatment. Box plot illustrates minimum to maximum mean A-Score for each condition. Dots represent each animal. Symbols indicate significant interactions: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$. Color of * represents comparator condition **(c)**.

5.3.5 Zolpidem did not alter levodopa-induced striatal dopamine efflux

As we demonstrated in Chapter IV, L-DOPA-induced behavioral asymmetry is highly correlated with DA efflux within the lesioned striatum. It follows that a drug with anti-dyskinetic properties may potentially exert this influence via a reduction in the conversion or release of L-DOPA-derived DA from striatal 5-HT terminals. Given the expression of zolpidem-sensitive $GABA_A$ receptors within the raphe nuclei, a zolpidem-induced reduction in striatal DA efflux—resulting in reduced L-DOPA-induced behavioral asymmetry—must be ruled out.

Similar to our finding in Section 4.3.8, co-administration of L-DOPA (10 mg/kg) and zolpidem (0.5 mg/kg) resulted in robust increases in striatal DA (**Fig 5.5**). Analysis of DA efflux over the course of the experiment using a One-Way Repeated Measures ANOVA (Geisser-Greenhouse correction) yielded a statistically significant main effect ($F_{1.486,\ 7.43} = 50.24$, $p = 7.65 \times 10^{-5}$; **Fig 5.5a**). We then compared each time point to mean baseline DA efflux using multiple comparisons *post hoc* tests (Bonferroni). It was found that DA efflux remained increased at a statistically significant level from 15 to 165 min post-DOPA ($p_{15\text{-}30} = 0.0265$, $p_{30\text{-}45} = 0.003$, $p_{45\text{-}60} = 7.69 \times 10^{-6}$, $p_{60\text{-}75} = 0.0007$, $p_{75\text{-}90} = 0.0004$, $p_{90\text{-}105} = 0.0007$, $p_{105\text{-}120} = 0.0022$, $p_{120\text{-}135} = 0.0012$, $p_{135\text{-}150} = 0.0007$, $p_{150\text{-}165} = 0.0013$).

Similar results were found for DOPAC. Analysis of DOPAC efflux over the course of the experiment using a One-Way Repeated Measures ANOVA yielded a statistically significant main effect ($F_{1.461,\ 7.306} = 33.72$, $p = 3.2 \times 10^{-4}$; **Fig 5.5b**). We then compared each time point to mean baseline DOPAC efflux using Bonferroni-corrected multiple comparisons *post hoc* tests. It was found that DOPAC efflux remained

increased at statistically significant levels from 45 to 180 min post-DOPA ($p_{45\text{-}60}$ = 0.0278, $p_{60\text{-}75}$ = 0.0081, $p_{75\text{-}90}$ = 0.0024, $p_{90\text{-}105}$ = 0.002, $p_{105\text{-}120}$ = 0.0073, $p_{120\text{-}135}$ = 0.0019, $p_{135\text{-}150}$ = 0.0014, $p_{150\text{-}165}$ = 0.0011, $p_{165\text{-}180}$ = 0.0024).

Analysis of 5-HIAA efflux over the course of the experiment failed to find a statistically significant main effect ($F_{1.3,\ 6.499}$ = 0.9494, p = 0.3924; **Fig 5.5c**). As a result, the relationship between 5-HIAA efflux and L-DOPA-induced behavioral asymmetry was not investigated further.

To determine whether zolpidem altered DA, DOPAC, or 5-HIAA efflux, we compared the *in vivo* microdialysis data presented here to that obtained in Chapter IV (**Fig 4.5**). A Repeated Measures Two-Way ANOVA failed to find a statistically significant main effect of drug treatment for DA ($F_{1,\ 10}$ = 0.6514, p = 0.4384; **Fig 5.6a**), DOPAC ($F_{1,\ 10}$ = 1.628, p = 0.2308; **Fig 5.6b**), nor 5-HIAA ($F_{1,\ 10}$ = 0.4624, p = 0.5119; **Fig 5.6c**). This data suggests that it is unlikely that the zolpidem-induced changes in L-DOPA-induced behavioral asymmetry described above can be attributed to changes in the conversion, release, nor metabolism of L-DOPA-derived striatal DA.

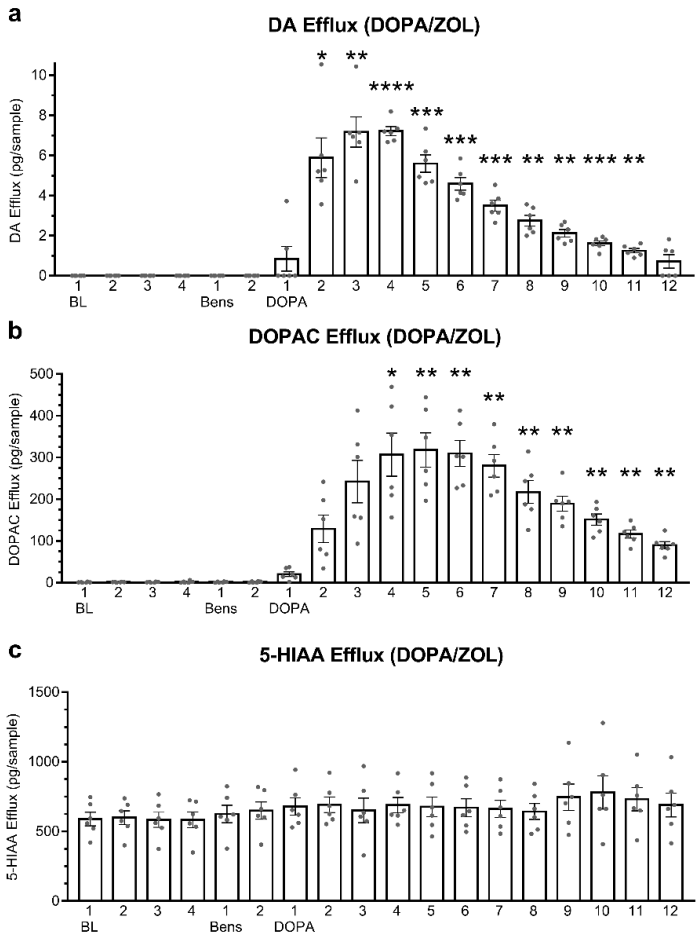


FIGURE 5.5. **L-DOPA-induced DA, DOPAC, and 5-HIAA efflux following concomitant administration of zolpidem.** Animals (n = 6) were implanted with microdialysis probes in dorsolateral striatum. Following 1 h baseline sample collection (BL1 - 4), animals were pretreated (Bens 1 – 2) and given L-DOPA (10 mg/kg, i.p.) concomitantly with zolpidem (0.5 mg/kg, i.p.). Samples (20 µL) were collected every 15 min over 3 h post-DOPA (DOPA 1 – 12) and immediately analyzed via HPLC-ED. Repeated Measures ANOVAs found statistically significant increases in DA **(a)** and DOPAC **(b)** efflux, but not 5-HIAA **(c)**. Bars represent mean ± *SEM*. Symbols indicate statistically significant interactions (compared to baseline): * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

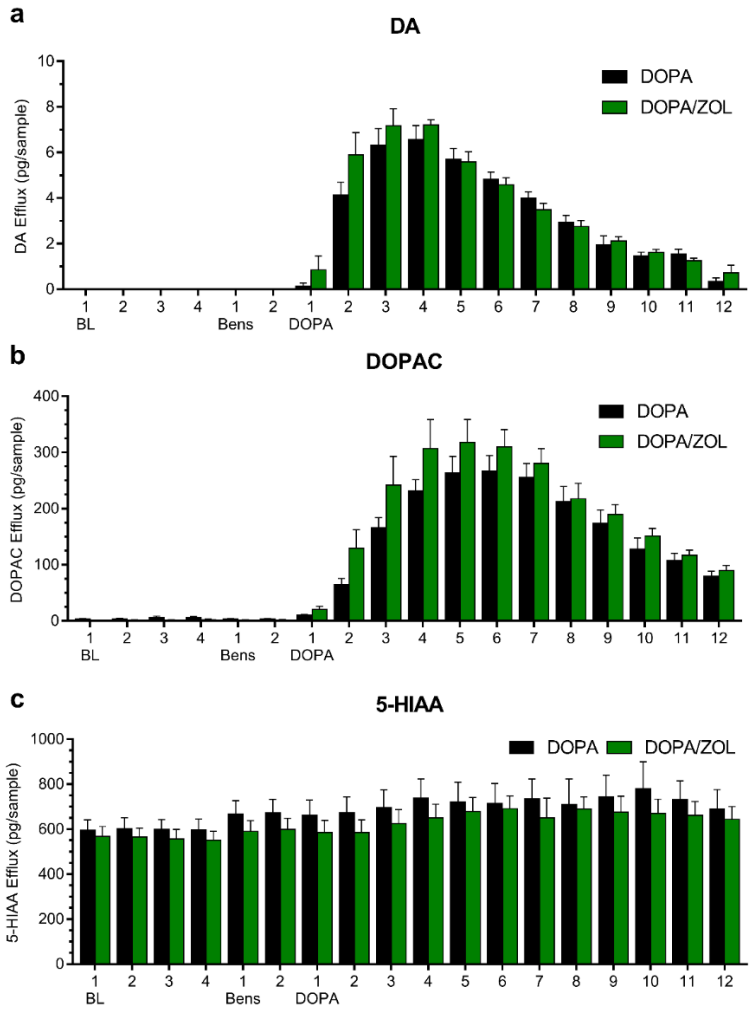


FIGURE 5.6. **Comparisons of DA, DOPAC, and 5-HIAA efflux.** Animals (n = 13) were implanted with microdialysis probes in dorsolateral striatum. Following 1 h baseline sample collection (BL1 - 4), animals were pretreated (Bens 1 – 2) and given L-DOPA (10 mg/kg, i.p.; n = 7; black bars), or L-DOPA concomitantly with zolpidem (0.5 mg/kg, i.p.; n = 6; green bars). Samples (20 µL) were collected every 15 min over 3 h post-DOPA (DOPA 1 – 12) and immediately analyzed via HPLC-ED. Repeated Measures Two-Way ANOVAs failed to find a statistically significant difference in DA **(a),** DOPAC **(b)**, nor 5-HIAA **(c)** efflux between conditions. Bars represent mean ± *SEM*.

5.3.6 Concomitant administration of zolpidem altered levodopa-induced rotations

Our experimental design also provided an opportunity to assess the utility of concomitant administration of zolpidem on L-DOPA-induced behavioral asymmetry Analysis of rotational behavior using a Repeated Measures Two-Way ANOVA revealed a statistically significant main effect of both time ($F_{13, 130} = 9.749$, $p = 5.9 \times 10^{-14}$) and dose ($F_{1, 10} = 5.238$, $p = 0.0451$**; Fig 5.7a**). The dose effect was further deconstructed using Bonferroni-corrected multiple comparisons at each time point post-injection. The sole time bin at which statistically significant difference we observed was at 90-105 min post-injection ($p_{90\text{-}105} = 0.0366$; **Fig 5.7d**).

We also compared mean rotational behavior across the entirety of the post-injection period. For this analysis, mean net contraversive rotations over the course of the experiment (for each animal) served as the dependent variable. A Mann-Whitney U test comparing mean rotational behavior between experimental conditions failed to find a statistically significant difference ($p = 0.0714$, **Fig 5.7b**).

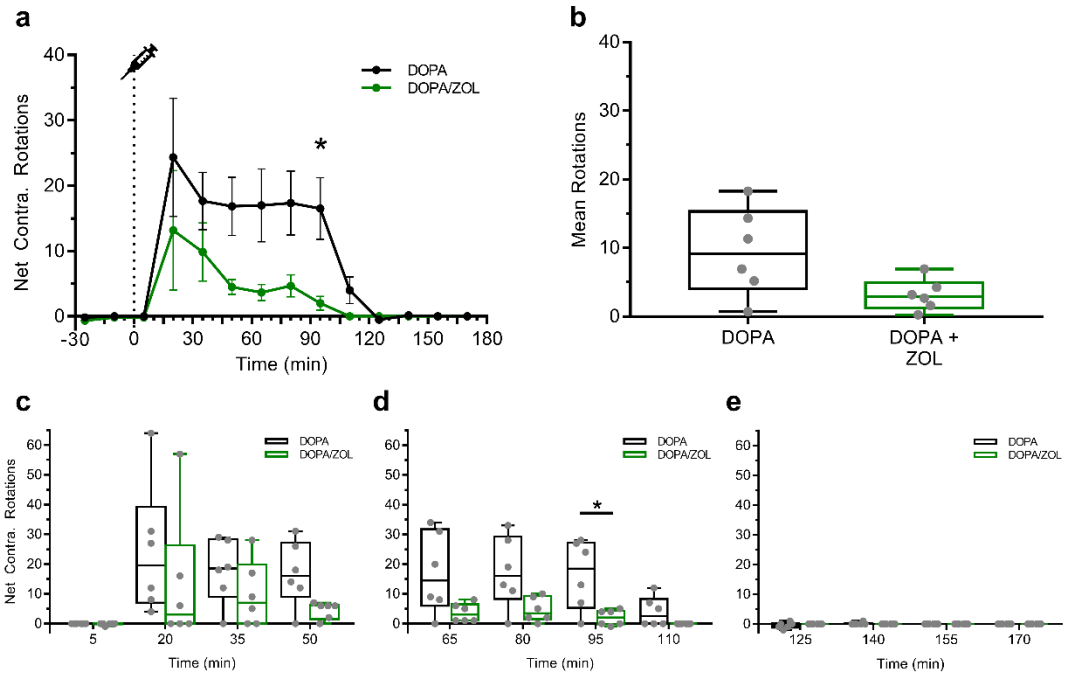


FIGURE 5.7. **Effects of concomitant administration of zolpidem on net contraversive rotations**. Behavior was analyzed over 5 min, every 15 min, coinciding with the central 5 min of each dialysate sample. **(a)** Net contraversive rotations by treatment, over course of experiment. Vertical dotted line denotes time of L-DOPA/zolpidem administration (i.p.). Lines and symbols represent mean net contraversive rotations at given time point, error bars represent *SEM*. **(b)** Mean overall net contraversive rotations by treatment. Box plot illustrates minimum to maximum mean rotations for each condition. Dots represent each animal. **(c-e)** Number of net contraversive rotations at each analyzed time point. **(c)** Hour 1, **(d)** Hour 2, **(e)** Hour 3. Box plot illustrates minimum to maximum net contraversive rotations. Dots represent each animal. Symbols indicate statistically significant interactions: * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$.

5.3.7 Concomitant administration of zolpidem did not alter dyskinesia-like stereotypy nor overall levodopa-induced behavioral asymmetry

A Repeated Measures Two-Way ANOVA of time spent displaying dyskinesia-like stereotypy failed to find a statistically significant main effect of treatment ($F_{1, 10}$ = 0.0566, p = 0.8167; **Fig 5.8a**), but did find an effect of time ($F_{11, 110}$ = 40.06, $p < 10^{-15}$). Similarly, analysis of A-Scores failed to find a significant main effect of treatment ($F_{1, 10}$ = 0.7934, p = 0.394, **Fig 5.9a**). There was, however, a statistically significant main effect of time ($F_{11, 110}$ = 54.66, $p < 10^{-15}$).

As with rotational behavior, we also sought to examine whether zolpidem affected mean time spent displaying dyskinesia-like behavior. A Mann-Whitney U test of comparing DOPA and DOPA/ZOL conditions failed to find a statistically significant difference ($p > 0.99$; **Fig 5.8b**). A similar analysis of mean A-Score over the course of the post-injection period also failed to find a statistically significant difference (p = 0.9372; **Fig 5.9b**).

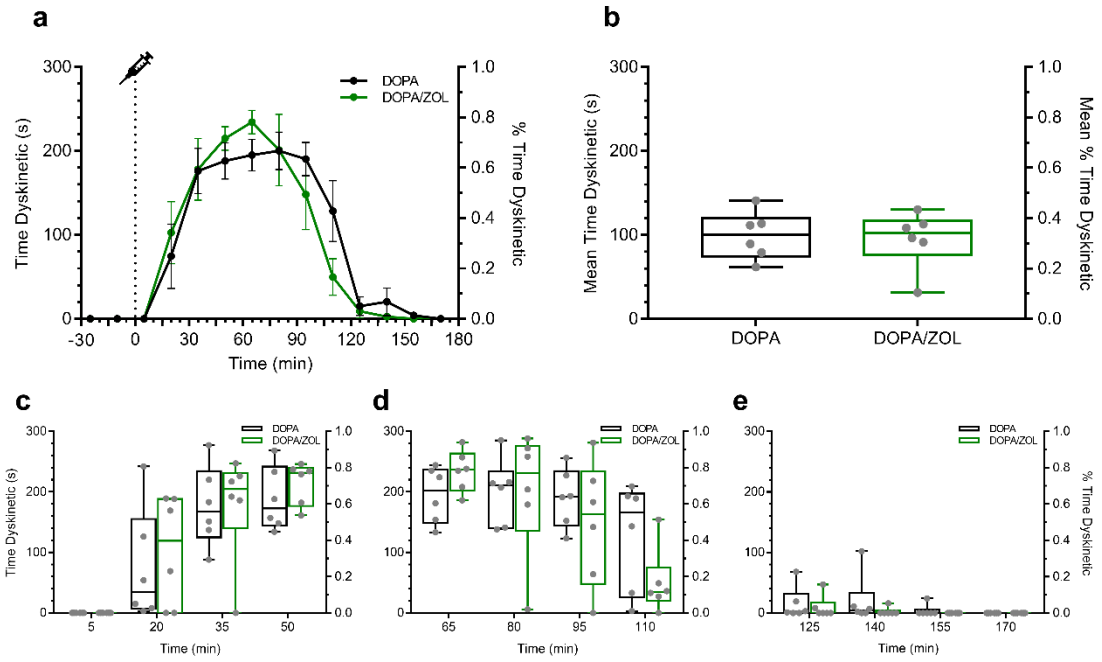


FIGURE 5.8. **Effects of concomitant administration of zolpidem on time spent displaying dyskinesia-like behavior**. Behavior was analyzed over 5 min, every 15 min, coinciding with the central 5 min of each dialysate sample. **(a)** Time spent displaying dyskinesia-like behavior by treatment, over course of experiment. Vertical dotted line denotes time of L-DOPA/zolpidem administration (i.p.). Lines and symbols represent mean time spent displaying dyskinesia-like behavior at given time point, error bars represent *SEM*. **(b)** Mean time spent displaying dyskinesia-like behavior by treatment. Box plot illustrates minimum to maximum mean time spent displaying dyskinesia-like behavior for each condition. Dots represent each animal. **(c-e)** Time spent displaying dyskinesia-like behavior at each analyzed time point. **(c)** Hour 1, **(d)** Hour 2, **(e)** Hour 3. Box plot illustrates minimum to maximum time spent displaying dyskinesia-like behavior. Dots represent each animal.

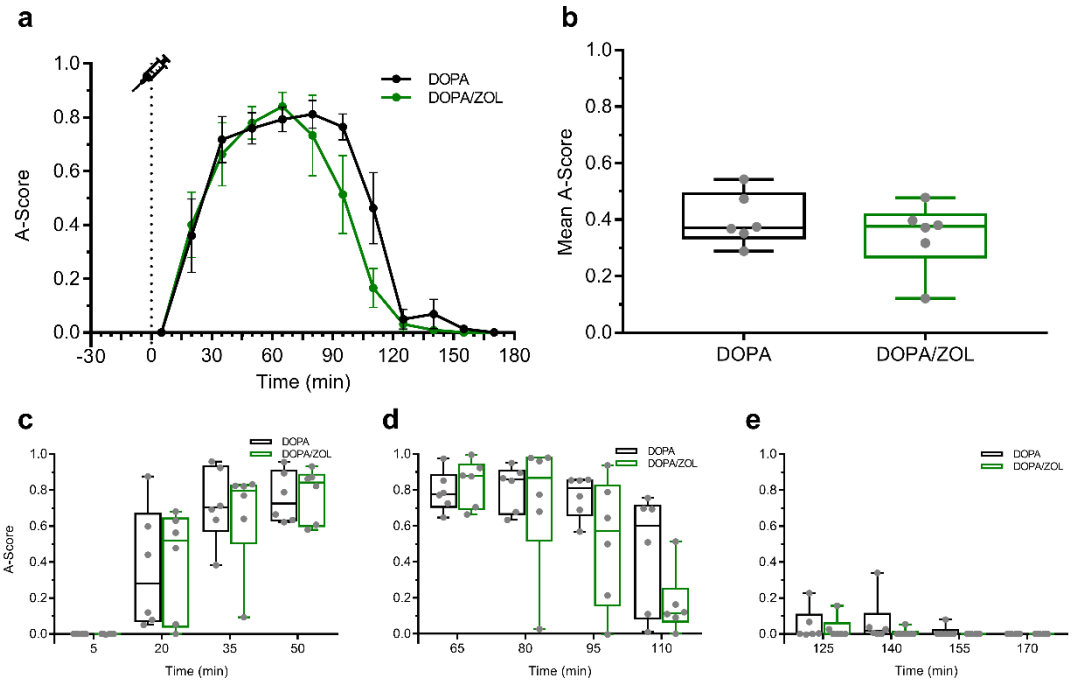


FIGURE 5.9. **Effects of concomitant administration of zolpidem on A-Score**. Behavior was analyzed over 5 min, every 15 min, coinciding with the central 5 min of each dialysate sample. **(a)** A-Score by treatment, over course of experiment. Vertical dotted line denotes time of L-DOPA/zolpidem administration (i.p.). Lines and symbols represent mean A-Score at given time point, error bars represent *SEM*. **(b)** Mean A-Score by treatment. Box plot illustrates minimum to maximum mean A-Score for each condition. Dots represent each animal. **(c-e)** A-Score at each analyzed time point. **(c)** Hour 1, **(d)** Hour 2, **(e)** Hour 3. Box plot illustrates minimum to maximum A-Score. Dots represent each animal.

5.3.7 Zolpidem altered the relationship between striatal dopamine and levodopa-induced behavioral asymmetry

The data described above show that concomitant administration of 0.5 mg/kg zolpidem with 10 mg/kg L-DOPA did not alter overall behavioral asymmetry. Still, the possibility remains that zolpidem altered the impact of striatal DA on this behavior. For example, zolpidem appears to have altered the pharmacokinetics/pharmacodynamics of the L-DOPA response. Indeed, peak DA efflux shifted earlier from 45-60 min to 30-45 min post-DOPA (**Fig 5.6a**). It should be noted, however, that DA efflux remained comparable 45-60 min post-DOPA in the zolpidem condition.

Zolpidem also appears to have shifted the peaks of L-DOPA-induced behavior, as well as the relationship of that peak to peak striatal DA efflux. Latency to peak rotational behavior did not change (**Fig 5.10a**), but concomitant administration of zolpidem shifted the latency to peak dyskinesia-like behavior from 45-60 min (**Fig 4.6**) to 60-75 min post-DOPA (**Fig 5.10c**). Similarly, peak A-Score occurred 60-75 min post-DOPA in the zolpidem condition (**Fig 5.10e**), compared to 45-60 min following L-DOPA alone (**Fig 4.6e**). This is remarkable, given the shift in striatal DA efflux to an earlier time bin. This indicates that zolpidem exerted an influence on the impact of L-DOPA-derived DA efflux, which is further explored below.

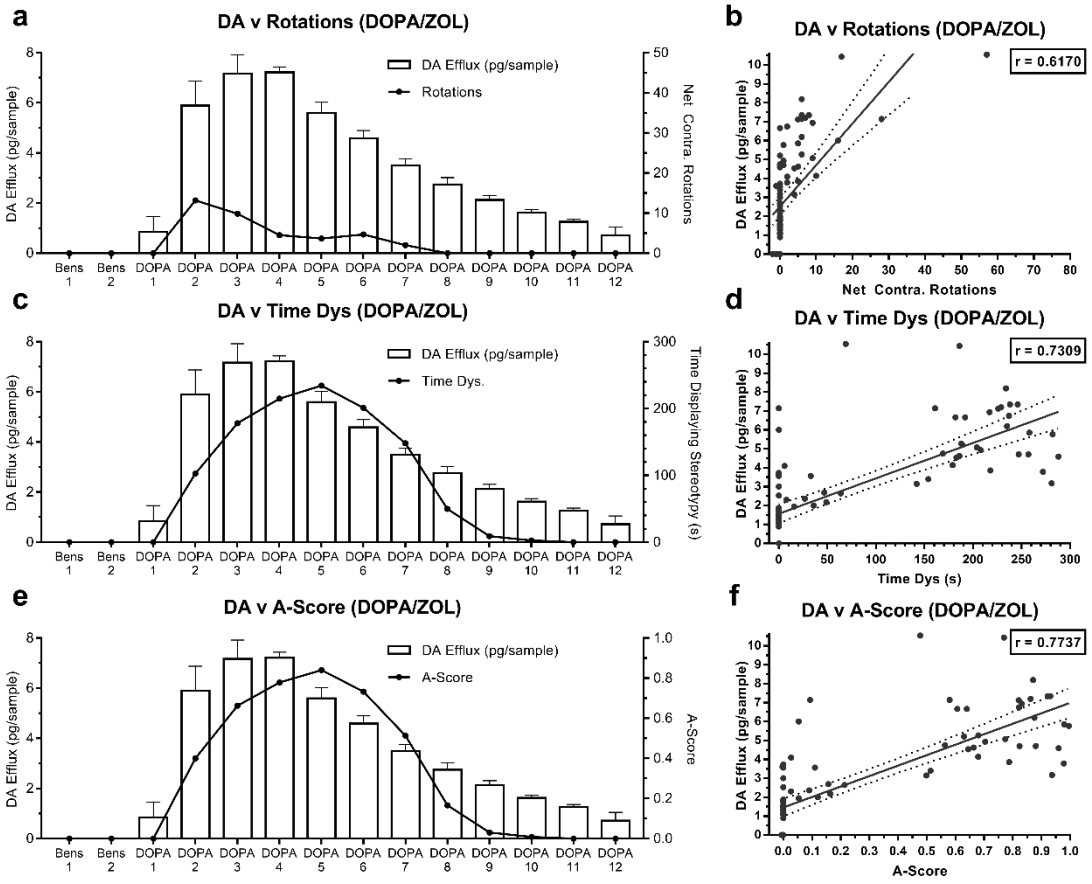


FIGURE 5.10. **Relationship between DA efflux and L-DOPA-induced behavioral asymmetry following L-DOPA and zolpidem. (a, c, e)** DA efflux versus rotational behavior **(a)**, dyskinesia behavior **(c)**, and A-Score **(e)** over course of experiment. **(b, d, f)** Correlational analysis of the relationship between L-DOPA-induced DA efflux and net contraversive rotations **(b)**, time spent displaying dyskinesia-like behavior **(d)**, and A-Score **(e)**. All correlations were statistically significant ($p < 0.05$). Lines represent best fit for the relationship; dotted lines denote 95% confidence interval for the fit line.

To investigate whether zolpidem opposed the effects of L-DOPA-derived DA on overall behavioral asymmetry, we assessed the relationship between these parameters. We found that concomitant administration of zolpidem with L-DOPA resulted in a reduction in the relationship between striatal DA efflux and rotations ($r = 0.6170$, $p = 4.12 \times 10^{-10}$, **Fig 5.10b**), as well as time spent displaying dyskinesia-like stereotypy ($r = 0.7309$, $p = 3 \times 10^{-15}$, **Fig 5.10d**). When time bins in which there 0 recorded rotations were removed, the correlation coefficient actually increased ($r = 0.6332$, $p = 0.0004$). In contrast, when time bins in which we observed no time spent displaying dyskinesia-like behavior were removed the relationship became weaker ($r = 0.4537$, $p = 0.0033$). Interestingly, addition of zolpidem also resulted in a reduction in the relationship between A-Score and DA efflux ($r = 0.7737$, $p < 10^{-15}$, **Fig 5.10f**). Similar to dyskinesia-like behavior, when time bins in which we observed an A-Score of 0 were removed, the strength of this relationship decreased ($r = 0.7171$, $p = 1.82 \times 10^{-11}$).

Finally, we sought to directly compare the two experimental condition in order to ascertain whether zolpidem altered the influence of striatal DA efflux on overall behavioral asymmetry. A direct comparison of the two experimental conditions using an Analysis of Covariance supported this hypothesis. The slopes of best-fit lines showed were not significantly different ($p = 0.1177$, **Fig 5.11**). However, a comparison of the elevations/y-intercepts of the best-fit lines revealed that zolpidem resulted in a statistically significant reduction in elevation ($p = 0.0416$).

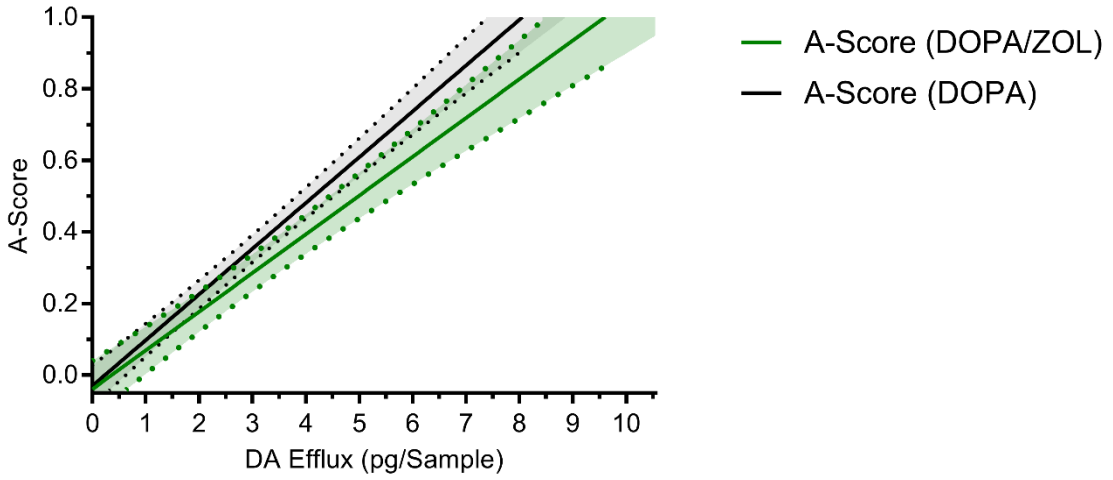


FIGURE 5.11. **Zolpidem reduced the influence of L-DOPA derived striatal DA on behavioral asymmetry**. Comparison of lines of best fit (DA vs. A-Score) in DOPA and DOPA/ZOL conditions using ANCOVA revealed that zolpidem reduced the slope of best fit line, indicating an opposition to the effects of striatal DA on behavioral asymmetry. Lines represent best fit for the relationship; dotted lines and shaded regions denote 95% confidence interval for the best fit line.

5.4 Summary of Findings

The overall goal of the experiments described in this chapter was to determine the effect, if any, of zolpidem on the manifestation of behavioral asymmetry produced by L-DOPA in unilaterally 6-OHDA-lesioned rats. First, we found that delayed systemic administration of zolpidem dose-dependently reduced dyskinesia-like behavior as well as overall behavioral asymmetry as measured by our BASS assay. This was evident in the observed reduction in the amount of time spent displaying unilateral stereotypy, A-Scores, and overall mean A-Score. Given that we have observed a strong positive

relationship between L-DOPA-induced striatal DA efflux, we then sought to determine whether a zolpidem-induced reduction in striatal DA could explain this effect using *in vivo* microdialysis. We found that concomitant administration of zolpidem did not change striatal DA efflux. In contrast, this observation was accompanied by a lack of effect of zolpidem on behavioral asymmetry when administered concomitantly with L-DOPA, as measured by BASS. However, we also observed that zolpidem altered the relationship between L-DOPA-derived DA efflux and behavioral asymmetry, such that a larger amount of DA was needed to produce increased behavioral asymmetry in the zolpidem condition. Taken together, this data suggests that zolpidem may provide an opposing force on the impact of L-DOPA-derived DA on BG circuit output, as measured by overall behavioral asymmetry. Further. targeting of extrastriatal $GABA_A$ receptors with a drug such as zolpidem may compete against the circuit-level consequences of restored DAergic tone following DA denervation.

CHAPTER VI

GENERAL DISCUSSION

The experiments presented in this dissertation were designed to investigate whether zolpidem, a GABAergic PAM with selective affinity for extrastriatal BG nuclei, possesses antiparkinsonian and antidyskinesia properties. As a corollary, this work aimed to provide the first ever preclinical "proof of concept" evidence in the validation of the α_1-subunit expressing $GABA_A$ receptor as a potential target in the treatment of PD motor symptoms. These experiments also explored the relationship between striatal DA efflux and L-DOPA-induced behavioral asymmetry in a hemiparkinsonian rodent model. In doing so, we have validated a novel quantitative behavioral assay of L-DOPA-induced behaviors both pharmacologically and neurochemically.

First, the data in Chapter III indicate that zolpidem does indeed possess antiparkinsonian properties, as systemic administration of 0.1 mg/kg produced an improvement in both locomotor coordination and volitional movement. Next, the Behavioral Asymmetry Scoring System (BASS)—a novel behavioral assay designed in the course of completing this dissertation—was validated pharmacologically using a dose-response paradigm. BASS was shown to be superior to either rotational behavior or dyskinesia-like stereotypy alone in assessing severity of L-DOPA-induced behavioral asymmetry. Further, BASS was validated neurochemically, as this measure was found to be strongly correlated with L-DOPA-derived DA efflux in the lesioned neostriatum. We also found correlations between striatal DA with both rotational behavior and dyskinesia-like stereotypy. Finally, using BASS, we found that 0.5 mg/kg zolpidem reduced L-DOPA-induced behavioral asymmetry in a delayed administration paradigm but not when

given concomitantly with L-DOPA. This could not be attributed to altered DA efflux in the lesioned neostriatum, as we found no difference between L-DOPA- and L-DOPA/zolpidem-treated animals. In contrast, we found that zolpidem may exert the observed antidyskinesia effect via a reduction in the impact of L-DOPA-derived DA on the manifestation of dyskinesia-like behavior.

6.1 Zolpidem dose-dependently compromised locomotion, but subsedative doses possessed antiparkinsonian properties

The experiments documented in Chapter III investigated whether zolpidem-sensitive $GABA_A$ receptors may serve as a potential novel target in the treatment of motor symptoms of PD. First, it was found that systemic administration of a zolpidem significantly impaired performance on rotarod at doses above 0.5 mg/kg, compared to an undrugged state. Accordingly, this value was used as a threshold for therapeutic dose investigation. It was also found that a subthreshold dose of zolpidem reduced motor dysfunction in 6-OHDA rodents using rotarod. This effect was dose-dependent in that therapeutic effects were limited to one of the tested doses (0.1 mg/kg), with a higher dose (0.5 mg/kg) resulting in a decrease in motor coordination. We also found that zolpidem (0.1 mg/kg) improved forelimb use symmetry in unilaterally lesioned 6-OHDA rodents following acute systemic administration. Notably, the overall number of forepaw contacts were similar in both zolpidem and vehicle conditions, indicating that the observed improvement was not due to increased "weight" of each individual contact in the zolpidem condition.

6.1.1 Increased sensitivity to zolpidem induced by lesion of nigrostriatal dopamine

When compared to the results obtained in intact rats, the data obtained from 6-OHDA-lesioned rats indicate an increased sensitivity to a dose of 0.5 mg/kg. Compensatory alterations in ambient GABA and $GABA_A$ receptor expression levels induced by DA depletion in the BG may play a role. For example, Pan *et al* (1985) showed that $GABA_A$ receptor binding is reduced in striatum and GPe but increased in BG output nuclei (EPN/GPi, SNr) 5 months after unilateral 6-OHDA lesion in rats. Similarly, $GABA_A$ receptor α_1-subunit mRNA has been shown to be reduced in GPe but upregulated in STN, EPN, and SNr 3-5 weeks following unilateral 6-OHDA lesion (Chadha *et al*, 2000; Yu *et al*, 2001). Investigations using *in vivo* microdialysis have shown that levels of ambient GABA are increased 2-fold in the BG as a consequence of 6-OHDA lesion (Bianchi *et al*, 2003). Interestingly, Ruano *et al* (1993) found that zolpidem binding increases as a function of ambient GABA concentration. Consequently, it is possible that DA depletion could increase the sensitivity of the BG to zolpidem, such that a dose that showed no discernible behavioral effect in intact rats (0.5 mg/kg) could negatively impact performance following 6-OHDA lesion.

It follows that PD patients may also possess an enhanced sensitivity to GABAergic drugs such as zolpidem. It also follows that the therapeutic dose likely varies between individuals, as some animals benefitted from both 0.1 and 0.25 mg/kg. Further, the one animal that was considered a “non-responder” in the rotarod study exhibited a dose-dependent decrease in proficiency. The possibility exists that this animal may have benefitted from a lower dose (such as 0.05 mg/kg) that was not tested in this study. Since this animal exhibited a similar degree of DA depletion to animals that

benefitted, this may also be mediated by individual differences in GABAergic compensation to the lesion. Conversely, there may be a subset of individuals that may not benefit from zolpidem at all. As such, clinical studies should take this into consideration when designing dosing regimens since the length of disease course—and thus, degree of DA depletion—could potentially influence the effect of larger doses and mask any therapeutic effects.

6.1.2 Antiparkinsonian properties of zolpidem supported by clinical observations

To date, the face validity of this concept has not been thoroughly investigated, and direct translational insights are absent in the literature. In PD patients, single-patient and small-cohort case studies have shown that subhypnotic doses of zolpidem reduce Unified Parkinson's Disease Rating Scale (UPDRS) scores and reduce dyskinetic side effects associated with DA replacement (Daniele *et al*, 1997; Ruzicka *et al*, 2000; Chen and Sy, 2008; reviewed in Daniele *et al*, 2016). More recently, Hall *et al* (2014) reported that zolpidem improves UPDRS scores and modifies cortical beta oscillations in early-stage PD patients. Combined with the results presented here, the existing literature supports the hypothesis that zolpidem may indeed be a valid pharmacological intervention for PD, particularly when unwanted side effects of DA replacement therapies become problematic.

6.1.3 Validity of the unilateral 6-hydroxydopamine lesion model

For an animal model to be considered comparable to the condition observed in humans, thus allowing the findings in that model to be readily translatable to the clinic, specific criteria of validity must be satisfied. Among these subtypes, face, predictive, and construct validity are of most importance. Face validity requires that the deficits observed in the animal model resemble those in the clinic. In the case of PD, a valid animal model would present robust locomotor deficits including difficulty initiating movement, bradykinesia, as well as some non-motor deficits in cognition and gastrointestinal function. Indeed, it has been well-described that unilaterally 6-OHDA lesioned rats display all of these symptoms (Iancu *et al*, 2005; Glajch *et al*, 2012; Carvalho *et al*, 2013; Campos *et al*, 2013; Toti and Travagli, 2014; reviewed in Schwarting and Huston, 1996b; Deumens *et al*, 2002; Tieu, 2011). Furthermore, these motor symptoms have been shown to manifest as a direct result of DA denervation as well as pathophysiological changes in the BG circuit later observed in the PD patient population (Section 1.3.5).

To be considered to have predictive validity, an animal model must respond to known treatments for the disease. In other words, are the clinical benefits of known efficacious treatments also observed in this model? Satisfaction of these criteria not only solidify translatability of treatment, but also lend credence to the hypothesis that the prospective model and clinical population share a similar pathophysiology. This appears to be the case, as the unilateral 6-OHDA model responds favorably to subdyskinetic doses of L-DOPA (~2 mg/kg; Winkler *et al*, 2002). In fact, studies have used L-DOPA as a positive control variable in assessing the efficacy of novel compounds (Iderberg *et*

al, 2015a, b). As described in detail in Section 1.4, this animal model has also been shown repeatedly to manifest similar dyskinetic side effects of DA replacement therapies.

The most common criticism of the unilateral 6-OHDA lesion model of PD is an apparent lack of construct validity. For an animal model to be considered to have construct validity, the underlying pathology to be addressed should manifest in a manner similar to that observed in the clinic. For example, a valid translational model of the flu would require the manifestation of flu-like symptoms as a result of exposure to a flu virus. An issue that arises in the study of PD is that the mechanisms by which SNc neurons are lost in PD is not completely known. We do know, of course, that PD patients have not been administered 6-OHDA intracerebrally. It is rarely noted by those dismissing this model, however, that 6-OHDA can occur naturally and has been observed in both the caudate and the urine of PD patients (Curtius *et al*, 1974; Andrew *et al*, 1993). Combined with the known intracellular mechanisms of 6-OHDA outlined above, as well as the evidence for a role of Ca^{2+}-related neurotoxicity in both α-synuclein animal models and idiopathic PD patients, the 6-OHDA lesion model cannot be thrown out in a wholesale manner due to a lack of construct validity (Parihar *et al*, 2008; Surmeier *et al*, 2017ab; Ludtmann and Abramov, 2018).

On the contrary, it must be acknowledged that the rapid onset of SNc neuronal death does not accurately model the progressive nature of clinical PD. As described in Section 1.3.1, SNc neurodegeneration can begin >10 years prior to the emergence of PD motor symptoms. From this, it could be argued that PD manifests from slowly developing abnormalities in nigrostriatal input, and that this cannot be accurately mimicked in an acute lesion model. This argument, however, conflicts with several lines

of evidence: First, SNc neurodegeneration appears to follow an exponential decay function (Fearnley and Lees, 1991; Darmier *et al*, 1999; reviewed in Biju and de la Fuente-Fernandez; 2009). Second, the nigrostriatal DA system appears to be able to compensate for nigral cell loss until a critical threshold is met (Bernheimer *et al*, 1973; Robinson and Whishaw, 1988; de la Fuente-Fernandez *et al*, 2001; Assous *et al*, 2014; Section 1.3.3 above). It is not until this point, when the BG circuit is truly and irreversibly DA deprived, that PD motor symptoms emerge both in the clinic and 6-OHDA model. Second, the neurophysiological hallmarks of PD motor symptoms are directly related to said deficits (Section 1.3.5). Further, some rodent models said to mimic the progressive nature of SNc neurodegeneration and slowly accumulating locomotor phenotype fail to show these hallmarks, despite showing locomotor deficits likened to those of PD (Lobb *et al*, 2013).

Based on these insights, it can be argued that there is a threshold level of nigrostriatal denervation necessary to trigger endogenously irreversible DA depletion within the BG. Subsequently, the neurophysiological consequences of chronic DA depletion trigger compensatory mechanisms in amino acid neurotransmitter systems (discussed in Section 1.5.4). A new stasis is reached, which manifests behaviorally as the parkinsonian motor phenotype. Based on this, the 6-OHDA lesioned rodent could be considered an ideal and valid translational model for PD motor symptoms.

6.2 BASS effectively measures severity of levodopa-induced behavioral asymmetry

We have devised a novel approach in quantifying L-DOPA-induced behavioral asymmetry in unilaterally 6-OHDA-lesioned rats: the BASS method. In the studies outlined in Chapter IV, the data indicate that this assay can discriminate varying levels behavioral asymmetry as a function of dose. BASS was also found to be strongly correlated with L-DOPA-induced striatal DA efflux, making this measure unique among more commonly utilized assays. Of the 3 methods tested herein, both % time displaying dyskinesia-like behavior and BASS were superior to rotational behavior alone in discriminating doses. It was also found that BASS was superior to dyskinesia-like behavior in predicting this dose-response relationship, which was reflected in our investigation of the relationships between these measures and L-DOPA-induced increases in striatal DA efflux. In sum, we have shown that BASS can be used as a quantitative and DA-correlated means of measuring L-DOPA-induced behavioral asymmetry in unilaterally 6-OHDA-lesioned rodents.

BASS effectively weights L-DOPA-induced changes in behavioral output, placing emphasis on dyskinesia-like behavior and taking rate of rotational response into account by isolating the possible time available for rotation. This is critical, since the number of contraversive rotations and the time spent displaying axial and limb-based abnormal involuntary movements appear to be negatively correlated (**Fig 4.1b**). Our observations indicate that rotational behavior cannot occur simultaneously with axial or limb-based abnormal involuntary movements, thus estimating/quantifying the relative

amount of time displaying each behavior during a given epoch—as in commonly-utilized assays—cannot accurately describe L-DOPA-induced behavioral asymmetry.

6.2.1 BASS highlights the relationship between dopamine and dyskinesia

The necessity for this behavioral weighting becomes clearer if one were to compare L-DOPA-induced behavioral asymmetry to amphetamine-induced behaviors in intact rodents. DA-related motor behaviors seem to manifest in a dose-dependent continuum of responses, such that low doses of amphetamine induce a hyperlocomotive/hyperkinetic state. As the dose increases, intact rodents display some hyperlocomotive behavior, but motor stereotypies (i.e. grooming behaviors) occur with increasing frequency (Sharp *et al*, 1987). This interpretation can also be applied to L-DOPA-induced behavioral responses in the unilaterally lesioned rat, such that lower doses result in predominantly hyperlocomotive activity (i.e. rotational behavior) and higher doses result in the more robust manifestation of motor stereotypies (i.e. dyskinesia-like behavior).

The data presented here reflect this interpretation, with a dose of 5 mg/kg L-DOPA resulting in a similar amount of net contraversive rotations compared to higher doses. Similarly, a 5 mg/kg dose resulted in less time displaying dyskinesia-like behavior, which resulted in a lower overall A-Score than higher doses tested. Had rotational behavior been treated with equal weight to dyskinesia-like stereotypy, an inflated A-Score would result, thus reducing the sensitivity of the measure to dosing. If rotations had been treated similarly to the currently used rating scales—with time spent

displaying rotational behavior being the dependent measure—an artificially inflated score would result since the animal would be exhibiting rotational behavior for the majority of the time scored.

Our data investigating the latency to onset of each component behavior, as well as the relationship between these behaviors and striatal DA efflux, also yield additional insight. As described above, the emergence of rotational behavior consistently preceded the onset of dyskinesia-like stereotypy (**Fig 4.1c**). Additionally, peak rotational behavior consistently preceded both peak stereotypy as well as peak striatal DA efflux (**Fig 4.6**). Further, we observed a linear relationship between onset latency for both component behaviors and dose, such that larger doses resulted in a more immediate behavioral onset. These findings are consistent with those of unilateral electrical stimulation of the nigrostriatal projection, such that the threshold for rotational behavior was greater than that for DA-mediated increased in sniffing behavior (Arbuthnott & Ungerstedt, 1975). It follows that directed sniffing, rotational behavior/hyperlocomotion, and dyskinesia-like stereotypy are likely distinct behaviors that represent an underlying continuum, progressing as a function of the circuit-wide consequences of DA receptor stimulation within the BG circuit.

6.2.2 Is the hemiparkinsonan rodent a valid model of levodopa-induced dyskinesia?

As discussed in Section 6.1.3, there is an extremely large body of research validating the use of hemiparkinsonian rodents in the study of the neural correlates of PD motor symptoms. The study of LID in this rodent model, however, has been the subject

of intense debate. It has been argued that the sole quantifiable response to L-DOPA in this model is rotational behavior, and that other L-DOPA-induced involuntary movements are incompatible with motor abnormalities observed in patients and primates (Chase, 1998; Bezard *et al*, 2001; reviewed in Cenci *et al*, 2002).

Several lines of evidence contradict this view. First, the time course for L-DOPA-induced behavioral asymmetry and peak-dose dyskinesia in PD patients are very similar. The data presented in Chapter IV illustrate that the onset of both rotations and LID-like stereotypy rely on a rapid rise in striatal DA, and that peak stereotypy occurs coincident with peak DA (**Fig 4.6**). Second, as described in Chapter I, metabolic studies have shown concordance between the neurophysiological correlates of LID in both rodents and primates. Additionally, chronic administration of subthreshold doses of L-DOPA have been shown to gradually induce behavioral asymmetry in 6-OHDA-lesioned rodents (Winkler *et al*, 2002). Considering also that the manifestation of behavioral asymmetry is contingent on sufficient DA lesion, and is dependent on the administration of L-DOPA, this would likely satisfy the criteria for construct validity. Third, compounds that have shown antidyskinesia properties in PD patients and primates have also shown similar effects in rodents (Lundblad *et al*, 2002; Breger *et al*, 2016; Tamte *et al*, 2016; Chapter V). These findings lend credence to the predictive validity of this model.

The predominant issue taken with rodent models of LID is an apparent lack of face validity in this model. It is typically argued that there is a lack of physical similarity in L-DOPA-induced behaviors in rodents and choreic dyskinesia in PD patients, such that those observed in rodents constitute stereotypy (i.e. purposeless repetitive movements)

and not chorea (Bezard *et al*, 2001). Regardless of qualitative description, however, behaviors elicited by robust DA receptor stimulation have been described as similar between rodents and humans. For example, punding behaviors—also purposeless repetitive movements—have been observed in both cocaine users and L-DOPA-treated PD patients (Rylander, 1972; Evans *et al*, 2004). Cocaine-induced stereotypy, including head bobbing, sniffing, and grooming, has been shown to be mediated by similar neural circuitry in rodents as in humans despite a vast disparity in their appearance (Aliane *et al*, 2009). It follows that although these behaviors appear dissimilar, the underlying neural mechanisms are not (Canales and Graybiel, 2000). Therefore, this rodent model is clearly useful in the study of LID.

6.3 Zolpidem opposed dyskinesia, but only following delayed administration

Using our novel assay, validated in Chapter IV, the experiments described in Chapter V aimed to test the antidyskinesia potential of zolpidem. First, potential antidyskinesia properties were tested in a vehicle-controlled study using a delayed administration paradigm (45 min post-DOPA). This delay was chosen to ensure that the maximal in-brain concentration of both zolpidem and L-DOPA-derived DA were coincident (Durand *et al*, 1992; Trenque *et al*, 1994; Tamte *et al*, 2016). Zolpidem was found to dose-dependently reduce both the amount of time spent displaying dyskinesia-like behaviors and overall A-Scores, such that a dose of 0.5 mg/kg produced statistically significant reduction. This dose (0.5 mg/kg) also produced a statistically significant reduction in mean A-Scores compared to both DOPA and DOPA/VEH conditions. A

dose of 0.1 mg/kg also produced a reduction in both dyskinesia-like behavior and A-Scores, but this did not reach statistically significant levels.

In Chapter IV, the data show that there is a strong correlation between A-Scores and L-DOPA-derived striatal DA efflux. Knowing this, as well as the expression of zolpidem-sensitive $GABA_A$ receptors in the raphe nuclei, we sought to rule out the possibility that the zolpidem-induced reduction in A-Scores was attributable to reduced DA efflux using *in vivo* microdialysis (Pirker *et al*, 2000). In this experiment, zolpidem (0.5 mg/kg) and L-DOPA (10 mg/kg) were given concomitantly. First, it was found that there was no difference in striatal DA, DOPAC, or 5-HIAA efflux between conditions. It cannot be ruled out, however, that striatal DA efflux following lower doses of L-DOPA could be altered by 0.5 mg/kg zolpidem.

With respect to L-DOPA-induced behavioral asymmetry, it was found that zolpidem significantly reduced net number of contralateral rotations, but did not alter time spent displaying dyskinesia-like behaviors nor A-Scores. This experimental design also provided the unique opportunity to assess whether zolpidem opposed the influence of DA on L-DOPA-induced behavioral asymmetry. In comparing the relationships between striatal DA efflux and A-Scores across conditions, it was found that zolpidem reduced the impact of DA on these behaviors. In other words, it required more DA to produce a similar A-Score compared to L-DOPA alone.

6.3.1 Conflicting evidence concerning the role of subthalamic nucleus in dyskinesia

As reviewed in Chapter I, the role of STN in the manifestation of PD motor symptoms has been well described. According to the literature outlined in Section 1.3.5, the role of the cortico-subthalamic pathway appears to be to provide excitation to BG output nuclei, acting as a potent opposing force to striatonigral influences. The net result of this excitation, if BG output nuclei respond with an increase in activity, is the inhibition of a movement or a cancellation of a planned movement (Schmidt *et al*, 2013, Dunovan *et al*, 2015). In PD, NMDA-mediated cortico-subthalamic hyperexcitation propagates to BG output nuclei, resulting in a bradykinetic/akinetic locomotor phenotype (Tai *et al*, 2012; Pan *et al*, 2014; Bhattacharya *et al*, 2018). It follows logically that excessive inhibition of STN would likely result in a hyperkinetic phenotype.

Indeed, work by Wichmann and colleagues (1994) showed that pharmacological inhibition of primate STN resulted in dyskinesia behavior. Considering the effects of local DA agonist infusion, DA-mediated inhibition of STN neuronal activity supports this circuit model (Hasani and Feger, 1999). Further, it is well known that drugs such as cocaine and amphetamine, which increase extracellular DA, produce hyperlocomotor and stereotypy behavior (Sharp *et al*, 1987). From this, it can be proposed that LID-like behavior likely manifests as a result of excessive DA-mediated inhibition of the cortico-subthalamic pathway, leaving the potent inhibition of BG output nuclei unchecked.

While this makes sense from a circuit level perspective, it conflicts with much of the literature outlined in Chapter V. For example, metabolic studies have found that VA/VL regions of the thalamus—which receive dense GABAergic projections from GPi—show marked reductions in 2-deoxyglucose (2-DG) activity in MPTP-treated

primates that exhibited LID-like behaviors versus those that did not (Mitchell *et al*, 1992). Similarly, Trugman (1995) found that systemic administration of L-DOPA induced increases in 2-DG levels in EPN, SNr, as well as STN in unilaterally 6-OHDA-lesioned rats. Similarly, a study using *in vivo* microdialysis found that acute systemic L-DOPA administration elicited significant increases in extracellular glutamate levels in the SNr of unilaterally 6-OHDA-lesioned rats (Robelet, 2004). Further, optogenetic and pharmacological inactivation of STN has been shown to reduce DA agonist/precursor related behavioral asymmetries in unilaterally 6-OHDA-lesioned rodents (Petri *et al*, 2013; Yoon *et al*, 2016).

Thus, it makes sense that a GABAergic PAM such as zolpidem reduced L-DOPA-induced behavioral asymmetry, as we observed. Yet, this is ostensibly incompatible with BG circuit models, the actions of DA in STN, and the effects of inhibition of STN in primates. How can enhancing inhibition in STN, which has been associated with hyperkinesia/dyskinesia, also reduce the hyperlocomotive effects of L-DOPA-derived DA?

An explanation of this phenomenon may lie in the combination of GABAergic control of the STN by GPe, as well as the intrinsic membrane properties of these neurons. Neurons in the STN have been shown repeatedly to be capable of rebound bursting following periods of potent inhibition (Beurrier *et al*, 1999; Bevan *et al*, 2000, 2002). These bursts appear to be mediated by voltage-gated Ca^{2+} channels ($Ca_v1.2$-1.3, Ca_v3), as well as Ca^{2+}-mediated K^+ conductances (SK channels; Hallworth *et al*., 2003). It is well known that the GABAergic projections from GPe are well capable of altering pacemaking activity as well as the inhibition of STN neurons altogether (Hallworth and

Bevan, 2005). Interestingly, GABAergic IPSPs from these GPe afferents have been shown to have an equilibrium potential that is sufficiently hyperpolarized to trigger such rebound bursts upon their offset (Bevan *et al*, 2000). Combined with the observed effects of DA in GPe, as well as the observed relationship between LID behavior and excitation of GPe/GPi, it is possible that GPe and DA-mediated inhibition of STN produce an exacerbated pause-burst pattern (Boraud *et al*, 1998; Boraud *et al*, 2001; Mamad et al, 2015). In this pattern, periods of inhibition may encourage bouts of dyskinesia, while rebound bursts may revert the behavioral influence of the BG to bouts of rotational behavior or, even further, a normalization of the interhemispheric balance in BG output. In turn, the observed zolpidem-mediated reduction in L-DOPA-induced behavioral asymmetry may rely on the potentiation of striatopallidal synapses, reducing the inhibitory influence of GPe afferents. This has not been directly studied, to our knowledge, and therefore cannot be confirmed until direct manipulations of these variables are conducted.

6.4 Debate concerning the mechanism of action of zolpidem

It is generally hypothesized that drugs selective for the benzodiazepine site act as a partial agonist, or positive allosteric modulator, at the $GABA_A$ receptor. In this case, binding at the benzodiazepine site induces an alteration in the conformation of the subunit structures, with an end result of potentiated GABA-dependent IPSCs. This differs from full agonists at this receptor, like muscimol, that can activate/open the Cl^- channel independent of GABA. Alternatively, it has been proposed that some partial agonists may exert an effect by enhancing the binding affinity of GABA for the $GABA_A$

receptor. This has been a topic of debate in the case of zolpidem, as there is conflicting evidence in the literature.

Perrais and Ropert (1999) recorded rat layer V pyramidal neurons *ex vivo*, and found that zolpidem (10 μM) did not increase the frequency of IPSCs, confirming a post-synaptic mechanism. Further, they found that zolpidem increased both the amplitude and duration of IPSCs. In an outside-out patch configuration, zolpidem again enhanced IPSC amplitudes and durations in response to nonsaturating pulses of GABA (100 and 300 μM), but remained constant at when GABA was saturated (10 mM). From this, they concluded that zolpidem did not enhance the probability of channel opening, but that the increase in both IPSC amplitude and duration is attributable to an enhanced affinity of GABA for the zolpidem-bound $GABA_A$ receptor. These findings and subsequent interpretations were echoed—in a cell-type-specific manner—in similar recordings of various mouse cell types (Hajos *et al*, 2000).

The conclusions of Perrais and Ropert, echoed by Hajos *et al*, are difficult to reconcile with that found within zolpidem-sensitive BG nuclei. First, each study outlined in Section 1.6.2 concluded that zolpidem has no effect on the number of synaptic events (Chen *et al*, 2004; 2007; Zhang *et al*, 2008). This can be considered evidence against the increased affinity hypothesis, such that more events would be expected. Alternatively, the possibility remains that the presence of zolpidem at the benzodiazepine site may enhance the strength of the bond between GABA and the $GABA_A$ receptor. Consequently, the necessity of increased interacting forces to dislodge bound GABA from the receptor could result in increased duration of Cl^- channel opening. This hypothesis could potentially provide additional insight regarding the observed increase in

IPSC amplitude within STN. Interestingly, the equilibrium potential of $GABA_A$ IPSCs is extremely hyperpolarized (-83 mV; Bevan *et al*, 2000; Bevan *et al*, 2007). In instances of increased duration of channel opening, the increased driving force resultant from a more hyperpolarized reversal potential may yield an increased IPSC amplitude.

6.5 Potential sites of action

The literature described in this dissertation has provided direct insight as to the most likely pathophysiology underlying PD motor symptoms. Aberrant cortico-subthalamic excitation likely propagates to BG output nuclei, increasing inhibition of thalamocortical disfacilitation of the cerebral cortex. Additionally, this excitation can also propagate to pallidonigral GPe neurons, potentially coactivating flexor and extensor muscles, resulting in a bradykinetic/akinetic locomotor phenotype. According to the "3 pathway race" hypothesis, a loss of DA-mediated inhibition within STN would increase the physiological salience of the cortico-subthalamic pathway and bias the responsiveness of BG output nuclei toward inputs from STN.

All of these extrastriatal BG nuclei express zolpidem-sensitive GABAA receptors. However, it is difficult to pinpoint whether the observed antiparkinsonian properties of zolpidem can be attributed to actions within a specific nucleus. Indeed, activation of $GABA_A$ receptors within either GPe or STN is sufficient to reduce PD motor symptoms (Tachibana et al, 2011). Knowing this, the possibility remains that the observed effects of zolpidem may in fact be mediated by effective coincident binding at all of the aforementioned sites, or perhaps any permutation of these regions. In fact, it has been

shown that direct brain stimulation of various BG nuclei can be successful in reversing motor symptoms (Hashimoto *et al*, 2003; Shi *et al*, 2006; Vitek *et al*, 2012; Cleary *et al*, 2013). Additionally, stimulation of other regions such as the zona incerta and pedunculopontine nucleus have been shown to possess therapeutic benefit (Plaha *et al*, 2006; Thevathasan *et al*, 2018). Interestingly, these regions are also zolpidem-sensitive (Pirker *et al*, 2000). Considering this, combined with the wide distribution of zolpidem-sensitive receptors throughout the central nervous system, the localization of a critical site of action will likely remain elusive without extensive future research.

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