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Attentional dysfunction in Parkinson's disease: The role of central amygdala dopamine and possible treatment options

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Attentional dysfunction in Parkinson's disease: The role of central amygdala dopamine and possible treatment options

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Dedication

To my parents for encouraging and supporting all of my pursuits.

To my sister for always being proud yet unsurprised.

To all of my teachers in all forms for fostering my curiosity.

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Attentional dysfunction in Parkinson's disease: The role of central amygdala dopamine and possible treatment options

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While it is known that Parkinson's disease (PD) results in motor dysfunction, there exist many cognitive symptoms including impairments in attention. PD patients demonstrate impaired ability to switch attention between tasks, rules, or stimuli, ignore irrelevant stimuli, and sustain attention to stimuli or tasks. Dysfunction of the mesocortical system is suggested to be responsible for these attentional deficits. However, attentional impairments surface in the early stages of the disease and cortical areas are among the last regions to show pathology. Further, it is not well understood how effective common dopamine replacement therapy (L-dopa) is in restoring attentional dysfunction. Recent work suggests that L-dopa may only improve some aspects of attentional function in PD, thus making further examination of the effects of L-dopa on attentional function important. And as L-dopa also has many other limitations (e.g. possible development of unwanted motor side effects), it is also necessary to investigate other possible treatments for these dysfunctions.

In this dissertation, I first examined the role of dopaminergic function in the central amygdala (CeA) in the regulation of attentional processes in rodents. I found that dopaminergic input into the CeA mediated by D1 receptors is necessary for attention switching (i.e. disengagement behavior) and selective and sustained attention in rodents.

Then I investigated the effects of L-dopa on these two different types of attentional deficits in a rodent model of PD in which dopamine is depleted unilaterally using the neurotoxin, 6-hydroxydopamine. While L-dopa was able to recover basic attentional switching, more complex attentional processes (i.e. selective and sustained attention) were not recovered. In an attempt to find a better treatment for these deficits, I used methylene blue (MB), a metabolic enhancer and antioxidant, to target mitochondrial dysfunction, a characteristic of all compromised dopamine cells. While MB was able to provide moderate neuroprotection in this model of PD, it was unable to recover attentional function. Taken together, my dissertation work demonstrates that attentional function is partly regulated by sub-cortical CeA dopamine mechanisms and that PD-related attentional dysfunction may require a multi-faceted treatment approach.

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Chapter 1: Background

Parkinson's disease (PD) is a neurodegenerative disorder that results in progressive cell loss in many brain regions. Most prominently, dopamine cells within the substantia nigra pars compacta (SNc) die, which is responsible for the hallmark motor dysfunctions including akinesia, rigidity, tremor, bradykinesia and postural instability (Fahn & Sulzer, 2004; Fahn, 2003). However, there are also a host of cognitive deficits in PD which include attentional impairments (Dubois & Pillon, 1997; Pfeiffer, Løkkegaard, Zoetmulder, Friberg, & Werdelin, 2014a; Weintraub et al., 2015; Wu et al., 2012). This attentional dysfunction can include difficulty in switching tasks or rules, sustaining attention, and selectively attending to relevant stimuli while ignoring irrelevant stimuli. It is not well understood how these problems with attention arise in PD. The neuropathological progression of the disease is such that areas commonly implicated in executive function (prefrontal cortex) do not show degeneration until the later stages of the disease while attentional impairments can occur very early on (Braak, Ghebremedhin, Rüb, Bratzke, & Del Tredici, 2004; Filoteo et al., 1997; Zhou et al., 2012). Therefore, it is logical to postulate that other areas affected in PD may cause these symptoms. In order to test this hypothesis, I first examined the potential involvement of the central amygdala dopamine system in attentional functions. Then, I investigated the nature of attentional deficits in a rat model of PD and the efficacy of traditional dopamine replacement therapy. Finally, I evaluated a potential novel treatment using the compound, methylene blue to globally enhance neurological function.

1.1 ATTENTIONAL DEFICITS IN PARKINSON'S DISEASE

It is common for patients with PD to exhibit non-dementia-related cognitive dysfunction. Sixty-five percent of patients show difficulty with at least one of the

following: executive function, working memory, and attention (Wu et al., 2012). Furthermore, almost half of these patients experience more than one of these dysfunctions. Impairments in attentional function are common in PD patients and are usually one of the first cognitive impairments to arise (Chaudhuri, Healy, & Schapira, 2006; Chaudhuri & Schapira, 2009). However, attention is usually impacted in several, specific ways. Patients most prominently display impairments in sustained attention, selective attention, and shifting attention (Wu et al., 2012). Anecdotally, patients with PD have reported difficulty in either stopping one task to start another or terminating attention to a stimulus to attend to another stimulus. This has been further corroborated by empirical work demonstrating that patients do indeed show impaired task switching behavior (Ravizza & Ciranni, 2002).

Further work has been done to assess the ability of PD patients to shift implicitly learned rules or strategies. This ability to shift response strategy or 'set' (also known as set-shifting) is usually tested in humans using the Wisconsin Card Sorting Task or a related variant. In these tasks, the participant implicitly learns a response rule and once proficient in this response strategy, the rule is changed (unbeknownst to the participant) such that now the patient must either apply the same rule to a new set of stimuli (i.e. intradimensional shifting) or shift to a new response rule requiring attention to another aspect/dimension of the stimuli (i.e. extradimensional shifting). PD patients generally show impairments in extradimensional shifting (shifting to a new response rule) and commonly perseverate on a previously learned rule instead of shifting attention to another dimension of the stimuli (Lees & Smith, 1983; Cools, Barker, Sahakian, & Robbins, 2001; Cools, Clark, Owen, & Robbins, 2002; Ravizza & Ciranni, 2002).

Another common attentional dysfunction in PD is an inability to sustain attention. First empirically reported in 1989, PD patients demonstrated "increased distractibility"

and “difficulty holding attention” relative to healthy controls (Levin, Llabre & Weiner, 1989). Others have expanded on these findings and have further shown that impairments in signal/stimulus detection increase relative to the length of the time delay from the beginning of the trial to the stimulus presentation (Zhou et al., 2012). In addition to sustaining attention to a stimulus, patients also show deficits in selective attention, that is attending only to specific stimuli while ignoring other irrelevant stimuli (Filoteo et al., 1997; Yamaguchi & Kobayashi, 1998). Zhou et al. (2012) found dysfunction of the ‘orienting network’ in which a person must orient or attend to stimuli before being able to respond but not in the ‘executive network’ (the ability to choose a response) in patients with mild PD. Others have shown similar findings of selective attention deficits and further note that these deficits can be seen in early-to-moderate PD especially when the attentional demands are high (Filoteo, et al., 1997; Yamaguchi & Kobayashi, 1998).

Deficits in set-shifting, selective attention and sustained attention can also predict freezing of gait severity, gait speed, and falls in PD patients (Allcock et al., 2009; Lord, Rochester, Hetherington, Allcock, & Burn, 2010; Naismith, Shine, & Lewis, 2010; Rochester et al., 2004; Shine et al., 2013; Smulders, Esselink, Bloem, & Cools, 2015; Stefanova et al., 2014). This suggests that understanding the underlying cause of attentional dysfunction in PD will not only lead to improved cognitive outcomes but also better treatment of some motor aspects of this disease.

1.2 Modeling attentional deficits in PD

Several attempts have been made to model cognitive dysfunction in animal models of PD. Most commonly, rodents or non-human primates are exposed to neurotoxins to specifically deplete dopaminergic cells. The neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP) and 6-hyrodroxydopamine (6-OHDA) are

generally used for this purpose. Both aggregate in dopaminergic cells, inhibit mitochondrial respiration thus causing an increase in oxidative stress and subsequently apoptosis (Glinka, Tipton, & Youdim, 1996; Glinka, Tipton, & Youdim, 1998; Kupsch et al., 2014; Mizuno et al., 1998; Nicklas, Vyas, & Heikkila, 1985). As expected, this model results in PD-like motor deficits (bradykinesia, akinesia, etc.) in both rodents and non-human primates. However, the extent to which this preparation impairs cognitive, and more specifically attentional dysfunction has been understudied.

There has been wide range of work examining the role of dopamine in attentional processing and it has been demonstrated that dopamine, especially in the striatum is necessary for selective and sustained attention (Agnoli & Mainolfi, 2012; Chudasama & Robbins, 2004; Domenger & Schwarting, 2006; Granon et al., 2000; Rogers, Baunez, Everitt, & Robbins, 2001; Winstanley et al., 2010). However while these studies imply the incursion of attentional dysfunction in dopamine depletion models of PD, very few studies have actually been conducted to examine both the cognitive and attentional deficits in animal models of PD. Most prominently, the effects of MPTP administration in macaques on attention have been studied. Originally it was demonstrated that MPTP exposure leads to cognitive and attentional deficits and that these deficits onset before motor dysfunction when MPTP is slowly administered over weeks (Schneider, Sun, & Roeltgen, 1994; Schneider & Kovelowski, 1990). And when specifically studying attention using a cued reaction time task using the same model, Decamp & Schneider (2004) found that performance was markedly impaired in monkeys receiving MPTP compared to monkeys that had not received the neurotoxin. Furthermore, in these same macaques, MPTP treatment caused mild impairments in set-shifting similar to the pattern of deficits seen in patients with PD. Additionally, other aspects of cognitive function including spatial working memory have been studied, and it has been demonstrated that

neurotoxin models of PD in rats and macaques do indeed impair cognitive function similar to that of human PD after controlling for motor deficits (Decamp & Schneider, 2004; Ramirez-Garcia et al., 2014).

1.3 Potential role of the central nucleus of the amygdala in PD-related attentional deficits

Currently, the etiology of these attentional deficits in PD is not well understood. The prefrontal cortex (PFC), a structure important for higher cognition, executive function and some attentional functions, is generally considered to be responsible for the attentional dysfunction in PD (Cools et al., 2002; Ravizza & Ciranni, 2002). Even though the PFC plays an important role in attentional processing, compromised PFC function may not be the reason for all of the attentional deficits seen in PD. Attentional dysfunction in PD normally occurs early in the disease progression while pathological changes in the PFC do not occur until later stages of the disease (Alafuzoff et al., 2009; Braak et al., 1994; Braak et al., 2004). Furthermore, attempts to recover cognitive function in PD by targeting the PFC specifically have proven ineffective in improving attentional symptoms (Lewis, Slabosz, Robbins, Barker, & Owen, 2005; Owen et al., 1993). For these reasons, another circuit impaired early in PD might be responsible for the attentional deficits in PD. One area that could be responsible for part of the attentional dysfunction in PD is a subnuclei of the amygdala, the central nucleus of the amygdala (CeA). Pathological changes including an increase in the presence of Lewy bodies and Lewy neurites in the amygdala and specifically the CeA are seen early in the progression of PD, much earlier than PFC pathology (Braak et al., 1994).

The CeA has been shown to be involved in several attentional processes in a rat model. Specifically, the CeA is necessary for conditioned orienting (behavioral manifestation of enhanced attention to biologically relevant cues; Chachich & Powell,

1998; Gallagher, Graham, & Holland, 1990), and surprise-induced enhancement of learning (Holland & Gallagher, 1993; Lee, Youn, Gallagher, & Holland, 2008;Lee, Youn, O, Gallagher, & Holland, 2006; Maddux, Kerfoot, Souvik, & Holland, 2007). Most relevantly, the CeA plays an important role in mediating selective and sustained attention (P C Holland, Han, & Gallagher, 2000; Maddux et al., 2007). To assess these aspects of attention, the five choice serial reaction time task is commonly used. In this task, the rat is required to attend to multiple locations in order to detect the stimulus. The rat is required to wait several seconds for the stimulus to appear (sustained attention) and must ignore irrelevant stimuli while waiting (selective attention). Lesions of the CeA in rats causes impairments in this selective and sustained attention task when the attentional load is high (Holland et al., 2000; Maddux et al., 2007). This pattern of deficits is similar to what is seen in PD. Patients with PD show some deficits in selective and sustained attention but these deficits are most pronounced when the attentional demand is high (Brown & Marsden, 1990; Maddox, Filoteo, Delis, & Salmon, 1996; Taylor, Saint-Cyr, & Lang, 1986).

Also important to consider is that the CeA has a large reciprocal connection with the SNc (Fudge & Haber, 2000). It is widely known that dopaminergic denervation of the SNc is the hallmark pathology of PD but it is mainly discussed within the context of the nigrostriatal pathway responsible for motor function. However, it is important to consider that CeA-dependent attentional processes discussed earlier are also dependent on the connection with the SNc (El-amamy & Holland, 2010; Lee, Youn, Gallagher, & Holland, 2008; Lee, Youn, O, Gallagher, & Holland, 2006). So it would be expected that depletion of SNc dopamine would result in the altered CeA function possibly leading to impaired attentional processing. Therefore, understanding the role of CeA dopamine

mechanisms in attentional processing is crucial for understanding the attentional deficits seen in PD.

1.4 USE OF L-DOPA FOR THE TREATMENT OF ATTENTIONAL DYSFUNCTION IN PD

Patients with PD are most frequently treated with Levodopa (L-dopa), the chemical precursor to dopamine. L-dopa administration is highly effective at treating the hallmark motor dysfunctions of the disorder, but the efficacy of L-dopa in ameliorating cognitive/attentional deficits is not well understood. Most consistently, improvement of set-shifting deficits is reported with L-dopa usage. Patients typically show improvement compared to counterparts off L-dopa as well as never-treated newly diagnosed patients (Hornykiewicz et al., 1974; Cools et al., 2001; Cools et al., 2002; Owens et al., 1993). It seems that working memory can also be improved by L-dopa however it is less consistently seen (Fuhrer et al., 2014; Lewis et al., 2005; Marini, Ramat, Ginestroni, & Paganini, 2003).

While deficits in set-shifting can be restored with L-dopa, other new cognitive deficits emerge with the use of L-dopa. Namely, while L-dopa restores the ability to shift to a new response strategy in PD patients, the ability to reverse a rule (i.e. shift response strategy to a previously incorrect one) becomes impaired while on L-dopa (Owens et al., 1993, Cools et al., 2002). To explain this enhancement of some function but decrements in others, an overdose hypothesis has been formulated. It postulates that impairments in reversal learning are caused by an overdose of dopamine to the not-yet-depleted ventral striatum of early-moderate PD (Cools et al., 2006). However recently it has also been shown that L-dopa can exacerbate sustained attentional deficits in a non-human primate model of PD. Schneider and colleagues (2012) found that the dose effective for treating

the motor symptoms further impaired performance in a sustained attention task. Therefore, while the overdose hypothesis may account for some L-dopa-decrements in attentional and cognition, it cannot account for all of them.

Further, in some instances, no impact of L-dopa, either positive or negative, on attentional function has been observed. Specifically, selective attention remains impaired in patients on L-dopa compared to patients who have abstained from their L-dopa regimen (Lewis et al., 2005; Moustafa, Sherman, & Frank, 2008). Even more, these deficits remained unimproved while other working memory deficits improved considerably with L-dopa administration. Therefore, while some success is seen in using L-dopa to treat cognitive deficits, it may be that different domains of attention/cognitive impairments might require different therapies. For these reasons, it is imperative to study the nature of L-dopa's influence specifically on attentional deficits associated with PD.

While L-dopa may prove effective in treating attentional dysfunction seen in PD, it may still not be the ideal means for treating both the motor and cognitive deficits of PD simultaneously. L-dopa has several shortcomings that greatly impact PD patients. Firstly, the dosage necessary to relieve motor symptoms increases over time due to a development of tolerance as well as receptor density changes, and at later stages of the disease L-dopa becomes less effective simply due to the severity of dopamine depletion (Lessner, Fahn, Snider, Cote, Isgreen, & Barrett, 1979). The enhancement of dopamine synthesis and transmission is global and not simply confined to the depleted nigrostriatal pathway, which has been shown to be problematic for other dopaminergic functions such as reward learning. There is a significant incidence of impulse control disorders including gambling addiction in patients on L-dopa and dopamine agonists (Leeman & Potenza, 2011). Furthermore, L-dopa at large doses can cause severe adverse effects either after prolonged use or once a high dose of L-dopa is reached. Most commonly,

extramotor side effects known as L-dopa induced dyskinesias (LIDs) are seen in PD patients and are defined as involuntary repetitive-like movements of the face or limbs (Rajput et al., 2002). LIDs can become very severe and can interfere with a patient's ability to function (Ahlskog, 2011). Therefore, it could be problematic if a high dose is needed to remediate attentional dysfunction because of the possible development of these LIDs or other adverse outcomes.

1.5 Potential use of methylene blue for the treatment of multiple classes of symptom in PD

Because of L-dopa's adverse effects, investigating other treatment options is essential. Instead of targeting a specific neurotransmitter system (i.e. dopamine) it may be more efficacious to target the system as a whole. Neurodegenerative diseases (including PD) are commonly marked by mitochondrial dysfunction and oxidative stress (Banerjee et al., 2014; Barnham, Masters, & Bush, 2004; Fukae, Mizuno, & Hattori, 2007; Schapira, 2008). Therefore, treating the widespread cellular dysfunction may prove to enhance a wider range of symptoms. One potential way to enhance mitochondrial function is through the administration of methylene blue (MB). MB is a compound that acts by shuttling electrons to the electron transport chain, thus enhancing the activity of cytochrome *c* oxidase and subsequently enhancing metabolism of the cell (Visarius, Stucki, & Lauterburg, 1997). MB is also an antioxidant as a consequence of its rapid-cycling redox properties. Therefore, MB is desirable for the treatment of PD because a hallmark pathological trait is oxidative stress, and apoptosis due to oxidative stress is the primary cause of dopaminergic cell loss in PD (Kanthsamy, Borowitz, G., & Isom, 1994; Pallanck & Greenamyre, 2006; Schapira, 2008; Visarius et al., 1997). Recently, Hochgräfe and colleagues (2015) demonstrated that MB can slow the rate of protein aggregation when given preventatively (i.e. prior to reaching symptom threshold) in a

transgenic model of Alzheimer's disease. Therefore, MB may be useful not only in enhancing function of a compromised cell via enhanced metabolism, but also in decreasing the presence of reactive oxidative species (oxidative stress) thereby increasing the likelihood of cell survival.

Additionally, boosting metabolic function via methylene blue has been shown to be beneficial for cognition. In naive animals, MB has been shown to increase cognitive abilities specifically in learning and memory paradigms (Martinez et al., 1978, Riha, Bruchey, Echevarria, Gonzalez-Lima, 2005; Callaway, Riha, Wrubel, McCollum, Gonzalez-Lima, Callaway, Riha, Bruchey, Munchi, Gonzalez-Lima, 2004; Wrubel, Barret, Shumake, Johnson, Gonzalez-Limam 2006; Wrubel, Riha, Maldonado, McCollum, & Gonzalez-Lima; Rojas et al., 2012). And recently, MB has been studied in several neurodegenerative diseases and aging-related conditions. MB was shown to improve motor function and decrease tau aggregations in a pesticide model of PD (Wen, et al., 2011) and improve cognition and decrease pathological presence in a model of Alzheimer's disease (Medina et al., 2011). Most recently, discrimination learning was shown to be enhanced in a rat model of cerebral hypoperfusion with the administration of MB (Auchter, Williams, Barksdale, Monfils & Gonzalez-Lima, 2014). MB can also be used chronically without the induction of adverse side effects (Naylor et al., 1986). In sum, MB has been shown to be effective in rat models of neurodegenerative disorders for treating cognitive function. Furthermore MB has the potential to be useful in treating multiple classes of deficits simultaneously because MB is activity dependent and aggregates in areas where the demand is high (Barrett, Shumake, Jones, & Gonzalez-Lima, 2003; Quirk, Garcia, & González-Lima, 2006). However, no research has been conducted yet to investigate the use of MB to improve attentional function alone or in conjunction with motor function in a PD model.

Chapter 2: The role of the central amygdala dopamine in disengagement behavior

2.1 ABSTRACT[1]

Unilateral nigrostriatal dopamine depletion in animals induces contralateral sensorimotor deficits that are like symptoms associated with Parkinson's disease (PD). Unilateral nigrostriatal dopamine depletion also causes a contralateral deficit in disengagement behavior (e.g., ability to stop an ongoing activity to orient/attend to a new stimulus). This disengagement deficit has been shown to be resistant to treatments that rescued other motor and somatosensory deficits. Thus, disengagement behavior may involve unique sensorimotor information integration potentially important for attentional allocation and may rely strongly on a mechanism that includes extranigrostriatal circuitry. The central nucleus of the amygdala (CeA) and its connections with the nigral dopamine system have been reported to modulate cognitive processes dependent substantially on attentional allocation. CeA dopamine function might be also important for disengagement behavior. In Experiment 1, rats received microinfusions of 6-hydroxydopamine unilaterally to induce dopamine terminal loss in the CeA and were tested for disengagement behavior in addition to several sensorimotor functions. These rats showed deficits in contralateral disengagement behavior and an asymmetry in adhesive dot removal from the paws, but not in forelimb use in a cylinder or amphetamine rotation. In Experiment 2, rats received D1 or D2 antagonists into the CeA unilaterally prior to behavioral tests. The D1 antagonist disrupted disengagement behavior without affecting the other sensorimotor tests examined. The D2 antagonist had no effects on any of the

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behaviors tested. Our results suggest that CeA dopamine function is involved in modulation of disengagement behavior.

2.2 INTRODUCTION

Parkinson's disease (PD) is best recognized as a movement disorder triggered by a significant loss of nigrostriatal dopamine input, but is accompanied by various non-motor symptoms including cognitive related dysfunction (Marsh & Berk, 2003; McDowell & Chesselet, 2012). In particular, PD patients have difficulty shifting attention between two tasks (Cools et al., 2001; Naismith et al., 2010; Ravizza & Ciranni, 2002; Yamaguchi & Kobayashi, 1998). Different prefrontal and parietal cortical subregions are known to be involved in attentional shifting (Corbetta & Shulman, 2002; Fox, Barense, & Baxter, 2003; Ng, Noblejas, Rodefer, Smith, & Poremba, 2007; T W Robbins, 2007). However, the prefrontal and parietal cortical regions show pathological changes in late stage of PD (Braak et al., 2004) while deficits in attentional shifting appear in early stage of PD and do not respond well to treatment targeted to improve frontal cortical function (Lewis et al., 2005; Owen, Roberts, Hodges et al., 1993). Thus, other brain regions likely contribute to the difficulty that PD patients have in shifting attention between tasks.

One of the cognitive operations involved in shifting attention is the ability to disengage from the currently focused event and orient to a different event (Posner, Walker, Friedrich, & Rafal, 1984). Using an animal model for PD, Schallert and Hall (1988) first reported that nigral dopamine depletion resulted in impaired disengagement behavior. Rats normally show orienting to tactile-perioral stimulation by disengaging from on-going activities such as eating and drinking. However, rats with unilateral partial dopamine depletion due to infusion of 6-OHDA into the medial forebrain bundle

did not disengage from eating or drinking to orient to the tactile-perioral stimulation in the contralateral side. This disruption in disengagement was not due to simple somatosensory deficit because the same rats showed robust orienting to the same stimulation in the absence of on-going activities. Interestingly, behavioral training or tissue grafting that aimed at restoring nigrostriatal-dependent sensory and motor deficits in the PD animal models did not rescue this disengagement deficit (Mandel, Brundin, & Björklund, 1990; Nikkhah, Duan, Knappe, Jo¨dicke, & Bjo¨rklund, 1993; Schallert & Hall, 1988). This suggests that disengagement behavior is likely to rely on a circuitry other than (or in addition to) the nigrostriatal one important for sensorimotor processing.

The central nucleus of the amygdala (CeA) has reciprocal connections with the substantia nigra pars compacta, SNc, (Cheung, Ballew, Moore, & Lookingland, 1998; Fudge & Haber, 2000; Gonzales & Chesselet, 1990; Lee et al., 2005; Ottersen, 1981) and destroying nigral dopamine cells results in significantly reduced dopamine fibers in the CeA (Schober, Hertel & Unsicker, 2005). Several animal studies already showed that the CeA and its connections with the SNc were important for attentional allocation (Gallagher et al., 1990; Han, McMahan, Holland, & Gallagher, 1997; Holland et al., 2000; Lee et al., 2008; Lee et al., 2005, 2006). These studies do not necessarily address the exact nature of the information processing between the CeA and the SNc (i.e., from the CeA to the SNc, vice versa or both), but clearly support the idea that communication between these two structures is essential for some aspects of attentional processing. It is then possible that CeA dopamine function might be involved in disengagement behavior. We addressed this possibility by depleting dopamine input more specifically to the CeA (experiment 1) and temporarily inactivating CeA D1 and D2 receptors (experiment 2). Additional tests were administered to examine whether the CeA dopamine system would affect other somatosensory and motor functions.

2.3 METHOD

2.3.1 Subjects

Long Evans male rats (Charles River, Wilmington, MA), weighing between 250-275g at the beginning of the experiment were housed individually in a colony room on a reversed light-dark schedule with the lights off between 10 A.M. and 8 P.M. and the room was maintained at a constant temperature (72° F). All animals were monitored daily and allowed ad libitum access to food. Water was only restricted for the 24 hours directly preceding the behavioral tests. All experiments were conducted according to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and all protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

2.3.2 Experiment 1

2.3.2.1 Surgery.

Six rats were anesthetized using 2-5% isoflurane gas (Abbott Laboratories, Abbott Park, IL) and were placed into the stereotaxic frame (Kopf Instruments, Tujunga, CA). Each animal then received unilateral infusion of 6-hydroxydopamine (6-OHDA; Sigma Aldrich, St. Louis, MO) into the central nucleus of the amygdala (CeA). A 28-gauge needle (PlasticsOne, Roanoke, VA) was placed targeting the CeA (AP = -2.0mm and -2.4mm, ML = ± 4.2 mm, DV = -8.2mm) and 0.2-0.4 μl of 6 μg/μl 6-OHDA in PBS (i.e., 0.1 M phosphate buffer with 0.9 % saline) with 0.1% (w/v) ascorbic acid was infused at 0.1 μl/min rate. Each rat received two injections at two different AP coordinates. The injected site was counterbalanced so that half of the animals received

the lesion on the right CeA and the other half received it on the left CeA. Once the infusion was completed, the needle was left in place for five more minutes to allow the solution to diffuse, so that there would be no solution taken up to the striatum when the needle was removed. All rats were allowed to recover for two weeks before testing.

2.3.2.2 Behavioral Measures.

The disengagement test, an adhesive removal test, forelimb use in a cylinder and an amphetamine-induced rotation test were conducted. For all the tests, the experimenter was blind to the lesion condition. For the 24 hours immediately prior the disengagement test, all rats were water deprived so that they would be motivated to drink when the testing began. Individually, each animal was allowed to drink from the water spigot at the back of the home cage. While engaged in drinking, each rat was stimulated periorally using a cotton swab. Three trials were conducted on each side, in which the order of stimulation was randomized. The number of times the animal disengaged from drinking after the perioral stimulation was recorded for each side and percentages were calculated. Animals were also periorally stimulated when they were not engaged in drinking to measure baseline reaction to sensory stimulation.

In the adhesive removal test, round adhesive dots (1.3 cm diameter, Office Depot, Austin, TX) were placed on each forepaw pad. After being placed back in the home cage, the rat was allowed to remove the adhesive dots immediately without a limited hold (i.e., without having to wait for a certain time to initiate the response). The order (left vs. right forepaw) and latency in which the rat made contact with the dots and removed the dots were recorded over five trials. Percentage of trials where the ipsilateral paw to the lesioned CeA (i.e., good/unaffected paw) was contacted first and the dot on the ipsilateral paw was removed first were calculated.

In the cylinder test (as described in Schallert et al., 2000), a plastic cylinder (20 cm in diameter and 30 cm in height) was stood up on one end. Rats were placed into the cylinder and spontaneous forepaw touches to the cylinder during rears/exploration of the vertical wall were recorded. If only one paw was placed on the cylinder, it was recorded as independent use of that limb. If both paws were placed on the cylinder simultaneously, or if one paw was placed on the wall and then the other was immediately placed on the wall and alternated with the other limb during stepping, it was recorded as simultaneous use of both limbs. A total of 20 instances of left, right, and simultaneous (both) forelimb use were recorded during exploration of the cylinder wall in either horizontal or vertical planes. Preference for the limb use ipsilateral to the lesioned side was calculated by using the following equation previously established by Schallert et al. (2000): Ipsilateral paw touches + (.5) both paw touches divided by Total number of touches (ipsi + contra+ both)

For the rotation test, each rat was given an intraperitoneal injection of amphetamine (Sigma Aldrich) at a dose of 1mg/kg. Twenty minutes afterwards, the rat was placed into a plastic bowl (60 cm in diameter, 43 cm in height) with tape delineating four quadrants. The rat was allowed to move about the bowl for five minutes and each quarter turn was recorded for the ipsilateral and contralateral sides in relation to the CeA lesion.

2.3.2.3 Histology.

Rats received an overdose of pentobarbital (86 mg/kg) and phenytoin (11 mg/kg) mix (Euthasol® by Virbac Animal Health, Fort Worth, TX) and then were perfused transcardially with 0.9% saline followed by 4% Paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were extracted and placed into a 20% sucrose PFA solution overnight. The next day brains were rapidly frozen using powdered dry ice and stored at -80ºC. Brains were sliced at 30 μm using a sliding microtome and sections

containing the striatum, the amygdala and the midbrain dopamine regions were collected in four series.

One series was used for tyrosine hydroxylase (TH) staining and an adjacent series was used for Nissl staining. For the TH staining, the tissue was first treated with 0.3% hydrogen peroxide in PBS. Then the tissue was placed into a 6% normal goat serum (NGS) in PBS with 0.3% Triton (PBST) for one hour after rinsing in PBS. Immediately afterwards the tissue was incubated in 0.15% PBST solution containing 3% NGS and mouse TH antibody (1:5000, ImmunoStar, Hudson, WI) for 72 hours at 4 ºC. After rinsing, the tissue was incubated with biotinylated goat anti-mouse (1:250, Vector Laboratories, Burlingame, CA) for 1 hour and then with avidin-biotin conjugate (PK-6100, Vector Laboratories) for 1 hour, and reacted using 3,3-diaminobenzidine (DAB; Sigma Aldrich).

TH density was assessed after the TH-stained tissue had been mounted and cover-slipped. Sections were imaged using a microscope (Olympus BX61) at a magnification of 10x. The Nissl stained sections were used for anatomical orientation and for determination of the size and shape of the central amygdala. Using the Nissl section, a border was created around the CeA and then transferred to the appropriate TH-stained section and the density measure was taken within that border. Two samplings were also taken from the CeA at levels 25-28 (in accordance with Swanson Rat Atlas, 2003), using a circle with a radius of 100 μm (Fig 1). For the striatal reading, two dorsal and two ventral measurements (140 μm length square) were taken from the caudate putamen (CP) at levels 11, 15, and 18 and one dorsal and one ventral measurements were taken at level 22. In addition, CP area immediately adjacent to the CeA was sampled at levels 25-28 for possible spread of 6-OHDA. TH density in the SNc and ventral tegmental area (VTA) was assessed by placing a circle with a radius of 100 μm over the SNc and VTA. Two

samplings of each structure were taken at levels 36-39. Furthermore, two samplings in the midbrain reticular nucleus (MRN) area dorsal to the SNc were taken at levels 38 and 39 for baseline readings of non-dopaminergic area. Mean gray value measurement in Image J was used to calculate the brightness of image pixels (Rasband, 1997-2001, NIH).

2.3.3 Experiment 2

2.3.3.1 Surgery.

For the second experiment, a separate group of rats (N= 51) was used. All of the animals had bilateral cannula (26 gauge, PlasticsOne, Roanoke, VA) implants targeting the CeA (AP = -2.0, ML = ±3.6, DV = -7.2). These animals were previously used in a different task that measured attentional processing (i.e., 5-choice serial reaction time task) and had received a total of 4 bilateral infusions of saline, D1 antagonist SCH 23390, or D2 antagonist Raclopride. For the current study, animals' prior behavioral and drug experience were balanced and equally represented in each group. Even though animals had bilateral cannula implants, they only received unilateral manipulations for the current study.

2.3.3.2 Behavioral Measures.

The disengagement, adhesive dot, and limb use in cylinder exploration tests were conducted twice in the same manner as described for Experiment 1. The first set of tests was a baseline measure with unilateral saline infusion and the second set of tests involved an infusion of either a D1 antagonist SCH 23390 or D2 antagonist Raclopride (Sigma) to the same unilateral side where saline was infused. The interval between the saline and drug infusions was 24-48 hrs, and all the infusions were given in 0.2 μl over 2-min via 33-gauge infusion needle that extended 1 mm beyond the guide cannula. Two doses of SCH 23390, 0.5 μg and 1.0 μg, and two doses of Raclopride, 0.25 μg and 0.75 μg were

tested. In total, there were four groups of rats in which a given rat only received a total of two infusions, one saline infusion to serve as its own control and one drug infusion. For each group, the infusion site was counterbalanced so that half of the animals received infusion on the right CeA and the other half received it on the left CeA. Fifteen minutes after the saline/drug infusions, each rat was tested on all three tests, which took an average of 15 minutes to complete.

2.3.3.3 Histology.

Rats were perfused transcardially with 0.9% saline and 10% formalin. The brains were extracted, placed into 20% sucrose formalin solution overnight, and then frozen the following day. Brains were sliced using a sliding microtome at a thickness of 40 μm and sections containing the CeA were saved. Mounted sections were Nissl-stained to verify cannula placements. Cannulas were considered to have good placements if the guide cannula track was visualized within 1 mm above the CeA or if the injection needle track was seen within the CeA.

2.4 RESULTS

2.4.1 Experiment 1

2.4.1.1 TH Density.

TH density measurements were taken in the CeA, CP, SNc, and VTA. The sampled areas are illustrated in Fig 2.1 and representative pictures of the sampled areas from a single animal are shown in Fig 2.2. The density measures seen in Figure 2.3 correspond to the mean grey value in Image J, which reports brightness value of the sampled areas, thus higher numbers represent areas with low/reduced TH density. The

measurements of the MRN area (used for baseline reading of non-TH stained area) just dorsal to the SNc averaged 50 x 10^3. The measurements in the interested regions revealed decreased TH density in the lesioned CeA (i.e., the side that received 6-OHDA infusion) and the SNc ipsilateral to the lesioned CeA. However, there were no differences in the CP and the VTA between the two hemispheres. Paired sample t-tests comparing the structure ipsilateral and contralateral to the lesioned side were conducted for each brain region. In the CeA and the SNc, there was a significant difference between the lesioned and intact sides, $t(5) = 2.87$, $p < 0.05$ and $t(5) = 3.05$, $p < 0.05$, respectively. For the CP and VTA, there were no statistically significant differences between the lesion and intact sides, $t(5) = 1.08$, $p > 0.3$ and $t(5) = 0.27$, $p > 0.5$, respectively. Additional analyses only on the caudal CP areas dorsal to the CeA showed that TH density was still not significantly between the two hemispheres ($p>0.2$).

2.4.1.2 Disengagement Test.

Animals with 6-OHDA infusion in the CeA showed deficits in disengagement behavior. When the perioral stimulation was given on the side contralateral to the lesioned CeA, they failed to disengage from drinking and did not orient towards the stimulation (Fig 2.4A). In contrast, when the rats were tested while not in the act of drinking they always oriented when touched on either side. When they were periorally stimulated on the ipsilateral side to the lesion, they readily disengaged from drinking and oriented towards the stimulation. A paired sample t-test was conducted on disengagement behavior (i.e., percentage of trials when the animals disengaged from drinking) between the sides ipsilateral and contralateral to the CeA lesion. All rats

disengaged significantly more to ipsilateral perioral stimulation than to contralateral perioral stimulation, $t(5)=7.51$, $p = 0.01$.

2.4.1.3 Adhesive Dot Test.

When the adhesive dots were placed on the forepaw pads, animals readily contacted and removed the dots using their teeth. However, they showed ipsilateral paw bias in which they first contacted and removed the dot placed on the paw ipsilateral to the lesioned CeA before contacting and removing the dot placed on the contralateral paw. Two one-sample t-tests were conducted, one on ipsilateral dot contact and one on ipsilateral dot removal to determine if either of these behaviors occurred more than would be expected by chance (i.e. 50%). The rats contacted the ipsilateral dot first more frequently than would be expected due to chance, $t(5)=8.56$, $p < 0.001$; Fig 4B, and they also removed the ipsilateral dot first more than would be expected due to chance, $t(5) = 6.27$, $p < 0.01$; Fig 2.4B.

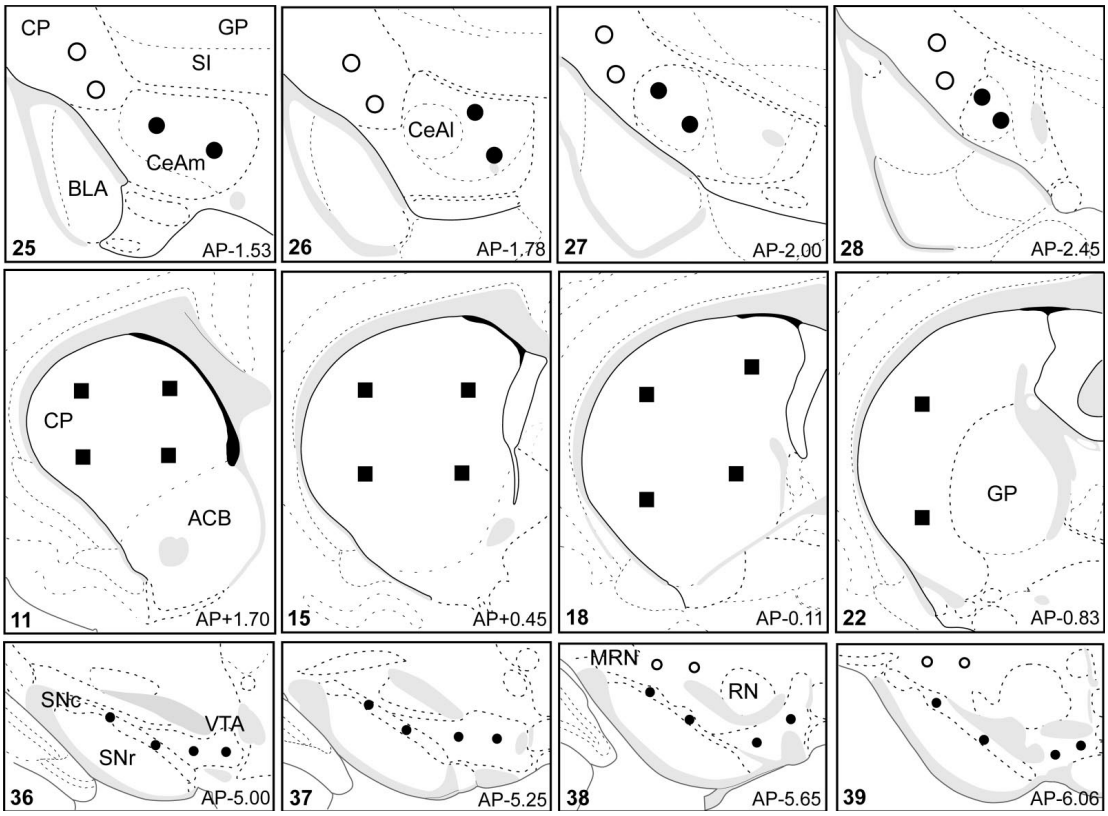
2.4.1.4 Cylinder Test.

Rats showed no motor impairment as measured by bias in the forepaws use to touch the cylinder wall when rearing/exploring. Animals used both forepaws equally whether it was independent or simultaneous use of the paws. Bias for the ipsilateral limb was calculated using the equation denoted in the methods section. A one-sample t-test confirmed that ipsilateral paw use was not different from the 50% chance level, $t(5) = 0.38$, $p > 0.5$; Fig 2.4C.

2.4.1.5 Amphetamine-Induced Rotation Test.

Typically, animals with nigrostriatal dopamine depletion show ipsilateral rotation bias when injected with amphetamine (Choi-Lundberg et al., 1998). However, rats with the CeA dopamine depletion showed no preference in their rotational behavior towards the ipsilateral side (Fig 2.4D). A paired sample t-test confirmed that there was no bias in the direction of the rotations, $t(5)= 0.74$, $p > 0.1$.

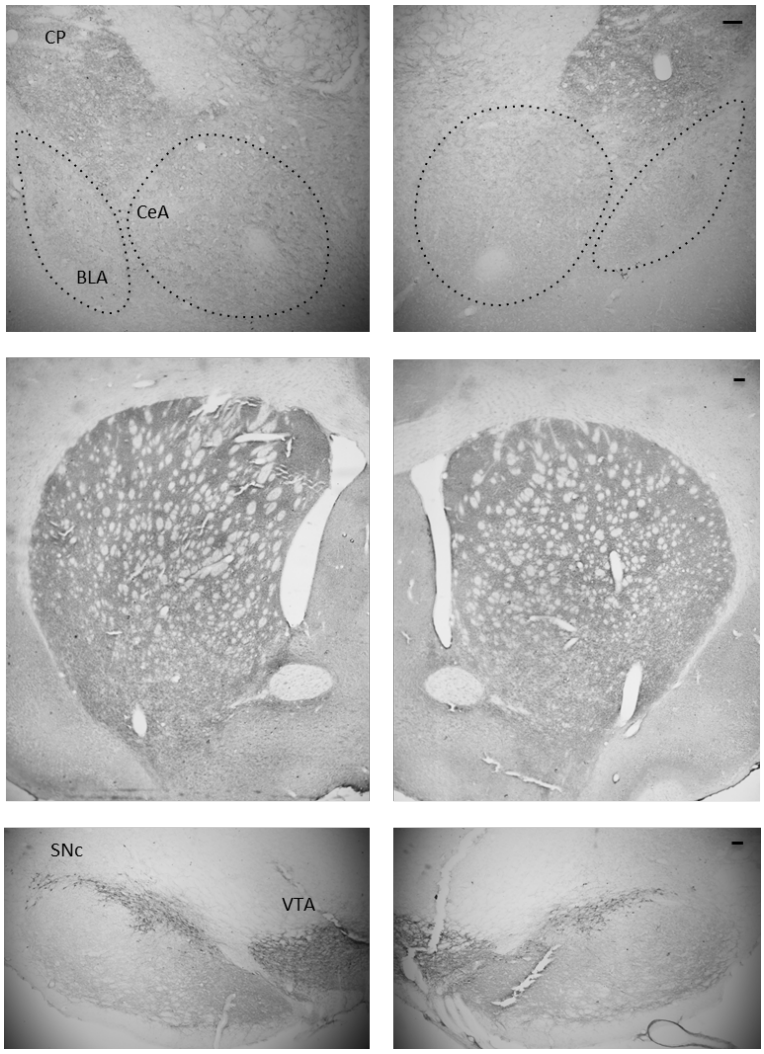
Figure 2.1 Sampling areas used to measure density in TH-stained tissue



Diagrams are adapted from Swanson (2003) to represent the sampled areas to measure density in tyrosine hydroxylase (TH)-stained tissue. In the diagrams of the top row, black and open circles represent sampled areas in the central amygdala (CeA) and in the adjacent caudate putamen (CP) area, respectively. Diagrams in the middle row show sampled striatal areas as black squares. Diagrams at the bottom row show sampled areas of the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) as black circles and sampled midbrain reticular nucleus (MRN) as open circles. ACB, nucleus

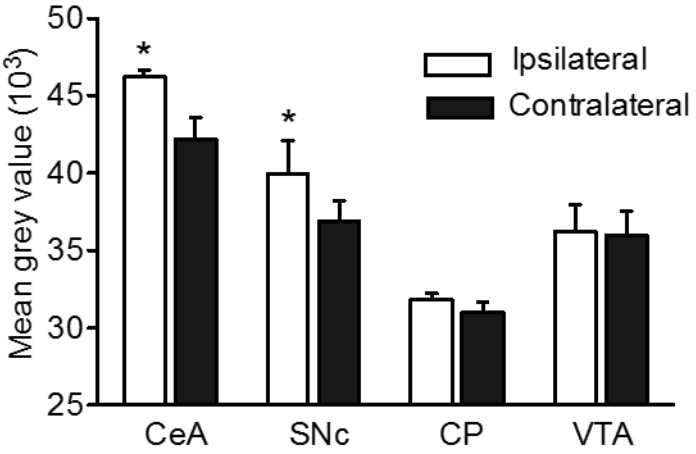
accumbens; BLA, basolateral amygdala nucleus; CeAl, central amygdala nucleus lateral part; CeAm, central amygdala nucleus medial part; GP, globus pallidus; SI, substantia innominate. Adapted with permission from Brain Maps: Structure of the Rat Brain (3rd ed., pp. 39, 47, 53, 61, 67, 69, 71, 73, 89, 91, 93, 95), by L. W. Swanson, 2003, San Diego, California:

Figure 2.2 Photomicrographs of TH-stained sections



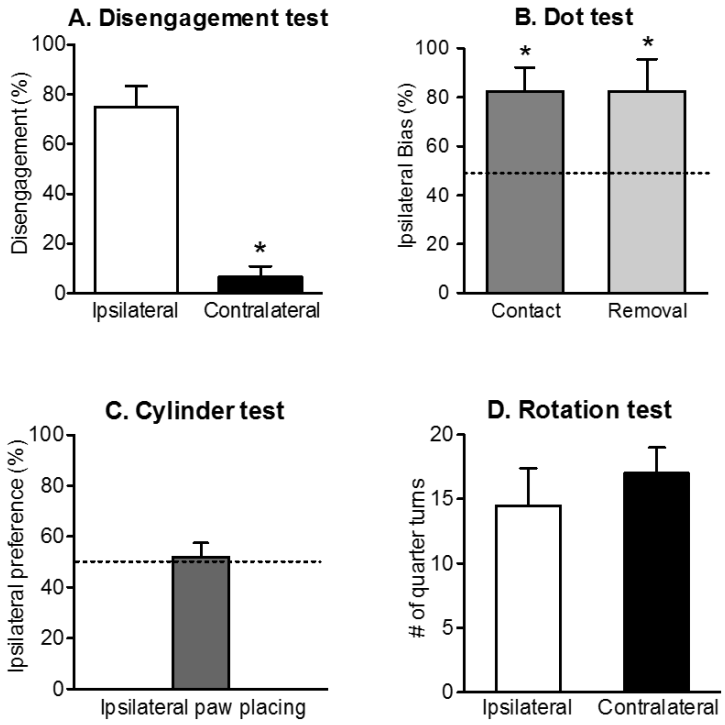
Photomicrographs of the tyrosine hydroxylase (TH)-stained brain sections taken from an animal that received 6-hydroxydopamine (6-OHDA) infusions into the right central amygdala (CeA). The right CeA as well as the right substantia nigra pars compacta (SNc) are visibly lighter compared with ones on the left whereas the caudate putamen (CP) regions do not show obvious differences between two hemispheres. Horizontal bars on the upper right corner in each row indicate 100 μm.

Figure 2.3 TH density quantification



Mean (± SEM) gray value measure in the tyrosine hydroxylase (TH)-stained tissue. The sampled areas are the central amygdala (CeA), substantia nigra pars compacta (SNc), caudate putamen (CP), and ventral tegmental area (VTA). TH density measures are compared between the ipsilateral (i.e. the same side in which the CeA was infused with 6-hydroxydopamine [6-OHDA]) and contralateral (i.e. the side with intact CeA) regions. As indicated by higher brightness value, TH density was significantly lighter in the lesioned CeA and the SNc ipsilateral to the lesioned CeA in comparison to their own contralateral regions. *$p < .05$.

Figure 2.4 Performance in motor, sensorimotor, and disengagement tests



(A) Mean (± SEM) percentage of disengagement from drinking to perioral stimulation on ipsilateral and contralateral sides in relation to lesioned central amygdala (CeA). (B) Mean (± SEM) percentage of trials with first contact and removal of the adhesive dot placed on the ipsilateral paw. (C) Mean (± SEM) bias of ipsilateral paw use in the cylinder test. (D) Mean (± SEM) quarter turns induced by amphetamine. * $p \leq 0.01$

2.4.2 Experiment 2

2.4.2.1 Cannula placement.

Cannulae were judged as being acceptable if the center of the cannula placement was between AP -1.53 and 2.00 mm and at the most 1 mm above the CeA. Nineteen rats were excluded from the experiment for inaccurate cannula placement leaving 32 rats with good placement with the breakdown between the groups being n = 7 in the 0.5 µg SCH 23390 group, n = 10 in the 1.0 µg SCH 23390 group, n = 7 in the 0.25 µg Raclopride group, and n = 8 in the 0.75 µg Raclopride group. Figure 2.5 depicts each cannula placement in relation to the CeA. Each point represents the cannula tip at its centermost point with the grey ovals representing animals in the 0.5 µg SCH 23390 group, the black ovals representing animals in the 1.0 µg SCH 23390 group, the grey diamonds representing animals in the 0.25 µg Raclopride group, and the black diamonds representing animals in the 0.75 µg Raclopride group.

2.4.2.2 Disengagement Test.

When the animals were tested for disengagement behavior after unilateral infusion of saline, they all readily disengaged from drinking with perioral stimulation (white bars in Fig 2.6A and 2.6B). When the animals were tested again after 24-48 hrs with D1 or D2 antagonists, only the rats that received unilateral infusion of 1.0 µg D1 antagonist, SCH 23390, showed impaired disengagement (Fig 2.6A). Orienting was not diminished at all if the rats were not drinking. Interestingly, even though the disengagement deficits were substantial in the contralateral side of the infusion, some deficits were observed in the ipsilateral side as well. Animals that received the lower

dose of D1 antagonist SCH23390 or either doses of D2 antagonist Raclopride did not show any impairment in disengagement behavior (Fig 2.6A and 2.6B). Paired t-tests comparing baseline to test performance confirmed these observations. With the larger dose of SCH 23390, there was a significant difference between the baseline and test performance ipsilaterally, $t(9)= 2.34$, $p = 0.04$, and contralaterally, $t(9)= 3.85$, $p < 0.01$. There was no difference between baseline and test performance at the lower dose of SCH 23390 on the ipsilateral side, $t(6)= 1.00$, $p > 0.1$, and the contralateral side, $t(6)= 1.55$, $p > 0.1$. There was no difference within the 0.25 µg Raclopride group in disengagement between saline and infusion days on the side ipsilateral to the infusion, or on the side contralateral to the infusion, $t(6) = 1.00$, $p > 0.1$. There was no t-test conducted between saline and infusion day on the ipsilateral side because the means were identical. Additionally, there was also no difference in disengagement seen in the 0.75 µg Raclopride group between saline and infusion day on the side ipsilateral to the infusion, $t(7) = 1.24$, $p > 0.1$, or the side contralateral to the infusion, $t(7) = 1.43$ $p > 0.1$.

We also ran additional analyses to confirm that animals' prior drug experiences (as mentioned in the methods) did not influence the current results. There were no differences in disengagement behavior as well as dot and cylinder tests among the rats that had received saline, D1, and D2 antagonists prior to the current study. Furthermore, there was no interaction effect between their prior drug exposure and the current drug exposure.

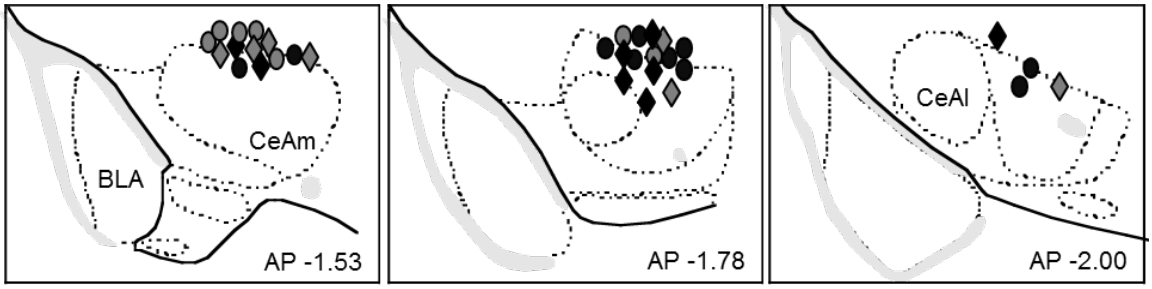
2.4.2.3 Adhesive Dot Test.

When animals had adhesive dots placed on their forepaws after saline infusion, both dots were readily removed across all 5 trials. There was no preference for one paw as shown with 53% preference for the ipsilateral dot touched, suggesting no sensorimotor deficits and no dominant paw use as a result of saline infusion. Additionally, 24-48 hrs later when the animals were given an infusion of D1 antagonist, SCH 23390, there was no paw preference in which of the dots were first touched, at the 0.5 or 1.0 µg doses (all ps > 0.1). When given an infusion of the D2 antagonist, Raclopride, there was again no preference at the 0.25 or 0.75 µg doses for which of the dots was first touched or removed, all ps > 0.05. The ipsilateral bias for dot touched was at a chance level ranging 50-58% among the drugs and doses tested.

2.4.2.4 Cylinder Test.

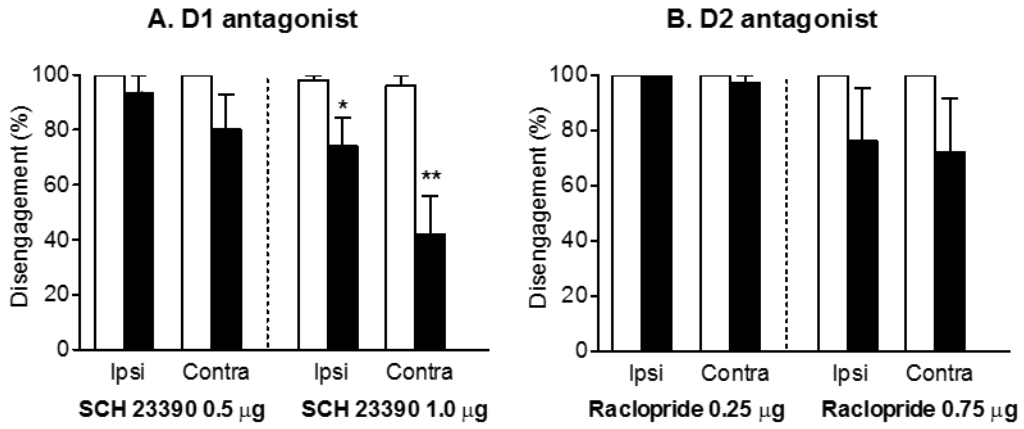
After the saline infusion, all animals showed no bias or preference for one limb use over another when contacting the sides of the cylinder. When tested 24-48 hrs with a D1 antagonist SCH 23390 or D2 antagonist Raclopride infusion, neither drug caused a preference for which forepaw was used to contact the cylinder when rearing, all ps > 0.1. Ipsilateral preference was at a chance level ranging 46-52% among the drugs and doses tested.

Figure 2.5 Cannula placements within the central amygdala (CeA) between AP -1.53 and -2.00.



Cannulations were judged as acceptable if the tip of the cannula track was at most 1 mm above the CeA. Each oval or diamond represents a cannula tip at its most central point for each animal. Ovals represent the animals receiving SCH 23390 with the gray representing the lower dose (0.5 μg) and the black representing the higher dose (1.0 μg). The diamonds represent the animals receiving Raclopride with the gray representing the lower dose (0.25 μg) and the black representing the higher dose (0.75 μg).

Figure 2.6 Disengagement function after CeA D1 or D2 antagonism



Mean (± SEM) percentage of disengagement from drinking to perioral stimulation on ipsilateral and contralateral sides to the central amygdala (CeA) that received infusions of D1 (A) or D2 (B) antagonists. White bar represents disengagement on the day of saline infusion and black bar represents disengagement on the day of drug infusion. * p □ .05. ** p □ .01.

2.5 DISCUSSION

In the first experiment, animals with 6-OHDA lesions of the CeA failed to disengage from drinking in order to orient to perioral stimulation on the contralateral side of the lesion, but showed intact disengagement behavior bilaterally when not engaged in drinking. All rats also showed normal orienting to ipsilateral stimulation whether or not they were engaged in drinking at the time. In the second experiment, rats with infusions of the D1 antagonist SCH 23390, but not D2 antagonist Raclopride, directly into the CeA showed deficits in disengagement behavior. In both experiments, animals did not show any deficits in the sensorimotor tests with the exception of the adhesive dot test in experiment 1. They contacted and removed dots from the ipsilateral paw to the lesion first. Taken together, the current study suggests that the CeA is necessary for disengagement behavior and may mediate this behavior through a D1 mechanism.

Previous work on rodent models of PD demonstrated that the deficit in disengagement behavior was not rescued by treatments that restored sensorimotor deficits. For example, Schallert and Hall (1988) were able to train animals with unilateral nigrostriatal dopamine depletion to recover from somatosensory neglect. However, the same animals continued to show complete failure to orient to the contralateral perioral stimulation if they were engaged in eating, drinking or grooming behavior. In other studies, rats with grafts of dopamine-rich fetal ventral mesencephalon in the dopamine depleted striatum recovered from the earlier sensorimotor deficits, but continued to show deficits in disengagement behavior (Mandel et al., 1990; Nikkhah et al., 1993). These findings suggest that the striatal dopamine circuitry that is important for sensorimotor functions might not mediate the disengagement behavior. In fact, our current study showed that an extra-nigrostriatal circuitry (i.e., the CeA dopamine function) was important in mediating disengagement behavior without affecting orienting response to

stimulation in the absence of drinking behavior. It is unlikely that disengagement deficits seen in our study are entirely due to the potential damage to the striatum in the course of CeA manipulations. In experiment 1, the overall TH density in the CP area was not different between the two hemispheres. Even though some lighter TH density was observed occasionally in the caudoventral CP area near the lesioned CeA, there were no obvious differences between the two hemispheres. In experiment 2, there were intact disengagement and sensorimotor functions on the contralateral side of saline or D2 infusions as well as on the ipsilateral side (i.e., the side being modulated by the hemisphere with the cannula implant without infusion). Thus, potential mechanical damage to the striatum could not be responsible for disengagement deficits seen in our study.

Failure to disengage from an event/task is not limited to drinking behavior. It can occur in the presence of eating or grooming (Schallert and Hall, 1988). Animals may also show disengagement deficits to a different event other than perioral stimulation. Whishaw and Tomie (1988) reported that unilateral nigrostriatal 6-OHDA lesioned animals were unable to stop eating in order to dodge when other rats approached them from the contralateral side. It is interesting to note that visual disengagement deficits have also been reported in PD patients (Sacrey, Travis, & Whishaw, 2011; Sacrey & Whishaw, 2012). In a reach-to-eat task, in which subjects are required to reach for a small food item (e.g., Cheerio) in order to eat it, visual attention is usually engaged as the subject reaches for food. However, once the subject reaches the target and begins to grasp the food, visual attention is quickly disengaged, possibly in order to engage somatosensory attention to finish the task. Unlike typical subjects, PD patients were not able to disengage visual attention at the point of grasping the food and took much longer to finally disengage. Thus, it is likely that disengagement deficits seen in our study are

not due to impaired mechanisms for processing drinking behavior and perioral stimulation per se. As originally suggested by Schallert and Hall (1988), the disengagement deficit may reflect impaired capacity to simultaneously monitor multiple sensory events and to direct attention appropriately.

The current study suggests that intact CeA dopamine function is important for disengagement behavior. It is likely that the CeA influences disengagement behavior in conjunction with or by modulating other areas such as the striatum and cortex. Anatomical connections suggest possible information flow among these areas via limbic-cortical-striatal "spiral loops" (Fudge & Haber, 2000; Haber, Fudge, & McFarland, 2000). Several studies demonstrated that CeA's interaction with nigrostriatal function was important for behaviors reflecting different aspects of attention, including attentional shifting (Lee et al., 2008; Lee et al., 2005, 2006). In particular, the role of the CeA-nigrostriatal circuitry in conditioned orienting behavior is notable (Han et al., 1997; Lee et al., 2005). In an appetitive conditioning, animals often acquire conditioned orienting response to the conditioned stimulus such as light. This behavior is interpreted as reflecting acquired/enhanced attention to the stimulus via conditioning (Holland, 1977). Compromise in the CeA-nigrostriatal function impairs conditioned orienting behavior without affecting unconditioned orienting displayed in the absence of conditioning. Thus, it is plausible that the CeA-nigrostriatal projection might be important in attentional allocation in situations that require more complex information integration whether it is orienting to perioral stimulation while drinking or orienting to light during conditioning. CeA may also influence prefrontal and parietal cortical functions via its connections to cortical projecting cholinergic cells in the substantia innominata (Holland, 2007; Maddux, Kerfoot, Chatterjee et al, 2007). It is notable that attentional disengagement during covert orientation was impaired among people with parietal

cortical damage (Posner et al., 1984). In addition, rats with posterior parietal cortical damage were impaired in attentional set-shifting (Fox et al., 2003). Thus, it is possible that the CeA might interact with the parietal cortex to mediate disengagement behavior.

Even though the SNc is best known for its dopaminergic projections to the striatal area that is involved in motor and related functions, some studies showed that the SNc is involved in other information processing. In particular, it has been suggested that a key function of dopamine neurons in the midbrain area is to direct or enhance attention to biologically significant events or cues that predict significant events (Redgrave, Prescott, & Gurney, 1999; Wolfram Schultz & Dickinson, 2000). Dopamine neurons in the SNc and VTA respond to stimuli that signal biologically significant events such as food (W Schultz, Dayan, & Montague, 1997) or aversive airpuffs to the eye (Matsumoto & Hikosaka, 2009) and to salient and arousing events independent of reward value (Horvitz, 2000). Thus, the nigral dopamine input to the CeA may be important for disengagement behavior in our current study. In addition, the dopamine input from the VTA to the CeA, even though it is sparse, should also be considered (Pitkänen, Pikkarainen, Nurminen, & Ylinen, 2000; L. W. Swanson, 1982). Our histological analyses of TH density in experiment 1, however, showed that depleting dopamine input in the CeA via 6-OHDA infusion only reduced TH density in the SNc, but not in the VTA. Therefore, the SNc dopaminergic input to the CeA seems to be the main source in regulating CeA dopamine function important for disengagement behavior.

Our second experiment suggested that a D1, but not D2, mechanism in the CeA was important for disengagement behavior. This finding is in line with what is typically known about the D1 and D2 functions. In the striatum, D1 and D2 receptors generally have opposite/different effects on neural mechanisms such as adenylate cyclase, synaptic plasticity, and learning. For example, D1 is reported to enhance adenylate cyclase, long-

term potentiation, and appetitive conditioning while D2 either impairs or has no effects on these (Calabresi, Pisani, Centonze, & Bernardi, 1997; Eyny & Horvitz, 2003; Kerr & Wickens, 2014; Yamamoto et al., 1999). Striatal D1 and D2 receptors also have opposite/different responses to dopamine depletion or L-DOPA treatment. D1 receptor density and mRNA levels are generally reduced after the loss of nigrostriatal dopamine input, while D2 receptor density and mRNA levels tend to increase (Gerfen et al., 1990; Joyce, 1991; Marshall, 1979). Chronic L-DOPA treatment seems to enhance D1 signaling or binding whereas the effects on D2 are minimal with some reporting down regulation (Aubert et al., 2005; Darmopil, Martín, De Diego, Ares, & Moratalla, 2009; Xu, Zhang, Qin, Papa, & Cao, 2009). However, some evidence suggests that CeA D1 and D2 mechanisms might differ from those in the striatal or cortical areas. For example, D1 and D2 receptors in the amygdala, including the CeA, are not linked to adenylate cyclase activity (Kilts, Anderson, Ely, & Mailman, 1988; Leonard et al., 2003). Some studies showed that CeA D1 and D2 influence learning in the same way: intra-CeA infusions of D1 or D2 antagonists impaired fear conditioning and conditioned place preference while D1 and D2 agonists improved both learning tasks (Guarraci, Frohardt, Falls, & Kapp, 2000; Guarraci, Frohardt, & Kapp, 1999; Rezayof, Zarrindast, Sahraei, & Haeri-Rohani, 2002; Zarrindast et al., 2003). It is possible that the effects of D1 and D2 mechanisms in the CeA are more cognitive domain specific. In addition to the disengagement behavior, we also found that D1, but not D2, mechanisms were important for the five choice attentional task (Smith et al., 2011).

In both experiments, animals generally did not show some typical sensorimotor deficits linked to extensive striatal DA neuron loss as measured by several behavioral tasks. During the adhesive dot test in the first experiment, however, the rats consistently made contact with and removed the ipsilateral dot first, even though the contact and

removal of the ipsilateral dot were followed by the contact/removal of the contralateral dot. The latencies to remove the dots in the ipsilateral vs. contralateral paws were 32-sec and 39-secs, respectively, and were not statistically different ($p>0.1$). Unlike the temporal D1 inactivation in the CeA, which resulted in no ipsilateral bias in the dot test, depletion of CeA dopamine resulted in reduced TH density in the SNc, presumably reflecting reduced dopamine function in the SNc. This could have potentially influenced the nigrostriatal dopamine activity, which is suggested to be important for the adhesive dot test. Even though our data suggested that there was no difference in striatum TH density between the ipsilateral and contralateral sides, subtle changes in nigrostriatal activity undetected by TH density might have been enough to cause ipsilateral bias. However, it is not known whether the nigral dopamine cells send collateral projections to the CeA and striatum. Based on our TH density data, retrograde-depletion of nigral dopamine input to the CeA did not seem to affect nigral dopamine input to the striatum, possibly suggesting separate SNc projections to the CeA and the striatum. We are currently conducting an anatomical study to address this issue.

Together, our current study shows that the nigral-CeA dopamine circuitry may be involved in disengagement behavior. This finding may have significant implications for understanding one of the most consistent characteristics in PD: inability to engage in (or shift attention between) two tasks (Filoteo et al., 1997; Naismith et al., 2010; Ravizza & Ciranni, 2002; Yamaguchi & Kobayashi, 1998). Accumulating evidence suggests that failure in attentional allocation in PD patients cannot be simply explained by deterioration of cortical functions (Heiko Braak et al., 2004; Lewis et al., 2005; Woodward et al., 2002). Interestingly, the CeA shows the most consistent pathological changes among the amygdala subnuclei in PD patients (H. Braak et al., 1994; Heiko

Braak et al., 2004; Harding, Stimson, Henderson, & Halliday, 2002). Furthermore, the pathological changes in the CeA occur as the SNc shows the first sign of pathogenesis, prior to cortical changes. Thus, CeA dopamine dysfunction may contribute to attentional disengagement problems seen in PD patients.

Chapter 3: The roles of central amygdala D1 and D2 receptors on attentional performance in a five choice task

3.1 ABSTRACT

The central amygdala (CeA) has been shown to play an important role in mediating several attentional processes including selective and sustained attention. Emerging evidence suggests that the connections between the CeA and the midbrain dopamine areas are important for attentional processing. However, little is known about the role of dopaminergic input into the CeA in mediating attentional processes. To investigate how dopamine activity in the CeA modulates attentional processing, CeA D1 and D2 receptors were temporarily inactivated during testing in a five choice task. In this task, rats were trained to detect one of five recessed ports that briefly illuminated in order to receive a food reward, therefore requiring the rats to successfully sustain their attention to monitor all five ports and selectively attend to the lit port. Then, rats were tested in several altered versions of the task to increase attentional load (e.g. variable ready period). In two experiments, the D1 antagonist, SCH 23390, or the D2 antagonist, raclopride, were infused into the bilateral CeA preceding the test sessions. D1 but not D2 inactivation reduced performance in the more demanding versions of the five choice task. Therefore, CeA D1 receptors might mediate attentio[2]nal functions important for visual cue detection in a five choice task.

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3.2 INTRODUCTION

The central nucleus of the amygdala (CeA) plays an important role in appetitive learning and attentional processing (Peter C. Holland & Gallagher, 1999). The CeA is necessary for several forms of attention such as enhanced orienting to a conditioned stimulus (e.g., rearing towards an appetitively conditioned light stimulus; Gallagher et al., 1990; McDannald, Kerfoot, Gallagher, & Holland, 2004) and enhanced attention to a conditioned stimulus as a result of an unexpected outcome contingency (P C Holland & Gallagher, 1993). Further, the connections between CeA and midbrain dopamine areas have been implicated in these attentional processes (El-Amamy & Holland, 2006; Han et al., 1997; Lee et al., 2008; Lee, Wheeler, & Holland, 2011; Lee et al., 2005, 2006).

The CeA also modulates selective and sustained attention as measured by the ability to attend to a specific visual cue in the presence of multiple cues. Holland and colleagues found that bilateral CeA lesions impaired performance in an operant multiple-choice reaction time task (Holland et al., 2000). In this task, rats were trained to nose poke into a port that was briefly illuminated (500msec) among 3 ports to receive a food reward. The impending port illumination was signaled by the house-light for 5 sec. When the attentional load was increased by shortening the port illumination to 100 msec or by varying the duration of the house-light signal, rats with CeA lesions showed reduced accuracy and response time. In subsequent studies, Holland and colleagues also showed that the CeA's connections to the cholinergic substantia innominata/nucleus basalis magnocellularis (SI/nBM) and the cholinergic input to the medial prefrontal cortex

(mPFC) were important for mediating the attentional performance in a similar multiple-choice reaction task (Holland, 2007; Maddux et al., 2007).

However, it is currently unknown whether the midbrain dopamine connections with the CeA, previously implicated in enhanced attentional processing of conditioned cues (Lee et al., 2005, 2006) also play an important role in mediating attentional performance during the multiple-choice reaction task. Anatomically, the CeA and the midbrain dopamine cells have reciprocal connections (Asan, 1997; Cheung et al., 1998; Fudge & Haber, 2000; Haber et al., 2000; Hongjoo J. Lee et al., 2011; Hongjoo J Lee et al., 2005; L. W. Swanson, 1982), suggesting that dopamine can have a direct influence on CeA function. In fact, several studies showed that manipulations of dopamine receptors in the CeA altered learning and memory such as fear conditioning and conditioned place preference (Guarraci et al., 2000, 1999; Rezayof et al., 2002; Zarrindast et al., 2003). Our recent study also suggests that dopamine function in the CeA is critical for a particular type of attentional processing (Smith, Geissler, Schallert, & Lee, 2013). Either permanent dopamine depletion or temporary blockage of D1 receptors in the CeA produced deficits in the rat's ability to disengage from an ongoing activity and attend to an incoming stimulus. Therefore, we examined whether dopamine functions in the CeA are important for attentional performance during a five choice task by temporally inactivating CeA D1 receptors (Experiment 1) or CeA D2 receptors (Experiment 2).

3.3 METHOD

3.3.1 Subjects

One hundred and three male Long-Evans rats (Charles River) weighing 250-275 g upon arrival were housed in a vivarium with a reversed 14 hour light: 10 hour dark cycle with lights off at 10 AM. One week after arrival, rats were food restricted to maintain 90% of their free-feeding body weight but had constant access to water. Rats were allowed *ad libitum* access to food and water during the one-week recovery period after surgery. All behavioral training and testing occurred during the dark phase. All experiments were conducted according to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and all protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

3.3.2 Surgery

Rats were anesthetized using 2-5% isoflurane gas (Abbott Laboratories) and were placed into the stereotaxic frame (Kopf Instruments, Tujunga, CA). All the rats were implanted with bilateral guide cannulae (26 gauge, PlasticsOne) to target the CeA (AP = -2.0, ML = ±3.6, DV = -7.2). Once the cannulae were in place, dental acrylic (Lang Dental Manufacturing Co., Wheeling, IL) was poured onto the skull to create a head cap with 4 jewel-screws anchored to the skull. After the dental acrylic had completely dried, dummy cannulae were inserted into the guide cannulae. The rats received a subcutaneous injection of buprenorphine hydrochloride (0.01 mg/kg; TW Medical, Denver, CO) and were given one week to recover from surgery.

3.3.3 Apparatus

Five choice training and testing was conducted in 8 operant boxes with aluminum side walls and ceiling, and clear acrylic front and back walls (30.5 cm W × 25.4 cm D × 30.5 cm H; Coulbourn Instruments, Whitehall, PA). One of these side walls was concave

and contained five recessed ports (each 2.5 cm in diameter) with each of the recessed ports 3 cm from the grid floor (stainless steel rods 0.5 cm in diameter, parallel, spaced 1.0 cm apart) and had 3 red LED lights inside to illuminate the port at the appropriate time. Additionally, the ports were equipped with infrared beams that detected nose pokes. Opposite the concave wall was a 2-watt house light (centered on the wall, 26 cm from the floor) and a recessed food cup (centered, 2 cm from the floor) equipped with an infrared beam to detect entries. On the top of the box was an activity monitor (Coulbourn Instruments) that measured activity through infrared beam breaks. Each box was housed in a light- and sound-attenuating chamber (58.4 cm × 61 cm × 45.7 cm; Coulbourn Instruments) and interfaced with a computer using GraphicState 3.1 (Coulbourn Instruments).

3.3.4 Five choice task

3.3.4.1 Shaping

In order to train the rats on the five choice task, two shaping procedures were conducted. First, rats underwent a magazine-shaping session in which they were trained to eat a single grain pellet (45 mg grain tablet, Test Diet, Richmond IN) delivered to a food cup located within the conditioning chamber. A total of 30 pellets were delivered at a variable interval (averaging 60 sec) over a 30 min session. After this session, all rats reliably retrieved grain pellets from the food cup. Second, the rats went through a nose poke-shaping session in which they were trained to make a nose poke response to the ports. All five ports were illuminated for 30 sec and a nose poke to any port during this time resulted in the delivery of a grain pellet in the food cup. This daily session was continued until the rats met criterion of 80% or more responses over 30 trials with a variable intertrial interval (ITI) of 30 sec. After completing the shaping sessions, the rats began training in the five choice task.

3.3.4.2 Training

Rats were trained to the baseline task gradually. The beginning of a trial was signaled by the house light. After 5 sec of constant illumination (i.e., ready period), one of the five target ports was illuminated. Rats first had to detect (i.e., nose poke) a port that was illuminated for 30 sec. Once the rats reached the criterion (80% trials with correct responses), the port light duration was shortened successively to 20 sec, 10 sec, 5 sec, 3 sec, 1 sec, and then finally to 500 msec once they met the criterion at each stage. Regardless of the port light duration the session time was 30 min, but as port light duration decreased, the number of trials increased to accommodate the 30 min training window. Therefore, the total trials for each stage ranged from 30 trials (for 30 sec port light) to 60 trials (for 500 msec port light). The rats had a total of 5 sec (i.e., response period) from the time the port light was illuminated to make a nose poke response to the target port unless the port light duration was longer than 5 sec, in which case the rats were allowed to make a nose poke for the entire duration the port was illuminated. A correct nose poke resulted in an immediate delivery of a grain pellet and darkening of the house light (and the port if still illuminated). If no correct response was made during the 5 sec response period, the house light was darkened. Responses to the non-target ports (i.e., the other 4 that were not illuminated) during the 5 sec response period were recorded as errors but resulted in no consequences. In addition, nose poke responses to any of the 5 ports during the 5 sec ready period were recorded as premature responses but did not have any consequences. The baseline task consisted of 60 trials in a 30 min session (variable ITI of 30 sec) and each port was illuminated equally (i.e., 12 times per session) on a semi-random schedule (i.e., no port was lit more than two times consecutively). This procedure was adapted from Holland and colleagues (Holland, 2007; Holland et al., 2000; Maddux et al., 2007), which differ from the typical 5 choice serial

reaction time task (5CSRTT) procedure (Robbins, 2002). The initiation and termination of the trials are independent of the rats' response in our procedure. Therefore, premature response during the ready period does not delay or cancel port illumination. In addition, an incorrect response during the response period does not terminate the trial allowing the rats an opportunity to correct their mistakes and make a correct response. Nevertheless, this modified version of the five choice task was sensitive to attentional challenges and manipulations of the CeA and its connections to the cortico-cholinergic system (Holland, 2007; Holland et al., 2000; Maddux et al., 2007)

In order to finish training on the 500 msec baseline task, the rats had to reach the criterion twice during 3 consecutive training days. Then, the rats received bilateral cannulae implantation, were given a week to recover, and were retrained on the 500 msec task to the same criterion as before (See Table 3.1).

3.3.4.2 Testing

Once rats reached criterion on the 500 msec task post-surgery, they received a handling procedure for two days before testing. The handling procedure consisted of a mock drug infusion process in which the dummy cannulae were removed, the rats were gently held for a couple of minutes, and the dummy cannulae were replaced. The testing phase was 7 days long with 4 infusion/test days. These infusion tests consisted of one baseline test and three challenge tests (all 60 trials each). In the first challenge, the port light duration was shortened from 500 msec to 100 msec for all trials. In the second challenge, the typically constant 5 sec ready period varied to 1, 5, or 9 sec. The third challenge was a blink condition in which the house light blinked during the 5-sec ready period. Half of the rats were run in the order of 500 msec baseline task, shortened port light challenge, variable ready period challenge, blink challenge. The other half were run in the reverse order (blink, variable ready period, shortened port light, baseline). In

between these four test days, the rats underwent the baseline task with no infusion to ensure that there were no lasting effects of the drugs (See Table 3.2).

3.3.5 Infusions

Rats were assigned to receive 0.9% saline (Experiments 1 and 2), a D1 antagonist SCH23390 (Sigma Aldrich, St Louis, MO; Experiment 1), or a D2 antagonist raclopride (Sigma Aldrich; Experiment 2). All the infusions were given fifteen minutes before the start of testing. Bilateral infusions (0.2 µl each) were delivered over 2-min via 33-gauge infusion needle (PlasticsOne) that extended 1 mm beyond the guide cannula using an infusion pump (Harvard Apparatus, Holliston, MA) and a Hamilton syringe (Hamilton, Reno, NV). Two doses of SCH 23390 (0.5 µg and 1.0 µg given bilaterally) and two doses of raclopride (0.25 µg and 0.75 µg given bilaterally) were used. Therefore, there were three groups per experiment; (1) Saline, SCH 23390 0.5 µg, SCH23390 1.0 µg; (2) Saline, raclopride 0.25 µg, and raclopride 0.75 µg.

3.3.6 Histology

After behavioral testing was complete, rats were perfused transcardially with 0.9% saline and 10% formalin. The brains were extracted, placed into 20% sucrose formalin solution overnight, and then frozen the following day. Brains were sliced using a sliding microtome at a thickness of 40 µm and sections containing the CeA were saved. Every fourth section was mounted and Nissl-stained to verify cannula placements. Cannulae were considered to have good placements if the guide cannula track was visualized within 1 mm above the CeA (as defined by Swanson, 2004) as the infusion cannula extended 1 mm past the tip of the guide cannula or if the injection needle track was seen within the CeA.

3.3.7 Statistical Analyses

All statistical analyses were conducted in PASW version 18. All measures of performance in the task were assessed using a repeated measures 3x4 analysis of variance (ANOVA) with the between group factors of drug assignment (control and two different doses of drugs) and the within factors of testing sessions (baseline and three different attentional challenges). Four *post-hoc* One-Way ANOVAs were conducted (when appropriate) on each testing session.

3.4 RESULTS

3.4.1 Experiment 1: CeA D1 Antagonism

3.4.1.1 Cannula placement verification

Cannula placements were considered acceptable if the guide cannula track was visualized within the CeA or up to 1 mm above the CeA (as defined by Swanson, 2003). The number of rats with acceptable cannula placements was 10 in the saline control condition, 8 in the SCH 23390 1.0 µg group and 6 in the SCH 23390 0.5 µg group (Figure 3.1). Six rats in the saline group included in the analyses only had acceptable unilateral cannula placements. However, their performance levels were not different from the ones with acceptable bilateral placements at all 4 tests with saline infusions (all ps > 0.1). Unacceptable placements were either too dorsal or too rostral in relation to the CeA and located in the striatum, substantia innominata or the intercalated nucleus. Additionally, one of the rats in the saline group with acceptable unilateral placements received a mock infusion prior to testing (due to blocked cannula), in which the rat was just gently held on the experimenter's lap for the duration of the actual infusion.

3.4.1.2 Post-surgery training

Rats were re-trained daily on the baseline 500 msec task post-surgery until they met the criterion of 80% correct performance (i.e., trials with correct nose poke to the target port) twice within 3 consecutive days of training. To determine if there were any pre-existing differences between the groups prior to drug infusions, two measures were examined: (1) the average percent correct trials on the days the rats met criterion and (2) the number of days necessary to meet criterion. When a One-Way ANOVA was run on average percent correct trials, there was a main effect of group, $F(2,21) = 4.34$, $p = 0.026$. *Post-hoc* Tukey's HSD revealed that the average percent correct trials for the SCH 23390 1.0 µg group (M = 88.85 SEM = 1.03) was significantly higher than the control group (M = 84.16, SEM = 0.97). However, when a one-way ANOVA was run on the amount of training days needed to reach criterion, there was no difference between groups, $F(2,21) = 1.15$, $p = 0.34$.

3.4.1.3. Infusion tests

Accuracy. Accuracy in each test was measured as percentage of trials with correct responses out of a total 60 trials (Figure 3.2A). The results show an overall main effect of drug, $F(2,21) = 6.598$, $p = 0.006$, and an interaction of the drug and testing, $F(6,63) = 3.389$, $p = 0.006$ while the overall testing effect was marginally significant, $F(3,63) = 2.537$ $p = 0.065$. To further examine the interaction, separate One-Way ANOVAs were conducted for each testing session. These revealed that the D1 antagonist, SCH 23390 had no effect on performance in the 500 msec baseline test [$F(2,21) = 0.645$, $p = 0.54$] or in the 100 msec test [$F(2,21) = 1.087$ $p = 0.36$]. However, D1 receptor antagonism did impact performance at the variable ready period test [$F(2,21) = 4.539$, $p = 0.02$)] in which the higher dose of SCH 23390 (1.0 µg) significantly lowered accuracy in comparison to the control group (Tukey's HSD). In the blink challenge test, there was

also a significant effect of group assignment [$F(2,21) = 9.661$, $p = 0.001$] with the rats receiving the higher dose of SCH 23390 displaying reduced accuracy in comparison to control rats and to rats receiving the lower dose of SCH 23390.

Correct Response Latency. Performance in the five choice task was also assessed using latency to respond correctly (Figure 3.2B). The overall results show a main effect of testing, [$F(3,63) = 4.354$ $p = 0.008$], a trend of a main effect of drug [$F(2,21) = 3.325$, $p = 0.056$], and a significant interaction of testing and drug [$F(6,63) = 2.84$, $p = 0.016$]. Further analyses revealed that SCH 23390 had no effect on response latency in the 500 msec test [$F(2,21) = 0.175$, $p = 0.84$], the 100 msec test [$F(2,21) = 0.177$, $p = 0.84$], or the variable ready period test [$F(2,21) = 1.771$, $p = 0.20$] . However, the D1 antagonist did have an effect on response latency in the blink challenges, $F(2,21) = 11.673$ $p < 0.001$ in which the rats receiving the higher dose of SCH 23390 were slower to respond correctly than the rats receiving the lower dose or saline.

Omissions. Omissions were defined as trials in which no nose poke response (correct or incorrect) to the ports was made during the 5-sec response period (Figure 3.2C). The data show a main effect of testing [$F(3, 63) = 3.128$, $p = 0.032$], a main effect of drug [$F(2,21)= 12.516$, $p < 0.001$], and an interaction of testing and drug [$F (6, 63) = 2.904$, $p = 0.015$]. One-way ANOVAs revealed that there was a main effect of drug in all three attentional challenges [100 msec: $F(2,21) =6.81$, $p = 0.005$; variable ready period: $F (2,21) = 4.782$, $p = 0.019$; blink: $F(2,21) = 8.373$, $p = 0.002$] but not in the 500 msec task [$F(2,21) = 0.692$, $p = 0.512$, Figure 2C]. Tukey's *post hoc* tests demonstrated that at all attentional challenges, rats receiving the higher dose of SCH 23390 had a higher rate of omissions than the other two groups.

Premature Responses. Premature responses were defined as nose poke responses to the ports during the ready period prior to the illumination of a port (Figure 3.2D).

There was a main effect of testing, [$F(3, 63) = 19.832$, $p < 0.001$], a main effect of drug, [$F(2,21) = 6.302$, $p = 0.007$] and an interaction of testing and drug [$F(6, 63) = 3.741$, $p = 0.003$]. Further analyses elucidated that there was a main effect of drug at all attentional challenges [100 msec: $F(2,21) = 4.414$, $p = 0.025$; variable: $F(2,21) = 6.757$, $p = 0.005$; blink: $F(2,21) = 8.487$, $p = 0.002$] but not at the 500 msec task [$F(2,21) = 2.436$, $p = 0.112$]. Rats receiving the higher dose of SCH 23390 committed fewer premature responses in comparison to control rats at all three attentional challenges as well as fewer premature responses compared to rats receiving lower dose of SCH 23390 in the blink condition.

Locomotor Activity. To quantify locomotor activity during testing sessions, beam breaks from an infrared activity monitor were recorded for the duration the rats were in the operant boxes. Locomotor activity (seen in Table 3.3) was not affected by the application of a D1 antagonist as demonstrated by a lack of significant main effect of drug [$F(2, 21) = 0.893$, $p = 0.424$] as well as no main effect of testing [$F(3,63) = 0.861$ $p = 0.466$] or interaction of the two factors [$F(6,63) = 1.45$, $p = 0.21$]. The results eliminate the possibility of motor impairment as an explanation for the decrements in performance seen in the attentional challenges. Furthermore, we analyzed the latency to retrieve the food pellets once the rats made the correct nose poke response (Table 3). There were no differences among the groups at any testing point: drug [$F(2, 21) = 0.53$, $p = 0.535$], testing [$F(3,63) = 0.454$ $p = 0.715$], and interaction [$F(6,63) = 0.905$, $p = 0.497$]. These results further rule out motor as well as motivational factors for the decreased performance seen in the attentional challenges.

Table 3.1 Timeline of experiment progression

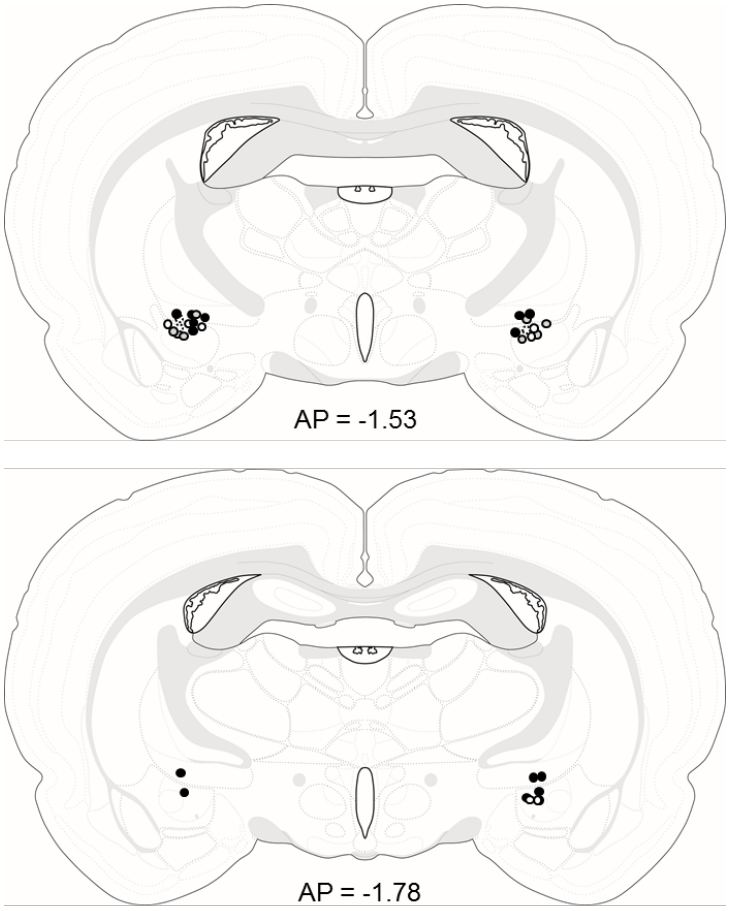
Event	Number of Days	Criterion TO PROGRESS
Pre-surgery training	10-14	≥80% correct trials 2 out of 3 consecutive days
Surgery and Recovery	7	n/a
Post-surgery training	2-10	≥80% correct trials 2 out of 3 consecutive days
Testing	7	No criterion – rats moved through testing schedule regardless of performance

Table 3.2 Testing orders for attentional challenges

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
Order 1	500 msec	Baseline	100 msec	Baseline	Variable	Baseline	Blink
Order 2	Blink	Baseline	Variable	Baseline	100 msec	Baseline	500 msec

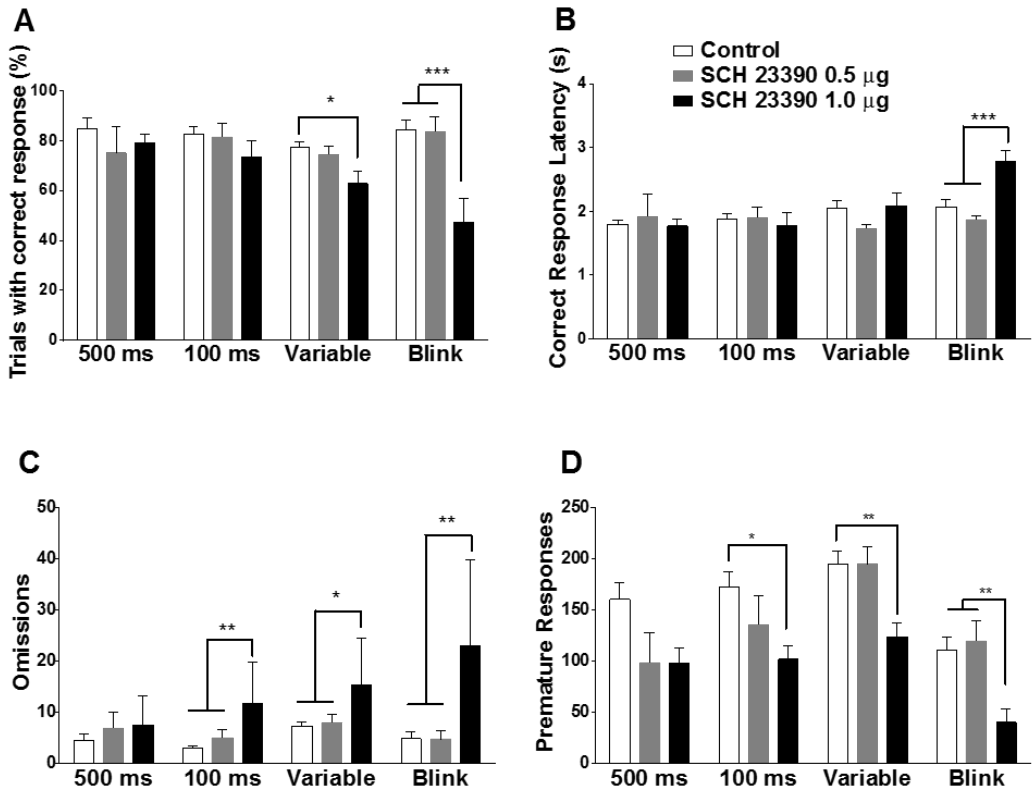
Note: Rats received infusions every other day starting day 1 (gray shading) and received no infusion on three baseline days interspersed between infusion days (no shading).

Figure 3.1 Cannula placements within the CeA between AP -1.53 and -2.00.



Each oval represents a guide cannula tip at its most central point for each animal. Filled ovals represent the animals receiving SCH 23390 with the grey representing the lower dose (0.5 μg) and the black representing the higher dose (1.0 μg) while open (white) ovals represent animals in the control group. Dotted ovals represent the placements of rats with unilaterally acceptable placements.

Figure 3.2 Five choice task performance after CeA D1 antagonism



A. Mean (± SEM) percent trials with correct responses across all tests. B. Mean (± SEM) latency to make the first correct response. C. Mean (± SEM) numbers of trials with omissions (no nose pokes during the response time) across all tests. D. Mean (± SEM) numbers of premature responses across all tests. Premature responses were defined as nose pokes to any ports during the ready period directly prior to the port light illumination. *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 3.3. Locomotor activity and food cup latency after CeA D1 antagonism

	Group	500 msec	100 msec	Variable	Blink
Activity (# of beam breaks)	Saline	491 ± 91.60	525.3 ± 73.75	419 ± 84.14	467.7 ± 70.04
	SCH 0.5μg	446 ± 96.43	525.33 ± 94.54	544.67 ± 125.28	676.67 ± 135.67
	SCH 1.0 μg	393.5 ± 52.15	416.75 ± 94.14	462.42 ± 60.74	377.63 ± 54.66
Food Cup Latency (sec)	Saline	1.55 ± 0.24	1.28 ± 0.07	1.40 ± 0.18	1.45 ± 0.245
	SCH 0.5μg	1.67 ± 0.12	1.74 ± 0.24	1.67 ± 0.19	1.67 ± 0.14
	SCH 1.0 μg	1.52 ± 0.11	1.49 ± 0.12	1.55 ± 0.10	1.52 ± 0.11

3.4.2 Experiment 2: CeA D2 Antagonism

3.4.2.1 Cannula placement verification

Cannula placements were again considered acceptable if the cannula track was visualized within the CeA or up to 1 mm above the CeA (see Figure 3.3). In Experiment 2, the number of rats with acceptable placements were six in the control condition, seven in the raclopride 0.25 µg group, and six in the raclopride 0.75 µg group. Four of the rats in the saline group only had acceptable unilateral cannula placements. Unacceptable cannula placements were located dorsal to the CeA in the striatum or rostral to the CeA in the striatum or substantia innominata. Additionally, two of these four rats only received a mock infusion due to blocked guide cannulae.

3.4.2.2 Post-surgery training

Rats were re-trained daily on the baseline 500 msec task after cannula placement surgery until they met the criterion of 80% correct performance twice within 3 consecutive days of training. To determine if there were any preexisting differences between the groups prior to drug infusions, two measures were examined: (1) the average percent correct trials on the days the rats met criterion and (2) the number of days necessary to meet criterion. One-way ANOVAs were conducted for each variable with drug group assignment as the between subjects factor. There were no differences in average percent correct trials [$F(2,16) = 0.332$, $p = 0.722$] and in number of training days needed to meet criterion [$F(2,16) = 2.32$, $p = 0.13$].

3.4.2.3 Infusion Tests

Accuracy. Performance on the infusion tests was first measured by accuracy (i.e., percentage of trials with correct response). The results (Figure 3.4A) suggest that D2 antagonist, raclopride, did not influence accuracy as seen by no effects of drug [$F(2,16) =$

1.643, p = 0.224], or testing [F(3,48) = 1.302, p = 0.29] or an interaction of those two [F(6, 48) = 0.52, p = 0.79].

Correct Response Latency. Performance in each of the infusion day tests was also assessed using response latency to nose poke the correct port. There was only a main effect of testing [F(3,48) = 3.244, p = 0.03], but no main effect of drug [F(2,16) = 1.311, p = 0.28] and no interaction of drug and testing [F(6,48) = 0.559, p = 0.76, Figure 3.4B]. Upon conducting six paired samples *t*-tests comparing performance at the different infusion tests with a bonferroni-adjusted significance level of p = 0.008, no significant differences emerged (all *p*s > 0.02). While there were no significant differences across *t*-tests, it is likely that the original main effect of testing is due to overall slightly longer latencies in the three challenge versions of the task in comparison to the 500 msec baseline task.

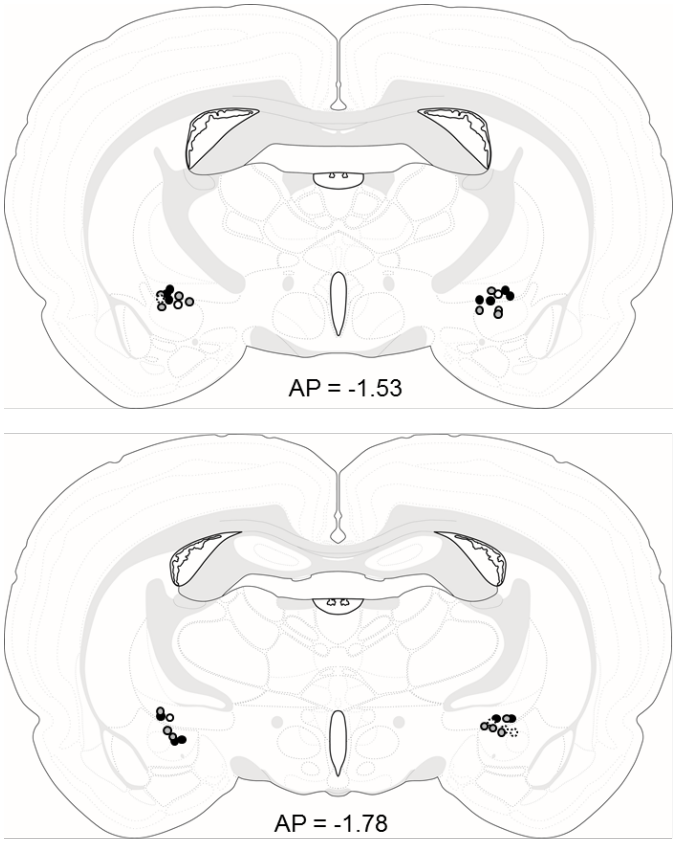
Omissions. The overall omission rates were low across all the testing sessions and did not differ among the drug groups (Figure 3.4C): testing [F(3, 48) = 0.972, p = 0.414], drug [F(2,16) = 0.802, p = 0.466] , an interaction [F(6,48) = 0.519, p = 0.791].

Premature Responses. Raclopride also did not influence the nose pokes to the ports made during the ready period (Figure 3.4D). There was only a significant main effect of testing, F (3,48) = 24.018, p <0.001, but no drug [F(2,16) = 0.417, p = 0.67] or an interaction [F(6,48) = 0.985, p = 0.446]. *Post hoc* paired samples *t*-tests were conducted with a bonferroni-adjusted significance level of p = 0.008, and revealed greater levels of premature responses made in the variable ready period test compared to all other tests: 500 msec [t(18) = -5.164, p < 0.001], 100 msec [t(18) = -6.174, p , 0.001], and blink [t(18) = 9.279, p < 0.001].

Locomotor Activity. Regardless of test or drug assignment, activity levels were comparable across all rats (Table 3.4). There were no effects of drug [F(2, 16) = 0.751, p

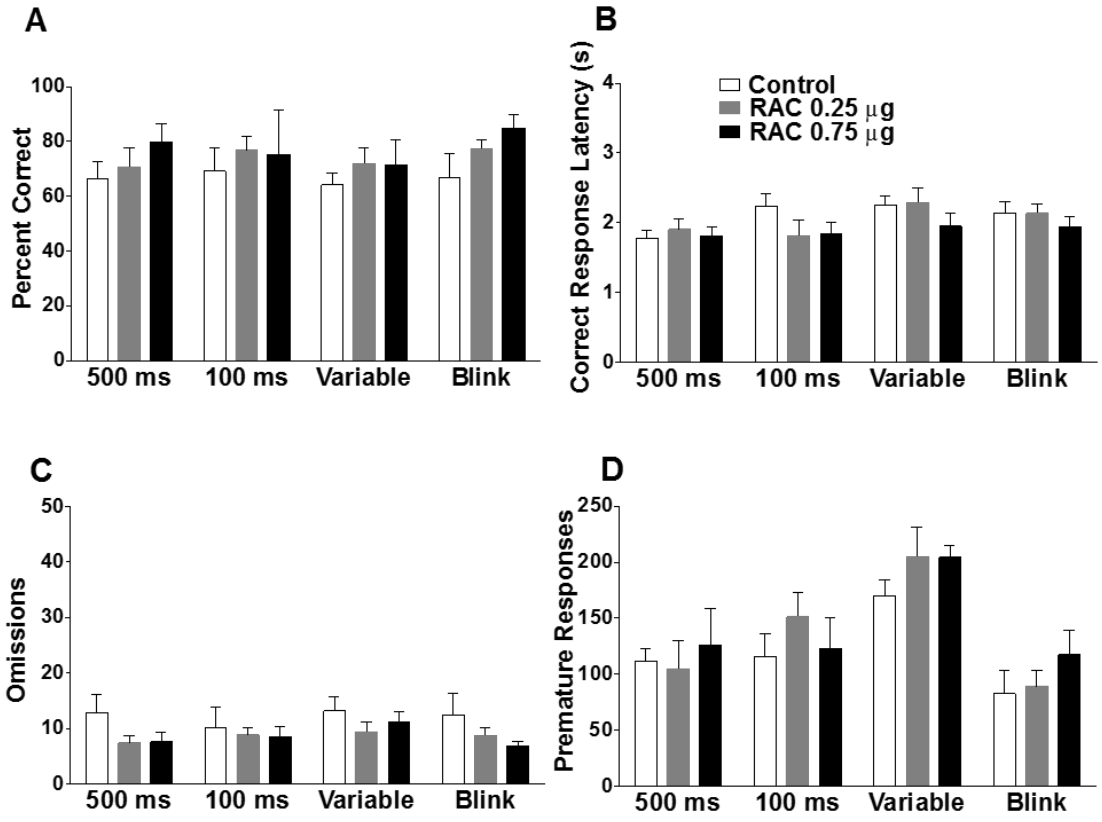
= 0.488], testing [$F(3,48) = 1.684$, $p = 0.183$] or interaction of these two variables [$F(6,48) = 0.57$, $p = 0.752$].

Figure 3.3 Cannula placements within the CeA between AP -1.53 and -2.00.



Each oval represents a guide cannula tip at its most central point for each animal. Open ovals represent animals receiving saline and filled ovals represent the animals receiving Raclopride with the grey representing the lower dose (0.25 μg) and the black representing the higher dose (0.75 μg). Dotted ovals represent the placement for animals with unilaterally acceptable placements.

Figure 3.4 Five choice task performance after CeA D2 antagonism.



A. Mean (± SEM) percent trials with correct responses across all tests. B. Mean (± SEM) latency to make the first correct response. C. Mean (± SEM) numbers of trials with omissions (no nose pokes during the response time) across all tests. D. Mean (± SEM) numbers of premature responses (nose pokes to any ports during the ready period) across all tests. No significant differences were detected.

Table 3.4. Locomotor activity (beam breaks) after CeA D2 antagonism

Group	500 msec	100 msec	Variable	Blink
Saline	470 ± 67.32	637.5 ± 70.58	581 ± 97.12	559.67 ± 64.28
Raclopride 0.25 μg	533.43 ± 62.28	578 ± 86.64	570 ± 93.03	552.57 ± 88.04
Raclopride 0.75 μg	617.67 ± 64.53	652.83 ± 80.42	666 ± 88.33	716.5 ± 78.08

3.5 DISCUSSION

Here we have demonstrated that CeA D1 but not D2 receptors are important for maintaining adequate performance in this five choice task. When D1 receptors in the CeA were temporarily inactivated using SCH 23390, response accuracy decreased when the ready period became variable from trial-to-trial or when the house light blinked during the ready period. Furthermore, D1 receptor antagonism in the CeA resulted in more omissions and fewer premature responses in all three attentional challenges as well as increased correct response latency when the house light blinked during the ready period. This impairment in performance was significant in the group that received a higher dose of D1 antagonist SCH 23390 even though this group showed better performance during the baseline training prior to the infusion. Thus, the pre-existing differences were not likely to confound the subsequent results showing impairing effects of SCH 23390. Therefore, taken together, these data suggest that CeA D1 receptors play an important role in modulating performance during the five choice task.

The increased omission rates observed in the current study falls within the range of what others have seen in cases when accuracy has been impaired as well as when accuracy has been relatively unaltered (e.g., Baunez & Robbins, 1999; Passetti, Dalley, & Robbins, 2003). Increased omission is generally interpreted as reflecting impaired attention when other measures such as food cup latency are not affected (as was the case in our study). However, it can also reflect non-attentional aspects such as 'response vigor' described by Robbins (2002) as reflected by response latencies and rates of omissions. In addition, a premature response in our study is likely to reflect the overall

response vigor more than impulsive behavior because in this procedure, premature responses do not result in any consequences (i.e. termination of the trial). Generally, in other versions of this task, the rat must withhold responding during the ready period in order to avoid a 'time-out' period, therefore premature responses are used to gauge inhibitory control over impulsivity (T. W. Robbins, 2002). However, in our version of the task, responses made during the ready period were not followed by time-out and therefore premature responses are not necessarily due to a failure to inhibit responding and cannot be interpreted strictly as a measure of impulsivity. And as a consequence of this modification, the number of premature responses tends to be higher in our procedure but comparable to other data sets using this same protocol (Olshavsky et al., 2014).

It is likely that the CeA D1 receptors are important for modulating other aspects of five choice task in addition to attentional processing. Studies have demonstrated dissociation between accuracy and response vigor. For example, systemic inactivation of DA receptors can result not in deficits in accuracy but in response vigor (Harrison, Everitt, & Robbins, 1997; Weed & Gold, 1998). At the local level, D1 receptors within the medial prefrontal cortex (mPFC) and striatum are necessary for task accuracy while D2 receptor roles differ depending on the area under examination. In the mPFC, D2 receptor inactivation has little-to-no impact on performance in the 5-CSRTT (Granon et al., 2000) while striatal D2 receptors impact response latency (Agnoli & Mainolfi, 2012). Within the nucleus of accumbens, D1 receptors appear to be important for performance during the 5-CSRTT (Pezze, Dalley, & Robbins, 2007): D1 receptor partial agonist SKF 38393 improved the accuracy and reduced omission, whereas D1 receptor antagonist

SCH 23390 decreased accuracy, and increased both omissions and correct response latencies. In contrast, D2 receptors in the nucleus of accumbens do not seem to directly modulate attentional function but influence the general performance including perseverative responses and food cup latency. Our findings align with existing work showing the importance of D1 receptors in attentional performance and more ambiguous role of D2 receptors.

Reduced performance by D1 receptor blockade in the CeA is not likely due to motor impairment or a simple inability/unwillingness to perform the basic task. The activity levels examined during the sessions were comparable across all three groups. The Coulbourn activity monitor used in the current study is commonly used to measure activity levels in the conditioning chamber. For example, freezing behaviors are recorded using the same kind of monitors (Lee, Choi, Brown, & Kim, 2001; Lee & Kim, 1998). We have also detected enhanced activity levels in the same set up. Systemic injection of D1 agonist, SKF 829580 (0.25 mg/kg), significantly increased activity levels during a classical appetitive conditioning (unpublished data): Saline group (514±59 beam breaks), D1 agonist group (831±54 beam breaks), $p<0.005$. Therefore, we believe our activity monitoring system is sensitive enough to record potential differences in activity levels. Further, the overall response latency of the rats was under 2 seconds and 2-3 seconds when the performance was impaired even though the allotted response time was 5 seconds demonstrating that the rats had enough time to complete the task. The range of the response latency seen in the current study is very similar to the ones reported by Holland et al. (2000) who initially used the modified version of the five choice task.

Their reported response latency was generally just below 2 seconds for control and CeA-lesioned groups and was within 2-4 seconds for CeA-lesioned group when performance was impaired. In addition, the rats infused with D1 antagonist in the current study were not any slower in collecting the food pellet once it was dispensed into the food cup. This suggests that the overall motivational levels were similar in terms of their willingness to retrieve the food reward. While an effect of multiple infusions cannot be completely ruled out given that each rat received a total of 4 infusions of the same drug and dose, this experiment was designed to minimize any possible multiple infusion effects. Firstly, we used two different orders of the attentional challenges as seen in Table 2 and confirmed that order of attentional challenge tests did not result in differences in performance levels. Furthermore, in addition to having two testing orders, we spaced infusions 48 hours apart with the intent of minimizing any potential effect of repeated infusions.

It has been previously demonstrated that D1 and D2 receptors are important for learning. Rats with systemic injections of D1 and D2 antagonists during a Pavlovian learning paradigm showed respective impairment and enhancement of conditioned food cup approach when tested 24-hours later drug-free (Eyny & Horvitz, 2003). However, other work has shown that within the CeA, D1 and D2 receptors operate similarly to affect fear conditioning and conditioned place preference (CPP). Intra-amygdala (mostly targeting CeA) infusions of 2 μg SCH 23390 (D1 antagonist) and 1 μg ecticlopride (D2 antagonist) prior to fear conditioning and/or prior to a retention test 24-hours post-conditioning resulted in reduced freezing at the retention test (Guarraci et al., 2000, 1999). Similarly, intra-CeA infusions of 1.0 μg SCH 23390 or 0.5 μg sulpiride (D2

antagonist) resulted in decreased acquisition of morphine-induced CPP (Rezayof et al., 2002; Zarrindast et al., 2003). Instead of having opposing roles on Pavlovian appetitive learning as is the case at the systemic level, D1 and D2 receptors within the CeA have similar roles in modulating fear and reward learning. Comparatively, we find that a 1.0 μg dose of SCH 23390 in the CeA causes a decrement in attentional function while neither dose (0.25 or 0.75 μg) of the D2 antagonist, raclopride had an impact on attentional functioning as measured by the five choice task. The findings of our current study also align with our recent work examining CeA D1 and D2 receptor functions in another behavioral process called disengagement behavior (Smith et al., 2013). Disengagement behavior is the ability to stop or disengage from an ongoing behavior (i.e. drinking) to attend to an incoming stimulus such as perioral stimulation (Schallert & Hall, 1988). Depletion of dopamine input in the CeA caused a disruption in disengagement but not simple spontaneous orienting to perioral stimulation in the absence of ongoing activity, suggesting that CeA dopamine function is important for an attentional component of this behavior without affecting sensory information processing (Smith et al., 2013). In addition, using the same doses and antagonists used in the current study, we demonstrated that CeA D1 receptor antagonism impaired disengagement behavior while D2 antagonism within the CeA had little-to-no impact on disengagement behavior. It cannot be completely ruled out that higher doses of D2 antagonist, raclopride might influence the performance during the five choice task. However, in a study that examined the role of CeA D2 receptors in fear and anxiety, intra-CeA infusions of 0.75 μg raclopride (the same dose used in the current study) yielded the same results as higher

doses (de la Mora, Gallegos-Cari, Arizmendi-García, Marcellino, & Fuxe, 2010). For example, intra-CeA infusions of either 0.75 μg or 2 μg raclopride increased the latency for the rats to bury an electrified probe while the same two doses and a higher dose (4 μg) had no effect on the rats' behavior on elevated plus maze. Thus, the maximal effect of raclopride in the CeA D2 receptors seems to have been achieved with 0.75 μg.

The midbrain dopamine inputs to the CeA arise from both the SNc and the ventral tegmental area (Oades & Halliday, 1987; L. W. Swanson, 1982). However, our previous study suggests that the nigral dopamine input might be more important for modulating CeA's function. Injection of 6-hydroxydopamine into the CeA, which caused disengagement deficits, resulted a decrease in dopaminergic cells in the SNc but not in the VTA suggesting that dopaminergic input from the SNc is important for successful disengagement behavior (Smith et al., 2013). We have also shown that intact nigral dopamine and CeA connections are crucial in processing enhanced attention driven by prediction error (Lee et al., 2006; Lee et al., 2008). Furthermore, blocking the communication between the VTA and the CeA does not result in the same deficits in enhanced orienting to a visual conditioned stimulus as when the SNc-CeA communication is blocked (Lee et al., 2005; Lee, et al., 2011). Others have also shown that MPTP injections that caused significant reduction in the nigrostriatal dopamine fibers in mice also resulted in significant reduction of dopamine fibers in the CeA (Schober, Herfel, & Unsicker, 2005). While the VTA's input to the CeA cannot be ruled out, it is likely that the SNc dopaminergic input into the CeA plays an important role in attentional processing.

Among the many neural circuits that are involved in regulating attention, the CeA might modulate attention through the basal forebrain cholinergic system, known to play a crucial role in attentional function (Harati, Barbelivien, Cosquer, Majchrzak, & Cassel, 2008; McGaughy, Dalley, Morrison, Everitt, & Robbins, 2002; Risbrough, Bontempi, & Menzaghi, 2002). The CeA can influence the basal forebrain cholinergic system via its direct projections to the SI/nBM (Fritz, Yilmazer-Hanke, Roskoden, Schwegler, & Linke, 2005) which, as a part of the basal forebrain system, in turn sends cholinergic projections to cortex, including the PFC (Mesulam, Mufson, Wainer, & Levey, 1983). The SI/nBM and its projections to the PFC are known to play a crucial role in visual attention during the 5CSRTT (McGaughy et al., 2002). Further, it has been shown that the connections between the CeA and SI/nBM are also necessary for maintaining accuracy in a similar task (Holland, 2007) and the elimination of cholinergic inputs to the PFC as well as lesions of the CeA produce similar deficits in the multiple choice reaction task (Maddux, et al., 2007). Similarly, cholinergic cells in the SI/nBM and their projections to the posterior parietal cortex as well as the CeA play an important role for enhanced attentional processing driven by prediction error (Bucci, Holland, & Gallagher, 1998; Chiba, Bucci, Holland, & Gallagher, 1995; Maddux et al., 2007), the same attentional processing modulated by the SNc-CeA connections (Lee et al., 2006; Lee et al., 2008). Therefore, the CeA might modulate attentional function via its connections to the cortical-cholinergic system.

However, the role of CeA's substantial reciprocal connections to the SNc cannot be ruled out (Fudge & Haber, 2000; Gonzales & Chesselet, 1990). It is possible that the

CeA could be modulating attentional function through the nigrostriatal dopaminergic system, which in concert with the PFC is also crucial for maintaining adequate performance in the 5-CSRTT (Christakou, Robbins, & Everitt, 2001; Rogers et al., 2001). Nigrostriatal dopamine depletion (Baunez & Robbins, 1999), but not necessarily ventral striatal dopamine depletion (Cole & Robbins, 1989) impairs accuracy when the presentation of the port light becomes unpredictable. In the current study, the higher dose of D1 antagonist SCH 23390 also significantly impaired accuracy when the ready period varied. Reducing temporal predictability of the visual targets is likely to require higher levels of readiness or alertness to respond. Therefore, CeA D1 receptors might be important for modulating this aspect of attentional processing. In addition to the modulation of attentional function, the CeA's connections to the nigro-striatal pathway can also influence other factors influencing performance during the five choice task. Bilateral lesions of the medial part of the striatum significantly affected all aspects of performance during 5CSRTT including 'response vigor' (Rogers et al., 2001). In the same study, lateral striatal lesions resulted in severe performance deficits (i.e., increased omission) that precluded them from completing the task. More specific nigro-striatal dopamine depletion also increased omission and correct response latency with a minor impact on accuracy (Baunez & Robbins, 1999). Therefore, the CeA might also play a role in modulating response vigor via its connections to the nigro-striatal pathway.

The current experimental preparation used to study the roles of CeA dopamine receptors in attention may give insight into diseases that are characterized by aberrant dopaminergic function. Specifically, Parkinson's disease (PD), primarily known as a

motor disorder due to the hallmark nigrostriatal dopamine loss, also features attentional dysfunction even in the early stages of disease (Filoteo et al., 1997; Swainson, Rogers, Sahakian, Summers, & Polkey, 2000; Woodward et al., 2002; Yamaguchi & Kobayashi, 1998; Zhou et al., 2012). Dysfunction of the mesocortical system is suggested to be responsible for PD-related cognitive problems; however, cortical areas typically are among the last regions to show pathological changes in PD (Alafuzoff et al., 2009; Braak et al., 2004), and a drug therapy targeted to restore prefrontal function is not always effective at improving attentional deficits in PD patients (Lewis et al., 2005; Owen et al., 1993). Thus, extra-cortical regions are likely to contribute to attentional deficits, especially in the early stages of disease. Therefore, the loss of SNc dopaminergic input into the CeA might contribute to the attentional dysfunctions associated with PD. This work also has implications for other disorders beyond PD. Dysfunction of dopamine system is often seen in disorders accompanied by attentional issues, such as ADHD, depression, and schizophrenia (Abi-Dargham, 2004; Aleman & Kahn, 2005; Braak et al., 1994; Di Michele, Prichep, John, & Chabot, 2005; Laurens, Kiehl, Ngan, & Liddle, 2005; Swanson et al., 2000). Thus, examining dopamine functions in the CeA will broaden our understanding of the neural mechanisms responsible for attentional deficits associated with PD and various other mental disorders.

Chapter 4: The impact of L-dopa on attentional impairments in a rat model of Parkinson's disease

4.1 ABSTRACT

Deficits in attention including difficulty switching attention between tasks or rules, sustaining attention, and selectively attending to specific stimuli are commonly seen in patients with Parkinson's disease (PD). Further, while these deficits are frequently reported, it is unclear how traditional dopamine replacement therapy such as L-dopa affects these deficits. Therefore, in rat model of PD in which dopamine is unilaterally depleted using 6-hydroxydopamine, we first examined the impact of acute and chronic L-dopa treatments on attentional switching as modeled by disengagement behavior (i.e. the ability to disengage from an on-going behavior such as eating or drinking to attend to perioral stimulation). Then, in a separate experiment, we evaluated the use of L-dopa for treating selective and sustained attention deficits using a five choice task. Our data suggest that the L-dopa dose necessary to recover motor function can also successfully restore attention switching behavior (i.e. disengagement behavior) in the short term. However, the dose of L-dopa useful for recovering disengagement behavior and motor function further worsened performance in the selective and sustained attention task. Furthermore, this same dose was responsible for inducing dyskinesias in rats given chronic daily injections. Taken together, these findings demonstrate that simple dopamine replacement therapy may not be sufficient for treating all types of attentional dysfunction occurring in PD.

4.2 INTRODUCTION

While Parkinson's disease (PD) is primarily characterized by the cardinal motor dysfunction, cognitive and attentional deficits are also commonly present (Pfeiffer, Løkkegaard, Zoetmulder, Friberg, & Werdelin, 2014b; Weintraub et al., 2015). Patients with PD demonstrate difficulty with executive function, working memory, as well as several types of attention. For example, PD patients have difficulty dividing attention between multiple stimuli, sustaining attention for a prolonged period of time, and selectively attending to relevant stimuli and ignoring irrelevant stimuli (Filoteo et al., 1997; Yamaguchi & Kobayashi, 1998; Zhou et al., 2012a). Furthermore, PD has also been shown to result in difficulty switching behavioral rules or tasks (Cools, Barker, Sahakian, & Robbins, 2003; Cools, Clark, Hornykiewicz, 1974; Owen, & Robbins, 2002; Ravizza & Ciranni, 2002).

However, the ability of traditional dopamine replacement therapy (i.e. levodopa; L-dopa) in recovering these attentional difficulties in PD is not well understood. In a review by Hornykiewicz (1974), L-dopa is said to improve attentional switching behavior in cases of mild PD, however no study has been conducted in severe PD to study the ability of L-dopa to improve attention switching. In the case of set-shifting, L-dopa recovers the ability to shift response rules (i.e. Wisconsin card sorting task) but causes new impairments in reversal rules (i.e. reversal of reward probability; Cools et al., 2002, 2003; Dujardin et al., 2013). Further, there are conflicting reports on whether selective and sustained attention can be improved with L-dopa. For example, Dujardin et al. (2013) demonstrated that PD patients on L-dopa continue to show impairments in choice

reaction time tasks in comparison to healthy controls (Dujardin et al., 2013) while Zhou et al. (2012) showed no improvements in selective or sustained attention with L-dopa and Moustafa, Sherman, and Frank (2008) demonstrated persistent selective attention deficits (but not worsened) with L-dopa administration. These varied results may be partly due to the nature of clinical research. Patients with PD are commonly on other dopaminergic and non-dopaminergic medications in addition to L-dopa. The disease severity also varies between studies and L-dopa may be differentially effective depending on disease severity.

Therefore, it is pertinent to study the effects of dopamine replacement therapy on attentional impairments in animal models of PD. While attentional dysfunction in PD-modeled animals is not well characterized, there is some work demonstrating in both non-human primate and rat models of PD that attentional deficits do exist (Schallert & Hall, 1988; Decamp and Schneider, 2004). Specifically, rats with unilateral dopaminergic depletion of the nigrostriatal pathway fail to disengage from an on-going behavior (e.g., eating, drinking, grooming) to attend to perioral stimulation even though they have intact sensory ability to detect the perioral stimulation (Schallert & Hall, 1988). This behavior is thought to be analogous to basic attention switching behavior in humans (Posner et al., 1984). Non-human primates exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) also show impairments in sustained attention and attentional shifting behavior (Decamp & Schneider, 2004).

While the therapeutic value of L-dopa has been extensively characterized, little work has been done to assess the efficacy of L-dopa in the treatment of attentional

dysfunction in models of PD. Recently, work by Schneider, Pioli, Jianzhong, Li and Bezard (2012) found that the dose necessary to improve motor function in the MPTP-exposed primates further impaired sustained attention. That is, when asked to sustain attention and indicate which of 3 cues was lit, macaques that received a dose of L-dopa optimized for improving motor impairments, decreased the rate of correct responses. Beyond this, no other work to date has assessed the impact of L-dopa on a spectrum of attentional processes in animal models of PD. Therefore, we designed two experiments to determine the impact of L-dopa treatment on attentional switching behavior (Experiment 1) and selective and sustained attention (Experiment 2) in a rat model of PD.

4.3 Experiment 1: Acute and chronic effects of daily L-dopa administration on disengagement behavior

In this experiment, all rats were given unilateral infusions of 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle. After post-surgery recovery, rats received daily L-dopa or vehicle (saline) injections for 4 weeks and were tested for disengagement behavior along with motor function at Day 1, Week 1, Week 2, and Week 4 (Figure 4.1).

4.3.1 Method

4.3.1.1 Subjects

Male Sprague-Dawley rats (300-400g, Harlan) were triple-housed in a vivarium with a reversed 14 hour light: 10 hour dark cycle with lights off at 10 AM. Rats had *ad libitum* access to food for the duration of the experiment and water access was only restricted 24 hours prior to disengagement testing. All behavioral training and testing occurred during the dark phase. All experiments were conducted according to the

National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and all protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

4.3.1.2 Surgery

Rats were anesthetized using 2-5% isoflurane gas (Abbott Laboratories) and were placed into the stereotaxic frame (Kopf Instruments, Tujunga, CA). All rats were given unilateral infusion of 6-Hydroxydopamine (6-OHDA) into the medial forebrain bundle (AP = -3.3, ML = ±1.7, DV = -8.5). A concentration of 7µg/µl 6-OHDA was prepared in PBS with 0.1% ascorbic acid (Sigma Aldrich, Milwaukee, WI) and 1.0 µl was infused at a rate of 0.1 µl per minute (Hamilton syringe; Harvard Apparatus infusion pump). After the infusion was finished, the skull incision was sutured shut and rats received a subcutaneous injection of buprenorphine hydrochloride (0.01 mg/kg; TW Medical, Denver, CO). Rats were then placed on a heating pad and once awake and mobile, they were returned to their home cage and given a two week recovery period.

4.3.1.3 L-dopa injections

There were three separate groups of injection conditions. For two groups of rats, L-dopa methyl ester hydrochloride (Sigma Aldrich, Milwaukee, WI) was given either at 5 mg/kg or 10 mg/kg. For both groups, benserazide (10 mg/kg; Sigma Aldrich, Milwaukee, WI) was given 15 minutes prior to L-dopa injection to block decarboxylation of L-dopa peripherally. The third group was a vehicle group that was further divided into two subgroups: one group received two saline injections spaced 15 minutes while the other

group received an injection of benserazide (10 mg/kg) and then saline injection 15 minutes apart. However, no difference between these two subgroups existed so they were collapsed to form the vehicle group. The attentional disengagement test followed by a motor (cylinder) test was conducted 15 minutes after the second injection (i.e., either saline or L-dopa).

4.3.1.4 Behavioral testing

Disengagement Test. For the 24 hours immediately prior the disengagement test, all rats were water deprived so that they would be motivated to drink when the testing began. For testing, each animal was allowed to drink from the water spigot at the back of the home cage. While engaged in drinking, each rat was stimulated periorally using a cotton swab. Five trials were conducted on each side, in which the order of stimulation was randomized. The number of times the animal disengaged from drinking after the perioral stimulation was recorded for each side and percentages were calculated. Rats were also periorally stimulated when they were not engaged in drinking to measure baseline reaction to sensory stimulation. Rats were first tested prior to L-dopa injection to assess baseline deficits and additionally tested at 4 different time points over the course of 4 week injection period (Fig 1).

Cylinder Test. In the cylinder test to measure forelimb use (as described by Schallert, Fleming, Leasure, Tillerson, & Bland, 2000), a plastic cylinder (20 cm in diameter and 30 cm in height) was stood up on one end. Rats were placed into the cylinder and spontaneous forepaw touches to the cylinder during rears/exploration of the vertical wall were recorded. If only one paw was placed on the cylinder, it was recorded

as independent use of that limb. If both paws were placed on the cylinder simultaneously, or if one paw was placed on the wall and then the other was immediately placed on the wall, it was recorded as simultaneous use of both limbs. A total of 20 instances of left, right, and simultaneous (both) forelimb use was recorded during exploration of the cylinder wall in either horizontal or vertical planes. The rat was removed from the cylinder either after 20 instances of forelimb use or 5 minutes has passed. The cylinder test was conducted immediately after the disengagement test. Preference for the limb use contralateral to the lesioned side (i.e., affected limb by the dopamine lesion) was calculated by using the following equation previously established by Schallert et al. (2000):

$$\frac{\text{Contralateral paw touches} + (.5)\ \text{both paw touches}}{\text{Total number of touches (ipsi + contra+ both)}}$$

*L-dopa induced dyskinesias***.** Rats were assessed at each testing session for L-dopa induced dyskinesias (LIDs), using a modified abnormal involuntary movement (AIM) rating system adapted from Cenci & Lundblad (2007). Rats were scored on a scale from 0-4 in four different AIM categories: limb (i.e. hyperkinetic movement of usually the forepaws), axial (i.e. torsion or flexion of the trunk), motor (i.e. increased locomotion/turning towards the side contralateral to the lesion, and oral (i.e. chewing motions, twitching of facial muscles). A score of 0 indicated no presence of the AIM while scores 1-4 indicated presence of dyskinesia to varying degrees. A score of 1 signified a present but infrequent movement and a score of 2 indicated a movement present for more than half of the observed time. A score of 3 was given for a

continuously present dyskinesia that was suppressible by external stimulation such as a tap on the cage. If the movement was continuous and unable to be suppressed by external stimulation, it was given a score of 4. Ratings were given for all four AIM categories and then added together for a total AIM score. If LIDs were severe enough to impair or prevent performance in the behavioral tasks, the rat was not tested for the remainder of the experiment.

4.3.1.5 Immunohistochemistry

At the conclusion of the experiment, rats received an overdose of pentobarbital (86 mg/kg) and phenytoin (11 mg/kg) mix (Euthasol® by Virbac Animal Health) and then were perfused transcardially with 0.9% saline followed by 4% Paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were extracted and placed into a 20% sucrose PFA solution overnight. The next day brains were rapidly frozen using powdered dry ice and stored at -80ºC. Brains were sliced at 30 µm using a sliding microtome and sections containing the midbrain dopamine regions were collected in four series.

For the tyrosine hydroxylase (TH) staining, the tissue was first treated with 0.3% hydrogen peroxide in PBS. Then the tissue was placed into a 6% normal horse serum (NHS) in PBS with 0.3% Triton (PBST) for one hour after rinsing in PBS. Immediately afterwards the tissue was incubated in 0.15% PBST solution containing 3% NHS and mouse TH antibody (1:5000; ImmunoStar, Hudson, WI) for 72 hours at 4 ºC. After rinsing, the tissue was incubated with biotinylated horse anti-mouse IgG (1:250, Vector Laboratories) for 1 hour and then with avidin-biotin conjugate (PK-6100, Vector Laboratories) for 1 hour, and reacted using 3,3-diaminobenzidine (DAB; Sigma Aldrich).

Once the TH-stained tissue was mounted and cover slipped, sections were imaged using a microscope (Olympus BX61) at a magnification of 10x. In order to determine the extent of dopamine depletion, basic 2-D stereology was conducted. The number of TH+ cells in the SNc was counted across four sections of tissue (levels 36-39; Swanson, 2003). These numbers were averaged and compared lesion to intact side. In addition to TH+ count in the SNc, integrated optical density in the SNc and ventral tegmental area (VTA) was also measured using ImageJ (Rodbardt function; Rasband, 1997-2001; NIH). Optical density was measured by sampling two locations (radius 100μm) for each structure at four levels (SNc: 36-39; VTA: 37-40; Swanson, 2003).

4.3.2 Results

4.3.2.1 Dopamine depletion and lesion verification

Quantification of dopamine depletion for lesion verification purposes was calculated two ways (Figure 4.2A). First, TH immunoreactive (ir) cells were counted in the entire SNc across four sections. These numbers were averaged and overall dopamine depletion was calculated by obtaining the percent difference score between the intact and lesioned sides. Secondly, optical density measures were taken across the same sections, averaged and the same percent difference score was calculated. For this study, only rats with 50% or greater DA depletion were included in the statistical analyses. Using either measure resulted in the exclusion of (the same) five rats. Of the rats included in analyses (VEH: n=7; L-dopa 5 mg/kg: n=7; L-dopa 10 mg/kg: n=7), there was an 77% reduction in TH-ir positive cells in the SNc with no significant group differences [$F(2,18) = 1.45$ $p > 0.2$]. Using the optical density measurement, overall lesion severity was lower (86%), however no differences between these groups were detected [$F(2,18) = 1.85$, $p > 0.15$].

TH optical density was also quantified in the VTA and on average there was 65% dopamine depletion with no group differences in optical density between groups [$F(2,18) = 0.18$, $p > 0.5$].

4.3.2.2 Dyskinesia development

As a consequence of daily L-dopa injections, some rats developed L-dopa induced dyskinesias (Table 4.1) and were unable to be tested thus changing the n values as the experiment progressed (Table 4.2). For the rats that developed dyskinesia, most were first observed to have dyskinesia at the Week 1 testing time point. Most commonly, rats displayed a forelimb dyskinesia precluding them from being tested in either behavioral test. While the maximum possible score was a 16 (4 sub-ratings all 0-4), no rat scored above an 8 at onset. While all sub-categories of dyskinesias were observed, typically only one or two of the subcategories were observed in each rat. Forelimb dyskiniesias were most commonly seen and present in the majority of rats. On average, forelimb dyskinesia at onset was rated at 2 and 2.25 (4 max) for the L-dopa 5 mg/kg group and L-dopa 10 mg/kg group respectively. The overall LID scores at the onset for both treatment groups were relatively similar (L-dopa 10 mg/kg: 2.8 ± 0.6; L-dopa 5 mg/kg: 2.4 ± 0.8).

4.3.2.3 Disengagement behavior

To determine if L-dopa administration had an impact on disengagement behavior on the affected side (contralateral side), one-way ANOVAs were conducted among three groups at each testing time point. At baseline (prior to L-dopa injection), all rats showed low or non-existent levels of disengagement behavior on the affected side and these severe deficits in disengagement were not different among three groups as expected [$F(2, 21) = 0.41$, $p = 0.67$; Figure 4.2B]. On the first day of injection, rats in the saline

group and 5 mg/kg L-dopa group continued to show severe disengagement deficits. However, the rats that received 10 mg/kg L-dopa showed significant improvement in disengagement behavior [$F(2,18) = 17.92$, $p < 0.001$]. At Week 1, no differences in the rate of disengagement are seen amongst the groups [$F(2,12) = 1.25$, $p = 0.32$] even though both L-dopa groups show more disengagement than the saline group. It is likely to be caused by the overall increase in variability of the disengagement behaviors in rats across all groups. Only one rat in the L-dopa 10 mg/kg group was able to be tested at Week 2 and no differences between the L-dopa 5 mg/kg group and the saline group were observed [$F(2,8) = 2.5$ $p = 0.14$]. At Week 4, only one rat in the L-dopa 10 mg/kg group and two in the L-dopa 5 mg/kg group had not developed dyskinesias and therefore no analyses were conducted.

4.3.2.4 Motor function

The cylinder test was conducted to assess motor function in all rats. In order to determine if there was any preference in paw usage in the cylinder, one-way t-tests with a test value of 50% (chance usage of one paw) were conducted for each group at each time point. To allow for group comparisons, one-way ANOVAs at each time point were also conducted. No analyses were conducted for the week 4 time point due to the majority of L-dopa treated rats having developed dyskinesias by that point. Across all time points, rats receiving vehicle showed either a significant decrease in bad paw usage or a trend of decreased bad paw usage [Baseline: $t(6) = 5.21$, $p < 0.001$; Day 1: $t(6) = 7.12$, $p < 0.001$, Week 1: $t(5) = 2.30$, $p = 0.07$; Week 2: $t(5) = 2.40$, $p = 0.06$; Figure 4.2C]. L-dopa 5 mg/kg did not improve the use of bad paw as they continued to display impaired usage of

the bad paw across all time points as well [Baseline: $t(6) = 12.466$; Day 1: $t(6) = 22.36$, $p < 0.001$; Week 1: $t(5) = 3.06$, $p = 0.028$; Week 2: $t(4) = 6.14$, $p < 0.01$]. However, the L-dopa 10mg/kg group was able to recover initially impaired bad paw usage [Baseline: $t(6) = 20.221$, $p < 0.001$] after a single injection of 10 mg/kg L-dopa [Day 1: $t(6) = 0.63$, $p > 0.5$]. This improvement was still observed after a week of injections [Week 1: $t(4) = -0.42$, $p > 0.5$]. No statistic was calculated at Week 2 as only 2 rats could be tested due to dyskinesia development. Furthermore, when comparing across groups, no differences in bad paw usage were seen at baseline [$F(2, 18) = 0.83$, $p > 0.4$] but at Day 1 administration of L-dopa 10 mg/kg resulted in increased bad paw usage compared to vehicle treatment [Day 1: $F(2,18) = 3.53$, $p = 0.05$]. However, this effect went away at Week 1 [$F(2,14) = 2.27$, $p = 0.1$].

4.3.3 Experiment 1 Discussion

Acutely, L-dopa 10 mg/kg, but not 5 mg/kg, is effective in restoring disengagement and motor function of the affected side. However, rats receiving either dose of L-dopa readily develop dyskinesias thus precluding any potential chronic benefits of L-dopa. While it appears that L-dopa 10 mg/kg becomes less effective at Week 1, the rats remaining at Week 1 show similar motor and disengagement performance from Day 1 to Week1 suggesting no decrease in efficacy.

Figure 4.1 Experiment 1 design: Acute and chronic effects of L-dopa on disengagement behavior and motor function

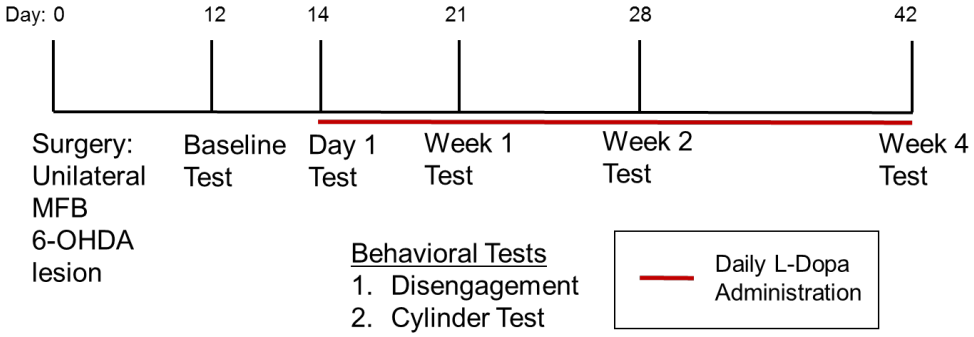


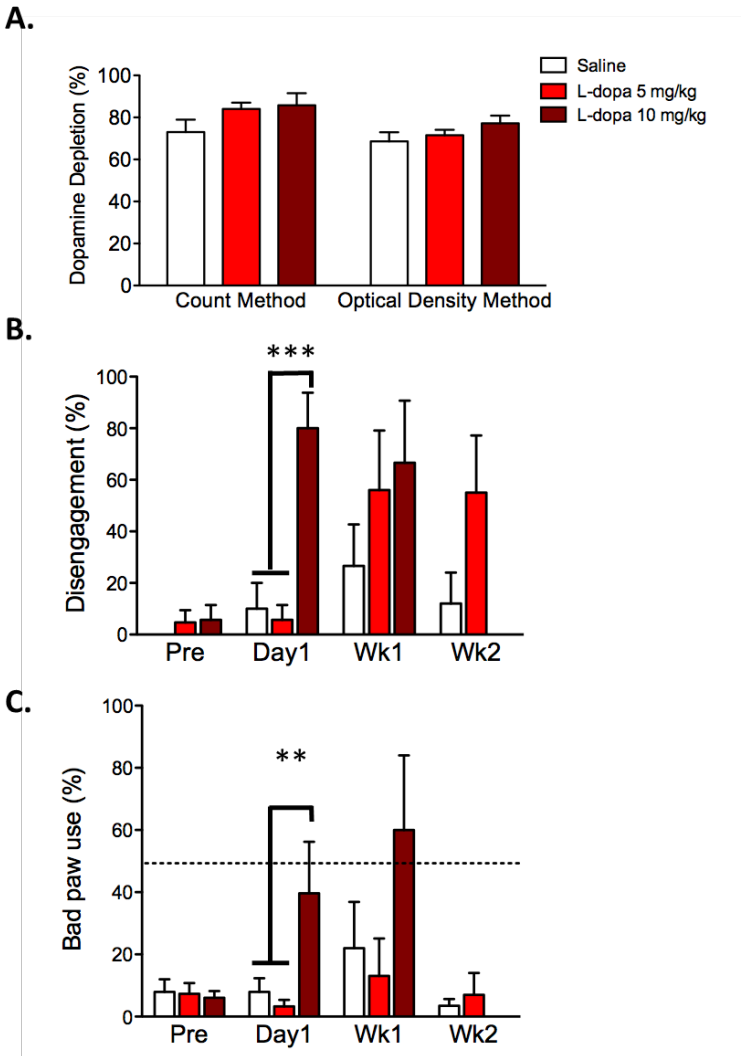
Table 4.1 Rate and severity of dyskinesia in L-dopa-treated rats

	total *n*	number of rats that developed LID	number of rats with LID at Week 1	average onset of LID (days) [a]	average LID severity at onset[b]
Saline	7	0	0	-	-
L-dopa 5 mg/kg	7	6	4	8.4 ± 1.4	2.4 ± 0.8
L-dopa 10 mg/kg	7	6	5	8.2 ± 1.1	2.8 ± 0.6

[a] Rats were discontinued from testing once LIDs precluded rats from being tested.

[b] While all possible scores ranged 0-16 (combination of four 0-4 sub-ratings), no rat was scored above an 8 at onset. Generally rats exhibited only 2 (any 2) of the 4 sub-types of dyskinesia when scored.

Figure 4.2 Dopamine depletion, disengagement behavior, and motor function



Mean (± SEM) dopamine depletion in the SNc as measured by counting of TH+ cells and sampling of optical density. B. Mean (± SEM) disengagement behavior on the side contralateral to 6-OHDA lesion. C. Mean (± SEM) usage of the bad (contralateral) paw in the cylinder test, chance usage = 50%. ** $p < 0.01$, *** $p < 0.001$

4.4 EXPERIMENT 2: THE IMPACT OF L-DOPA ADMINISTRATION ON FIVE CHOICE TASK PERFORMANCE

In experiment 1, many rats developed dyskinesias within 1-2 weeks of L-dopa injections preventing further testing to examine the chronic effects. Therefore, only the acute effects of L-dopa on selective and sustained attention were examined using a five choice task (Figure 3A). The rats were trained on this task prior to surgery (unilateral 6-OHDA infusion into the MFB), and then re-trained after recovering from the surgery (Figure 4.3). Performance in the five choice task of these rats on and off L-dopa were then compared.

4.4.1 Method

4.4.1.1 Subjects

Male Sprague-Dawley rats (300-400g, Harlan) were triple-housed in the same condition as in Experiment 1 with a reversed 14 h light: 10 h dark cycle with lights off at 10 AM. Rats were food restricted to maintain 90% of free-feeding weight except during the post-operative recovery period and had *ad libitium* access to water during the experiment. All behavioral training and testing occurred during the dark phase. All experiments were conducted according to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and all protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

4.4.1.2 Surgery

Rats were anesthetized using 2-5% isoflurane gas (Abbott Laboratories) and were placed into the stereotaxic frame (Kopf Instruments, Tujunga, CA). All rats were given

the same unilateral infusion of 6-OHDA into the medial forebrain bundle as done in Experiment 1 except a range of 0.6-1.0µl of the 7µg/µl 6-OHDA solution was given. Also unlike Experiment 1, rats were given 25 mg/kg dose of desipramine (Sigma Aldrich, Milwaukee, WI) prior to surgery to protect noradrenergic cells from 6-OHDA. They also received the same post-operative care as in Experiment 1.

4.4.1.3 L-dopa injections

The rats were divided into three injection groups and received identical injection procedures as in Experiment 1. Briefly, there were L-dopa 5 mg/kg and L-dopa 10 mg/kg groups, and vehicle group. Benserazide (10 mg/kg) was given 15 minutes prior to L-dopa injections or some saline injections. Unlike Experiment 1, the rats received the injection procedures only over three days during the final days of behavioral testing.

4.4.1.4 Five choice task

Apparatus. Five choice task training and testing was conducted in 8 operant boxes with aluminum side walls and ceiling, and clear acrylic front and back walls (30.5 cm W × 25.4 cm D × 30.5 cm H; Coulbourn Instruments, Whitehall, PA). One of these side walls was concave and contained five recessed ports (each 2.5 cm in diameter) that were located 3 cm from the grid floor (stainless steel rods 0.5 cm in diameter, parallel, spaced 1.0 cm apart) and had 3 red LED lights inside to illuminate the port at the appropriate time. Additionally, the ports were equipped with infrared beams that detected nose pokes. Opposite the concave wall was a 2-watt house light (centered on the wall, 26 cm from the floor) and a recessed food cup (centered, 2 cm from the floor) equipped with an infrared

beam to detect entries. Each box was housed in a light- and sound-attenuating chamber (58.4 cm × 61 cm × 45.7 cm; Coulbourn Instruments) and interfaced with a computer using GraphicState 3.1 (Coulbourn Instruments).

Shaping. In order to train the rats on the five choice task, two shaping procedures were conducted. First, rats underwent a magazine-shaping session in which they were trained to eat a single grain pellet (45 mg grain tablet, Test Diet, Richmond IN) delivered to a food cup located within the conditioning chamber. A total of 30 pellets were delivered at a variable interval (averaging 60 sec) over a 30 min session. After this session, all rats reliably retrieved grain pellets from the food cup. Second, the rats went through a nose poke-shaping session in which they were trained to make a nose poke response to port. All five ports were illuminated and a nose poke to any port resulted in the delivery of a grain pellet in the food cup. This daily session was continued until the rats met criterion of 80% or more responses over 30 trials with a variable intertrial interval (ITI) with an average of 30 sec. After completing the shaping sessions, the rats began the baseline training in the five choice task.

Training. In the baseline task, the beginning of a trial was signaled by the illumination of the house light. After 5 sec of constant illumination (i.e., ready period), one of the five target ports was illuminated for 500 msec. The rats had a total of 5 sec (i.e., response period) from the time the port light was illuminated to make a nose poke response to the target port. A correct nose poke resulted in an immediate delivery of a grain pellet and darkening of the house light (and the port if still illuminated). Responses to the non-target ports (i.e., the other 4 that were not illuminated) during the 5 sec

response period were recorded as errors but resulted in no consequences. In addition, nose poke responses to any of the 5 ports during the 5 sec ready period were recorded as premature responses but did not have any consequences. The baseline task consisted of 60 trials in a 30 min session (variable ITI of 30 sec) and each port was illuminated equally (i.e., 12 times per session) on a semi-random schedule (i.e. no port was lit more than two times consecutively). This procedure was adapted from Holland et al., (2000) and Maddux et al., (2007), in which the initiation and termination of the trials were independent of the rats' response.

Rats were trained to the baseline task gradually. Rats first started on a task where the port light was illuminated for 30 sec which allowed the rats 30-sec response period. Once the rats reached the criterion (80% trials with correct responses), the port light duration was shortened successively to 20 sec, 15 sec, 10 sec, 5 sec, 3 sec, 1 sec, and then finally to 500 msec. Regardless of the port light duration, the session time was 30 min resulting the number of trials for each program to change. Therefore, total trials started at 35 (for 30 sec port light) and increased to 60 as the port light duration shortened to 500 msec. In order to finish training on the 500 msec baseline task, the rats had to reach the criterion (80% correct response) twice during 3 consecutive training days. Then, the rats received unilateral 6-OHDA MFB lesions, were given two weeks to recover, and then retrained on the task to the same criterion as before.

Re-training and Testing. In our typical five choice task procedure, rats are briefly re-trained on the 500 msec protocol after a given surgery, and are further tested in more challenging protocols with higher attentional demands. However, rats with unilateral

dopamine depletion show significant behavioral deficits at 500 msec protocol. Thus, rats were re-trained on the task starting from the 30s stimulus duration and subsequent protocols as criterion was met. The majority of animals with acceptable lesions was unable to complete re-training to perform at 500 msec protocol. Therefore, these rats were discontinued from re-training after seven days of sub-criterion (< 80%) performance in the same training protocol (e.g., 5 sec port light protocol). Once this occurred, the rats were injected with saline or L-dopa and run again in the same training protocol that the rats stagnated on to test the possibility that L-dopa could improve the impaired performance levels. Rats were tested across three days with an injection of L-dopa or saline occurring prior to each session. Performance on these three days was compared to the last three days of training prior to the injections.

4.4.1.5 Immunohistochemistry

Brain tissue was collected and preserved, processed for TH in an identical manner as Experiment 1. The immunohistological staining process was the same except that a higher concentration of primary antibody (mouse anti-TH) was used (1:2500).

4.4.2 Results

4.4.2.1 Dopamine depletion and lesion verification

There were 32 rats with 50% or greater dopamine depletion. However, as there were so few rats with ≥50% dopamine depletion completing five choice training (n=7; mean dopamine depletion: 63%), we chose to focus on the rats unable to complete training. Therefore from here on, only data from the rats unable to complete five choice

training is presented. All groups showed comparable dopamine depletion of the SNc as measured by the optical density method described above [$F(2,22) = 0.54$, $p = 0.59$; Table 2]. Furthermore, all groups showed greater than 50% dopamine depletion of the VTA but no differences in the level of dopaminergic depletion among these groups [$F(2,22) = 1.44$, $p = 0.26$; Table 4.2].

4.4.2.2 Five choice task

After completing training in the five choice task, rats were given 6-OHDA infusions unilaterally into the MFB. Once two-weeks of recovery were complete, rats were re-trained using the same pre-surgery training method. The majority of rats were unable to re-train down to 500 msec port light protocol in the five choice task post-surgery (Fig 4.4A). Rats were considered to have failed re-training after 7 days of sub-criterion performance (< 80%) on the same training protocol. The median protocol they remained was at 5 sec port light duration. As these rats showed severe deficits in this task, we continued to train the rats for three more days and gave L-dopa (or saline) injections 15 minutes prior to training on those three days. Within the vehicle group, half of the rats were treated with benserazide and saline while the other half were treated with two injections of saline. No differences between these two subgroups existed so they were collapsed to form one vehicle group. Performance of these rats on L-dopa (or saline) for those three days (on) were then compared to their performance on the last three days prior to the injections (off).

Performance on and off L-dopa was examined across treatment groups using percentage of correct trials. The rate of correct responses when comparing pre-injection

vs injection days was differentially affected by the dose of L-dopa injected [$F(2,22) = 4.395$, $p = 0.025$; Figure 4.4B]. While there were no differences in performance between groups on the training sessions prior to injection, [$F(2,22) = 0.56$, $p = 0.58$] there was a difference in the rate of trials completed correctly in the testing sessions in which L-dopa injections were give directly prior [$F(2,22)= 13.77$, $p < 0.001$]. Additionally, rats receiving L-dopa 10 mg/kg performed significantly fewer correct trials in comparison to both rats receiving L-dopa 5 mg/kg and vehicle.

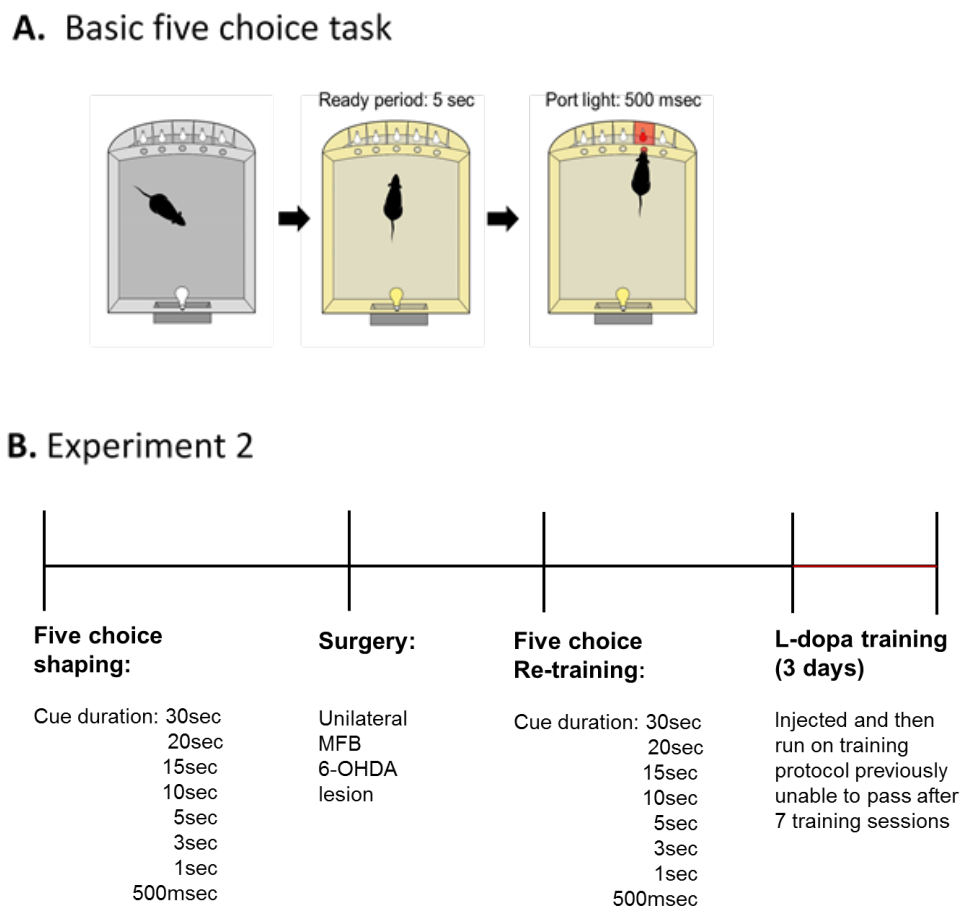
Because the rats received dopamine depletion unilaterally, we investigated the possibility that performance in the five choice task varied by port location. To do this, percentage of correct responses were calculated for the extreme left and right ports then were renamed the ipsilateral and contralateral ports in reference to the lesion location in the rat (Figure 4.4C & 4.4D). If the rat had a left hemisphere lesion, the right-most port was considered the bad-side (contralateral) port and the left-most port was considered the good-side (ipsilateral) port and vice versa for a right side lesion. A repeated measures ANOVA looking at performance pre- and post-infusion in both the good and bad side ports was conducted (between-subjects variable: time point; within-subjects variable: port location in reference to the lesion). L-dopa did not impair performance across the board [$F(2,22) = 1.00$, $p = 0.4$], but did impair performance depending on port location [$F(1,22) = 32.01$, $p < 0.001$] demonstrating that all lesioned rats show a lower correct response rate in the port contralateral to the lesion compared to the port ipsilateral to the lesion. There was also a trend of an interaction of port location and treatment assignment [$F(1,22) = 3.46$, $p = 0.07$]. Paired samples *t*-tests assessing performance with and without

injection treatment at each group level demonstrates that saline injection nor low dose of L-dopa injection does not change performance in either ports (all *t*s < 1, all *p*s> 0.4). However, while the high dose of L-dopa does not alter the rate of correct responses in the port contralateral to the lesion [$t(8) = 0.479$, $p = 0.65$] it does decrease the rate of correct responses in the port ipsilateral to the lesion [$t(8) = 3.64$, $p = 0.007$; Figure 4.4D].

4.4.3 Experiment 2 Discussion

As the majority of rats were unable to complete re-training in the five choice task, rats were assessed in the protocol they were unable to meet criterion on (80% correct trials) after seven training sessions. Acute L-dopa injections prior to training in this protocol resulted in a dose dependent decrease in the rate of correct responses. This overall decrease in correct responses is specifically due to decreased performance on the port most ipsilateral to the lesion (i.e., unaffected/good side). The compromised performance on the contralateral (i.e., affected/bad side) port was unaffected by L-dopa administration.

Figure 4.3 Experiment 2 design



A. Schematic representation of the five choice task with a cue (port light) duration of 500 msec. B. Experiment 2 design. Rats were trained on a series of task in which the port light was successively shortened as the rats showed proficiency in each cue duration protocol. After rats were trained on the task and recovered from surgery, the rats were retrained on the task. Rats were unable to re-train on the task, and after stagnating on the same training protocol for 7 days, rats were given L-dopa injections and then run through the same training protocol for three days and performance as assessed.

Table 4.2 Dopamine (DA) depletion in the SNc and VTA after 6-OHDA infusion

		DA depletion (%)	
	n	SNc	VTA
Saline	8	88.88 ± 1.60	72.83 ± 2.80
L-dopa 5 mg/kg	8	85.13 ± 1.20	64.95 ± 4.48
L-dopa 10mg/kg	9	88.71 ± 4.71	71.07 ± 2.69

Figure 4.4 Five choice task performance in 6-OHDA-lesioned rats

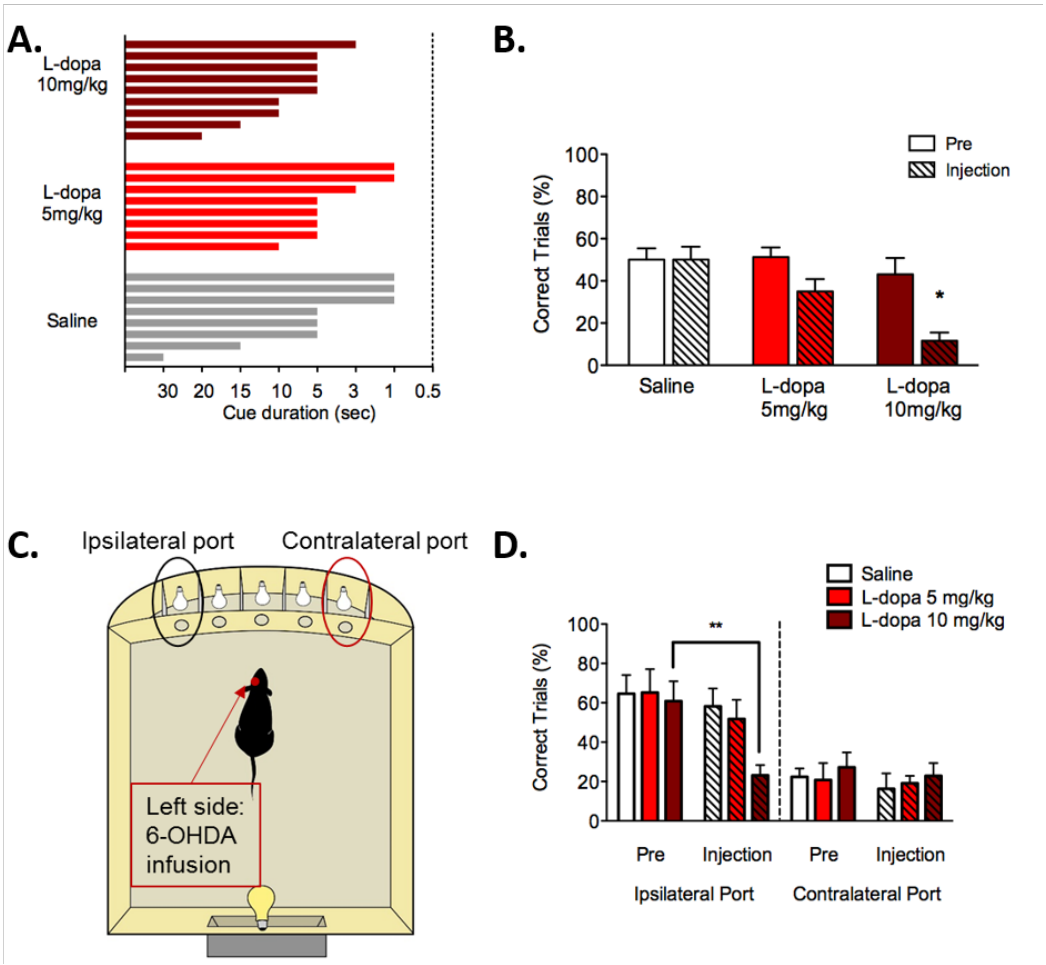


Figure 4.4 continued

A. Training progress for all rats. Each bar represents one rate. Bar terminates at the training protocol the rat was unable to complete to criterion (i.e. ≥80% correct). B. Mean (± SEM) percentage of trials completed correctly. C. Schematic demonstrating the contralateral port is the port opposite the lesioned hemisphere and the ipsilateral port refers to the port on the same side as the lesioned hemisphere. D. Mean (± SEM) percentage of correct trial for the ipsilateral and contralateral port pre-treatment and with L-dopa injection. E. Mean (± SEM) percentage of trials omitted. D. Mean (± SEM) premature responses (i.e. responses made during ready period). * $p < 0.05$, ** $p < 0.01$

4.5 General Discussion

These experiments are the first to extensively characterize deficits in attention in a rodent 6-OHDA model of PD. While disengagement deficits have been studied previously in the 6-OHDA model of PD (Mandel et al., 1990; Schallert & Hall, 1988), deficits in selective and sustained attention have not. Here we have demonstrated that 80% or greater unilateral dopamine depletion leads to profound difficulty in selective and sustained attention. While rats were unable to complete re-training to reach baseline performance (with 500 msec port light duration) that was achieved prior to 6-OHDA lesions, most made it at least half-way through re-training and stagnated with sub-criterion performance in the training protocol with a 5s port light duration. Furthermore, this decrement in performance was lateralized such that performance was specifically impaired in trials in which the lit port was contralateral to the lesion.

The findings from both experiments demonstrate that L-dopa can be acutely effective at returning some but not all attentional functions disrupted in this rat model of PD. After one injection, disengagement behavior was readily recovered with the same dose of L-dopa that recovered motor function of the affected paw. However, as time progressed rats receiving this 10 mg/kg dose developed dyskinesias readily. As a result, some of the rats treated successfully at Day 1 were unable to be tested at Week 1. But when comparing performance from Day 1 to Week 1 solely for the rats still able to be tested at Week 1, there is no decreased in efficacy. Further, the lower dose of 5 mg/kg was not successful in returning motor function but does show moderate improvement in disengagement behavior at Week 1.

Disengagement behavior has previously been shown to be dependent on nigrostriatal dopamine as well as SNc dopaminergic projections to the central amygdala (Mandel et al., 1990; Schallert & Hall, 1988; Smith, Geissler, Schallert, & Lee, 2013). Thus, it was anticipated that L-dopa administration would improve disengagement behavior. Because disengagement behavior was improved with the same dose of L-dopa as motor function, this adds further evidence that L-dopa use in humans may efficacious in improving both attention switching behavior and motor function at the same dosage.

While 10 mg/kg L-dopa was effective at improving disengagement deficits acutely after the first injection, the same dosage resulted in further impairment in already sub-optimal performance during the five choice task. This parallels similar research done in a non-human primate model of PD. Decamp & Schneider (2004) demonstrated that deficits in sustained attention do exist in monkeys exposed to MPTP and that these deficits can be exacerbated (Jay S. Schneider, Pioli, Jianzhong, Li, & Bezard, 2013) with L-dopa administration. However it is reasonable to expect that L-dopa would improve these functions as dopamine has also been shown to play an integral role in selective and sustained attention. Specifically, dopaminergic input into the medial PFC is integral for these aspects of attention (Granon et al., 2000; Winstanley et al., 2010). Further, it seems that striatal dopamine and it's input to the PFC is necessary for proficiency in a selective and sustained attention task as shown by profound deficits in accuracy after striatal lesions (Belinda J. Cole & Robbins, 1989; Rogers et al., 2001), impaired ability to reacquire the task after the lesion has been created (Rodgers et al., 2001), and even greater deficits when the striatum and PFC are disconnected (a Christakou et al., 2001).

We see a similarly profound difficulty in reacquiring the task after 6-OHDA infusion. Therefore, it was expected that L-dopa would enhance dopamine levels and restore functionality of the striatum which would then allow the striatum to successfully mediate attention through its cortico-striatal loops (a Christakou et al., 2001). However L-dopa does not improve selective and sustained attention deficits and instead further exacerbates these impairments.

There are several possible reasons for this lack of improvement in five choice task performance in unilateral nigrostriatal dopamine depleted rats after L-dopa administration. Firstly, L-dopa administration significantly increases extracellular dopamine in both hemispheres in a unilateral rat model of PD and increases D1 receptor occupancy (Orosz & Bennett, 1992; Robertson & Robertson, 1989). Previously, D1 receptor have been shown to contribute to attentional function and excessive D1 stimulation may lead to interference in attentional function. Furthermore, tonic and phasic signaling are known to play specific roles in dopaminergic modulation of several behavioral functions including reward processing. L-dopa has been shown to increase tonic firing, and in mild PD phasic signaling has been shown to be altered (Mouradian et al., 1988). Secondly, in addition to dopamine, norepinephrine also mediates attentional processes feeding into the prefrontal cortex (Carli, Robbins, Evenden, & Everitt, 1983; Newman, Darling, & McGaughy, 2008). Lesions of the dorsal noradrenergic bundle result in deficits in a five choice serial reaction time task designed to assess multiple aspects of selective, sustained, and divided attention (Carli et al., 1983). In PD, there is also a decrease in the presence of norepinephrine due to degradation of the locus

coeruleus (Chan-Palay & Asan, 1989) that occurs early in the progression of PD. However in our experimental preparation for Experiment 2 that examined five choice task performance, a noradrenergic blocker, desipramine was given prior to surgery to protect noradrenergic fibers. Further, it has been shown that the administration of L-dopa (the precursor to both dopamine and norepinephrine) not only increases dopamine levels but also norepinephrine levels albeit at a lower rate (Wiegand & Perry, 1961). Dopamine and norepinephrine levels show an inverted U-shaped relationship with attentional performance. Atomoxetine enhances the availability of both dopamine and norepinephrine in the PFC (Bymaster et al., 2002) and at moderate doses improves PFC-mediated attentional processes but causes impairments in these attentional processes at higher doses (Gamo, Wang, & Arnsten, 2010; Newman et al., 2008; Robinson et al., 2008). Therefore, it is plausible that the systemic administration of L-dopa in this model in which noradrenergic fibers were protected (via desipramine) that systemic L-dopa administration increased norepinephrine to an undesirable level which further disrupted lesion-induced selective and sustained attention deficits.

To further address the possibility that this experiment may have simply utilized L-dopa doses that were too high, we injected an even lower dose of L-dopa (2.5 mg/kg) to a separate group of rats with unilateral dopamine lesions and observed their performance on the five choice task. If it were the case that L-dopa could be useful for improving five choice performance and we were simply giving too much, a lower dose would have been effective. However, this low dose of L-dopa (2.5mg/kg) neither improved nor worsened

performance in this five choice task. Thus, this demonstrates that it is not the case that the negative impact of L-dopa was due to the dose being higher than necessary.

Due to the inability of a range of doses of L-dopa to improve performance and the pattern of deficits seen in the five choice task, it may be that the enhancement of dopamine (and possibly norepinephrine) in the intact hemisphere is causing these increased attentional deficits with L-dopa administration. While there is a decrement in performance from baseline with L-dopa injection, this decrement does not occur uniformly across all five ports in the five choice task. Specifically, accuracy data from the left-most and right-most ports (converted to ipsilateral and contralateral ports in relation to lesioned hemisphere) revealed that the impairment in performance on L-dopa is due to decreased accuracy in trials in which the port ipsilateral the lesion hemisphere were lit. Performance in the port ipsilateral to the lesioned hemisphere was significantly decreased while leaving contralateral port performance unchanged with L-dopa. And as dopamine's effect on attention and other cognitive tasks often show an inverted-U relationship (Gamo et al., 2010; Robinson et al., 2008; Vijayraghavan, Wang, Birnbaum, Williams, & Arnsten, 2007), this deficit suggests that a high dose L-dopa may be specifically impairing the ability of the intact hemisphere to mediate attentional function in the five choice task by enhancing dopamine to a detrimental level.

However, these deficits in selective and sustained attention may not simply be due to an increase in the neurotransmitters and brain areas directly related to L-dopa. Selective and sustained attentional is dependent on many neurotransmitters (e.g. dopamine, acetylcholine, norepinephrine, serotonin) and many brain areas which

commonly receive dopaminergic input from the SNc or striatum (T. W. Robbins, 2002). For example, in addition to dopamine and norepinephrine, the basal forebrain cholinergic system is highly important for selective and sustained attention and receives dopaminergic input from the SNc (McGaughy et al., 2002; Muir, Page, Sirinathsinghji, Robbins, & Everitt, 1993; Risbrough et al., 2002). Thus repairing such complex attentional function governed by such a large network may be unachievable simply by enhancing dopaminergic tone with L-dopa.

Because giving infusions of 6-OHDA into the MFB results in dopaminergic cell loss in the VTA in addition to the SNc, it should be considered whether these deficits are not solely attentional deficits but are partly due to reduced motivation. Previously, dopaminergic lesions of the VTA and nucleus accumbens have led to marked decreases in reward-seeking or motivated behavior (Adamantidis et al., 2011; Fields, Hjelmstad, Margolis, & Nicola, 2007; Weinberg, Nicholson, & Currie, 2011). Further, lesions of the SNc alone can decrease the number operant responses to obtain sucrose solutions, but in a sucrose preference test SNc-lesioned rats show no decrease in sucrose preference (Favier et al., 2014) . This suggests that while these rats show no impairments in reward processing, they do show impairments in motivation to pursue the reward. Therefore it is possible the deficits seen in the five choice task could be due in part to decreases in motivation as food rewards are used as motivation for performing the task. However, it is unlikely that these difficulties in re-training in the five choice task are due to decreased motivation. Most rats unable to complete training in the five choice task were able to complete at least half of the training protocols with 80% correct trials or better and

collected the food rewards for these trials. Furthermore, while these rats may have had decreases in motivation levels, motivation to work for a food reward may have been enhanced to a non-deficit level due to the mild food restriction all rats were maintained on during the experiment.

One major difference between Experiment 1 and Experiment 2 is the use of desipramine to protect noradrenergic fibers in Experiment 2 but not Experiment 1. As noradrenergic fibers neighbor dopaminergic fibers of the MFB (Jones & Moore, 1977), it is plausible that in Experiment 1 (in which desipramine was not used), there was norepinephrine depletion as a consequence of 6-OHDA infusion into the MFB. Therefore, it cannot be ruled out that the differences in L-dopa efficacy seen from Experiment 1 to Experiment 2 are due to norepinephrine. However, while it is a plausible argument, it seems unlikely that disengagement behavior would respond differently to L-dopa in a preparation that preserved norepinephrine. Previously, we have demonstrated that D1 receptors within the central amygdala are necessary for modulating the attentional component of disengagement behavior (Smith et al., 2013). Furthermore while norepinephrine has been implicated in attention, attention switching in humans has been shown to be mainly supported by dopamine and dopaminergic areas such as the striatum (Dove, Pollmann, Schubert, Wiggins, & Yves von Cramon, 2000; Luna et al., 2001; Sohn, Ursu, Anderson, Stenger, & Carter, 2000; Vaidya et al., 1998; Volkow, Fowler, Wang, Ding, & Gatley, 2002). Therefore, this gives us reason to conclude that norepinephrine is less crucial for attention switching compared to other more complex attentional functions.

These findings demonstrate that L-dopa is not sufficient for treating multiple types of attentional deficits in a rat model of PD. While the same dose of L-dopa is able to restore basic attentional switching function and motor function in a unilateral dopamine depletion model of PD, L-dopa administration was not able to restore more complex attentional function (i.e. selective and sustained attention). In addition to continuing to study the use of L-dopa to treat other cognitive and attentional deficits, more studies must be conducted to understand how dopamine transmission facilitates attention, and how L-dopa affects dopamine transmission in the context of attention. Furthermore, future work should also assess the possibility that attentional or cognitive functions such as selective and sustained attention that are reliant on many systems and neurotransmitters may be better treated with non-dopaminergic drugs that either target different neurotransmitter systems or enhance global neural function.

Chapter 5: An assessment of the ability of methylene blue to reduce behavioral and dopamine deficits in a 6-OHDA model of Parkinson's disease

5.1 ABSTRACT

Recently, alternative drug therapies for Parkinson's disease (PD) have been investigated because of several shortcomings of traditional dopamine-based therapies including difficulty treating cognitive and attentional dysfunction in PD. A promising therapeutic avenue is to target mitochondrial dysfunction in PD. One way to improve mitochondrial function is with the application of USP methylene blue (MB), an antioxidant and metabolic enhancer. MB has been shown to improve cognitive function in both intact rodents and rodent disease models. Therefore, we have investigated the ability of MB to treat attentional deficits as well as motor deficits in a rat 6-hydroxydopamine (6-OHDA) model of PD. MB also has neuroprotective capabilities that have specifically been demonstrated after neurotoxic insult so we also assessed the ability of MB to provide neuroprotection in this model. Through these experiments we have shown that daily administration of MB (4mg/kg) does not improve attentional processes such as attention switching (i.e. disengagement behavior) and selective and sustained attention (assessed using a five choice task; Experiment 2). Furthermore, MB was able to provide moderate neuroprotection in the SNc but this neuroprotection is dependent on the amount of 6-OHDA infused into the medial forebrain bundle. In conclusion, MB is not useful for improving performance in attentional tasks but is useful in preserving

dopaminergic fibers in this model. Future work should continue to study and optimize the abilities of MB for the treatment of PD.

5.2 INTRODUCTION

Currently, Parkinson's disease (PD) is most commonly treated pharmaceutically with levodopa (L-dopa), which while very effective at alleviating motor symptoms, has its shortcomings. For example, levodopa has been shown to be ineffective in restoring certain cognitive functions affected in PD (Cools et al., 2003; Dujardin, Degreef, Rogelet, Defebvre, & Destee, 1999; Lewis et al., 2005; Robbins & Cools, 2014; Schneider et al., 2013). Specifically, attentional processes such as attentional shifting (both task or rule/set shifting) and selective and sustained attention are common in PD and show varied responses to L-dopa. For example, the reduced ability to shift attention to a new rule or task in PD patients is improved with L-dopa (Cools et al., 2003, 2002). Among patients with mild PD, L-dopa has no impact on selective and sustained attention (Lewis et al., 2005; Moustafa et al., 2008). In addition to the conflicting effects of L-dopa on attentional functions, chronic L-dopa administration in patients and in animal models of PD can result in the development of L-dopa induced dyskinesias and impulse control disorders (Leeman & Potenza, 2011; Poletti & Bonuccelli, 2013; Rajput et al., 2002; Weintraub, 2008). For these reasons it is pertinent to investigate alternative treatments for PD.

Here we have investigated the possibility of using methylene blue (MB) to treat behavioral and neuronal deficits in a rat model of PD. MB is an antioxidant compound that also increases cell metabolism through the enhancement of mitochondrial activity at

the cytochrome oxidase complex (Lindahl & Öberg, 1961; Scott & Hunter, 1966; Visarius et al., 1997). MB has been shown to enhance cognitive function in both intact and disease-modeled rodents. A low dose of MB can facilitate learning and memory of intact rats in both appetitive and aversive contexts by increasing mitochondrial respiration (Callaway, Riha, Bruchey, Munshi, & Gonzalez-Lima, 2004; Callaway, Riha, Wrubel, McCollum, & Gonzalez-Lima, 2002; Martinez, Jensen, Vasquez, McGuinness, & McGaugh, 2013). Additionally, chronic MB administration enhanced spatial learning in a mouse model of Alzheimer's disease that exhibited mitochondrial dysfunction (Medina, Caccamo, & Oddo, 2011). Within the context of PD, MB was shown to restore motor function and preserve striatal cellular function in a rotenone model of PD (Wen et al., 2011), but MB's effects on cognitive functions in PD models are unknown.

Mitochondrial dysfunction is a common property of neurodegeneration seen in humans as well as animal models of neurodegenerative diseases including PD (Fukae et al., 2007; Janetzky et al., 1994; Kupsch et al., 2014; Mizuno et al., 1998). Therefore, MB also has the potential to be an effective neuroprotective agent by (1) enhancing cell metabolism and hence boosting the health of the cell and (2) reducing reactive oxidative species within and around the cell (Poteet et al., 2012). Oxidative stress is the primary cause of dopaminergic apoptosis in PD (Kanthsamy et al., 1994; Pallanck & Greenamyre, 2006; Schapira, 2008) thus potentially making MB a viable route of neuroprotection in PD. As proof of concept, infusion of MB into the striatum directly after an infusion of the neurotoxin rotenone to the same site significantly attenuated cell loss at the lesion site (Rojas et al., 2009)

However, as of yet, the ability of MB to restore cognitive and motor deficits and simultaneously provide neuroprotection in an animal model of PD has not been shown in the same experimental preparation. Therefore we have devised two experiments to examine the behavioral and neuronal effects of MB in a unilaterally dopamine depleted rat model of PD. In the first experiment, rats were tested for motor function and one type of attentional function (i.e., attentional disengagement) after MB treatment. In the second experiment, the impact of MB administration was assessed on more complex attentional function in a five choice task used to assess several aspects of attention including selective, sustained, and divided attention. In both experiments, the effects of MB on dopamine cell loss were measured.

5.3 METHOD

5.3.1 Subjects

Ninety-nine Sprague Dawley rats (350-450g; Experiment 1: N = 24; Experiment 2: N = 75; Harlan) were housed on a reversed 14 hour on: 10 hour off light cycle with lights turning off at 10 AM. In experiment 1, rats had *ad libitium* access to food for the duration of the experiment however, water access was periodically restricted for the 24 hours prior to disengagement testing only. In experiment 2, rats were food restricted to 90% of free-feeding weight for the duration of training and testing during the five choice task, and water access was also periodically restricted for 24 hours prior to disengagement testing. All behavioral training and testing occurred during the dark phase of the light cycle. All experiments were conducted according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals and all protocols were

approved by the Institutional Animal Care and Use Committee at The University of Texas at Austin.

5.3.2 Surgery

All rats underwent surgery to induce unilateral dopaminergic depletion or sham surgery. First, the rats were anesthetized using 2-5% isoflurane gas (Abbott Laboratories) and were placed into a stereotaxic frame (Kopf Instruments, Tujunga, CA). After a burr hole was created, 1.0µl (Experiment 1) or 0.6 µl (Experiment 2) of 7µg/µl 6-hydroxydopamine (6-OHDA; Sigma Aldrich, Minneapolis, MN) in 0.1 M phosphate buffered saline with 0.1% ascorbic acid was delivered into the medial forebrain bundle (AP= -3.3, ML = ±1.7, DV = -8.6) at a rate of 0.1µl per minute via 2 µl Hamilton syringe connected to Harvard apparatus infusion pump. Once the infusion was finished, the needle was slowly removed, the skull was cleaned, and the skin was sutured closed. Rats in Experiment 2 (but not Experiment 1) were also given 25 mg/kg dose of desipramine (Sigma Aldrich, Milwaukee, WI) prior to surgery to protect noradrenergic cells from 6-OHDA. At the completion of surgery, all rats received a subcutaneous injection of buprenorphine hydrochloride (0.01 mg/kg; TW Medical, Denver, CO) and were placed on a heating pad and returned to their home cage once awake.

5.3.3 Daily methylene blue feedings

Four mg/kg methylene blue (Faulding Pharmaceuticals; Aguadilla, PR) was given orally between 10 and 11 AM daily. Methylene blue (in 10% sucrose water) was mixed

with 2.6 g crushed Nilla® wafers to create a paste. The vehicle solution (10% sucrose water) had blue food coloring (Food, Drug, and Cosmetics Blue No.1) added to it.

5.3.4 Behavioral Tests: Experiment 1

5.3.4.1 Cylinder test

This test examines the use of the forelimbs since rats naturally explore the cylinder by rearing and contacting the cylinder wall with the forepaws. So, rats were placed into a Plexiglas cylinder stood on its side (20 cm in diameter and 30 cm in height). Rats were placed into the cylinder and spontaneous forepaw touches to the cylinder during rears/exploration of the vertical wall were recorded. Paw touches were scored in accordance with protocol described in Schallert et al., 2000. If only one paw was placed on the cylinder, it was recorded as independent use of that limb. If both paws were placed on the cylinder simultaneously, or if one paw was placed on the wall and then the other was immediately placed on the wall and alternated with the other limb during stepping, it was recorded as simultaneous use of both limbs. A total of 20 instances of left, right, and simultaneous (both) forelimb use was recorded during exploration of the cylinder wall in either horizontal or vertical planes. The rat was removed from the cylinder either after 20 instances of forelimb use or 5 minutes has passed. As the rat contacted the cylinder, the use of forepaws was recorded (left, right, or both) in accordance with previous methodology (Schallert, et al., 2000). Rats with unilateral dopamine depletion of the nigrostriatal pathway typically demonstrate a preference for using the paw ipsilateral to the lesion and use the paw contralateral very little when contacting the cylinder (Tillerson et al., 2001). Therefore, preference for the contralateral paw was calculated thusly:

$$\frac{\text{Contralateral paw touches + (.5) both paw touches}}{\text{Total number of touches (ipsi + contra+ both)}}$$

5.3.4.2 Disengagement test

Disengagement refers to the ability to discontinue an ongoing behavior to attend to perioral stimulation. Disengagement testing was conducted by restricting access to water for the twenty-four hours prior to testing. Rats were first tested for basic orienting behavior by stimulating on both sides of the face with a cotton swab out of the rats' sight to ensure no somatosensory deficits existed. During disengagement testing, rats were allowed to drink from a water spigot through a hole in the back wall of the home cage. As rats drank, an experimenter stimulated both sides of the face and whiskers using a cotton swab. If the rat stopped drinking to attend to the stimulation, this was recorded as a successful disengagement. If the rat continued to drink and did not stop to attend to the stimulation, it was recorded as an unsuccessful disengagement trial. Both sides were stimulated a total of 5 times with varying order. Disengagement was quantified by recording the percentage of the 5 trials in which disengagement occurred (5/5 = 100).

5.3.5 Behavioral Tests: Experiment 2

In addition to conducting the cylinder and disengagement tests, several other tests were conducted to assess motor and attentional function. These are described below.

5.3.4.1 Vermicelli handling test

The vermicelli handling test originally developed by Allred, et al. (2008) was used to assess forepaw function. Rats were trained to eat 7 cm long strands of uncooked vermicelli pasta (semolina pasta). Once rats readily ate the pasta, rats were given pasta

strands to eat in a testing cage and were filmed for 4 trials. An experimenter blind to the conditions scored the rats' behavior using video playback in half-speed. The experimenter recorded the number of paw adjustments made by both paws, the time it took to eat the entirety of the pasta, and abnormal behaviors exhibited during the trial such as touching the strand of pasta to the cage floor (Allred et al., 2008).

5.3.4.2 Five choice task

Apparatus. Five choice training and testing was conducted in 8 operant boxes with aluminum side walls and ceiling, and clear acrylic front and back walls (30.5 cm W × 25.4 cm D × 30.5 cm H; Coulbourn Instruments, Whitehall, PA). One of these side walls was concave and contained five recessed ports (each 2.5 cm in diameter) with each of the recessed ports 3 cm from the grid floor (stainless steel rods 0.5 cm in diameter, parallel, spaced 1.0 cm apart) and had 3 red LED lights inside to illuminate the port at the appropriate time. Additionally, the ports were equipped with infrared beams that detected nose pokes. Opposite the concave wall was a 2-watt house light (centered on the wall, 26 cm from the floor) and a recessed food cup (centered, 2 cm from the floor) equipped with an infrared beam to detect entries. Each box was housed in a light- and sound-attenuating chamber (58.4 cm × 61 cm × 45.7 cm; Coulbourn Instruments) and interfaced with a computer using GraphicState 3.1 (Coulbourn Instruments).

Shaping. In order to train the rats on the five choice task, two shaping procedures were conducted. First, rats underwent a magazine-shaping session in which they were trained to eat a single grain pellet (45 mg grain tablet, Test Diet, Richmond IN) delivered to a food cup located within the conditioning chamber. A total of 30 pellets were

delivered at a variable interval (averaging 60 sec) over a 30 min session. After this session, all rats reliably retrieved grain pellets from the food cup. Second, the rats went through a nose poke-shaping session in which they were trained to make a nose poke response to the ports. All five ports were illuminated for 30 sec and a nose poke to any port during this time resulted in the delivery of a grain pellet in the food cup. This daily session was continued until the rats met criterion of 80% or more responses over 30 trials with a variable intertrial interval (ITI) of 30 sec. After completing the shaping sessions, the rats began training in the five choice task.

Training and testing. Prior to surgery rats were trained to proficiency on the five choice task adapted from Holland, Han, & Gallagher (2000) and Maddux, Kerfoot, Chatterjee, et al. (2007) and described in Olshavksy et al., (2014). Rats were first trained on an easier protocol in which the port light duration was 30s. Once rats became proficient (80% correct response rate), the rats progressed to the next protocol with a shorter port light duration (i.e., 20, 15, 10, 5, 3,1, 500 msec). In the baseline task, a trial began with the house light illuminated to signal a 5 sec ready period. At the end of the ready period, one of the five recessed ports illuminated for 500 ms. Rats had a five second response period beginning with the port light illumination to make a nose poke into the correct port in order to receive a grain pellet into the food cup. Rats were allowed to nose poke incorrectly during the response period but if a correct response was not made within the 5 sec, the trial was ended, signaled with termination of the house light. After two training sessions (within a three day period; one training session per day) with 80% correct or better response rate, rats received surgery. Two weeks post-surgery, rats

began retraining in the same manner as before starting with the port light duration of 30 sec and successively moving to shorter port light durations exactly as prior to surgery. Once criterion was met on the 500 msec protocol again, rats moved onto the testing phase. Due to lesion severity, some rats were unable to re-train down to the 500 msec protocol. If the rats were unable to reach 80% correct response rate in a particular protocol for 7 consecutive days, training was then discontinued

Rats that completed retraining were tested in three versions of the five choice task in which the task was changed to increase attentional load in various manners: (1) the port light duration was shortened from 500 msec to 100msec, (2) the ready period between the start of the trial and the port light illumination varied from the previously fixed period of 5 sec to 1, 5, and 9 sec, and (3) the previously steady house light now blinked during the ready period.

5.3.6 Immunohistochemistry

After testing was concluded, rats were given an overdose of pentobarbital (86 mg/kg) and phenytoin (11 mg/kg) mix (Euthasol by Virbac Animal Health) and then were perfused transcardially with 0.9% saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). The brains were extracted and placed into 20% sucrose PFA solution overnight and were frozen on dry ice and then packaged for storage in the -80ºC freezer.

Brains were sliced at 30µm using a sliding microtome and sections containing the midbrain dopamine regions were collected in four series. One series was used for tyrosine hydroxylase (TH) staining and an adjacent series was used for Nissl staining. For the TH

staining, the tissue was first treated with 0.3% hydrogen peroxide in PBS. Then the tissue was placed into a 6% normal horse serum (NHS) in PBS with 0.3% Triton (PBST) for one hour after rinsing in PBS. Immediately afterwards the tissue was incubated in 0.15% PBST solution containing 3% NHS and mouse TH antibody (1:5000, ImmunoStar, Hudson, WI) for 72 hours at 4 ºC. After rinsing, the tissue was incubated with biotinylated horse anti-mouse IgG (1:250, Vector Laboratories) for 1 hour and then with avidin-biotin conjugate (PK-6100, Vector Laboratories) for 1 hour, and color reacted using 3,3-diaminobenzidine (DAB; Sigma Aldrich).

Once the TH-stained tissue was mounted and cover slipped, TH density of the substantia nigra (SNc) and ventral tegmental area (VTA) were measured. Photomicrographs of sections were taken using a microscope (Olympus BX61) at a magnification of 10x. Optical Density was measured (Image J) to assess the density of TH staining as a measure of dopaminergic enervation. TH density was assessed by placing a circle with a radius of 100 µm over the SNc and VTA. Two samplings of each structure using this circle were taken at levels 36-39 in relation to the Swanson Rat Atlas (Swanson, 2003).

5.3.7 Experimental designs

5.3.7.1 Experiment 1: The impact of daily methylene blue on motor function, disengagement behavior, and lesion severity.

All rats received 6-OHDA infusions unilaterally and were assigned to one of three treatment groups (see Figure 1a). One group began a daily feeding of MB one week prior to surgery and continued for the two weeks post-surgery (n=8). A second group was

exposed to MB only for the two weeks post-surgery and was given the vehicle for the week prior to surgery (n=8). The third group received the vehicle for the week prior and the two weeks after surgery (*n*=8) to examine the possibility that MB may provide more neuroprotective benefit if given prior to neurotoxic insult. All rats were tested for motor (cylinder test) and disengagement function two weeks post-surgery and 1 hour after MB/vehicle feeding. Then, MB/vehicle were discontinued for two-weeks and rats were tested again to assess any potential neuroprotective/long-lasting impact on behavioral deficits. Once testing was complete, rats were euthanized, and brains were extracted, preserved, and processed for TH immunohistochemistry.

5.3.7.2 Experiment 2: Assessment of neuroprotection of daily methylene blue after a smaller neurotoxic insult and its impact on associated attentional dysfunction

All rats were first trained on the five choice task (Figure 1b). Once the five choice task was mastered, rats had unilateral infusions of 6-OHDA or sham into the MFB. In this experiment, a smaller amount of 6-OHDA was infused with the intent of creating a less severe lesion to better model mild-moderate PD in which most attentional dysfunctions are reported (Filoteo et al., 1997; Hornykiewicz, 1974; Zhou et al., 2012a). Furthermore, a sham lesion group was added as a reference group for the performance levels expected in intact rats. Therefore, it was a 2 x 2 design with lesioned and sham groups receiving either MB or vehicle treatments (Sham + Vehicle: n = 11; Sham + MB: n = 11; Lesion + Vehicle: n = 19; Lesion + MB: n = 19). MB/Vehicle feedings began the day after surgery for all conditions. Two weeks post-surgery, motor and disengagement deficits were also assessed in these rats. Motor deficits were assessed using both the

cylinder test and the vermicelli handling test. Then, rats began re-training on the five choice task. Once training was complete, rats underwent 3 days of testing in 3 different attentional challenges. However, not all rats were able to complete post-surgery re-training. These rats were discontinued from training after 7 days of sub-criterion performance (< 80% correct trials) on the same training protocol. Once behavioral testing was complete, rats were euthanized, and brains were extracted, preserved, and processed for TH immunohistochemistry.

5.4 Experiment 1 Results

5.4.1 Dopamine depletion

Dopaminergic depletion was determined by quantifying TH staining and calculating the percentage of dopamine depletion by comparing the lesion side to the intact side for each rat. A value of 100% signifies complete dopamine depletion on the lesioned side while a value of 0% indicates no dopamine depletion. Analyses of percentage dopamine depletion were conducted on SNc and VTA (Figure 5.2). MB treatment either before or after the 6-OHDA lesions did not attenuate dopamine loss in the SNc [$F(2,21) = 0.33$. $p = 0.73$] and VTA [$F(2,21) = 0.003$, $p = 0.99$] as all three groups showed comparable dopamine depletion on the lesioned side.

5.4.2 Cylinder test

In order to assess paw preference in the cylinder, paw usage was calculated and one-sample t-tests were conducted to evaluate if the use of affected paw was at a chance level (50%). It was expected that MB administration would recover motor function, however this was not the case. At 2 weeks post-surgery, there was no discernable impact

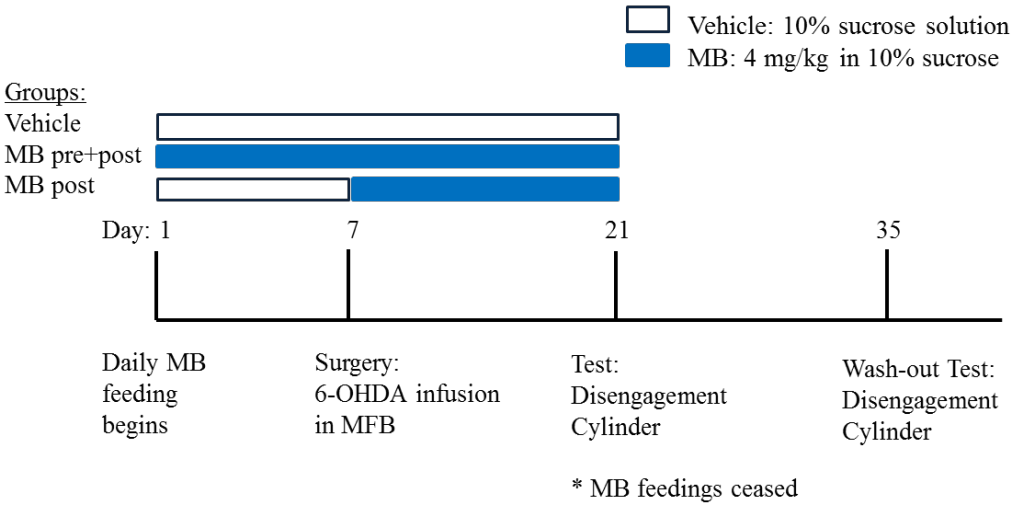
of MB (when on board) as rats in all three groups used their contralateral/affected paws significantly less (Figure 5.3A, left panel). All groups showed significantly lower levels of contralateral paw use compared to 50% level [Vehicle: $t(7) = 5.154$, $p = 0.001$; MB Pre & Post: $t(7) = 4.467$, $p = 0.003$; MB Post Only: $t(7) = 2.830$, $p = 0.025$]. This same trend continued when tested 2 weeks after cessation of daily MB feedings. Once again, all three groups continued to show a less than chance usage of the bad paw [Vehicle: $t(7) = 6.222$, $p < 0.001$; MB Pre & Post: $t(7) = 3.707$, $p = 0.008$; MB Post Only: $t(7) = 4.608$, $p = 0.002$; Figure 5.3A, right panel].

5.4.3 Disengagement test

As is the case with unilateral dopamine depletion, disengagement behavior was only impaired on the side contralateral to the lesion. MB on-board (two weeks post-surgery) significantly improved disengagement behavior on the side contralateral to the lesion (Figure 3B). Planned comparisons of the two treatment groups against the vehicle-treated group reveal a significant improvement in disengagement behavior in the MB post group [$t(14) = -2.84$, $p = 0.026$; Figure 5.3B left panel] but not the MB pre+ post group [$t(14) = -1.655$, $p = 0.12$]. At wash-out test (2 weeks after the last MB treatment), the disengagement levels among MB groups decreased a little and similar analyses revealed no significant differences in disengagement differences as a function of MB administration (all ps > 0.2; Figure 5.3B, right panel).

Figure 5.1 Experimental Designs

A. Experiment 1 Design



B. Experiment 2 Design

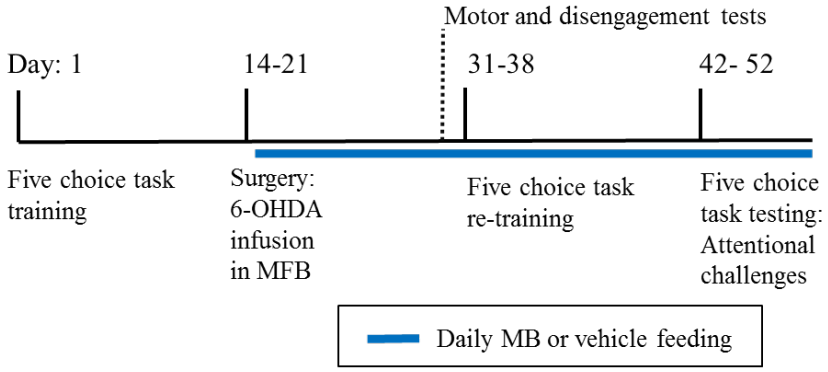
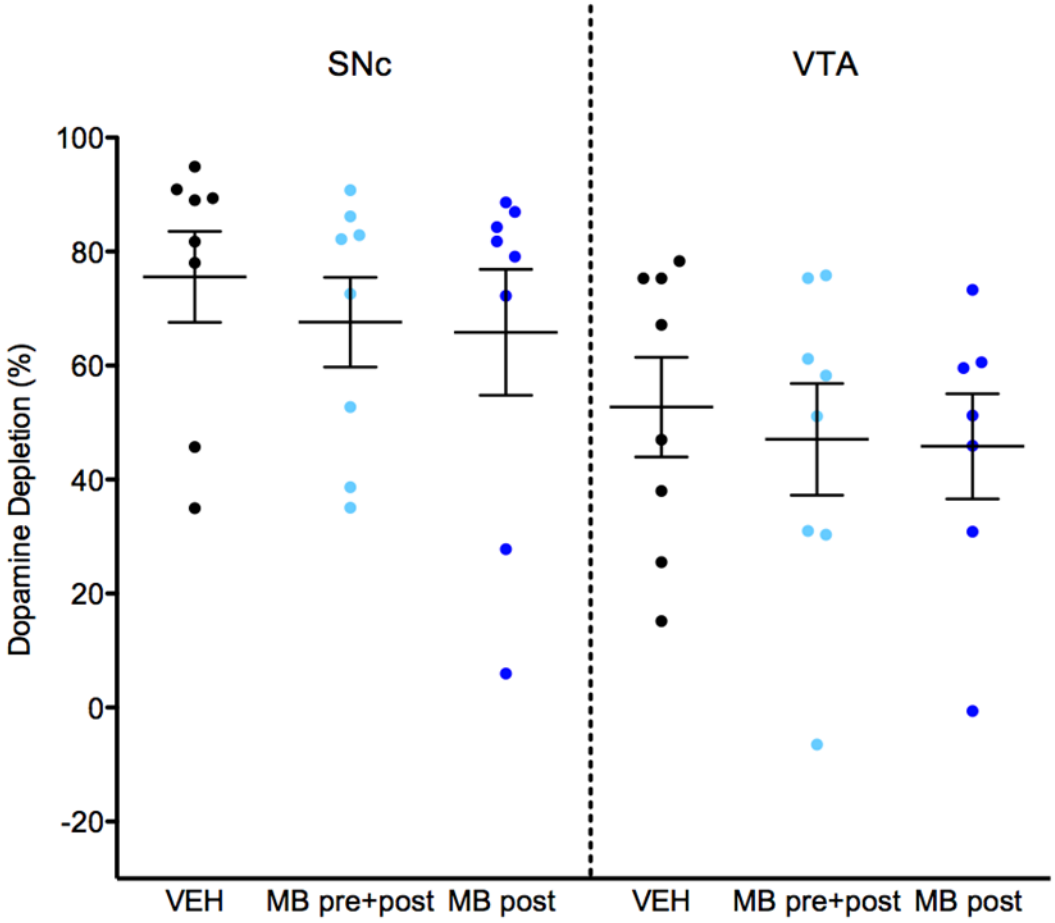
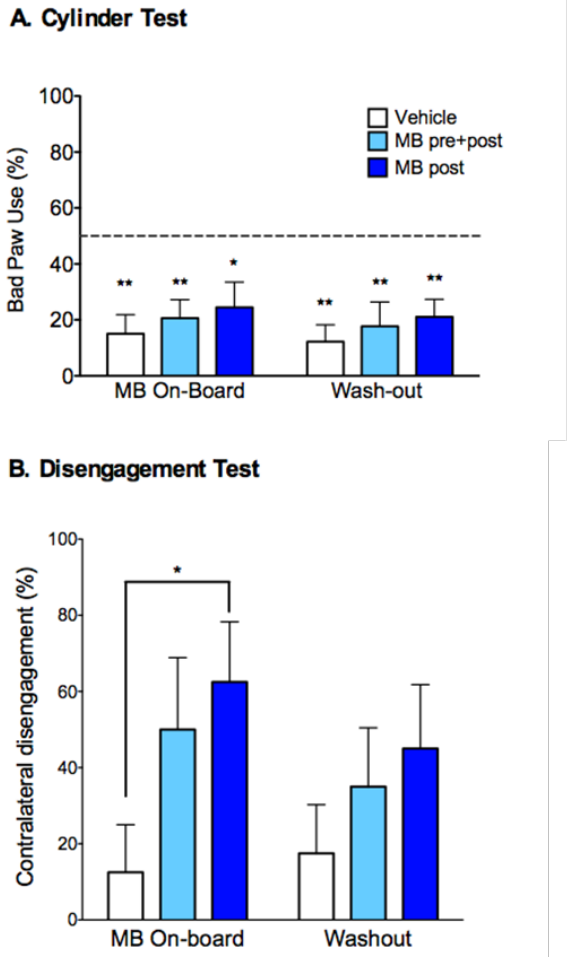


Figure 5.2 Dopamine depletion in the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA)



Mean (± SEM) dopamine depletion on the hemisphere that received a unilateral 6-OHDA MFB infusion in comparison to the intact hemisphere for the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA). There were no differences in dopamine depletion as a consequence of group assignment. All ps > 0.05.

Figure 5.3 Motor and disengagement function



A. Mean (± SEM) bad (contralateral) paw use in contacting the cylinder walls during exploration two weeks post-surgery with MB on board and two weeks after discontinuing MB/vehicle treatment. All groups showed decreased usage of the bad paw compared to chance level (50%). B. Mean (± SEM) disengagement behavior on the side contralateral to the 6-OHDA lesion two weeks post-surgery and two weeks after discontinuing MB or vehicle treatment. * $p < 0.05$, ** $p < 0.01$

5.5 EXPERIMENT 2 RESULTS

5.5.1 Dopamine depletion

TH density was measured in both the SNc and the VTA. The optical density was measured for both the intact and lesioned sides (averaged across 4 sections, 150 μm apart as done in Experiment 1) and then the percentage of dopamine depletion was calculated. A value of 100% signifies complete dopamine depletion on the lesioned side while a value of 0% indicates no dopamine depletion.

Simply by looking at the dopamine depletion measure, it is apparent that there is a proportion of rats in both lesion groups (i.e., lesion + vehicle and lesion + MB) with minimal dopamine depletion that is within the range of what is seen among sham rats (Figure 5.4). This seems to be a common property of 6-OHDA lesions in that either a large lesion is created or a small or no lesion is created. And instead of 6-OHDA dose having a linear relationship with lesion size, it affects the 'hit rate' or the amount of successful lesions. Therefore, as we used a moderate/small dose of 6-OHDA compared to what was used in Experiment 1, we had rats with severe dopamine depletion and rats with little-to-no dopamine depletion. In order to account for the unsuccessful lesions, the optical density for the lesioned side was plotted as a function of the intact side optical density for the SNc (Figure 5.5A) and the VTA (Figure 5.5C). Then, the distance (residuals) of each lesioned rat from the best-fit line for the sham groups (collapsed across treatment) was calculated. Rats in the lesion group with a residual no different than that of the sham groups (within the 95% confidence intervals for the sham group) were considered to have lesion size that was indistinguishable from rats with sham

lesions. Six rats from both lesion groups met this exclusion criterion and these rats were excluded from further analyses.

When looking solely at the significantly different residuals for both lesion groups in the SNc (Figure 5.5B; shaded area represents 1 standard error), it is apparent that the two lesion groups have different residual patterns. When an independent samples *t*-test is conducted on these two samples, the mean residual for the lesion + vehicle group is significantly greater than the mean residual for the lesion + MB group [$t(24) = 2.20$, $p = 0.013$]. Thus, while still significantly different from the sham groups, the lesion + MB has a significantly smaller mean residual demonstrating that there is a decrease in lesion severity with MB administration.

However, taking the same approach to dopamine depletion in the VTA does not reveal the same pattern (Figure 5.5D). After accounting for lesioned rats with the same residual to the best-fit line as the sham groups, both groups show overlapping residuals suggesting that there is no difference in VTA dopamine depletion as a function of MB.

5.5.2 Motor function

Two weeks post-surgery all rats were tested in both the cylinder and vermicelli handling tasks for motor impairments. Both tests demonstrate a motor deficit due to lesion and no recovery of function with MB. In looking at performance in the cylinder test (Figure 5.6A) using one sample *t*-tests, sham groups show normal chance (50%) usage of the contralateral paw [sham + veh: $t(11) = 0.33$; sham + MB: $t(10) = 0.32$, ps > 0.70] while both lesions groups show significantly less than chance usage of the contralateral/affected paw [lesion + veh: $t(12) = 4.21$; lesion + MB: $t(12) = 4.58$, ps ≤ 0.001]. Furthermore,

when examining the relationship of the independent variables (surgery and treatment assignment) using a 2 x 2 ANOVA, there is a significant effect of surgery [$F(1,45) = 19.02$, $p < 0.001$] but no significant effect of treatment or an interaction of the two variables [treatment: $F(1,45) = 0.01$, $p = 0.92$; interaction: $F(1,45) = 0.004$, $p = 0.95$]. Both lesions groups show decreased paw usage of the affected (contralateral) paw compared to both sham groups.

Examining paw adjustments made by both paws in the vermicelli handling task yields similar data. Firstly, while all rats were able to consume pasta in a reasonable amount of time, rats that received 6-OHDA were slower at consuming the pasta (M = 21.0 sec, SEM = 2.1) than sham rats (M =15.1 sec, SEM = 1.16). However, there were no differences in pasta consumption as a consequence of MB administration. While consumption latency is a useful measure, a more sensitive measure is the quantification of paw adjustments made while eating the pasta. Here we saw that lesion rats overall regardless of MB treatment exhibited more paw adjustments of the ipsilateral paw that sham rats (Figure 5.6B). A 2 x 2 x 2 mixed-design ANOVA was conducted with the between-subjects variables of surgery and MB treatment, and the within-subjects variable of paw sides. There was an overall difference in the amount of paw adjustments made between two paws [ipsilateral vs. contralateral; $F(1, 41) = 13.60$, $p < 0.001$] and also between the rats with 6-OHDA and sham lesions [lesion vs. sham; $F(1, 41) = 7.31$, $p = 0.01$]. However, these differences are mainly driven by the increased use of unaffected paw among lesioned rats as seen by the interaction effect of paw side and surgery condition [$F(1,41) = 9.22$, $p = 0.004$]. Looking at solely contralateral/affected paw use in

the vermicelli handling test, surgery status does not impact the number of paw adjustments made while consuming the dry pasta [$F(1,41) = 0.05$, $p = 0.83$]. However when only examining use of the paw ipsilateral to the lesion (unaffected paw), there is a significant effect of lesion demonstrating that lesioned rats commit more ipsilateral paw adjustments than their sham counterparts [$F(1,41) = 5.38$, $p = 0.025$]. This suggests that in order to facilitate successful pasta consumption, lesioned rats increased the amount of paw adjustments made by the unaffected paw. Furthermore, this corroborates findings from the cylinder test in which the rats relied more on the unaffected forelimb to contact the cylinder wall.

5.5.3 Disengagement test

In addition to testing motor function two weeks post-surgery, the impact of dopamine depletion and MB treatment on contralateral disengagement behavior was also evaluated (Figure 5.6C). A 2 x 2 ANOVA with between-subjects variables of surgery and treatment was used to assess disengagement behavior on the side contralateral to the lesion. This ANOVA revealed a significant effect of surgery condition [$F(1,45) = 7.53$, $p = 0.009$], but not an effect of MB treatment [$F(1, 45) = 0.35$, $p = 0.85$; Figure 6C] on disengagement behavior.

5.5.4 Five choice task

Although all rats received re-training in the five choice task after surgery, not all rats were able to complete training and move on to the testing phase. Of the sham animals, all but one (sham + veh) were able to move through training and complete

testing. However, the majority of the lesioned animals (9 of 13 lesion + veh rats and 6 of 13 lesion + MB rats) were unable to complete training. In Figure 5.7A, the top bar in each group represents the animals that completed the re-training down to 500 msec protocol while the rest of the bars each represent individual rats that did not complete and stagnated at a particular protocol. Rats were considered unable to complete training after spending 1 week (7 training sessions) on the same training protocol with sub-criterion performance. In both lesion groups, most rats stagnated on the 5s port light duration with a few advancing to 3s and 1s port light duration. There were no statistical differences in the rate of training success between the two lesion groups [X^2, (1, N = 26) = 1.42, $p >$ 0.20]. Because of this division, five choice data for the rats that failed to complete training was analyzed separately from the rats that successfully completed re-training and testing in the 3 attentional challenges. As expected, the lesioned rats that were able to reach 500 msec retraining and completed testing (i.e., Lesion+ VEH, n=4, and Lesion + MB, n=7) had significantly less severe dopamine depletion within the SNc than rats unable to retrain in the five choice task [$t(24) = 9.65$, $p = 0.005$].

Attentional Challenges. In order to assess the impact of dopamine depletion and MB treatment on performance in the five choice task, the percent trials with correct responses were measured and repeated measures ANOVA was conducted with a within variable of 4 levels (baseline task and 3 challenge conditions) and two between factors of surgery (lesion vs. sham) and MB treatment (MB vs. vehicle). The overall performance levels were reduced with the attentional challenges as seen by the main effect of attentional challenge [$F(3,90) = 17.97$, $p < 0.001$]. There was also a main effect of

surgery [$F(1, 30) = 4.29$, $p = 0.047$] suggesting that the lesioned rats generally performed worse than sham rats. No other significant effects were observed (all ps > 0.2; Figure 5.7B).

Discontinued rats. Rats failing to successfully retrain were discontinued after seven days of sub-criterion performance on the same training protocol (lesion + veh: n = 9; lesion + MB: n = 6). Only one sham animal was discontinued and for that reason, excluded from these analyses. Performance (as measured by percent correct trials) on the last day of training was assessed (Figure 5.7C). There was no impact of MB treatment as both lesioned groups showed similarly low levels of performance [$F(1,13) = 0.12$, $p = 0.74$].

Figure 5.4 Dopamine depletion in the SNc and VTA

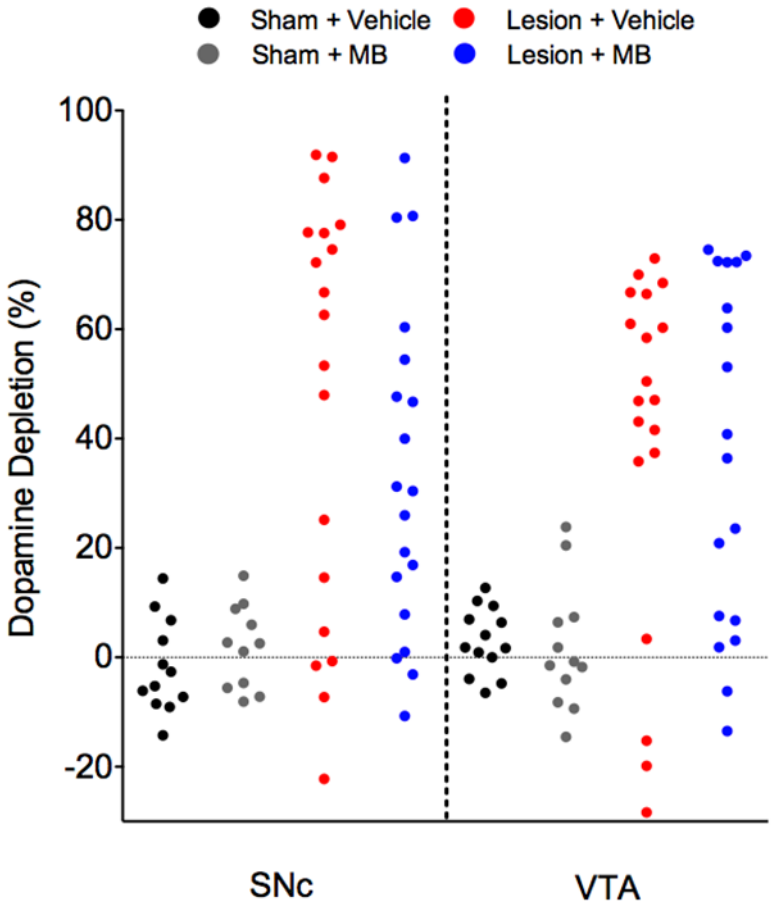


Figure 5.5 Lesion versus intact density in the SNc (top) and VTA (bottom)

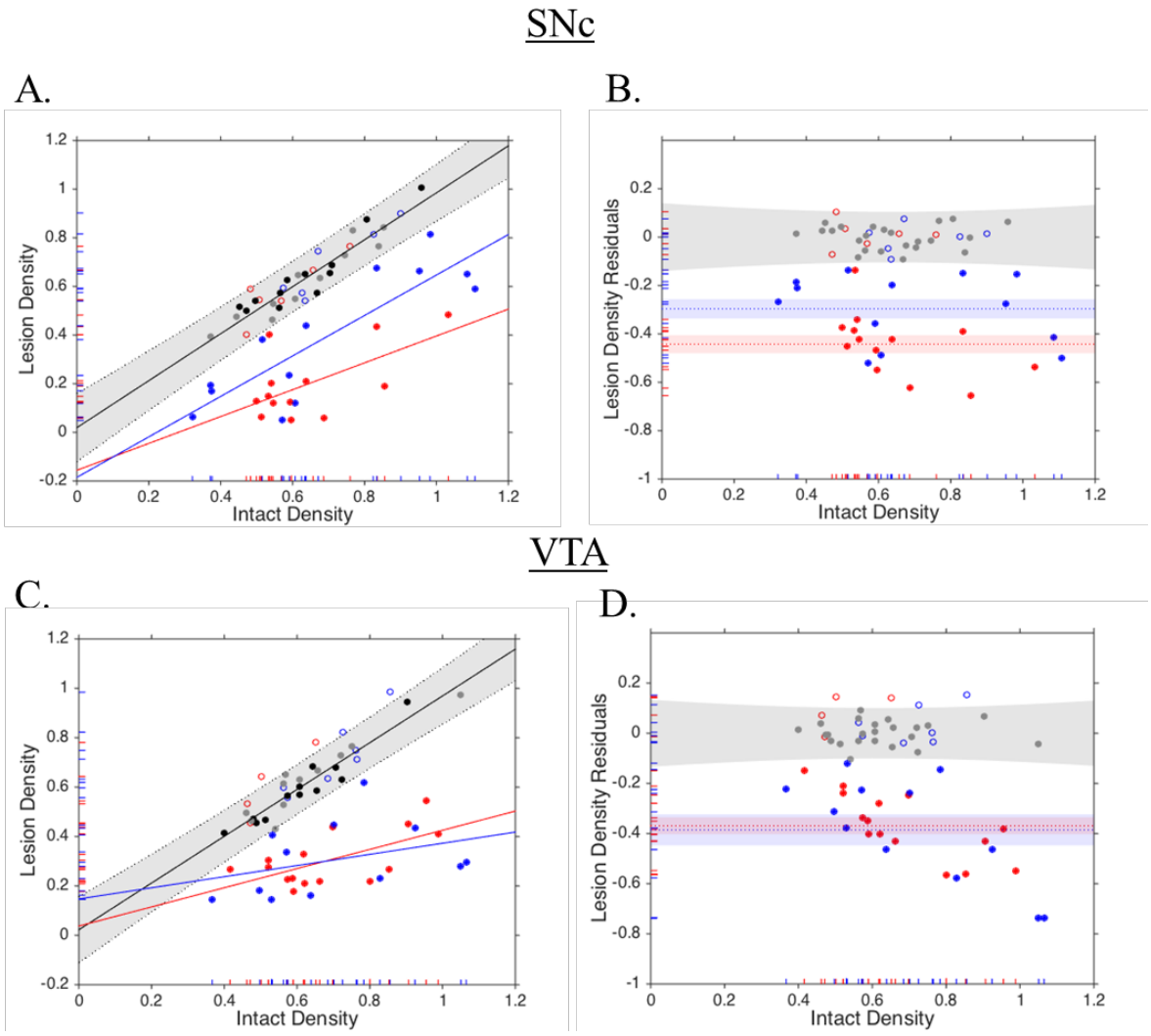


Figure 5.5 continued

A & C. Correlation of intact side optical density and infused/lesion side optical density in SNc (A) and VTA (C). Best fit line for the sham groups (collapsed) is in black with the gray surrounding area representing the 95% confidence interval. Best fit lines for both lesion + veh (red) and lesion + MB (blue) rats with significantly different residuals from that of the sham group only. Faded red and blue points represent subjects with lesion densities within the 95% confidence interval for the sham animals. These rats were considered to have failed lesions. B&D. Lesion density residuals plotted as a function of intact density for sham rats (gray; 95% confidence interval), lesion + vehicle (red), and lesion + MB (blue). Within the SNc (B) Mean lesion density residual ± SEM (line + shaded area) for both lesion groups are significantly different, $p < 0.01$.

Figure 5.6 Motor and disengagement function

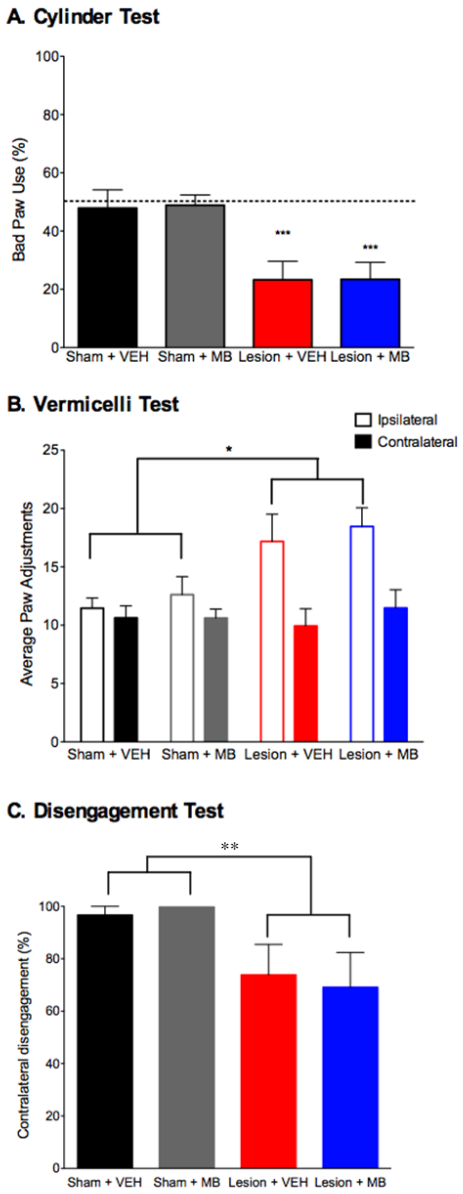


Figure 5.6 continued

A. Mean (± SEM) bad (contralateral) paw usage in contacting the cylinder while exploring. Both lesion groups show decreased paw usage than what would be expected due to chance (i.e. 50%). B. Mean (± SEM) ipsilateral and contralateral paw adjustments made while eating vermicelli strands. Lesioned rats made significantly more ipsilateral paw adjustments compared to sham animals. C. Mean (± SEM) contralateral disengagement behavior. * $p < 0.05$, ** $p < 0.01$,*** $p < 0.001$

Figure 5.7 Five choice performance

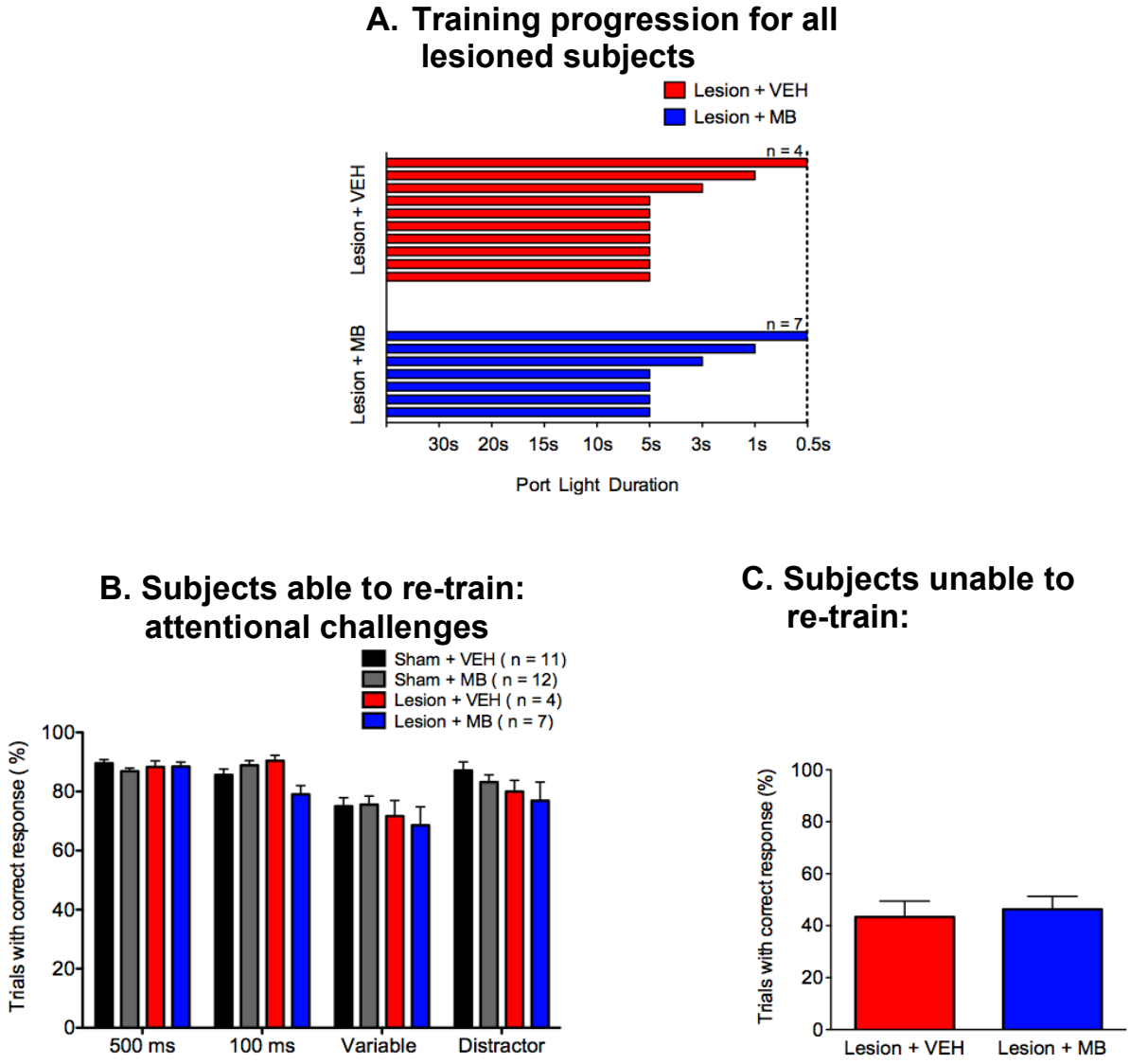


Figure 5.7 continued

A. The rate of training protocol completion for both lesion groups. Four lesioned rats receiving vehicle and seven lesioned rats receiving MB completed the five choice training protocol (represented by the top bar for each group). Rats that did not complete are all represented by one bar with the bar terminating at the training protocol unable to be mastered. B. Mean (± SEM) correct responses in the baseline five choice task (500 ms) and three attentional challenges for rats able to successfully re-train on the five choice task. C. Mean (± SEM) correct responses on the last day of training for rats that were unable to complete the re-training protocol. * $p < 0.05$

5.6 DISCUSSION

It is established that 6-OHDA administration causes dopamine depletion by disrupting mitochondrial function (Glinka, Tipton, & Youdim, 1996; Glinka & Youdim, 1995; Glinka, Tipton, & Youdim, 1998) and increasing the presence of reactive oxygen species (Kupsch, et al., 2014; Permual et al., 1989; Permual et al., 1992; Kumar et al, 1995). Furthermore, the application of antioxidants either *in vitro* or *in vivo* after 6-OHDA results in decreased presence of reactive oxidative species (Davison, Legault, & Steele, 1986; Mayo et al., 1998; Tiffany-Castiglioni, Saneto, Proctor, & Perez-Polo, 1982; Yamada et al., 1997). MB, not only enhances mitochondrial respiration (Callaway et al., 2004) through the electron transport chain but also has antioxidant properties (Salaris, Babbs, & Voorhees, 1991). Therefore we anticipated that chronic MB administration would lead to neuroprotection of SNc dopamine cells. Our data confirm this and demonstrate that an orally administered low-dose of MB can attenuate dopaminergic cell loss within the SNc after 6-OHDA infusion. Assessment of dopaminergic depletion within the VTA does not demonstrate a neuroprotective effect of MB. It has been demonstrated that the SNc is more susceptible to neurotoxin-induced degeneration than the VTA (Liang, Nelson, Yazdani, Pasbakhsh, & German, 2004; Speciale, Liang, Sonsalla, Edwards, & German, 1998). Our data also show slightly less dopamine depletion in the VTA than the SNc. This resilience may lie in the morphological differences of these cells compared to their SNc counterparts. It has been demonstrated that mitochondria within dopaminergic SNc and VTA cells differ. Specifically, mass of the mitochondria in SNc dopamine cells is smaller than that of VTA

dopamine cells mitochondria (Liang, Wang, Luby-Phelps, & German, 2007). While the functional significance of these differences in mitochondrial morphology has not been elucidated, it is possible that this difference may account for the difference in sensitivity to mitochondrial manipulations both good (MB) and bad (6-OHDA).

However, in comparison to previous work assessing the neuroprotective effect of MB, the effect seen in our experiments is rather small. These differences in effect size are likely due to (1) the experimental preparation and (2) the method by which MB was delivered/applied. Previously, MB infused directly into the striatum following a rotenone infusion to same location resulted in a markedly smaller lesion size as well as significantly greater mitochondrial function as measured by cytochrome oxidase activity (Rojas et al., 2009). Others have also show than MB can provide neuroprotection *in vitro* after rotenone application and when given orally *in vivo*, it can improve motor function in a rat neurotoxin model of PD (Wen et al., 2011). However, while Wen et al (2011) assessed the presence of reactive oxidative species *in vivo,* lesion severity and dopaminergic depletion were not quantified so the level of cell preservation is unknown. Therefore, we suspect that low-dose oral MB administration in combination with an acute, large neurotoxic infusion may not demonstrate the full capabilities of MB. The differences in the rate of dopamine depletion in our data provide some evidence for this. In the first experiment, we gave 7 µg of 6-OHDA to induce dopamine depletion in the SNc and saw no reduction in lesion severity with MB administration but in experiment 2, we gave a smaller amount of 6-OHDA (4.2µg) and saw MB-dependent neuroprotection. Recently, an Alzheimer's disease model in which transgenic mice showed that starting a

MB (20 mg/kg in saccharin water) regimen early was able to slow the rate of protein aggregation whereas starting MB treatment after reaching a threshold of protein aggregation that resulted in behavioral deficits was not effective (Hochgräfe et al., 2015). Therefore, we predict that neuroprotection would be greater in a model in which dopamine depletion is induced in a more progressive manner rather than with one acute neurotoxic event. Hence, either genetic models that induce protein aggregations leading to cellular dysfunction and death or neurotoxic models that gradually induce mitochondrial dysfunction and cell loss may be most useful in assessing neuroprotective value of MB.

While we did indeed see a mitigation of dopamine loss with MB, we did not see a corresponding mitigation of behavioral dysfunction as a result of this neuroprotection. Unilateral 6-OHDA-infused rats regardless of treatment assignment showed comparable performance across all motor and attentional tasks. If the neuroprotection due to MB did improve behavioral outcomes, we would have expected better performance across all measures in lesioned rats receiving MB compared to their vehicle-treated counterparts. It has been demonstrated that ~50% dopamine depletion is necessary to see motor impairments in both rodent and macaque models of PD as well as in human PD patients (Bernheimer, Birkmayer, Hornykiewicz, Jellinger, & Seitelberger, 1973; Riederer & Wuketich, 1976; Yuan, Sarre, Ebinger, & Michotte, 2005). Similarly, lesioned rats receiving MB had on average 49 % dopaminergic loss and still exhibited behavioral deficits. Therefore, this indicates that a larger preservation of dopaminergic cells is necessary to reach a sub-threshold level to see behavioral improvements.

Regardless of neuroprotective benefits, we expected that the acute metabolic effects of MB would also be able to enhance attention as others have routinely demonstrated cognitive enhancement with MB. In intact rats, MB has been shown to enhance extinction learning after fear conditioning (Gonzalez-Lima & Bruchey, 2004; Wrubel, Barrett, Shumake, Johnson, & Gonzalez-Lima, 2007). As well, performance in spatial working memory, discrimination learning, and novel object recognition can also be enhanced by MB (Deiana, Harrington, Wischik, & Riedel, 2009; Riha, Bruchey, Echevarria, & Gonzalez-Lima, 2005; Wrubel, Riha, Maldonado, McCollum, & Gonzalez-Lima, 2007). However we saw little enhancement of attentional function with MB administration. Sham rats receiving MB performed no better in the five choice task than their vehicle-treated counterparts.

Disregarding sham rats, we also hypothesized that MB would recover attentional function in lesioned rats by facilitating compensatory mechanisms/recovering metabolic functionality in lesioned rats. MB has been shown to improve behavioral outcomes in disease models of both PD and Alzheimer's disease. Motor function has shown to be improved with MB administration in a neurotoxic model of PD (Wen et al., 2011). And MB has improved spatial working memory performance in a rat model of Alzheimer's disease (Medina et al., 2011) and discrimination learning in a rat model of cerebral hypoperfusion (Auchter, Williams, Barksdale, Monfils, & Gonzalez-Lima, 2014). However, our data demonstrate that MB has little impact on attentional function in a neurotoxic model of PD. In Experiment 1, MB on-board showed mild enhancement of disengagement behavior on the impaired side relative to controls. However, in

Experiment 2, this effect was not replicated. It is likely that this effect is a result of several differentiating factors between the two experiments. Desipramine was given to protect noradrenergic fibers only in Experiment 2. It may be the case that depletion of some norepinephrine along with dopamine using 6-OHDA leads to a more profound disengagement deficit more susceptible to treatment with MB. Another factor is the lesion on average created in the Experiment 2 was smaller than Experiment 1 thus making the deficit less severe and possibly less sensitive to improvements as a consequence of MB administration. Further, among lesion rats in the five choice task, no improvements as a consequence of MB administration were observed either in rats able to complete training and the attentional challenges or in those rats that were unable to complete training. Taken together, this data suggests that MB is not useful for recovering attentional function.

In this experiment, a low dose of MB (4 mg/kg) was utilized and no behavioral improvements as a consequence of MB on board were observed. One cannot rule out that a higher dose of MB might be more effective in preserving dopamine cells and/or improving behavioral deficits. However, doses within the range of 1-4 mg/kg have been shown to improve learning and memory, but by 10 mg/kg, MB becomes ineffective (Bruchey & Gonzalez-Lima, 2008). And even higher doses can interfere with metabolic processes within the cell which can be more harmful (Bruchey & Gonzalez-Lima, 2008). However, higher doses (20 mg/kg) have been used in the treatment of Alzheimer's models with some success in improving behavioral and neural outcomes suggesting that the dosing parameters may be different in disease states (Hochgräfe et al., 2015).

The reason for which MB on board improves some cognitive tasks but not others may also lie in the role of memory. The distinguishing factor between cognitive tasks previously shown to be improved with MB administration and the tasks used in these current experiments is the necessity of memory. Pavlovian learning, working memory, and novel object recognition tasks require learning as the training is conducted over a period of days and the variables measured generally assess memory retention. It has also been shown that these same low doses of systemic MB as well as applying MB *in vitro* increases the rate of oxygen consumption within the cell (Riha et al., 2005; Callaway et al., 2004). Therefore, it is likely that MB exerts its beneficial effect by enhancing consolidation processes by increasing mitochondrial respiration necessary for memory formation. Here we have studied MB impact on attentional task requiring very little memory consolidation. Disengagement behavior is not a trained behavior, and while the five choice task requires an initial learning of the reward contingency, performance does not rely on the recruitment of short or long term memory processes. Subsequently, it may be simply that only cognitive processes relying on consolidation (or reconsolidation) may be enhanced with MB.

As we do not see any amelioration of behavioral deficits with MB alone, MB may work better in combination with traditional dopaminergic therapies. While MB can restore mitochondrial function, it cannot return dopamine levels to that of a sham condition. Therefore, MB in combination with L-dopa or other DA agonists may prove useful for more effectively treating both motor and cognitive deficits. It may be possible that by giving MB along with L-dopa, the dose of L-dopa necessary to recover behavioral

functions could be reduced. And as high doses of L-dopa typically cause unwanted side effects, this combination could not only prove useful in the treatment of a wider array of symptoms but also in causing fewer side effects.

Furthermore, it has been demonstrated that L-dopa can be toxic, possibly by increasing the rate of 6-OHDA generation (Maharaj, Sukhdev Maharaj, Scheepers, Mokokong, & Daya, 2005) and that these toxic effects can be mitigated with the application of antioxidants (Pardo, Mena, Casarejos, Paíno, & De Yébenes, 1995). Future work should investigate the possibility of this combination in restoring both motor and non-motor symptoms including cognitive and attentional dysfunction.

In sum, this work further validates metabolic enhancement as an effective and viable option for neuroprotection. While no data was collected in these experiments to quantify metabolic function or reactive oxidative species, we expect that these are the mechanisms by which MB is acting. Further, acute metabolic enhancement using MB does not enhance behavioral outcomes in this model. While this advances our working knowledge of the abilities of MB on board to impact behavior, future work should be aimed to assess the impact of MB in models more closely resembling PD neurodegeneration and pathology as well as other neurodegenerative disease such as mitochondrial dysfunction and oxidative stress are common features of neurodegenerative disorders.

Chapter 6: General Discussion

The goals of this dissertation work were (1) to elucidate the role of dopaminergic input into the central amygdala in regards to attention and (2) to understand how attentional deficits in a PD model are impacted by dopaminergic and non-dopaminergic therapies. In Chapter 2, I demonstrated that dopaminergic input (presumably from the SNc) into the CeA is indeed necessary for task switching behavior and specifically, this behavior is mediated by D1 receptors. I then found the same role of CeA D1 receptors in selective and sustained attention in Chapter 3. In Chapter 4, I assessed the ability of traditional dopamine replacement therapy with L-dopa to recover attentional dysfunction in a 6-OHDA model of PD and found that different types of attention respond to L-dopa administration differently. While attentional switching was able to be remediated in unilaterally dopamine depleted rats, more complex selective and sustained attention was worsened from the baseline 6-OHDA-induced deficits with the administration of L-dopa. Furthermore, chronic administration of L-dopa resulted in the development of dyskinesias precluding the rats from being able to perform the attentional tasks. In Chapter 5, I observed that methylene blue was able to provide mild neuroprotection to the dopamine cells in the 6-OHDA model of PD, but this wasn't sufficient to result in the overall attentional improvements.

To date, this is the first work to investigate the role of dopamine outside of the nigrostriatal pathway on attentional processes impacted in PD. Previously, attentional dysfunction has been attributed either directly to innate PFC dysfunction or degraded input from the striatum into the PFC (Cools, 2006; Ravizza & Ciranni, 2002). However, while the PFC does show pathological changes in PD, these pathological changes do not emerge until the last stages of the disease (Braak et al., 2004) whereas attentional

dysfunction emerges at the same time as motor dysfunction or prior to the onset of the motor deficits (Dubois & Pillon, 1997; Tadaiesky et al., 2008; Wu et al., 2012). Further, the striatal-cortical relationship has been widely implicated in attentional function thus leading to the hypothesis that PD-related attentional deficits are seen as a result of impaired communication of these areas (Agnoli & Mainolfi, 2012; Christakou, Robbins, & Everitt, 2001; Christakou, Robbins, & Everitt, 2004). While the degradation of this connection certainly contributes to attentional dysfunction seen in PD, this does not fully explain how these attentional deficits occur. For example, restoration of striatal function with cell graphs after MFB infusion of 6-OHDA does not recover disengagement behavior (Mandel et al., 1990). Therefore, it is pertinent to understand the role of other dopaminergic structures in attention.

It has previously been established that the CeA is necessary for several attentional processes including conditioned orienting, surprised-induced enhancement of attention, and selective and sustained attention, and that the reciprocal relationship of the SNc and the CeA must be intact for these attentional processes (El-Amamy & Holland, 2006, 2007; Lee et al., 2008; Lee, Gallagher, & Holland, 2010; Lee et al., 2006). Furthermore, projections emerging from the CeA have been well characterized in the context of these attentional processes. Projections from the CeA to the substantia innominata have been shown to influence cholinergic pathways regulating attentional function (Holland, 2007; Maddux et al., 2007). However, little work prior to this dissertation has investigated the importance of the SNc dopaminergic input into the CeA in these same attentional processes. This finding contributes to our understanding of attentional processes and the contribution of the nigral dopaminergic influence on CeA function.

In addition to implicating dopaminergic input into the CeA in attention, I have also demonstrated that D1, but not D2, receptors within the CeA are necessary for these

attentional functions. This aligns with previous research demonstrating a global need for D1 receptors in selective and sustained attention (Agnoli & Mainolfi, 2012; Pezze et al., 2007). Understanding how dopamine receptors regulate attention allows for the formulation of better treatment regimens for PD insofar that L-dopa and dopaminergic agonists are commonly used to treat motor and cognitive function in PD. Specifically, as the disease becomes severe and most dopamine cells are lost, patients become more reliant on dopamine agonist drug therapies. However, the majority of commonly prescribed dopamine agonists to treat PD are partial- or full-D2 or D2-like receptors agonists (Bonuccelli, Del Dotto, & Rascol, 2009). This could explain why some attentional dysfunctions can be treatment-resistant in patients with late-stage PD (Chaudhuri & Schapira, 2009; Dujardin et al., 2013). Thus, by understanding the contributions of different dopamine receptors to attention, treatments with different receptor sensitivities (especially higher D1 affinity) could be utilized to produce maximal benefits.

Because dopamine depletion via the medial forebrain bundle results in decreased attentional capacity, I hypothesized that basic dopamine replacement therapy with L-dopa would be able to repair attention switching as well as selective and sustained attention in the 6-OHDA model of PD. This was not entirely the case as attention switching (i.e. disengagement behavior) was easily recovered by L-dopa but selective and sustained attention was unable to be recovered. This difference in L-dopa's ability to recover different aspects of attention may be due to the complexity of the attentional processes being assessed. Disengagement behavior requires simple attentional orienting to the incoming stimulus and has previously been shown to be dependent on nigrostriatal as well as nigral-CeA function thus possibly lending itself to be easily repaired with L-dopa administration (Schallert & Hall, 1988; Smith et al., 2013). In contrast, while selective

and sustained attention is regulated by dopamine and the nigrostriatal pathway, it is also modulated by the basal forebrain cholinergic system (McGaughy et al., 2002; Muir et al., 1993; Risbrough et al., 2002; Robbins et al., 1989) and by dorsal noradrenergic fibers (Carli et al., 1983; Cole & Robbins, 1987). As a consequence, this highly intricate system may not simply be remediated with the restoration of dopamine levels.

In addition to demonstrating that L-dopa does not improve selective and sustained attention, I also demonstrated that L-dopa further exacerbates these deficits in a rat model of PD. It is likely that the exacerbation of attentional function seen with L-dopa administration may simply be due to an overdose of dopamine and/or norepinephrine. Previously, others have postulated that the incursion of new cognitive deficits with L-dopa usage may be due to an excess of dopamine in areas that have not yet experienced dopamine depletion (Cools, 2006). This may also be the case with selective and sustained attention. Within this model, it has been demonstrated that L-dopa enhances dopamine levels in both intact and denervated straita albeit at a lower rate in the intact striatum (Abercrombie, Bonatz, & Zigmond, 1990). While I did not collect any data in regards to dopamine level, the behavioral data suggests that this may be the case. The decreased overall accuracy in the selective and sustained attention task was specifically due to decreased accuracy in trials in which the port on the side ipsilateral to the lesion was the target (i.e. correct) port. This provides evidence for the argument that there is an optimum level of dopamine (and possibly norepinephrine) necessary for attentional function and that excessive levels cause impairments in attentional function. Thus, the findings in this dissertation work demonstrate that even in scenarios of simple dopamine depletion, application of L-dopa is detrimental possibly due to the incursion of supra-optimal levels of dopamine.

Overall, I have demonstrated that simple dopamine replacement therapy is not sufficient for restoring attentional function impaired in a rat model of PD. Due to the complexity of these cognitive processes as well as widespread pathology outside of the SNc in PD, I also assessed the usefulness of moving from a neurotransmitter-focused approach to a system-wide enhancement approach to restore cognitive function in this PD model. PD results in pathological changes of many areas including the dopamine-rich areas such as the SNc but also the locus coeruleus, and in later stages, cortical areas as well (Braak et al., 2004). These cells show abnormal mitochondrial function which leads to increased oxidative stress and finally, apoptosis (Fukae et al., 2007; Gubellini, Picconi, Di Filippo, & Calabresi, 2010; Schapira, 2008). Therefore, one means by which symptomology could be improved is by enhancing mitochondrial function of both compromised cells (restore function) and non-compromised cells (boost compensatory functions). Unilaterally depleting dopamine using 6-OHDA or other neurotoxins results in chronic mitochondrial dysfunction in the cells remaining after initial insult thus making this avenue feasible to pursue in this model of Parkinson's disease (Blum et al., 2001; Glinka & Youdim, 1995).

I have been the first to attempt to use the mitochondrial enhancer, MB to enhance attentional function in intact and PD-modeled rats. While MB has been shown to repeatedly improve learning and memory processes (Callaway et al., 2004, 2002; Gonzalez-Lima & Bruchey, 2004; Martinez et al., 2013; Riha et al., 2005; Wrubel, Riha, et al., 2007), until now it had not been assessed for its ability to improve attentional process. My work has demonstrated that MB on-board is only mildly effective at enhancing attention in contrast to the marked MB-related enhancements in learning and memory repeatedly demonstrated by others in intact and impaired rats. While attention is inherently necessary for effective learning and memory formation, these current findings

along with other evidence suggest that MB is beneficial for the formation of memories after learning but not the ability to initially attend in order to learn. Therefore, it is likely that the efficacy of MB lies in its ability to enhance consolidation processes (i.e. synaptic plasticity) which require mitochondrial synthesis of energy units such as ATP (Lim & Isaac, 2005). While MB (on-board) is marginally useful in restoring reduced attentional function, it may serve better as a treatment for mild cognitive impairment (which includes short-term memory problems) in PD.

Further, while MB's neuroprotective abilities have been assessed previously, it has not been assessed in this 6-OHDA model of PD. The neuroprotective effects of MB are most commonly studied *in vitro* (Nicklas, Vyas, & Heikkila, 1985; Wen et al., 2011). This work is the first to look at both behavioral and neuroprotective outcomes of MB administration in an *in vivo*/*ex vivo* model of PD. I demonstrated that orally-administered MB can provide neuroprotection under certain conditions. Only when the amount of 6-OHDA infused was lowered from 7 μg to 4μg was neuroprotection seen.

However, while I have demonstrated that oral methylene blue can provide some neuroprotection after an acute neurotoxic infusion into the MFB, this does not result in less severe behavioral impairments in these animals. Similar to patients diagnosed with PD, lesion models of PD only show behavioral deficits once a majority of dopamine cells are lost and these behavioral deficits show only a weak correlation with the amount of dopamine cell loss (Bernheimer et al., 1973; Riederer & Wuketich, 1976; Yuan et al., 2005). Even though MB provided mild neuroprotection in my study, it was unable to preserve the dopamine cells to levels more than 50% and support intact behavioral function. Consequently future endeavors to evaluate the neuroprotective potential of MB should utilize models of PD in which neurodegeneration or pathological changes occur gradually such as is common in transgenic mouse models or models in which neurotoxin

exposure is titrated over time. MB's neuroprotection might be more effective models with slower dopamine cell degeneration. While providing neuroprotection for PD could dramatically improve the lifespan as well as quality of life in PD patients, there is an inherent shortcoming in any attempt to provide neuroprotection in PD because symptomology and the ability to diagnose the disease does not emerge until substantial damage has occurred. Therefore the development of neuroprotective measures alone will not suffice. In order for any neuroprotective agent to be useful in improving quality of life or lifespan, the development of pre-clinical markers for PD is paramount so that cell loss can be slowed.

In the meantime, it would also be prudent to investigate the possibility of using MB and L-dopa simultaneously in attempt to better treat patients that have already experienced significant dopamine loss. As MB and L-dopa target different aspects of PD, it is likely that giving both would have a synergistic effect. Therefore, co-administering MB alongside L-dopa may result in better treatment of a wider array of symptoms as well as possibly less L-dopa necessary to ameliorate PD symptomology.

Together, this dissertation work yields substantial information in regards to attention impairments in PD. This work highlights the importance of understanding the nigral-CeA circuitry in the context of attentional function and suggests a role of this mechanism in PD-associated attentional dysfunction. This work also demonstrates that attentional dysfunctions in PD do not respond to treatment in the same manner as motor dysfunction and further highlight the need for continued study of the nature of cognitive dysfunction, the underlying circuitry, and its response to drug treatments. As treatment options improve for the motor dysfunction in PD with nuanced drug regimens and deep brain stimulation procedures, it is also important to continue to evaluate existing

treatments and if necessary, develop new treatment approaches for the non-motor symptoms of PD which can be debilitating even after motor function is improved.

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