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Mechanisms Underlying the Dysregulation of Postural Stability in Dopamine-Depleted Rats

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Mechanisms Underlying the Dysregulation of Postural Stability in Dopamine-Depleted Rats

by

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Dedication

This dissertation is dedicated to all of the people who told me I could do it, and to my grandmother, Shirley, who taught me how to "be a good boy". She was the very model of the kind of perseverance needed to pull this off.

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All of my undergrads: Ted Lin, Jitsen Chang, Michelle Dupre, Marnie Preston, Adva Buzi, Jennifer Gench, John Hong, Allison Ahrens, Candace Lam, and Yao Gbanaglo have all left their marks on this work, and it is built on their sweat. Thank you for your persistence and for treating me like a boss when I didn't even know how to be one.

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Jackie Kane deserves at least as much credit for this dissertation as I do, and has been a best friend and confidante to me throughout it all. I will be fortunate indeed if I get to work and laugh with more people like her in the future.

Tim Schallert's endless font of ideas inspired this work, and the long enthusiastic conversations kept the wheels turning despite the numerous ruts in the path. I thank him for helping me to realize the credibility of my own ideas, and all of my committee members for their teaching, answering, and support over the years.

Finally I thank my wife, Tomoko. She is my smile, she is my good mood when the going is tough, and we have done this together. Your turn.

Mechanisms Underlying the Dysregulation of Postural Stability in Dopamine-Depleted Rats

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The work described in this dissertation aims to understand how postural instability (PI), a troubling symptom of advanced Parkinson's disease (PD) in humans, develops from the degeneration of nigrostriatal dopamine neurons characteristic of PD. The studies herein (1) outline the development of clinically relevant methods for evaluating PI in experimental rodents, (2) indicate that PI may not result directly from disruption of dopamine systems but may instead arise from non-dopaminergic changes that occur subsequent to dopamine depletion, and (3) search for specific evidence of plasticity or degeneration outside of the damaged nigrostriatal dopamine system that may be linked to the development of PI. It is hoped that this work will help lay the foundation for the development of novel prophylactic treatments aimed at preventing the progression of PD to advanced stages where treatment-resistant symptoms such as PI appear.

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GENERAL INTRODUCTION

Parkinson's disease is the nation's leading neurological disorder of movement. According to the Parkinson's Disease Foundation, over one million Americans suffer from this disease for which there is currently no cure. Though symptomatic treatments exist, they often produce troubling side effects which can become more disabling than the disease itself. Persons affected with PD display a set of cardinal motor symptoms including tremor in the limbs, muscular rigidity, slowness of movement ("bradykinesia") or freezing ("akinesia"), and postural instability manifesting as impaired balance and an increased risk of falling.

There is a growing awareness that a constellation of other, generally non-motoric neurological symptoms may also be associated with the disease, and can often precede the motor signs which generally trigger initial doctor visits. These include sensory disturbances (poor olfaction and problems with color perception), mood disorders (especially depression), and certain sleep disorders (Wolters et al., 2000; Stiasny-Kolster et al., 2005). Though troubling, these prodromal symptoms may be useful in developing early detection strategies for the disease, allowing the application of neuroprotective therapies to help slow or halt the degenerative process before motor symptoms appear. However, at present, early detection tests for PD are not in wide clinical use, and so far there are no well-established treatments for halting the progress of PD, the root cause of which has yet to be elucidated

The symptoms of PD have long been thought to result primarily from the death of dopamine-producing neurons located in the *pars compacta* portion of the substantia nigra, reducing the release of dopamine onto the neurons of the caudate and putamen (known collectively as the striatum). This assumption has rested largely on two findings: (1) there is a marked loss of the heavily pigmented neurons of the nigra apparent upon postmortem examination of the brains of people who died with PD (Robinson and Rajput, 2005), and (2)

the use of drugs that enhance or replace dopaminergic activity (such as the mainstay therapy levodopa, the administration of which boosts dopamine synthesis) can, at least for a time, provide symptomatic relief from the disease (Goudreau and Ahlskog, 2005).

Of the cardinal symptoms of PD, PI is perhaps the most dangerous, and often leads patients to withdraw from their daily activities. PI, with its increased risk of falling, becomes most severe somewhat later in the disease course than other symptoms, and can result in a greatly increased risk of personal injury to the patient (Chou and Hurtig, 2005). That the different symptoms of PD often occur in distinct stages (as codified, for example, in widely used disease staging scales such as the Hoehn & Yahr rating scale and the UPDRS) raises the possibility that different levels or even types of brain pathology could be individually responsible for the different symptoms. PI is among the symptoms of PD that are most resistant to treatment with mainstay dopamine-replacement therapies (Zetusky et al., 1985; Horak et al., 1996; Hurtig, 1997; Jessop et al., 2006), although behavioral therapies involving exercise or balance training have shown some efficacy in ameliorating the symptom (Hirsch et al., 2003; Jobges et al., 2004; Protas et al., 2005).

Indeed, studies have indicated that some symptoms of PD are not well-correlated with the extent of dopaminergic depletion. Recent work by Bohnen et al. (personal communication; see also Bohnen et al. (2006)) has shown that while bradykinesia appears well-correlated with the extent of dopamine neuron loss (as measured by PET scanning of human PD sufferers following administration of radiolabeled ligands to the dopamine reuptake transporter), the symptoms of rigidity, fall risk, and tremor are not well correlated. In PD, intracellular inclusions known as Lewy bodies are seen upon postmortem histological examination, and these are associated with the areas of cellular degeneration seen in the disease (Robinson and Rajput, 2005). On the basis of this association, Braak et al. (2003) examined the distribution of Lewy bodies in the post-mortem brains of PD patients and

concluded that the loss of nigral dopaminergic neurons in PD is only a midpoint in a much more extensive degenerative process that actually begins in areas of the brainstem and olfactory bulb (which, interestingly, might explain the aforementioned prodromal olfactory symptoms). If the brain pathology of PD is indeed as widespread as this work suggests, the various motor and nonmotor symptoms of PD could be separately explained by degenerative processes going on in many different brain regions.

Furthermore, the symptoms of the disease respond variably to dopaminergic therapies. While treatment with levodopa or direct dopamine receptor agonists shows good efficacy in combating the symptoms of rigidity and bradykinesia, their effects on tremor are variable and their ability to treat postural instability is quite poor (Chou and Hurtig, 2005). As noted earlier, the correlation between structural markers of dopamine system integrity and clinical ratings of the severity of PD symptoms is similarly tenuous. A large multicenter clinical trial known as the ELLDOPA trial was recently undertaken to attempt to determine whether treatment with levodopa hastens the progression of PD (more on this later). Results from the study showed that patients treated with higher doses of levodopa throughout the study period ended with better clinical scores (after a period of drug washout), reflecting sparing of symptom progression, but hastened disease progression as measured physiologically with PET imaging of a marker associated with dopamine neurons—in this case, the dopamine reuptake transporter (Fahn, 2005). Though some have argued that this discrepancy may be due to a poor choice of the marker used to reflect the neural degeneration (Goudreau and Ahlskog, 2005), another explanation is that the integrity of the dopamine system is not as directly linked to the behavioral symptoms of PD as previously thought.

Though still underappreciated for its role in the symptoms of PD, it is established that degeneration occurs in several subcortical nuclei other than the substantia nigra in PD.

These include, to varying extents, "the dopaminergic mesocorticolimbic system, the noradrenergic locus ceruleus (oral parts) and motor vagal nucleus, the serotonergic raphe nuclei, the cholinergic nucleus basalis of Meynert, pedunculopontine nucleus *pars compacta*, Westphal-Edinger nucleus, and many peptidergic brainstem nuclei" (quoted from Jellinger (1991); see also Braak et al. (2004)). It remains to be seen to what extent various symptoms of the disease can be tied to degeneration in these structures. Lee et al. (2000), however, make a convincing case at least for the involvement of the pedunculopontine nucleus in several of the more treatment-resistant symptoms of PD.

One possibility is that chronic loss of dopamine input leads to plastic changes downstream from the dopamine terminals which could be more directly responsible for some PD symptoms. Following unilateral partial lesions of the nigrostriatal pathway using the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) in rats, Ariano et al. (2005) found that activated caspase-3 and elements of the apoptosome were elevated in the striatum deprived of normal dopamine input, suggesting that even relatively mild declines in dopamine transmission can adversely affect the dopamine-receiving cells of the striatum. In addition, dopamine loss has been shown to lead to a loss of dendritic spines and corticostriatal glutamatergic synapses onto one subpopulation of striatal medium spiny neurons, the primary cell type making up the striatum (Day et al., 2006). These neurons receive not only the nigrostriatal dopamine input but also glutamatergic input from overlying cortical areas, and experimental dopamine depletion (again, by lesioning with 6-OHDA, or due to chronic treatment with the vesicular monoamine uptake inhibitor reserpine) yields spine and synapse losses on the striatal neurons which in turn project to the globus pallidus. The authors of this study also discovered that inhibition of L-type calcium channels using the drug nimodipine ameliorated the loss of spines and synapses though it did not affect the extent of dopamine depletion. Again, it is possible that such "indirect" morphological

changes occurring secondary to dopamine depletion are responsible for some of the behavioral effects of dopamine loss, and part three of this dissertation assesses whether blocking such changes with nimodipine can lead to behavioral sparing in dopamine-depleted animals.

The possibility that dopamine depletion may not be the endpoint of a degenerative process in PD but rather a trigger for further pathological brain changes has major implications for pharmacotherapy in PD. Currently, most dopamine replacement therapies are only useful for a limited window of time in PD patients, after which they begin to produce troubling side effects. These include dyskinesias, "wearing-off" phenomena, and certain psychological abnormalities which can ultimately be as disabling as the underlying disease itself (Goudreau and Ahlskog, 2005). It is widely believed that the "timer" to the onset of these side effects begins with the onset of therapy with L-DOPA, which remains the most effective pharmacological treatment for the disease, though in fact the development of dyskinesias under drug therapy may be more closely connected with the progression of the underlying disease process as opposed to the duration of drug therapy (Papa et al., 1994). Nevertheless, for this reason L-DOPA therapy is often held in reserve until later in the disease process when a patient's symptoms no longer respond adequately to other medications such as direct dopamine agonists. In addition, some have posited that L-DOPA may itself hasten the progression of the disease, though this idea is still debated (Whone et al., 2003; Fahn, 2005; Goudreau and Ahlskog, 2005). If, however, diminution of dopamine signaling can trigger pathological brain plasticity, then the use of dopamine replacement therapies such as L-DOPA as early as possible in the disease process might be wise, providing not only therapeutic but possibly also prophylactic effect. For this reason, further research into the brain morphological sequelae of dopamine depletion, and their link to PD symptoms, is certainly warranted.

Initial pilot work in our lab suggested that in rat models of PD, some aspects of postural instability did not appear to arise directly from dopaminergic dysfunction, though the symptom was present in chronically dopamine-depleted animals. Given this finding and the studies mentioned earlier indicating that PI may not directly be a disorder of dopamine loss, we sought to refine methods for its evaluation in experimental rodents.

The first chapter of this dissertation details methods for evaluating PI in rats through the development of two behavioral tests, one of forelimb placing and another of reactivity to shifts in center of gravity. Because of accumulating evidence that the various symptoms of PD may have distinct pathological underpinnings, it is important that experimental models include behavioral analyses which can evaluate these different symptoms independently, and work in our lab has focused on this goal.

Chapter two presents evidence supporting the idea that at least one symptom of PD, postural instability, does not result directly from loss of dopamine innervation (even though dopamine loss may be a necessary component in its development). This is based on the original finding that strong antagonism of postsynaptic dopamine receptors does not produce PI, as measured with our test of forelimb placing, though animals chronically depleted of dopamine using 6-OHDA do show deficits. Through a series of small experiments this section attempts to rule out several potential explanations for this discrepancy, concluding that PI might result from a secondary plastic change or nonspecific damage brought on by the initial dopamine depletion.

Finally, in chapter three we attempt to not only identify changes in the brain outside of the nigrostriatal dopamine pathway in dopamine-depleted animals, but also seek to determine whether the aforementioned published reports of structural changes (spine and synapse loss) in the striatum of dopamine-depleted animals can explain the symptoms of PI seen in 6-OHDA lesioned rats. After administering the drug nimodipine to block these

plastic changes, we used the behavioral assays developed in chapter one to assess changes in the animals' behavior after lesioning to determine whether the development of PI symptoms is also blocked. We also investigate the integrity of two brain areas directly connected to the degenerating nigral dopamine cells to determine whether cell loss in these areas may be linked to the observed functional deficits.

It is hoped that building evidence indicating that some troubling symptoms of PD are not the direct result of dopaminergic dysfunction will lead to research into strategies for protecting neural systems other than (or in addition to) the nigrostriatal dopamine pathway, in which the development of neuroprotective therapies has thus far proven quite challenging. In addition, this work will further increase recognition of potentially significant nondopaminergic pathologies in PD and hopefully lead to more effective and individualized treatment of PD patients based on their unique needs and profile of symptoms.

Chapter One: Developing methods for evaluating postural instability in rodents

CHAPTER OVERVIEW

Rodent models are used widely in PD research. However, their success as such depends on the ability of investigators to carefully analyze the sensorimotor behavior of the animals and relate this to the symptoms of PD to understand how experimental therapies interact with the disease process (Cenci et al., 2002). At present, few behavioral tests have been developed to specifically evaluate PI in rats, though the work of Teitelbaum and colleagues on the pharmacological manipulation of what they termed "static stable equilibrium" provides an excellent starting point for the further study of PI (De Ryck et al., 1980; Morrissey et al., 1989). In humans with PD, PI manifests primarily as an increased propensity to falling, due to inadequate correction of postural imbalances.

We have developed two new behavioral assays of PI in rats. The first is a modification of a vibrissae-elicited forelimb placing test that has been used previously in rats with cortical or striatal damage or dopamine depletion (Barth et al., 1990a; Barth et al., 1990b; Woodlee et al., 2005b). This test can evaluate sensorimotor integration and its contribution to regaining stable postural support from an unsupported state. We have developed new rating scales appropriate to using this test in dopamine-depleted animals, which display patterns of behavior in the test which are distinct from animals with other types of lesions, and have characterized the placing behavior of dopamine-depleted rats. The second test evaluates an animal's reaction to experimentally-imposed offsets in its center of gravity and is thus analogous to the "push test" commonly used to evaluate PI in PD patients. We have termed this test the "postural instability test" (PIT) and have characterized how normal rats, dopamine-depleted rats, and rats under the influence of various dopaminergic drugs behave in the test.

The two experiments in this chapter have previously been published (Woodlee et al., 2005b; Woodlee et al., 2008), and the manuscripts represent our first published works on the use of the forelimb placing test (Experiment 1) and postural instability ("PIT") test (Experiment 2). The focus of the papers as they were published was not on the use of the tests for evaluating postural instability, though we quickly came to appreciate the utility of these tests as reflective of postural impairments, and subsequent chapters will focus on their use for this purpose.

EXPERIMENT 1: VIBRISSAE-ELICITED FORELIMB PLACING AS AN INSTRUMENT FOR ASSESSING POSTURAL INSTABILITY IN RATS

Introduction

In this experiment we worked with rats that had suffered occlusion of the middle cerebral artery (MCAo) or aspiration of the sensorimotor cortex (both of which are used as models of stroke), or that had been depleted of dopamine by injection of the catecholamine neurotoxin 6-hydroxydopamine. Our initial work with this test was to develop a cross-midline version in which stimulation of the vibrissae on one side of the body was used to elicit a placing response in the opposite forelimb. Later we began using the test more extensively in dopamine-depleted rats as a measure of postural instability, at which time we dropped use of the cross-midline variation and developed more sensitive rating scales for this application (described in more detail in Appendix 2). Here we describe the methods for using the test and the results of its application to animals with experimental stroke-like lesions and 6-OHDA lesions. We discovered that this test allowed us to discriminate readily between the three different lesion models employed, indicating that the test is useful for

investigating various aspects of sensorimotor integration in addition to its use as a test of postural instability.

Tests of forelimb placing ability have long been used in rats (Marshall, 1982; Wolgin and Kehoe, 1983; Barth and Stanfield, 1994; Felt et al., 2002). Intact rats, held aloft, instinctively attempt to place their forelimb(s) upon any nearby surface they sense. This tendency has been exploited to develop tests of sensorimotor integration or motor function in rat lesion models. Placing may be triggered visually (Marshall, 1982), by touching the forelimb to a surface (Wolgin and Kehoe, 1983), or by stimulation of the vibrissae (Barth and Stanfield, 1994). Traditionally, the sensory trigger is applied to the same side of the body as the forelimb being tested (e.g., the left vibrissae are stimulated to test for placing in the left forelimb).

The course of recovery following unilateral stroke in rats may be influenced by events occurring in the intact hemisphere (Jones and Schallert, 1992b, 1994). Evidence exists suggesting that anatomical reorganization takes place between the two hemispheres following brain damage. This is manifest, for example, in the presence of new transcallosal fibers in rats sustaining thermocoagulatory damage to the sensorimotor cortex (Napieralski et al., 1996; Uryu et al., 2001). Notably, though, some of these changes do not occur when an aspiration technique is used for creating the lesion (Carmichael and Chesselet, 2002; Voorhies and Jones, 2002). Therefore it would be interesting to see whether behaviors that are presumably dependent upon interhemispheric communication might be differentially affected by aspiration versus other types of lesions. There is, however, a relative dearth of motor behavioral tests useful for examining sensorimotor integration across the midline in rats.

In this experiment we present data based on a modification of the vibrissae-elicited forelimb placing test, in which rats are held sideways in order to stimulate the vibrissae

contralateral to the forelimb in which placing is being evaluated. By comparing results from this "cross-midline" placing test to those found when placing is triggered by ipsilateral stimulation, distinct and lesion-dependent patterns of placing recovery can be seen. Interestingly, in some cases, cross-midline sensory information can trigger placing in an impaired limb earlier than sensory information entering the impaired hemisphere can. This test can also detect chronic deficits in some lesion models. The nature of the deficits differs markedly between the models of stroke and Parkinson's disease investigated here, making the cross-midline test potentially useful in discriminating between these different conditions in rats.

Methods

Subjects

Male Long-Evans hooded rats from Harlan Laboratories (MCAo, MCAo shams, and 6-OHDA lesions) or Charles River Laboratories (aspiration lesions) were used. Rats were housed singly or in pairs in acrylic tub cages with wood shavings, and were maintained on a 12:12 h reversed light cycle. All testing was performed in the dark portion of the cycle. They were handled frequently, allowed to explore novel environments, and given food and water *ad libitum*.

Surgeries

Middle cerebral artery occlusion (MCAo) and shams

Rats (n=12 total; 6 MCAo and 6 shams) weighing 350 to 400 g were anesthetized with isoflurane. A temperature probe was inserted into the rectum and a heating pad was used to maintain rectal temperatures at 37°C to 37.5°C throughout surgery. MCAo was induced as described by Longa et al. (1989). Under an operating microscope, the right common carotid artery (CCA) was exposed through a midline neck incision and carefully

dissected free from the surrounding nerves and fascia between its bifurcation and the base of the skull. The occipital artery branches of the external cervical artery (ECA) were then isolated, dissected, and electrically coagulated. The ECA was dissected further distally and coagulated along with the terminal lingual and maxillary artery branches, which were then divided. The internal cervical artery (ICA) was isolated and carefully separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated close to its origin with a 5-0 nylon suture. Next, a 5-0 silk suture was tied loosely around the mobilized ECA stump, and a 4 cm length of 3-0 poly-L-lysine-coated monofilament nylon suture (Harvard Apparatus, Holliston, MA), blunted at the tip, was inserted through the proximal ECA into the ICA and thence into the circle of Willis, effectively occluding the MCA. The suture was inserted 18 to 20 mm from the bifurcation of the CCA, depending on the animal's body weight. After the intraluminal suture was placed, the neck incision was closed with a silk suture. The silk suture around the ECA stump was tightened around the intraluminal nylon suture to prevent bleeding.

The rat was allowed to recover from anesthesia and returned to its cage. One rat that did not demonstrate a left forelimb (contra to the right MCAo) paresis during this recovery period (at 60 min after the initiating the occlusion) was excluded from further study. After 75 minutes of MCAo, the rats were reanesthetized with the same anesthetic and the intraluminal suture was carefully removed. The CCA and ICA were then inspected to ensure the return of good pulsations. The neck incision was closed with silk sutures, and the animals were returned to their home cages.

Sham rats were subjected to the same anesthetic, mid-neck incision, and ECA and CCA isolation procedures, but did not receive MCAo.

6-hydroxydopamine (6-OHDA) lesions

Rats (n=6) were lesioned unilaterally with 10 μg 6-OHDA delivered into the medial forebrain bundle, as detailed in Appendix 2. These 6-OHDA rats were tested at chronic (>400 days post-operative) time points on the forelimb placing test, well after the time placing deficits stabilized in MCAo-lesioned rats. Lesions were confirmed by rotation to a challenge dose of 3 mg/kg d-amphetamine (Sigma, St. Louis), and only rats with a strong ipsilesional rotating bias were used in this study.

Aspiration lesions

Rats (n=12) were anesthetized with Equithesin and placed in a stereotaxic apparatus. The scalp was incised at midline and a rectangular craniotomy was created using a surgical drill. Coordinates for the craniotomy were between 0.7 mm posterior and 1.7 mm anterior to bregma and 2.8 to 4.7 mm lateral to the midline. Following skull removal, the dura was carefully excised. A 1.0 ml syringe was adapted to a rubber tube connected to a vacuum pump, and a 20-gauge blunt-ended needle was placed at the end of the syringe. Using a surgical microscope, the exposed cortical brain tissue was removed by suction down to the appearance of white matter. Following surgery, rats were sutured and given an atropine injection (1.0 mg/kg). All animals were placed in an incubator during recovery and then returned to the home cage.

Behavioral testing

All rats were tested on both the vibrissae-evoked forelimb placing and limb-use asymmetry tests (described in Appendix 2), except for 6-OHDA-lesioned rats, which were tested only chronically (>400 days post-operatively) with the placing test. MCAo-lesioned and sham rats were tested on post-op days 7, 10, 14, 17, 21, 28, 35, 49, and 93, and on day

120 for "chronic" data; aspiration-lesioned rats were tested on post-operative days 2, 5, 8, 11, 14, and 20 in the placing test and on days 3, 7, 14, and 21 for limb-use asymmetry.

Tissue preparation

MCAo and aspiration-lesioned rats were deeply anesthetized with pentobarbital (75 mg/kg, i.p.) and transcardially perfused with a 0.1 M phosphate buffer (PB) rinse followed by a solution of 4% w/v paraformaldehyde in 0.1 M PB as fixative. Brains were removed and postfixed at 4ºC for approximately 48 h in the same fixative. Brains were then sectioned coronally at 50 μm on a vibratome. Every sixth section was mounted on slides and stained with Toluidine blue (a Nissl stain) for determination of lesion extent and location.

Estimates of remaining tissue volume

Images of stained slices were projected at 20x and overlaid with a transparent grid with 20 mm spacing, in order to estimate the volume of remaining tissue in various brain regions using the method of Cavalieri (Henery and Mayhew, 1989). Volume was computed with the equation $V = T \times (a / p)^2 \times \sum P$, where T is the average distance between slices (approximately 300 μm in this case), a is the grid spacing (20 mm), p is the projection magnification (20x), and P is the total number of grid intersections ("points") observed to fall within the borders of the structure under analysis. For MCAo animals, the volume of each entire hemisphere was calculated between 4.0 mm anterior and 4.0 mm posterior to bregma. For aspiration-lesioned animals, the volume of the remaining neocortex and striatum was estimated in each hemisphere between 3.0 mm anterior and 1.0 mm posterior to bregma.

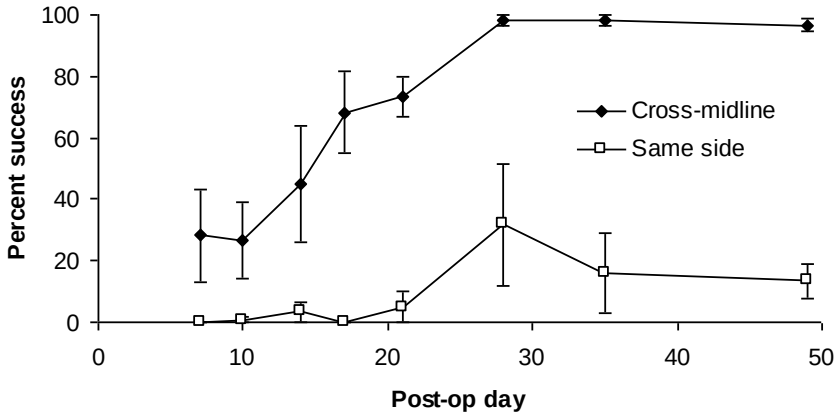


Figure 1.1 Same-side versus cross-midline placing in animals receiving occlusion of the middle cerebral artery

Middle cerebral artery occlusion is used as a model of stroke. Animals with this lesion show severe and enduring deficits for the contralateral forelimb in the placing test, but not if the vibrissae stimulated are the ones ipsilateral to the lesion. Data are means ± SEM.

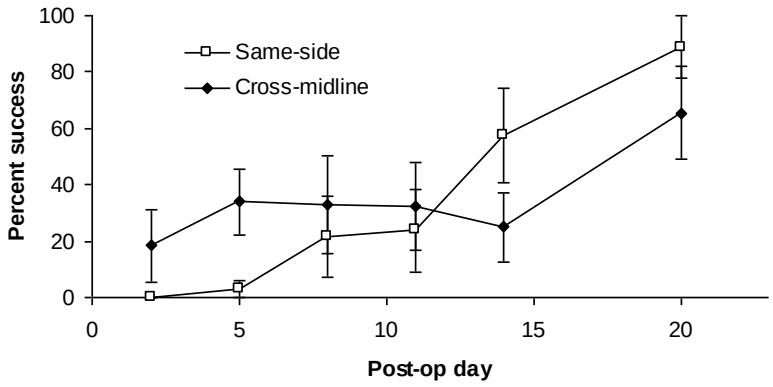


Figure 1.2 Same-side versus cross-midline placing following aspiration removal of the forelimb representation area of sensorimotor cortex

Animals with this lesion recover both same-side and cross-midline forms of placing at roughly equal rates. Data are means ± SEM.

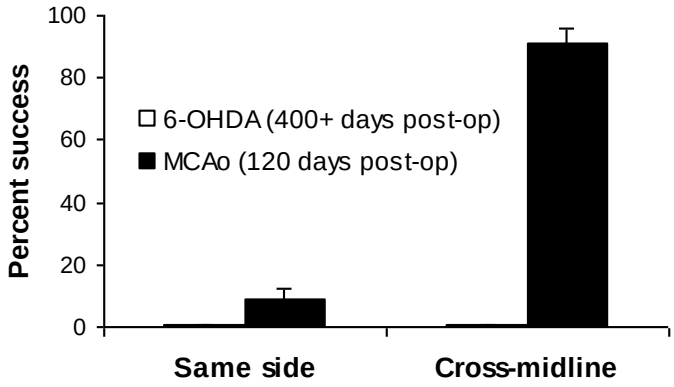


Figure 1.3 Forelimb placing success at chronic post-lesion time points

Though cross-midline placing has essentially returned to normal 4 months after MCAo, placing triggered by the contra-to-lesion vibrissae is still severely impaired in these rats. Rats lesioned with 6-OHDA show a complete absence of placing even >1 year after the lesion, regardless of the side to which stimulation is delivered. Data are means ± SEM.

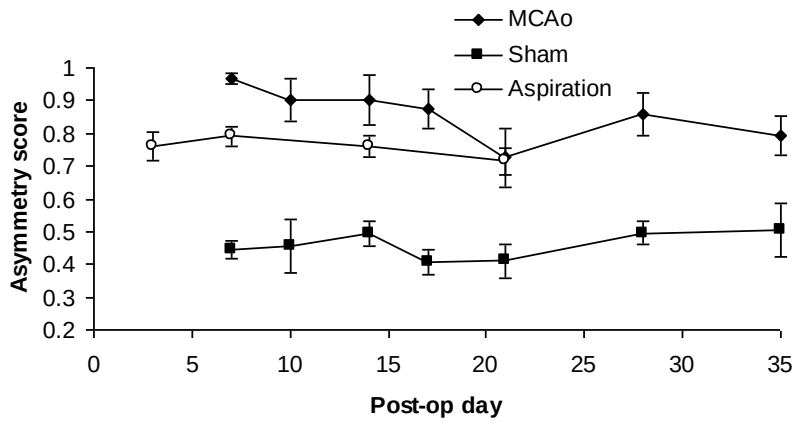


Figure 1.4 Performance in the limb-use asymmetry ("cylinder") test for rats lesioned via MCAo or sensorimotor cortex aspiration

Both lesions produce a significant asymmetry in spontaneous forelimb use compared to sham-operated animals. A score of 0.5 denotes symmetric forelimb use; scores >0.5 indicate increasing reliance on the forelimb ipsilateral to the lesion. Data are means ± SEM.

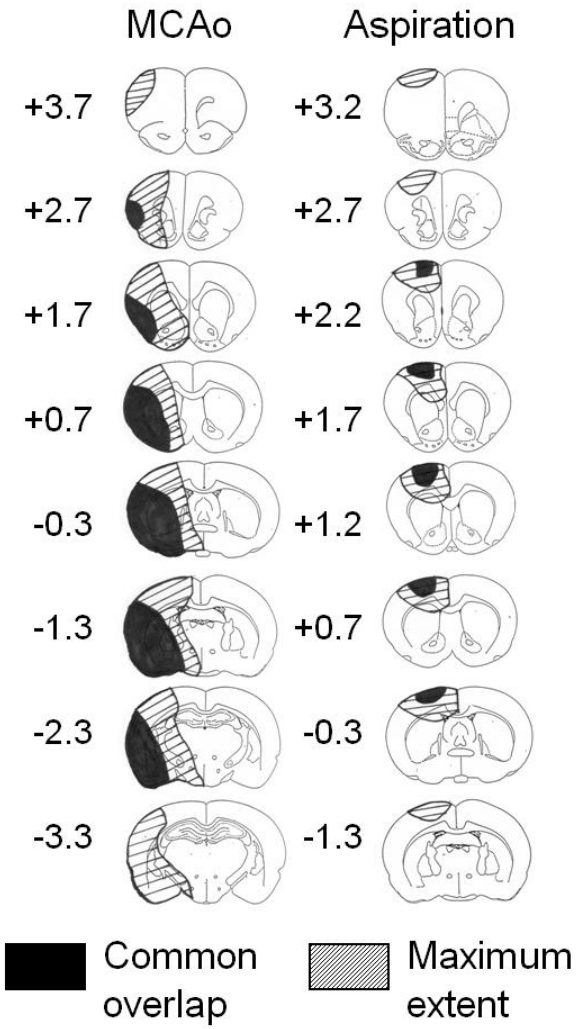


Figure 1.5 Diagram of the extent of the stroke-like lesions produced in Experiment 1

Same-side triggered forelimb placing did not recover in the animals with more extensive damage produced by MCAo.

Lesion type	Area examined	Between AP levels	Control volume†	Lesioned side volume	% tissue remaining
Aspiration	Neocortex	-1.0 to +3.0	98±2	85±2	87%*
MCAo	Whole hemisphere	-4.0 to +4.0	335±20	148±12	44%*
Sham (MCAo procedure)	Whole hemisphere	-4.0 to +4.0	322±23	324±21	101%

Table 1.1 Volumes of remaining tissue following the stroke-like lesions given in Experiment 1

AP levels indicated are in mm relative to bregma as determined by the atlas of Paxinos and Watson (1998). Volumes are expressed as mean mm^3 of tissue ± SEM. Aspiration-lesioned rats sustained damage only to the cortex; MCAo procedures damaged the cortex and large portions of the striatum. †Volume controls were the unlesioned hemispheres of the same rats for MCAo and sham animals, and a separate group of sham-operated controls for the aspiration animals. *Indicates a significant difference in tissue volumes with $p < .05$.

Lesion type	Average "advantage"	Across post-op days
Aspiration	2.3% ± 10.1%	2-20
MCAo	61.9% ± 8.1%	7-93

Table 1.2 The cross-midline "advantage"

These figures represent the average amount (in terms of percent success) by which cross-midline placing was more readily elicited than same-side placing over the post-op time period indicated. Cross-midline placing had a strong advantage for MCAo-lesioned rats, but virtually none for aspiration-lesioned rats. 6-OHDA-lesioned rats, in which neither cross-midline nor same side placing recovers, also show no "advantage." Error is expressed as SEM.

Results

Placing test: general

Pre-operatively in all rats, and post-operatively in sham-operated animals, placing responses were consistently elicited at success rates greater than 90% (usually 100%) in both forelimbs, regardless of the type of trigger (i.e., cross-midline or same side), so those results are not shown here. The ipsilesional (unaffected) limb of all lesioned rats maintained placing ability at similarly high levels, regardless of either lesion type or side of vibrissae stimulation used to elicit the placing. For the sake of brevity, these results also are not discussed further here. It is important to note, however, that the maintained ability of the contralesional whiskers to drive placing in the unimpaired forelimb, regardless of lesion type, indicates that vibrissae sensory dysfunction is not a major contributor to any deficits seen in this study.

Placing test: MCAo

Placing test data for MCAo-lesioned animals are graphed in Figure 1.1. A doubly repeated-measures ANOVA was run using SPSS, with post-op day and placing variant as within-subjects variables. This revealed a significant placing variant X post-op day interaction [$F(8, 40) = 3.84$, $p < .01$]. Therefore repeated measures ANOVAs were run separately for each placing variant. When this was done, a significant effect of post-op day was found for cross-midline placing [$F(8,40) = 11.15$, $p < .001$], but not for same-side placing [$F(8,40) = 1.72$, $p = 0.12$], the latter of which did not recover. Post-hoc Bonferroni-adjusted pairwise comparisons for each post-op day showed a significant ($p < .05$ after correction) difference between scores for the two kinds of placing on post-op testing days 17, 21, 35, and 49. In addition, there was no instance on any testing day in which any single rat scored higher in same-side placing than in cross-midline placing, indicating that the order of recovery (i.e., cross-midline before same-side) was maintained for all rats examined.

Placing test: Aspiration cortical lesion

Placing test data for aspiration-lesioned animals are graphed in Figure 1.2. Doubly repeated-measures ANOVA revealed a significant post-op day X placing variant interaction [$F(5, 45) = 5.41$, $p < .01$]. Examining the effect of post-op day for each type of placing individually revealed a significant effect of time for both kinds of placing [same side: $F(5, 45) = 11.36$, $p < .001$; cross-midline: $F(5, 45) = 4.46$, $p < .01$]. Post-hoc Bonferroni-adjusted pairwise comparisons of the two placing variants at each testing day showed no significant difference in scores between the two kinds of testing on any day.

Placing test: chronic deficits in 6-OHDA- and MCAo-lesioned rats

Chronic placing test data from 6-OHDA- and MCAo-lesioned animals are graphed in Figure 1.3. 6-OHDA-lesioned rats examined approximately 400 days post-operatively never placed the impaired forelimb in either the cross-midline or same-side trigger conditions. T-tests showed that this differed significantly from MCAo-lesioned rats tested 120 days post-op for both the cross-midline condition [93% success; $t(10) = -14.00$, $p < .001$ vs. 6-OHDA] and the same side condition [9% success; $t(10) = -2.80$, $p < .05$ vs. 6-OHDA].

Limb-use asymmetry

Data from the limb-use asymmetry test are graphed in Figure 1.4 for MCAo, MCAo sham, and aspiration-lesioned rats. Note that a score of 0.5 (50%) indicates symmetric limb use, while higher scores indicate greater reliance on the ipsilesional limb. Repeated-measures ANOVAs with post-op day as a within-subjects variable revealed a significant effect of post-op day on limb-use asymmetry in MCAo [$F(6, 30) = 5.00$, $p < .01$] and aspiration-lesioned rats [$F(3, 33) = 3.99$, $p < .05$], but not in sham-operated animals [$F(6, 30) = 0.73$, $p = 0.63$]. Multiple Bonferroni-corrected t-tests analyzed the difference between MCAo, sham, and aspiration-lesioned animals at post-op days 7, 14, and 21, and found that at day 7 all groups

differed significantly from each other; at day 14, aspiration and MCAo animals were significantly worse than shams but not significantly different from each other; and at day 21, the only significant difference was between aspiration-lesioned and sham animals (based on $p < .05$ after Bonferoni correction, using independent-samples t-tests).

Extent and location of lesions

Volumes of remaining tissue for animals in the various groups are shown in Table 1.1, and are depicted graphically in Figure 1.5. For all groups except shams [$t(10) = -0.04$, $p = 0.97$], t--tests indicated significantly less tissue remaining in the damaged hemisphere [MCAo, whole hemisphere: $t(10) = 7.85$, $p < .001$ relative to the unlesioned hemisphere; Aspiration, neocortex: $t(20) = 5.42$, $p < .001$ relative to a group of sham-operated controls]. MCAo lesions typically extended from 4.5 mm anterior to 4.5 mm posterior relative to bregma (as based on the atlas of (Paxinos and Watson, 1998) and destroyed most of the striatum and lateral regions of the neocortex, while leaving midline structures intact. Aspiration lesions were confined to the sensorimotor cortex and included damage through all six cortical layers down to the underlying white matter. In two (of 12) aspiration lesions the damage penetrated the white matter, producing some damage to the dorsal striatum. One of these rats, in which the striatal damage was most severe, also had the worst overall placing performance; the other striatally-damaged rat displayed performance similar to other animals in the aspiration-lesioned group.

Discussion

This study introduces a vibrissae-elicited forelimb placing test that can be used to track functional recovery after brain lesions and assess inter-hemispheric sensorimotor integration. By examining the response of the impaired forelimb to stimulation of both the contralesional and ipsilesional whiskers, distinct patterns of recovery can be revealed.

Importantly, these different patterns may be useful in determining the location of motor system lesions in the rat.

The major finding of this study is that MCAo lesions, which affect both the cortex and striatum, lead to enduring deficits in forelimb placing when triggered by the contralesional whiskers, but not if the placing is triggered by the ipsilesional whiskers (i.e., cross-midline). This cannot simply be attributed to a sensory deficit in the vibrissae, because stimulation of the "bad" vibrissae can easily elicit placing in the unimpaired forelimb. Therefore, the implication is that sensory information entering the intact hemisphere is somehow more able to access remaining motor programs in the lesioned hemisphere than can sensory information entering the lesioned side. Because the "impaired" forelimb can be induced to place when the cross-midline method is used, the placing deficit is not purely motor, either. Rather, the pattern of recovery for MCAo-lesioned rats suggests a deficit in sensorimotor integration that more heavily affects circuits that presumably remain within the damaged hemisphere.

In contrast to the effects of MCAo, rats rendered parkinsonian by infusion of 6-OHDA did not place the impaired forelimb regardless of the side to which sensory stimulation was presented. As in MCAo-lesioned rats, however, vibrissae on the impaired side could still trigger placing in the unimpaired forelimb of 6-OHDA rats. These results are consistent with parkinsonian akinesia and indicate a deficit of motor initiation. This is surprising given that the 6-OHDA lesions destroy only the dopaminergic nigral projections to the striatum, while the MCAo lesions destroy most of the striatum itself. Taken together with the picture for MCAo, this test is (as far as we are aware) the first diagnostic tool that can qualitatively distinguish between severe stroke and severe Parkinsonism in rats without the need for kinematic analysis (but see Metz and Whishaw (2002)).

With a smaller aspiration lesion, recovery on both forms of this test was complete and relatively rapid. However, following this lesion there was no clear difference in recovery between same-side and cross-midline placing as there was for MCAo-lesioned animals. Post-lesion plastic changes, including increases in layer V dendritic arborization in the undamaged, homotopic cortex (Jones and Schallert, 1994; Biernaskie and Corbett, 2001) and sprouting of transcallosal fibers from the intact cortex to the striatum underlying the lesion, normally occur following lesions in which the damaged tissue is left in place but do not occur following aspiration lesions (Napieralski et al., 1996; Voorhies and Jones, 2002). This may suggest a possible mechanism by which cross-midline placing gains its advantage. It is possible that new fibers and/or changes in the unlesioned hemisphere are partly responsible for allowing vibrissae input to the good hemisphere to access and trigger motor programs in the bad hemisphere responsible for forelimb placing. Another possibility, given that cross-midline placing is not a new behavior but one that intact normal rats can perform, is that the recovery of cross-midline placing may reflect the rebalancing of interhemispheric inhibitory influences that were made asymmetric by the lesion (Schallert et al., 1982; Wolgin and Kehoe, 1983; Schallert and Whishaw, 1984; Murase et al., 2004). Such rebalancing might similarly be subsumed by changes occurring in the intact hemisphere or between the hemispheres.

The differences in recovery patterns among lesion types suggest that intrinsic striatal circuits may be crucially involved in same-side vibrissae-elicited placing behavior. Deficits eventually abated when the injury was primarily cortical (aspiration lesions) but were more persistent when striatal tissue was severely damaged (MCAo), or when dopaminergic innervation of the striatum was disturbed (6-OHDA lesions). Another study produced similar findings using two different models of MCAo: longer-lasting and more severe deficits in forelimb and hindlimb placing elicited by stimulation of the same side were observed

following occlusion of the proximal MCA (causing striatal and cortical damage), compared to occlusion of the distal MCA, which caused only cortical damage (Roof et al., 2001).

Schmanke and Barth (1997) have shown that recovery of vibrissae-elicited placing can be influenced both by practice and by administration of amphetamine. Therefore, it may be possible to construe the "advantage" of cross-midline placing (see Table 1.2) over same-side placing as a matter of enhanced learning on the part of the intact hemisphere in lesioned rats. Indeed, the learning of a skilled reaching task is enhanced in the forelimb controlled by the intact hemisphere opposite a cortical lesion, when compared to sham-operated control rats (Bury and Jones, 2002). A number of plastic changes take place in the undamaged homotopic cortex in the days following a cortical insult (Jones and Schallert, 1992b, 1994; Kozlowski and Schallert, 1998; Jones, 1999; Schallert et al., 2003). Though the behavioral data presented here cannot yet be conclusively tied to these anatomical events, the development of tests, such as this one, that permit investigation of sensorimotor integration across the midline may prove useful in evaluating the behavioral correlates of such lesion-induced plastic changes.

In summary, the addition of a cross-midline variant to a battery of forelimb placing tests has revealed distinct stages of recovery and qualitatively different deficits that are dependent on the type of brain lesion sustained. The test can therefore be used to qualitatively distinguish certain kinds of brain lesions in the rat. In addition, our data demonstrate that chronic forelimb placing deficits following MCAo are neither outright sensory nor outright motor in nature, since these components can be examined separately with the test and were found to be intact. Though other sensory or motor asymmetries may exist in these animals (Bland et al., 2000), they do not affect placing of the unimpaired limb or cross-midline placing evoked by either set of vibrissae. Finally, the test should prove

useful for future investigations involving lesion-induced plastic events occurring across the midline and/or in the hemisphere opposite a lesion.

EXPERIMENT 2: A NEW TEST OF POSTURAL INSTABILITY BASED ON EXPERIMENTER-IMPOSED SHIFTS IN A RAT'S CENTER OF GRAVITY

Introduction

In Parkinson's disease (PD), postural instability is very common and contributes to the danger of frequent falls. Unfortunately, the impaired ability to make motor adjustments in response to involuntary changes in center of gravity is quite resistant to levodopa therapy (Bloem et al., 1996; Horak et al., 1996; Jessop et al., 2006). Methods for detecting postural instability are typically included in standard clinical exams. For example, in the "pull" or "push" tests the patient stands in front of the neurologist and is gently pushed or pulled by the shoulders forward or backward, shifting their center of gravity. The capacity to make catch-up steps of appropriate size to maintain center of gravity is assessed. In severe cases the feet seem almost glued to the ground and the patient needs to be caught by the neurologist to prevent falling. A component of the deficit is that the patient responds poorly even when center of gravity is only slowly displaced over a considerable distance.

However, despite the importance of this symptom in PD, there has been no convincing animal model of postural instability. Postural instability may underlie many of the behavioral deficits observed in common animal models of PD. In the hemi-Parkinson rat or mouse, there is a decreased reliance on the impaired forelimb for movements involving a response to weight shift. The animals preferentially initiate movement with the non-impaired forelimb, particularly for lateral movements during vertical exploration of surfaces (Schallert et al., 1997). By lifting the animal's hindlimbs and one forelimb off the

ground, each forelimb can be examined separately for impairments. In response to a rapidly imposed shift of weight on a smooth surface, parkinsonian rats often brace or drag the impaired forelimb instead of making adjusting steps, but step readily with the non-impaired forelimb to maintain center of gravity (Schallert et al., 1979; Schallert et al., 1992; Olsson et al., 1995). In this report we show that on a rough surface, which prevents the bracing reaction, moving the animal forward induces stepping with either the impaired or the unimpaired forelimb to regain center of gravity. However, in the impaired forelimb the distance moved per step is greatly exaggerated, indicating a deficit in response to imposed weight shift, as in PD. In contrast, the distance moved per step by the non-impaired forelimb was unexpectedly shorter than that of control animals, indicating adaptive enhanced reactivity and suggesting that compensatory neural plasticity in the intact hemisphere may have occurred.

Methods

Animals

Male Sprague-Dawley rats (n=14; 8 lesioned animals and 6 shams) obtained from an in-house colony were used for this experiment. They were kept in pairs in polycarbonate cages with sawdust bedding, on a 12:12 light:dark cycle with food and water available *ad libitum*. The animals were aged 3-4 months at the time of surgery.

Surgeries

Eight animals were lesioned with 10 μg of 6-OHDA delivered unilaterally into the medial forebrain bundle, in accordance with the procedures described in Appendix 2. Six sham-operated animals received the same treatment except that no solution was infused via the Hamilton needle, and the needle was retracted immediately following its lowering into the medial forebrain bundle.

Behavioral testing

Behavioral testing was performed by experienced testers blind to the treatment condition during the dark portion of the light cycle. Animals were tested in the postural instability (PIT), limb-use asymmetry, and forelimb placing tests as described in Appendix 2, and also in a test of amphetamine-induced rotation described below. All tests (except for amphetamine-induced rotation) were performed twice before the lesioning surgery as a baseline. Rats were also tested in the PIT test at 1, 2, 3, 4, 7, 10, and 14 days post-lesion; in the forelimb placing and limb-use asymmetry ("cylinder") tests on day 14; and in the rotation test (with sacrifice immediately afterwards) on approximately day 18 post-lesion. Performance in the PIT test under the influence of either haloperidol or apomorphine was assessed on the 8th and 9th post-operative days, with half of each experimental group receiving haloperidol on day 8 and apomorphine on day 9, and the other half vice-versa.

Measurements of striatal c-fos expression

Following all behavioral testing (2-3 weeks post-lesion), animals were treated with 3 mg/kg (i.p.) d-amphetamine sulfate (Sigma) to induce striatal c-fos expression in dopamine-receptive neurons (Graybiel et al., 1990). Two hours later the animals were anesthetized with 1.25 g/kg urethane and the chest cavity was exposed. The animals were transcardially perfused with 200 ml of heparinized (20 U/ml) 0.1 M phosphate buffer, pH 7.4, followed by 400 ml of 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer as a fixative. The brains were removed and postfixed in the same fixative for 48 hours. They were then sliced into serial 100 μm coronal sections using a vibratome. Every third section through the striatum was collected and stained immunocytochemically for c-fos as follows. Endogenous peroxidase was first inactivated by treatment with 0.3% hydrogen peroxide in 10 mM phosphate-buffered saline (PBS) for 30 min. The slices were then rinsed and transferred to a blocking solution consisting of 2% normal goat serum, 0.1% bovine serum albumin, and

0.4% Triton-X 100 in PBS, in which they were agitated for 90 min. They were then incubated in a primary antibody solution consisting of a 1:5,000 dilution of c-fos rabbit polyclonal antibody (catalog no. SC-52, from Santa Cruz Biotechnology, Santa Cruz, CA) diluted in the aforementioned blocking solution for 48 hours at 4ºC. Slices were next rinsed and incubated for 90 min in a secondary antibody solution of 1:200 goat anti-rabbit IgG (Sigma) in PBS containing 2% normal goat serum. Then, after another PBS rinse, slices were incubated for 90 min in a solution of 12 drops reagent A (avidin DH)and 12 drops reagent B (biotinylated horseradish peroxidase H) from a Vectastain Elite ABC kit, model PK-6100 (Vector Labs, Burlingame, CA), dissolved in 60 ml of PBS. Slices were then rinsed and developed with a solution of 0.7% (w/v) nickel ammonium sulfate and 0.05% (w/v) diaminobenzidine dissolved in Tris-buffered saline (TBS) containing 0.0013% freshly-added hydrogen peroxide. The reaction was monitored and stopped by repeated rinsing in TBS when c-fos-positive nuclei were easily visible under a microscope. Slices were then mounted on gelatin-coated slides, dehydrated, and coverslipped.

C-fos activity in the mounted slices was measured by automated counting of the darkly-stained c-fos-positive nuclei using NIH ImageJ software (http://rsb.info.nih.gov/ij/), version 1.33. Magnified digital images of a 1.9 mm-diameter circular region of the central striatum were captured using a microscope with 10X objective and Nikon Coolpix 4500 digital camera. Images were converted to 8-bit and thresholded with ImageJ such that the dark c-fos-positive nuclei were separated from the lighter background. Thresholded nuclei were counted using ImageJ's "Analyze Particles" feature and the total number of nuclei counted within the image frame for each striatum (left and right) in each slice was recorded. This counting was performed on every other c-fos-stained slice (i.e., on a 100 μm slice every 600 μm), beginning with the most anterior slice containing a joined corpus callosum and working posterior until nuclei had been counted from four slices. Results of this count are

expressed as the total number of nuclei counted within a given hemisphere from the four slices.

Behavioral testing with dopaminergic drugs

On the 8th and 9th days post-lesion, rats were tested 15 min following an injection of either the dopamine antagonist haloperidol (Sigma; 3 mg/kg i.p., dissolved in 0.3% w/v tartaric acid) or agonist (R)-apomorphine (MP Biomedicals; 0.5 mg/kg s.c. freshly dissolved in saline and injected immediately), to test the contributions of the dopamine system to the effects seen in the PIT test. In unilaterally 6-OHDA-lesioned animals, doses of apomorphine above 0.5 mg/kg often elicit dyskinesias in the impaired forelimb, thus the relatively low dose of 0.5 mg/kg was used. It should be noted, however, that at this dose apomorphine may have a greater effect on presynaptic dopamine autoreceptors than on postsynaptic receptors, possibly leading to a net *antagonistic* effect in terms of overall dopamine signal throughput.

Amphetamine-induced rotation

The number of turns clockwise and counter-clockwise that a rat made in a large circular bowl were counted for 5 min, beginning 20 min after an i.p. injection of 3 mg/kg d-amphetamine sulfate (Sigma; dissolved in saline). This was the same amphetamine injection that was used for inducing striatal c-fos expression (see above), and animals were subsequently sacrificed two hours post-injection. We used rotation together with results from the limb-use asymmetry test as an index of lesion severity and completely excluded from this study two 6-OHDA-infused animals that displayed neither robust rotation to amphetamine nor a forelimb use asymmetry in the cylinder test. We considered these animals to be either partial depletions or "stereotaxic misses", as often occurs with this

surgical technique due to the small size of the targeted medial forebrain bundle, and they are not part of the n of 8 lesioned rats.

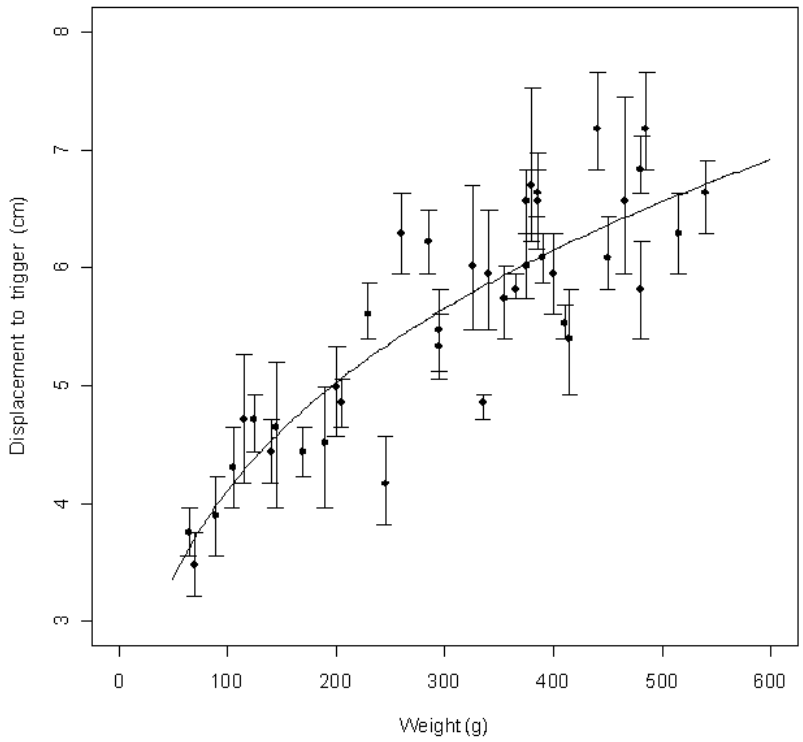


Figure 2.1 Dependence of PIT test results on animal weight

The displacement needed to trigger a catch-up step in a group of 40 intact male Sprague-Dawley rats of various weights is shown. Larger animals require greater displacement. Points are the individual animals' means (averaged across six trials—three on each of the two forelimbs), and error bars represent bootstrapped **95%** confidence intervals for retesting within a given animal. Curve formula is Displacement = $1.092(\text{Weight}^{0.288})$; $R^2 = 0.787$, $p < .001$.

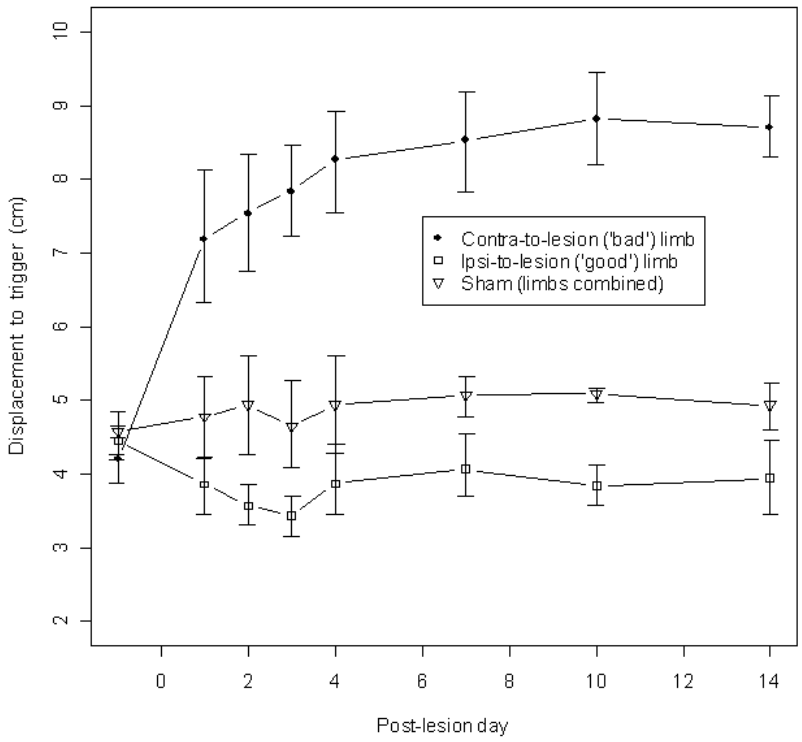


Figure 2.2 PIT test results following unilateral 6-OHDA or sham lesion

The ipsilateral limb of lesioned animals (n=8) becomes more reactive to experimenter-imposed shifts of the animal's center of gravity, while the contralateral limb (n=8) becomes less reactive relative to sham-operated animals (n=12 limbs [6 animals]). Data are means ± bootstrapped **95%** confidence intervals. Bonferroni-corrected t-tests showed that ipsilateral limb performance differed significantly from sham limb performance with $p < .05$ at all post-op time points (but not at the pre-op baseline).

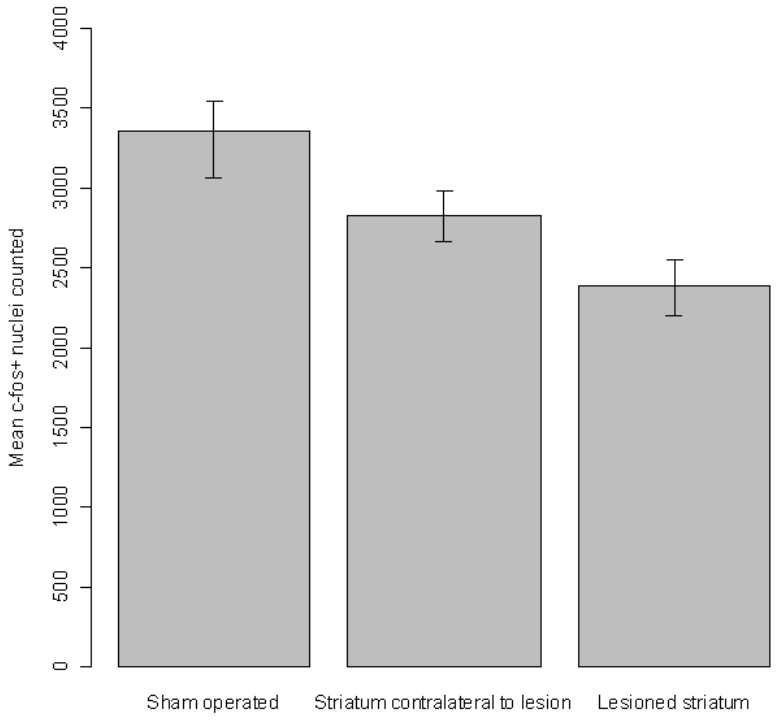


Figure 2.3 Amphetamine-induced striatal c-fos expression

C-fos induction in response to amphetamine challenge was reduced in both hemispheres of unilaterally lesioned animals (n=8) relative to sham controls (n=12 hemispheres). Data are means ± bootstrapped **95%** confidence intervals. T-tests showed all group comparisons to be significantly different with $p < .05$.

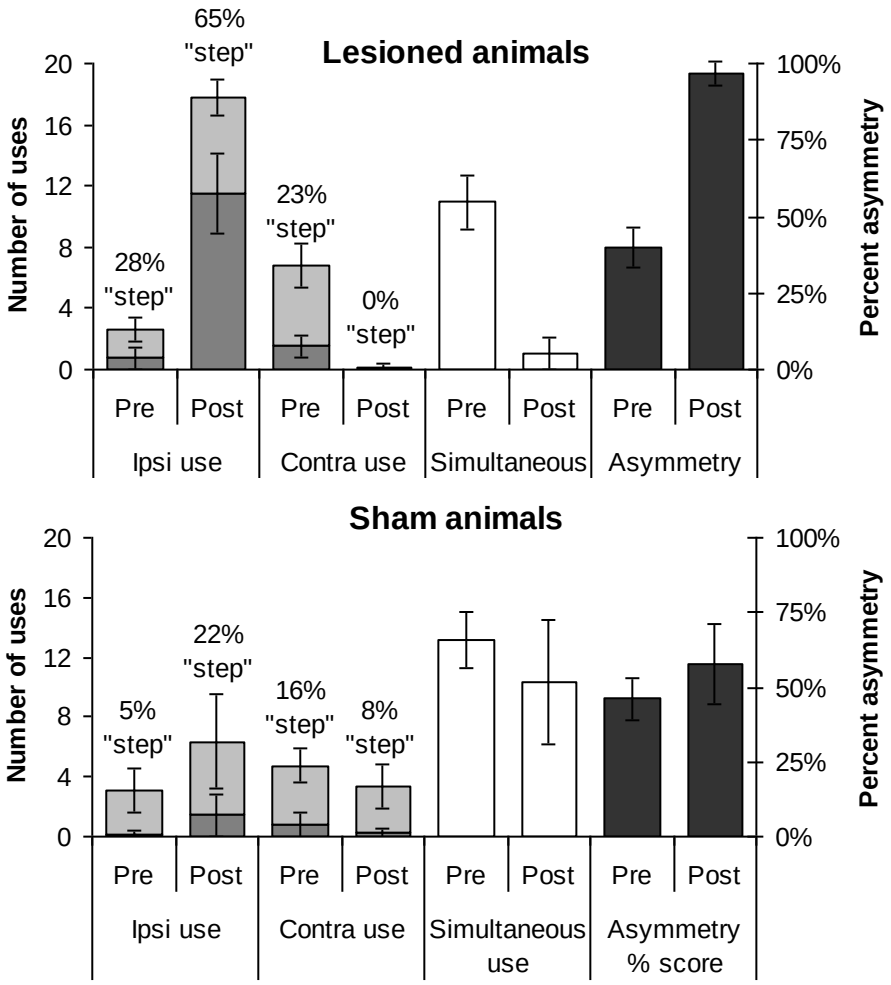


Figure 2.4 Limb-use asymmetry ("cylinder") test

These data show the number of ipsilateral limb, contralateral limb, or simultaneous limb use events recorded in the cylinder test both pre- and post-operatively (use the axis on the left for these), as well as the calculated percent asymmetry scores (use the axis on the right) in 6-OHDA versus sham-operated animals. For independent uses of the contra or ipsi limbs, the data are further broken down to show the percentage of such limb uses that were part of "serial-stepping" behaviors as described in the text (denoted by the portion of the bars that is darker-shaded). Data are means ± bootstrapped **95%** confidence intervals. The difference in proportion of serial-stepping behaviors between pre- and post-surgery tests differed significantly at $p < .01$ for the lesioned animals, but not for sham-operates.

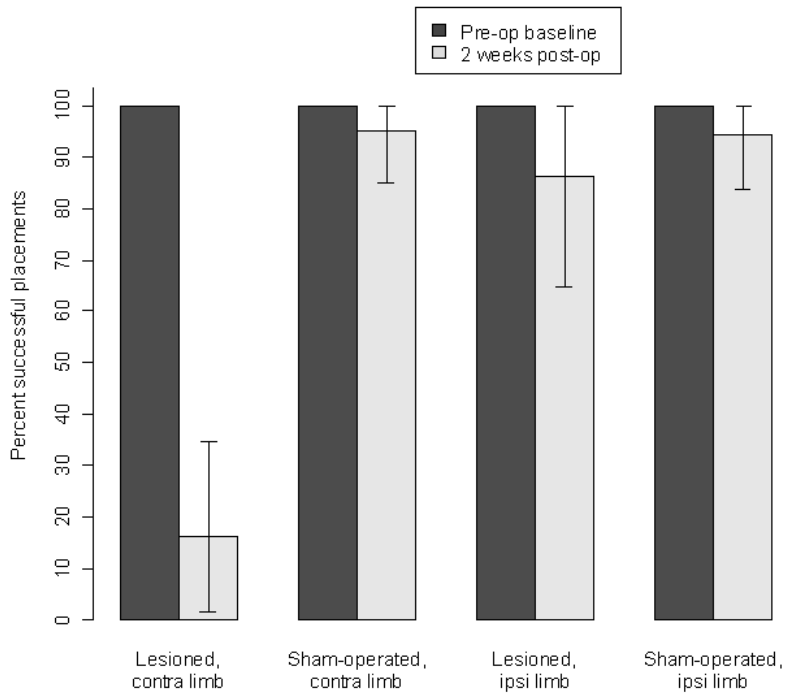


Figure 2.5 Vibrissae-evoked forelimb placing test

Data show the percent successful forelimb placing resulting from ten trials of vibrissae stimulation. The only significant anomaly in placing behavior was seen in the contra-to-lesion limb following 6-OHDA lesion. Data are means ± bootstrapped **95%** confidence intervals.

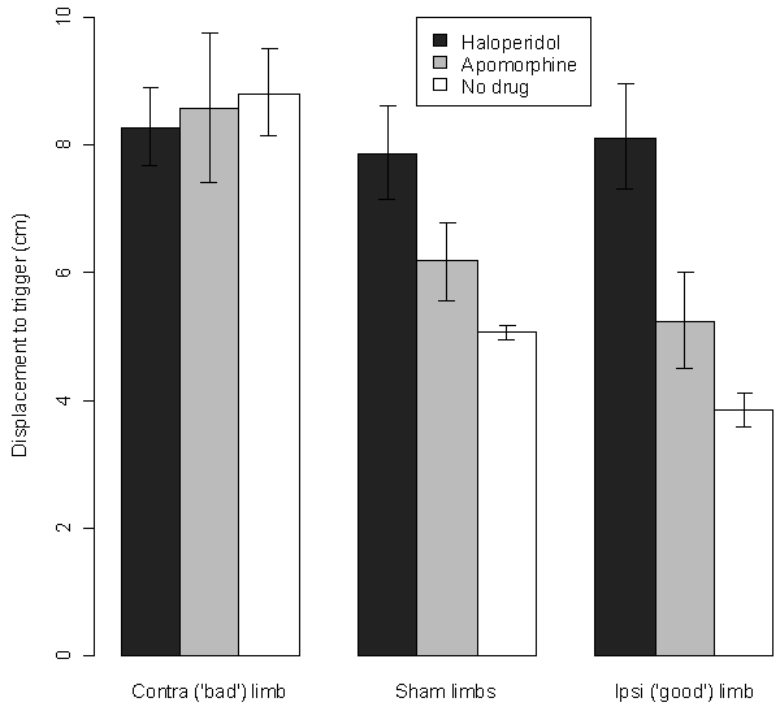


Figure 2.6 PIT testing under dopaminergic drugs

These data show the amount of displacement needed to trigger a catch-up step in the PIT test following systemic administration of either apomorphine or haloperidol. Data are means ± bootstrapped **95%** confidence intervals. Performance under apomorphine differed significantly from the no-drug condition in both sham limbs and ipsilateral limbs, with $p < .01$.

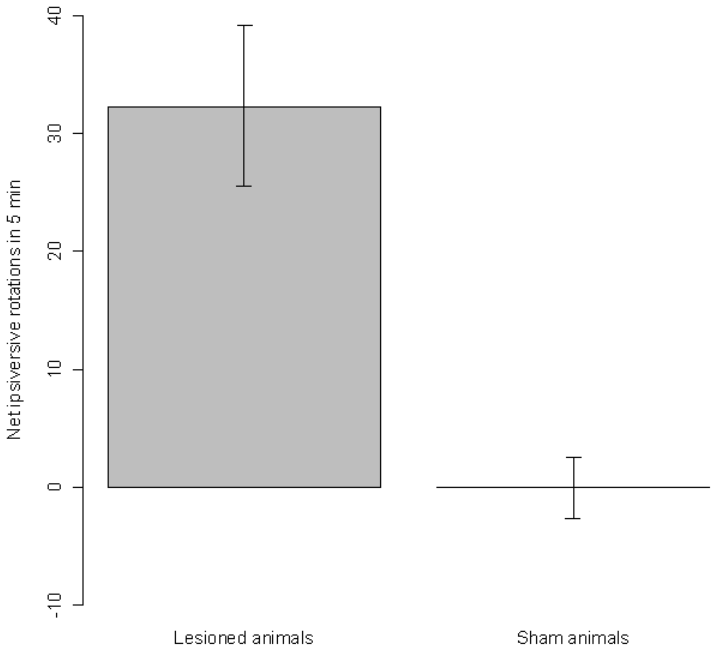


Figure 2.7 Rotational behavior in response to amphetamine injection

These data show the net number of ipsiversive rotations observed over a 5-minute time period, between 20-25 minutes following an injection of 3 mg/kg d-amphetamine sulfate. Data are means ± bootstrapped **95%** confidence intervals.

Results

PIT test

Data from the PIT test are plotted in Figures 2.1 and 2.2. Figure 2.1 shows the dependence of test results on the weight of the animal, as tested in a separate group of unmanipulated male Sprague-Dawley rats (n=40). Larger animals required a greater shift in the center of gravity over the planted forelimb in order to trigger a catch-up step. We also examined the effect as a function of animal age, but the correlation with weight was slightly better so only the latter is presented here (best curve fit: Displacement = 1.092(Weight0.288), $R^2 = 0.787$, $p < .001$). Figure 2.2 shows the results of PIT testing following unilateral 6-OHDA lesion (n=8). As early as day 1 post-lesion, and continuing throughout our observation period, the reaction to body mass displacement was impaired in the contralateral limb relative to that of sham-lesioned controls (n=12 forelimbs from 6 animals). When the center of mass was sufficiently shifted, the contralateral limb always responded by stepping, but the displacement required to elicit this response was consistently greater than the unimpaired forelimbs in every animal in either the control or 6-OHDA groups. Compared to either limb in the sham control group, the ipsilateral limb in 6-OHDA treated rats always showed increased reactivity to the experimenter-imposed weight shift. A repeated-measures ANOVA revealed a significant interaction between the effects of limb condition and post-operative time ($F(14,133) = 20.5$, $p<.001$), as well as main effects for both factors individually (Limb: $F(2,19) = 86.6$, $p<.001$; and Time: $F(7,133) = 20.2$, $p<.001$). Post-hoc Bonferroni-corrected t-tests were performed to compare the performance of the ipsilateral limbs of lesioned animals to the limbs of sham animals at each post-operative time point and indicated statistically significant differences at the $p<.05$ level on all post-operative days, but not at the pre-surgery time point.

Amphetamine-induced striatal c-fos expression

The results of counts of c-fos-positive nuclei in the striatum of rats that had been treated with d-amphetamine prior to euthanasia are presented in Figure 2.3. Amphetamine induced a robust expression of c-fos in sham-lesioned animals. In the dopamine-denervated (ipsilateral) striatum this expression was reduced to 72% of the control (sham-operated) level, while the striatum contralateral to the lesion displayed a reduction to 86% of the sham-operated level. Student's t-tests showed significant differences among all hemisphere conditions: ipsilateral vs. contralateral, $t = 3.32$, $p < .01$; ipsilateral vs. sham, $t = 5.99$, $p < .001$; contralateral vs. sham, $t = 3.41$, $p < .05$.

Limb use asymmetry test and "stepping" behaviors

Results from the limb-use asymmetry test are shown in Figure 2.4 for both the 6-OHDA and sham-operated sets of animals. The limb use behavior of sham-operated animals did not differ greatly between the pre-operative and 2 weeks post-operative testing points shown in the figure. The 6-OHDA animals, by contrast, showed a much greater reliance on independent use of the ipsilateral forelimb following the lesion, at the expense of both contralateral limb use and simultaneous limb use. This effect is summarized by the limb-use asymmetry score, which in lesioned animals rose from 40% at baseline to 97% post-lesion (using paired-samples t-tests to compare pre- vs. post-surgery conditions: 6-OHDA lesions, $t = 22.5$, $p < .001$; sham-operates, $t = 2.59$, $p = 0.049$). In addition, the percent of ipsilateral limb uses that were part of serial lateral stepping behavior increased dramatically after surgery (paired t-tests comparing percent ipsilateral stepping behavior pre- vs. post-surgery: 6-OHDA lesions, $t = 3.85$, $p < .01$; sham-operates, $t = 1.52$, $p = 0.19$).

Forelimb placing

Results from the vibrissae-elicited forelimb placing test are plotted in Figure 2.5. Pre-operatively, both limbs of all rats placed with 100% success. Post-operatively only small decrements of placing ability were seen in the ipsilateral limb of lesioned animals or either limb of sham animals. However, placing ability in the contralateral limb of lesioned animals declined such that only 16% of placing attempts were successful after surgery.

PIT test with dopaminergic drugs

Results from performing the PIT test under the influence of systemically administered apomorphine or haloperidol are charted in Figure 2.6. The administration of the dopamine antagonist haloperidol caused both limbs of animals, regardless of experimental group, to react more slowly to displacement (i.e., haloperidol caused all limbs to perform like the contralateral limbs of undrugged but lesioned animals). Surprisingly, administration of the dopamine agonist apomorphine actually slightly increased the displacement needed to trigger a catch-up step in sham animals or in the ipsilateral limbs of lesioned animals, while not having an effect on the contralateral (impaired) limb of lesioned animals (paired t-tests comparing no-drug vs. apomorphine conditions: sham limbs, $t = 3.30$, $p < .01$; limbs ipsilateral to lesion, $t = 3.92$, $p < .01$). As noted before, this may be because the dose used (0.5 mg/kg) could act preferentially on presynaptic dopamine autoreceptors, thereby reducing the firing rate of the dopaminergic neurons.

Rotation

Rotational behavior of the animals following an injection of 3 mg/kg d-amphetamine sulfate is summarized in Figure 2.7. As expected, lesioned animals displayed robust ipsiversive rotation in response to amphetamine administration, while sham-operated animals showed virtually no consistent rotation. These rotational responses, in conjunction

with performance in the limb-use asymmetry test, were used to verify that lesions were successful in the lesioned group.

Discussion

We observed a severe decrease in the reactive capacity of the forelimb contralateral to dopamine depletion in response to an involuntary shift of center of mass, which was not ameliorated by a dopamine agonist. This may model, at least in part, the postural instability in PD (Jessop et al., 2006), and may be reflective of the kinesthesia impairments that have been noted in the disease (Maschke et al., 2003). Marked changes in the motor behavior of the ipsilateral (unimpaired) forelimb were also found. These changes included an increased reactivity to experimenter-imposed displacements of the animal's center of gravity and an increased propensity to use the ipsilateral limb for lateral weight-shifting steps during rearing behaviors. Re-examination of data in the literature led us to find that the speed with which rats will react to remove a small sticky piece of tape placed on the wrist of their ipsilateral forelimbs is also enhanced when compared to unlesioned animals (Schallert et al., 1982; Schallert et al., 1983). In addition, under some conditions 6-OHDA rats can display a reduced latency to orient towards an ipsilateral perioral stimulus when compared to unlesioned animals (Schallert and Hall, 1988). However, changes ipsilateral to 6-OHDA lesions do not always appear to be "super-normal": it has been shown in testing skilled reaching for food pellets that 6-OHDA-lesioned rats display subtle impairments in the "good" limb which differ qualitatively from the type observed in the contralateral limb (Vergara-Aragon et al., 2003).

Changes in motor behavior ipsilateral to a brain lesion are not confined only to unilateral models of PD. Evidence supports changes in both behavior and the capacity to acquire new skills in the intact limb following unilateral injury to the motor cortex, used as a

model of stroke (Bury and Jones, 2002; Hsu and Jones, 2006). Research in this area indicates that the presence of a motor cortical lesion promotes increased brain plasticity especially in the motor cortex contralateral to the lesion which, when coupled with the increased reliance on the non-impaired forelimb displayed by rats after such an injury, may allow the animal to adapt to the loss of function in its impaired limb (Jones and Schallert, 1992a, 1994). Our behavioral evidence presented here suggests that similar phenomena may occur following lesions to the nigrostriatal pathway. The anatomical and/or neurochemical underpinnings of these changes remain to be assessed, though Miklyaeva et al. (2007) found an increase in dendritic arborization in the motor cortex contralateral to a dopamine depletion which may be linked to this. However, the compensatory behavioral changes were observed in the present study early after neurotoxin exposure, which perhaps is more consistent with rapid neural adaptations or with loss of inter-hemispheric inhibitory influences.

We measured c-fos expression in the striatum in response to challenge with d-amphetamine, a technique used to provide an index of dopaminergic signaling throughput dependent upon pre-synaptic release of dopamine by amphetamine. In so doing we did not find an upregulation of c-fos expression in the striatum contralateral to the lesion, as would be expected if increased dopamine innervation of or signaling within that striatum was the mechanism behind the ipsilateral behavioral changes we observed. In fact, we observed a slight decrease in c-fos signal in the unlesioned hemisphere following 6-OHDA, suggesting that whatever reorganization is responsible for the enhanced responsiveness of the ipsilateral side might be non-dopaminergic. Supporting this is our finding that administration of the dopamine agonist apomorphine did not relieve the contralateral postural stability deficit in the 6-OHDA animals, nor did it enhance the responsiveness of sham-operated animals' forelimbs. Possibly changes in corticostriatal connectivity or in striatal dendritic morphology, or changes in areas outside of the striatum, are responsible for the ipsilateral

motor effects. Extensive postoperative experience using the ipsilateral ("crutch") limb for compensation during weight shifting movements might serve to solidify the adaptive reactive capacity and ensure long-term postural stability. Indeed, if increased reliance on the ipsilateral forelimb following unilateral motor cortical injury can trigger plastic changes in the intact cortex, perhaps similar changes in behavioral demand following nigrostriatal injury could have effects on the motor cortex as well (Hsu and Jones, 2006).

On the other hand, evidence does support some changes in the nigrostriatal dopamine system contralateral to 6-OHDA lesion, which might be linked to the behavioral phenomena we observed in a way that is not revealed by measurement of c-fos expression. The finding of increased levels of extracellular dopamine in striatal dialysates contralateral to 6-OHDA (Robinson and Whishaw, 1988), which until the present study has not had a potential behavioral correlate, coupled with more recent evidence of bilateral changes in dopamine receptor density following unilateral 6-OHDA lesions (Ferre and Fuxe, 1992; Nikolaus et al., 2003; Xu et al., 2005; Waszczak et al., 2006) suggest that the "intact" striatum may have the capacity for increased dopaminergic signaling, possibly via the D2 pathway. Evidence also exists showing an upregulation in post-mortem striatal dopamine content (as measured by HPLC) in striata contralateral to a severe 6-OHDA lesion relative to the striata of sham-operated controls (Warenycia and McKenzie, 1987), though this is not consistently found. Finally, bilateral electrophysiological anomalies have been observed in the striatum, substantia nigra, and subthalamic nucleus of animals following unilateral 6-OHDA infusions (Warenycia and McKenzie, 1987; Breit et al., 2006; White-Cipriano and Waszczak, 2006). In this paper we found that systemic administration of the dopamine antagonist haloperidol did lead to a deficit in the PIT test, indicating that although non-dopaminergic changes might be responsible for enhanced reactivity on the unimpaired side following unilateral 6-OHDA lesion, intact dopamine systems are nevertheless important for normal execution of this task.

The presence of changes in the ipsilateral forelimb following a unilateral lesion indicates the need for careful behavioral analysis in such lesion models to partition out the contributions of possible changes in the unlesioned hemisphere. Though behavioral tests providing an asymmetry score (such as the limb-use asymmetry test) are convenient and allow for within-animal control, they must also be combined with tests which can evaluate function on each side of the body independently in order to distinguish between the contributions of the ipsilateral and contralateral sides to an asymmetry score. Even reaching tests, in which each forelimb is examined separately, do not allow one to evaluate the function of the non-impaired limb relative to that of control animals. During reaching behavior the opposite limb is required for adequate postural support; if it is impaired, this can influence reaching success and qualitative measures of reaching dynamics (Vergara-Aragon et al., 2003).

Of course one of the most straightforward ways to deal with the dilemma presented by potential changes in the contralateral hemisphere is to always include sham-lesioned or unlesioned control animals in studies that involve unilateral lesions. Indeed, more frequent use of such controls is likely to lead to discoveries about the ways in which the unlesioned hemisphere can plastically adapt to brain injury, and about mechanisms of brain plasticity in general.

Chapter Two: The demonstration of a non-dopaminergic component contributing to postural instability in dopamine-depleted rats

CHAPTER OVERVIEW

Having established the usefulness of the forelimb placing and PIT tests in assessing motor function following various types of brain lesions, we began experimenting with the effects that various pharmacological manipulations had on performance in these tests. Our preliminary studies indicated that pharmacological disruption of the dopamine system (using postsynaptic receptor antagonists or agents which interfere with presynaptic synthesis or release of dopamine) did not lead to profound disturbances of postural stability as measured with the forelimb-placing test, though animals sustaining 6-hydroxydopamine (6-OHDA) induced chronic dopamine depletion show severe and permanent PI symptoms as reflected in this test (Woodlee et al. (2005a); Experiment 3). This suggests that acute disruption of the dopamine system alone may not sufficient to account for all facets of postural instability in PD, and that chronic dopamine denervation may lead to additional brain changes which account for the emergence of PI symptoms.

The experiments in this chapter generally rule out several hypotheses that might explain the discrepancy in postural support behaviors observed between 6-OHDA-lesioned rats and rats subject to pharmacological dopamine blockade, including:

- The need for depolarization block (i.e. the cessation of presynaptic DA neuron firing; Experiment 4)
- The need for collective blockade of both D1-type and D2-type postsynaptic dopamine receptors (Experiment 4)
- Possible interference due to interhemispheric competition effects following unilateral 6-OHDA lesions (Experiment 5)

- The potential contribution of norepinephrine depletion, which does occur in PD and can be caused by 6-OHDA lesions (Fulceri et al., 2006; Fornai et al., 2007; Rommelfanger and Weinshenker, 2007); Experiment 6); and
- Differential behavioral effects depending on the location of experimental dopamine lesion (Experiment 7).

Though the hypothesis that norepinephrine might be involved in the pathogenesis of postural instability does gain some limited support in Experiment 6, we generally could not find evidence to support any of the above hypotheses. In eliminating these possibilities we conclude that a strong possibility is that postural instability in 6-OHDA-lesioned animals may result from a combination of dopamine depletion plus some non-specific degenerative or plastic process that occurs in response to loss of striatal dopamine innervation.

EXPERIMENT 3: THE CONTRAST BETWEEN PRESYNAPTIC DOPAMINE DENERVATION VERSUS POSTSYNAPTIC DOPAMINE RECEPTOR BLOCKADE IN PERFORMANCE ON TESTS OF POSTURAL INSTABILITY

Introduction

A number of techniques have been used to experimentally mimic the dopaminergic dysfunction that occurs in PD. One widely-used and reliable model employs stereotaxic infusion of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) to produce a selective loss of catecholaminergic neurons (Ungerstedt, 1968, 1971). A typical method infuses the toxin into either the substantia nigra *pars compacta* (where the dopaminergic cell bodies reside) or into the medial forebrain bundle (which carries the axons of these neurons towards the forebrain). Once infused, the toxin enters the neuron via catecholamine reuptake transporters (Ahlskog and Hoebel, 1973) and causes neuronal death via inhibition of mitochondrial respiratory chain complexes and the creation of large quantities of free

radicals (Glinka et al., 1997). When appropriate methods are used, 6-OHDA may be delivered in such a way as to produce a dopamine-specific lesion in the nigrostriatal pathway, mimicking the most overtly recognized pathology present in PD.

Prior to the discovery of 6-OHDA's usefulness, drugs which acted as antagonists of dopamine receptors were often used in PD models as it was noted that in sufficient doses they could produce the catalepsy, akinesia, and rigidity typical of advanced, untreated PD, furthering the argument that the primary symptoms of PD result from the loss of dopamine innervation. The present experiment produces evidence that performance on a test of postural instability is affected differently by administration of a dopamine antagonist (haloperidol) than by 6-OHDA lesions. Based on the presumption that the effects of haloperidol are acute and specific to dopamine, we conclude that the unique ability of 6-OHDA lesions to produce postural instability as measured in our test of forelimb placing may be due either to the chronically disruptive nature of 6-OHDA lesions, or to effects that the lesion has outside of the nigrostriatal dopamine system.

In addition, work in this Experiment seeks to more fully characterize the relationship between 6-OHDA-induced dopamine depletion and forelimb placing ability. To this end we also investigate the time course over which this symptom develops following dopamine depletion, and the relationship between symptom severity and the extent of dopamine loss.

Methods

Animals

Twenty-eight adult male Long-Evans rats from Charles River laboratories were used in the studies. They were housed in pairs in clear acrylic cages, on a 12:12 light:dark cycle with food and water available *ad libitum*. All testing was performed by an experienced tester blind to the rats' treatment conditions, during the dark portion of the rats' light cycle. All

animals were handled extensively and acclimated to the behavioral tests that would be used prior to any manipulations.

Surgeries

Eight animals (n=8) were lesioned unilaterally with 8 μg of 6-OHDA delivered into the medial forebrain bundle as described in Appendix 2. An additional 6 animals (n=6) were given sham lesions in which the needle was lowered into the MFB, but no solution was infused. All animals were baselined on the behavioral tests prior to surgery.

In addition, a separate group of 59 male adult Long-Evans rats was lesioned with 7 μg of 6-OHDA delivered into the MFB in conjunction with another experiment involving the effects of exercise (data not reported in this dissertation). Data from these animals, including placing performance and determination of the extent of dopamine depletion via HPLC (see Appendix 2) was used to create Figure 3.3 described below.

Drugs

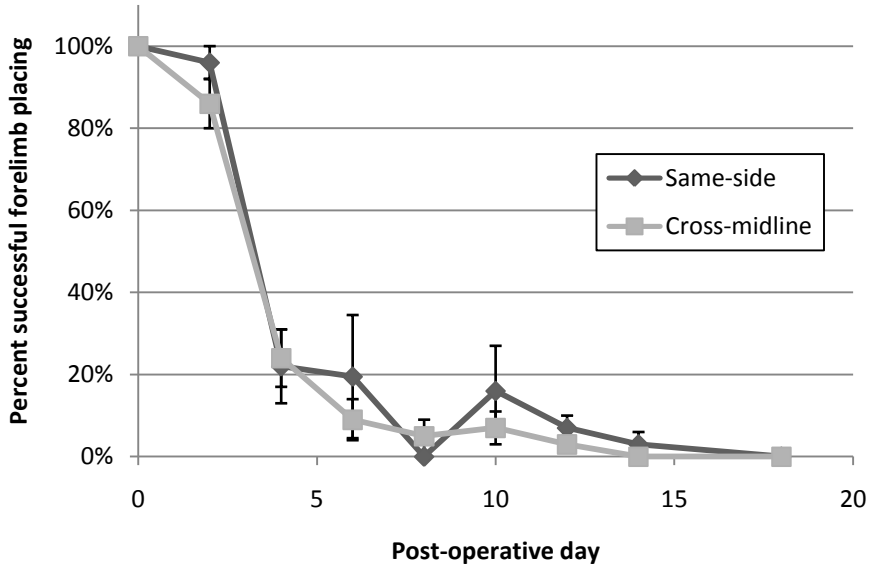
Eight intact animals that had previously been acclimated to the behavioral tests received an injection of 3 mg/kg haloperidol i.p., dissolved at 3 mg/ml in a solution of 0.3% tartaric acid. An additional group of 6 animals received vehicle injections (1 ml/kg of 0.3% tartaric acid). All animals were baseline tested within the week prior to receiving the injection, and again 15 min after the injection.

Rats that received 6-OHDA lesions as described above subsequently received injections of the dopamine agonists L-DOPA and apomorphine several months after the lesion, to evaluate the effects of these drugs on forelimb placing. The drugs were given on separate occasions at least one week apart. Pre-drug testing was performed within 2 d prior to the drug injections, and post-drug testing was carried out 30 min (for L-DOPA) or 15 min (for apomorphine) after the drug injection. Apomorphine (MP Biomedicals) was dissolved

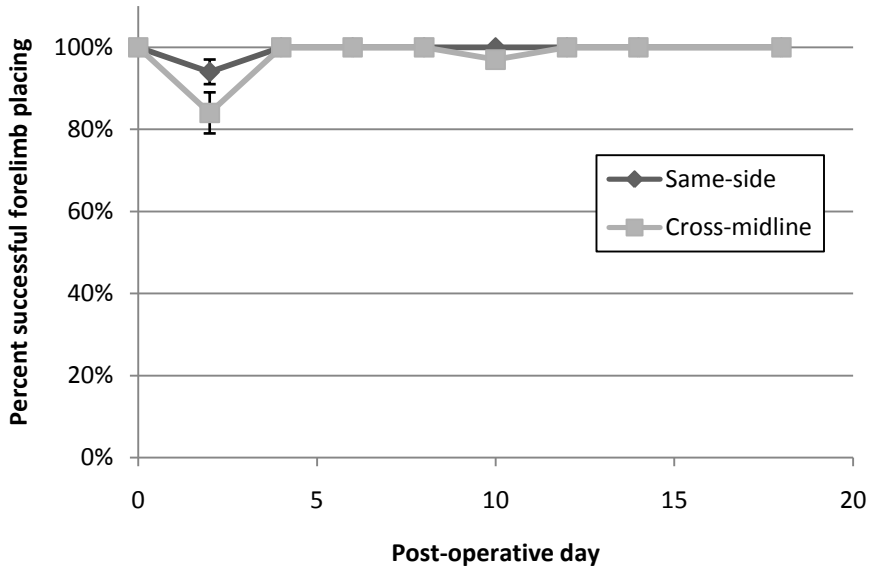
at 0.5 mg/ml in cold saline containing 0.05% w/v ascorbic acid and injected s.c. immediately at 1 ml/kg, for a dose of 0.5 mg/kg. For L-DOPA treatment, rats were first treated with benserazide (a peripheral AADC inhibitor), dissolved in saline and given at 15 mg/ml/kg i.p. Fifteen min after the benserazide injection, L-DOPA was administered at a dose of 30 mg/2 ml/kg i.p. L-DOPA was prepared by dissolving it at 150 mg/ml in 0.1N HCl then diluting it 9:1 with distilled water.

Behavioral testing

Animals in the surgery groups were tested on the forelimb placing test at baseline (pre-surgery), and on days 2, 4, 6, 8, 10, 12, 14, and 18 post-surgery. (For data on the time course of PIT test performance following 6-OHDA lesion, see Experiment 2, Figure 2.2; see Figure 2.6 for performance under haloperidol versus no drug in lesioned and unlesioned animals.)



(a)



(b)

Figure 3.1 Forelimb placing deficits in the contralateral limb following unilateral 6-OHDA lesion **(a)** or sham lesion **(b)**

The ipsilateral limb remains unaffected (i.e., performs at 100% success) in all circumstances. The deficit does appear to "develop" over the first few days post-lesion. Data are means ± SEM.

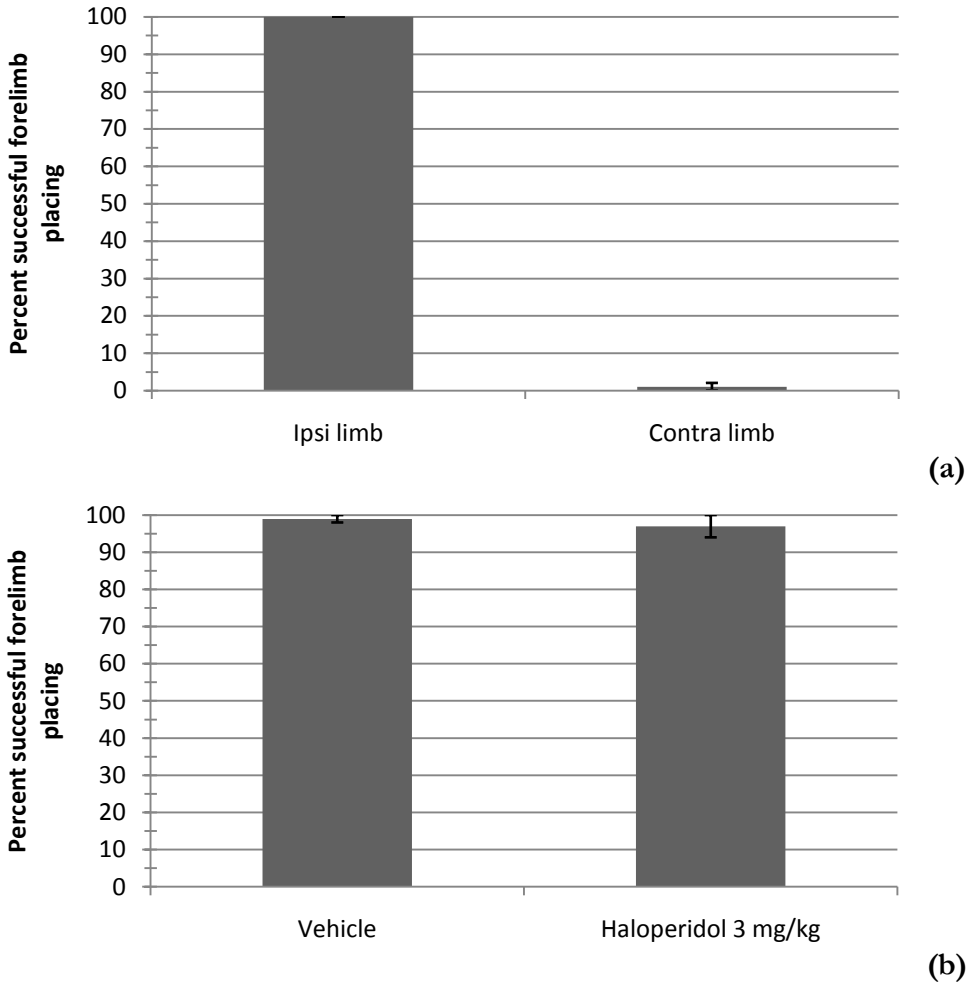


Figure 3.2 Forelimb placing test: effects of unilateral 6-OHDA lesion versus systemic administration of haloperidol

Data from the forelimb placing test for **(a)** animals lesioned unilaterally with 6-OHDA and then tested approximately one year post-lesion for the performance of each forelimb independently, and **(b)** normal (i.e., unlesioned) animals given a systemic injection of either 3 mg/kg of the dopamine antagonist haloperidol, or drug vehicle (performance of the two forelimbs is averaged together in this case). Note the marked contrast between the forelimb contralateral to a presynaptic 6-OHDA-induced dopamine depletion and the forelimbs of animals treated with haloperidol. This is despite the severe catalepsy and akinesia otherwise displayed by haloperidol-treated rats. Data are means ± SEM.

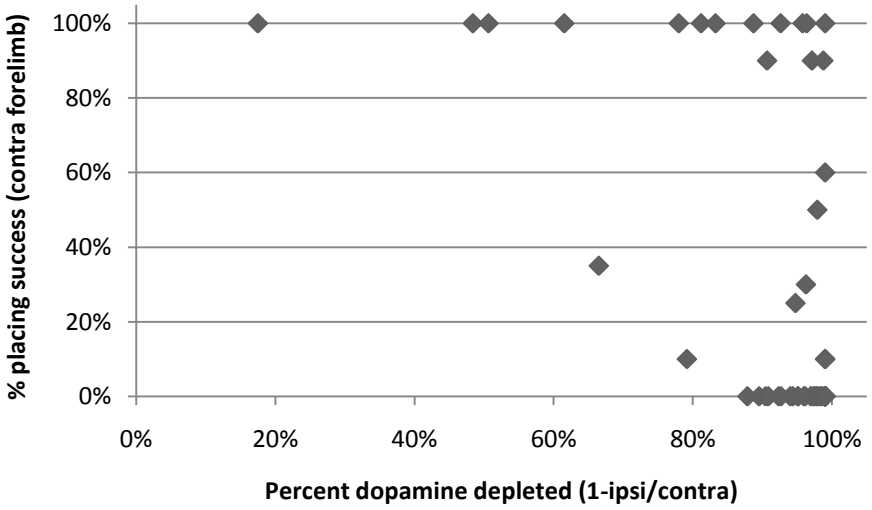


Figure 3.3 Relationship between dopamine depletion and contralateral forelimb placing ability

Forelimb placing ability is lost completely only in subjects in which dopamine has been depleted >87% relative to the unlesioned hemisphere. But even animals with such severe depletions sometimes still show placing ability in the forelimb contralateral to the lesion. Data are taken from a separate group of 59 animals not otherwise referred to in the dissertation.

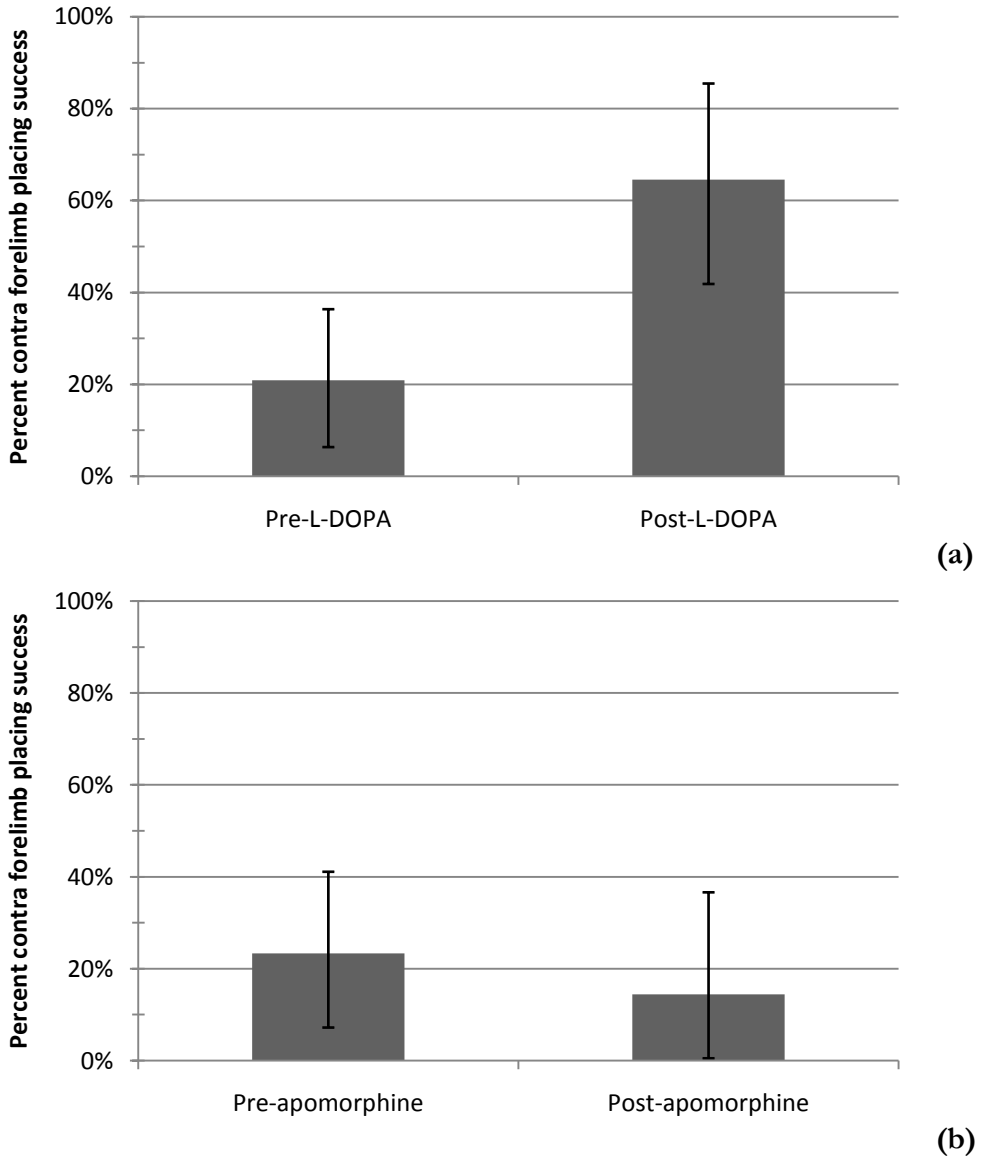


Figure 3.4 Effects of L-DOPA or apomorphine on forelimb placing ability in 6-OHDA-lesioned rats

The effects of administering the indirect dopamine agonist L-DOPA **(a)** or the direct dopamine receptor agonist apomorphine **(b)** to animals with an established forelimb placing deficit following unilateral 6-OHDA lesion. L-DOPA ameliorates the deficit ($p = .002$ by t-test), while apomorphine does not ($p = 0.18$). Data are means ± bootstrapped **95%** confidence intervals.

Results

Time course of placing deficit development

The time course of placing deficit development for animals lesioned with 6-OHDA is charted in Figure 3.1. As seen in Figure 3.1a, there appears to be no appreciable difference in same-side-triggered versus cross-midline-triggered placing in rats lesioned with 6-OHDA (cf. the stroke-like lesions from Experiment 1). The deficit takes up to two weeks to fully stabilize but is ultimately profound. Sham lesions (lowering the needle without infusing toxin; Figure 3.1b) produce only a transient placing deficit when measured two days post-op. In all cases, the ipsilateral limb retains normal placing ability.

6-OHDA lesion versus dopamine antagonist treatment effects

As seen in Figure 3.2a, even when measured more than 1 year post-lesion, rats have not recovered placing ability in the forelimb contralateral to the lesion, while maintaining normal placing on the ipsi side. In contrast, rats treated with high doses of the dopamine antagonist haloperidol show essentially normal placing compared to vehicle-injected rats (Figure 3.2b), despite being profoundly akinetic and cataleptic otherwise.

Relationship between dopamine depletion and forelimb placing

Figure 3.3 plots placing performance measured in the contralateral limb of 6-OHDA lesioned animals versus the level of post-mortem striatal dopamine depletion measured via HPLC. In this large dataset we see that absolute loss of placing doesn't occur until dopamine depletions reach >87%; however, even some animals with stronger depletions than this can maintain normal placing ability. The unreliability of this relationship at the highly-depleted end of the scale raises the possibility that some non-dopaminergic measure may be more directly related to forelimb placing ability.

Effects of dopamine agonist drugs on forelimb placing

Figure 3.4 shows the effects of administering either the presynaptically acting indirect dopamine agonist, L-DOPA, or the postsynaptic dopamine receptor agonist, apomorphine, in reversing placing deficits in 6-OHDA lesioned rats. L-DOPA is able to partially reverse the deficit (paired bootstrapped t-test: $t[10]=4.07$, $p=.002$), while apomorphine is not ($t[8]=1.02$, $p=0.18$).

Discussion

In this experiment we have shown that forelimb placing ability in rats, which we believe mimics some aspects of the postural instability seen in humans with PD, is affected differently by presynaptic lesion of dopamine neurons versus (primarily) postsynaptic blockade of dopamine receptors by the dopamine antagonist haloperidol. Evidence obtained from treating lesioned animals with two types of dopamine agonists suggests further that this behavioral deficit is not mediated solely by dopamine.

Following 6-OHDA infusion, deficits in forelimb placing take up to two weeks to become maximal, and remain confined to the forelimb contralateral to the lesion. In contrast to data obtained from animals with stroke-like lesions (Experiment 1), forelimb placing ability does not differ based on whether the vibrissae stimulated to trigger the placing are contralateral or ipsilateral to the limb being evaluated. Results gathered from rats in which striatal dopamine levels were evaluated via HPLC show that a substantial depletion of dopamine (>87% relative to the unlesioned hemisphere) is necessary to totally abolish the placing response; yet some rats with even more severe lesions maintain placing ability. This disconnect may indicate that though severe dopamine depletion in the dorsal striatum is necessary for the deficit to appear, another unknown condition may also need to be met before placing behavior is disrupted. (One possibility is that dopamine depletion in more ventral areas of the striatum, where we did not assess the extent of depletion, or regional

heterogeneity in the dorsal depletion which was not noticed due to the single measurement of a large area of dorsal striatum, may be more connected with the behavioral outcomes.)

In contrast to 6-OHDA-lesioned animals, rats treated with high doses of haloperidol showed no difficulty in performing placing behavior, despite the fact that most rats so treated were severely akinetic and cataleptic (e.g., they could be placed in an awkward pose on an open tabletop and would not move from that pose for a minute or more). Others have also found that treatment with haloperidol leaves various forms of postural-support behavior intact despite its otherwise profound effects (Morrissey et al., 1989; Cordover et al., 1993). This discrepancy suggests that disruption of the dopamine system alone, on which haloperidol is presumed to be acting specifically, is not sufficient as an explanation for why 6-OHDA produces deficits in the placing test.

When we attempted to "treat" placing deficits in lesioned rats with dopamine agonists, another surprise appeared: though the direct receptor agonist apomorphine (which, like L-DOPA, is often used to combat motor symptoms in the human disease) could not reverse the placing deficit, administration of the mainstay therapy L-DOPA did provide relief. The reason for this discrepancy is not clear, though at least two possible explanations exist. One is simply that the dose of apomorphine used was not optimal for providing relief. Comparing the dose used (0.5 mg/kg) to the literature indicates that, if anything, this dose was somewhat higher than those frequently used in 6-OHDA rat models; however, when depleted animals are treated with large doses of dopamine agonists, dyskinetic movements in the contralateral side often result (Cenci, 2007), and these were not observed to any appreciable extent in the apomorphine-treated animals.

Another possibility is that L-DOPA, as a precursor for dopamine, may also be having an effect on norepinephrine systems in other brain areas (since NE is synthesized

from DA), and this effect may be more directly tied to L-DOPA's ability to restore placing. This possibility is explored further in Experiment 6.

In the meantime, the possibility remains that haloperidol-treated animals do not display forelimb placing deficits because of the acute nature of the treatment used in this study, or because haloperidol does not effectively block all dopamine receptors (i.e., because it is somewhat selective for the D2-subtype of dopamine receptor)—we will further examine these possibilities in Experiment 4. In addition, via its action on presynaptic dopamine autoreceptors, haloperidol can increase presynaptic dopamine release (an effect which would not occur after 6-OHDA lesion), and through an unknown mechanism this may exert some positive effect on placing performance despite the concurrent postsynaptic blockade of dopamine receptors by the drug.

EXPERIMENT 4: THE ROLE OF D1- VERSUS D2-TYPE DOPAMINE RECEPTORS AND DEPOLARIZATION BLOCK IN PRODUCING DRUG-INDUCED POSTURAL INSTABILITY

Introduction

Based largely on the observation that the therapeutic effects of dopamine antagonists may take 2-3 weeks to "kick in" following the onset of their administration in humans (e.g., as a treatment for schizophrenia—note, however, that this phenomenon seems to be specific to the therapeutic use of dopamine *antagonists*, as opposed to dopamine agonists whose action is very rapid in most conditions for which they are indicated), some have theorized that chronic administration of dopamine antagonists leads to an eventual state of depolarization block in which the presynaptic dopamine neurons cease to release dopamine, though acute administration of such antagonists leads to an initial increase in the presynaptic firing rate (Grace and Bunney, 1986; Grace et al., 1997). It is possible that cessation of

presynaptic dopamine activity, analogous to what occurs following a 6-OHDA lesion, is necessary to produce a placing deficit. We tested this by administering dopamine antagonists daily for three weeks, a time period cited as being sufficient to produce the depolarization block state (Grace and Bunney, 1986; Lane and Blaha, 1987). If the onset of depolarization block is sufficient to produce deficits in postural stability, such deficits would be expected to appear during the third week of drug administration.

A second possibility for explaining the lack of effect that acutely-administered haloperidol has on forelimb placing is that though haloperidol antagonizes all known types of dopamine receptors to some extent, the drug is relatively selective for dopamine receptors of the D2 subfamily over those of the D1 subfamily (Kebabian et al., 1997). Receptors of both subtypes are expressed in the striatum, but distinct subpopulations of striatal projection neurons express more of one type of receptor than the other (though virtually all striatal neurons do express some amount of both receptors; Aizman et al., 2000; Mink, 1999), thus administering haloperidol may have a greater effect on only a certain subset of striatal neurons. However, since 6-OHDA exerts its effects presynaptically (by destroying the DA neuron itself), it would reduce signaling through both D1- and D2-driven systems. To test the hypothesis that concurrent antagonism of both D1- and D2-type receptors (or more selective antagonism of D1-type receptors alone) might be necessary to produce a placing deficit, we also used various combinations of different dopamine antagonists in a chronic-administration study. We gave normal rats the D1-selective antagonist SCH-23390 at doses (0.3 mg/kg i.p.) sufficient to block >95% of postsynaptic D1 receptors in the rat (Neisewander et al., 1998). We did this either alone or in combination with either high-dose haloperidol or another more selective D2-subtype antagonist, eticlopride, to also block D2-type receptors. As noted above, this regimen continued daily for three weeks, to exclude the possibility that the inception of depolarization block is necessary to produce a placing deficit.

Finally, another method of "quieting" dopamine neurons (somewhat analogous to using chronic antagonist administration to produce depolarization block) is to use very low doses of dopamine agonists, such that the agonist binds preferentially to presynaptic autoreceptors and calms firing via autoreceptor-mediated feedback mechanisms (Skirboll et al., 1979; Andersen and Gazzara, 1993). We attempted to create this condition by administering low doses of the dopamine agonist apomorphine, and tested animals on the PIT and placing tests after administration.

Methods

Groups for dopamine antagonist experiments

Male Sprague-Dawley rats aged 3-4 months were used (n=33 total; 9 for the first run using haloperidol, 24 for the second using eticlopride). They were obtained from a local colony and housed in pairs in clear acrylic cages, on a 12:12 light:dark cycle with food and water available *ad libitum*. All testing was performed by an experienced tester blind to the rats' treatment conditions, during the dark portion of the rats' light cycle.

In the first (pilot) run, 9 rats were divided into 3 groups of 3 and assigned to receive daily injections of haloperidol, SCH-23390, or a combination of the two drugs, administered as described below, for three weeks. In the second study, 24 rats were divided into 4 groups of 6 and randomized to receive 3 weeks of daily injections of either SCH-23390, eticlopride, a combination of the two antagonists, or saline, again as described below.

Drug administration

Haloperidol (Sigma) was dissolved at 3 mg/ml in 0.3% tartaric acid and injected i.p. at 1 ml/kg for a dose of 3 mg/kg. SCH-23390 HCl was dissolved at 0.5 mg/ml (free base weight) in saline and injected i.p. at 1 ml/kg for a dose of 0.5 mg/kg. Eticlopride was dissolved at 0.8 mg/ml (free base weight) in saline and injected i.p. at 1 ml/kg for a dose of

0.8 mg/kg. For groups receiving two drugs in combination, the drugs were prepared so that they were each given at the same dose, but as one single injection with the same total injection volume (i.e. 1 ml/kg).

Haloperidol acts as a moderately selective antagonist of D2-type dopamine receptors (Schotte et al., 1993; Kebabian et al., 1997), while SCH-23390 is specific for the D1-subtype. The doses used in this study have been reported to be sufficient to block >95% of their respective receptor subtypes in rat striatum (Neisewander et al., 1998; Crocker and Hemsley, 2001; Kapur et al., 2003). Eticlopride is a D2-type dopamine receptor antagonist that is more selective than haloperidol, which is why it was chosen for the follow-up study.

Behavioral testing

In the pilot study, the drugs (haloperidol, SCH-23390, or a combination) were administered once daily for 21 days, and performance on the PIT and forelimb placing tests (described in Appendix 2) was evaluated both just before and 15 minutes after drug administration on every weekday. Values from the five weekdays of each week were averaged to get a weekly mean score on each of the tests.

In the follow-up study, rats received the injections (eticlopride, SCH-23390, a combination of the two, or saline) daily for three weeks, and were tested both pre- and 15 minutes post-injection on the seventh day of each of the three weeks on both the forelimb placing test and a test of catalepsy, described below.

Catalepsy test

For the catalepsy test, rats were removed from their home cages and their forelimbs were placed onto a stable bar (about the diameter of a standard pencil) which was elevated 8 cm above the testing surface. As soon as the experimenter released the rat from this imposed stance, a timer was started, and the time taken for the rat to return both forelimbs

back to the tabletop was recorded. Catalepsy, in addition to being a symptom of Parkinson's disease, is a known effect of extrapyramidally active dopamine antagonists, and this test was used to confirm the efficacy of the kinds and doses of drugs used here, and as a contrast to the anticipated lack of impairment in the forelimb placing) test.

Low-dose apomorphine study

For this substudy, a separate group of eight adult male Sprague-Dawley rats were handled and acclimated to the PIT and forelimb placing tests for 3 days. On day 4, rats were tested on both tests prior to any manipulations, and they then received s.c. injections of 30 μg/kg apomorphine (MP Biomedicals; dissolved in saline at 30 μg/ml and injected immediately). They were then reevaluated on both tests at 10 min and 40 min after the injection. The dose of 30 μg/kg used here is autoreceptor-preferring and not thought to induce significant activation of post-synaptic dopamine receptors (Skirboll et al., 1979; Jeziorski and White, 1989; Andersen and Gazzara, 1993)

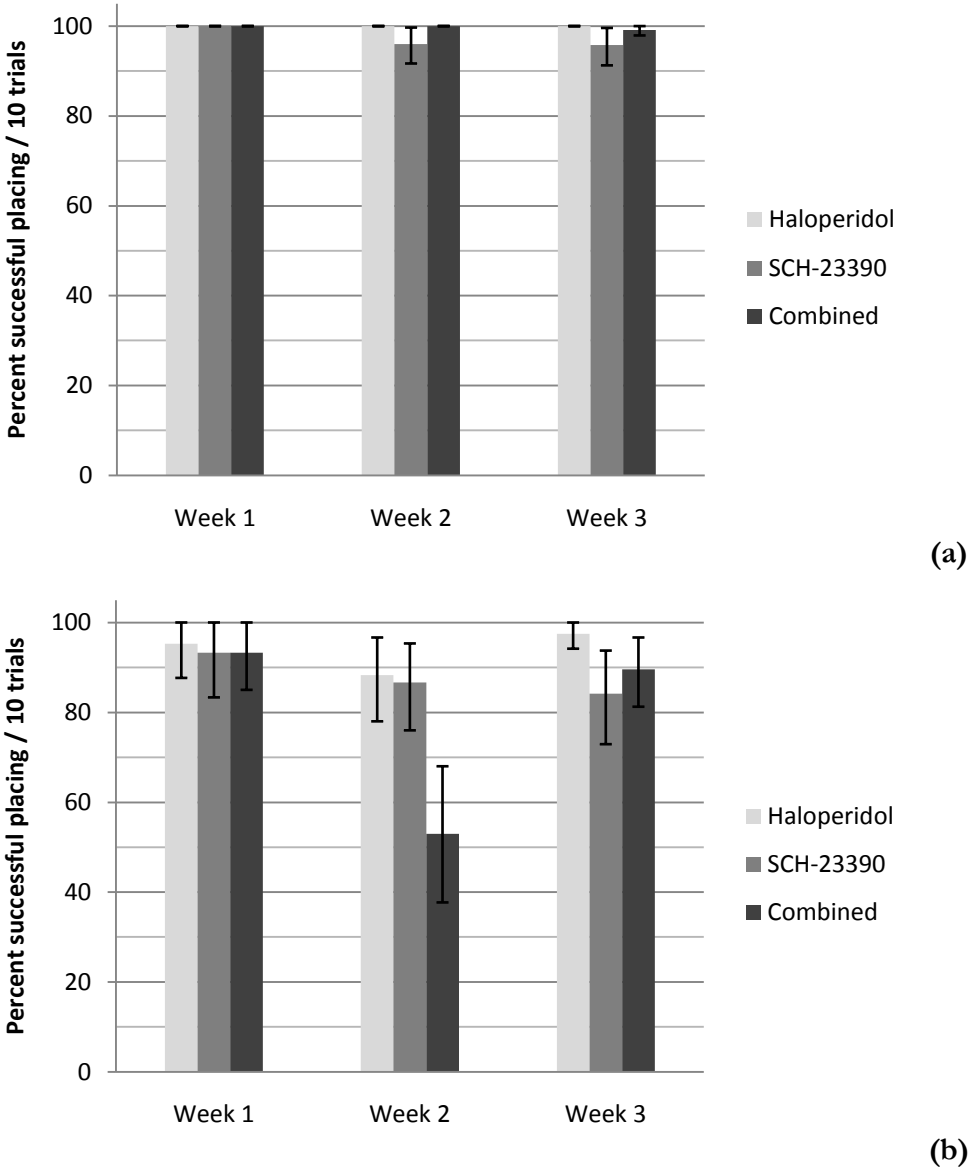


Figure 4.1 The effect of daily administration of dopamine antagonists on success in the forelimb placing task

Panel **(a)** shows mean performance just prior to daily drug administration while panel **(b)** shows performance measured 15 min after drug injection. In general, neither haloperidol nor SCH-23390 alone greatly affected forelimb placing. A combination of the two drugs does adversely impact scores during the second week of daily administration, but this largely recovers by the third week. Data are means ± bootstrapped **95%** confidence intervals.

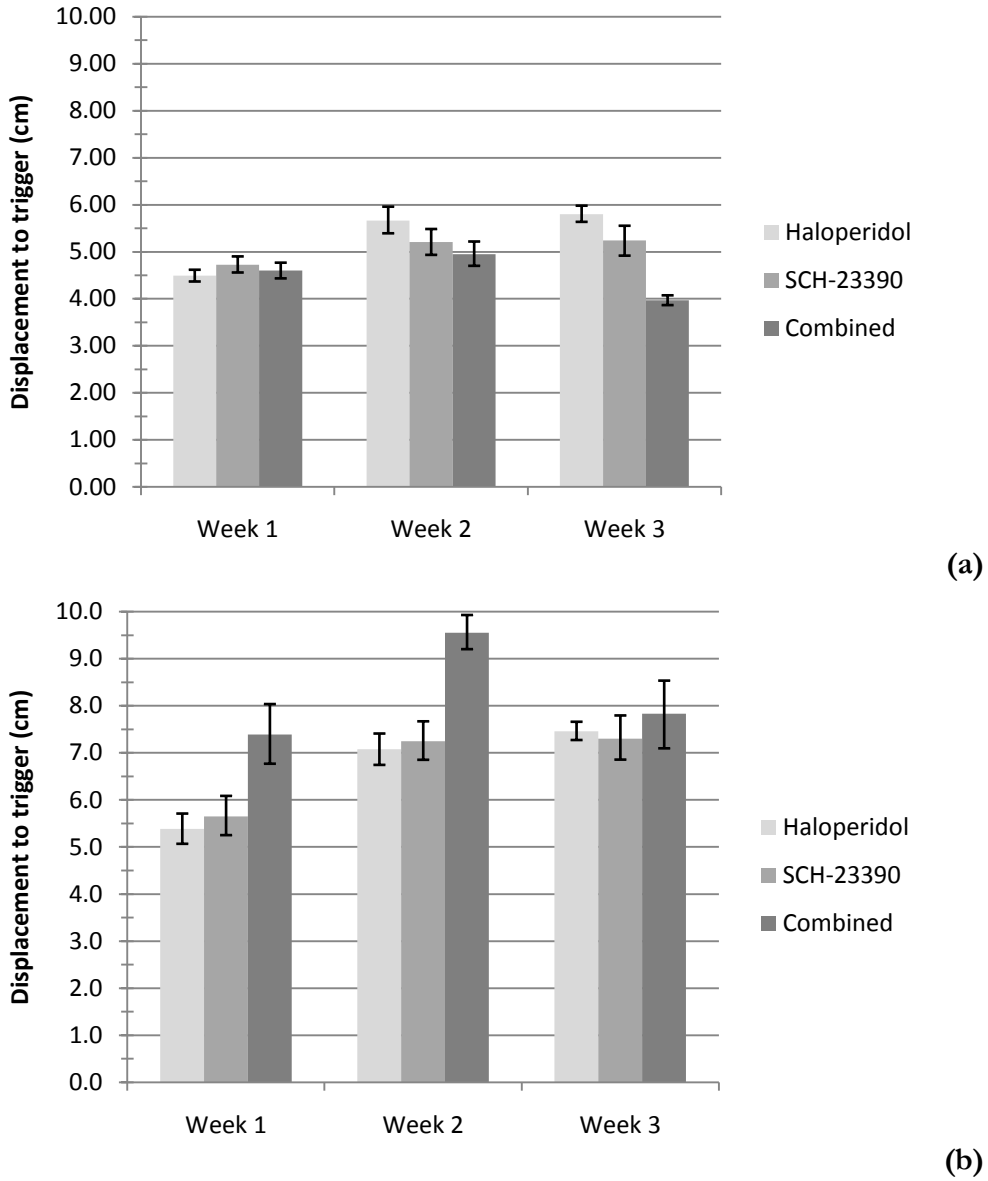


Figure 4.2 The effect of daily administration of dopamine antagonists on performance in the PIT test

Panel **(a)** shows mean performance just prior to daily drug administration while panel **(b)** shows performance measured 15 min after drug injection. In general, performance in this test appears to degrade with prolonged daily administration of the antagonist(s). A combination of the two drugs has a greater detrimental effect (at least upon post-administration testing) than does either antagonist given alone. Data are means ± bootstrapped **95%** confidence intervals.

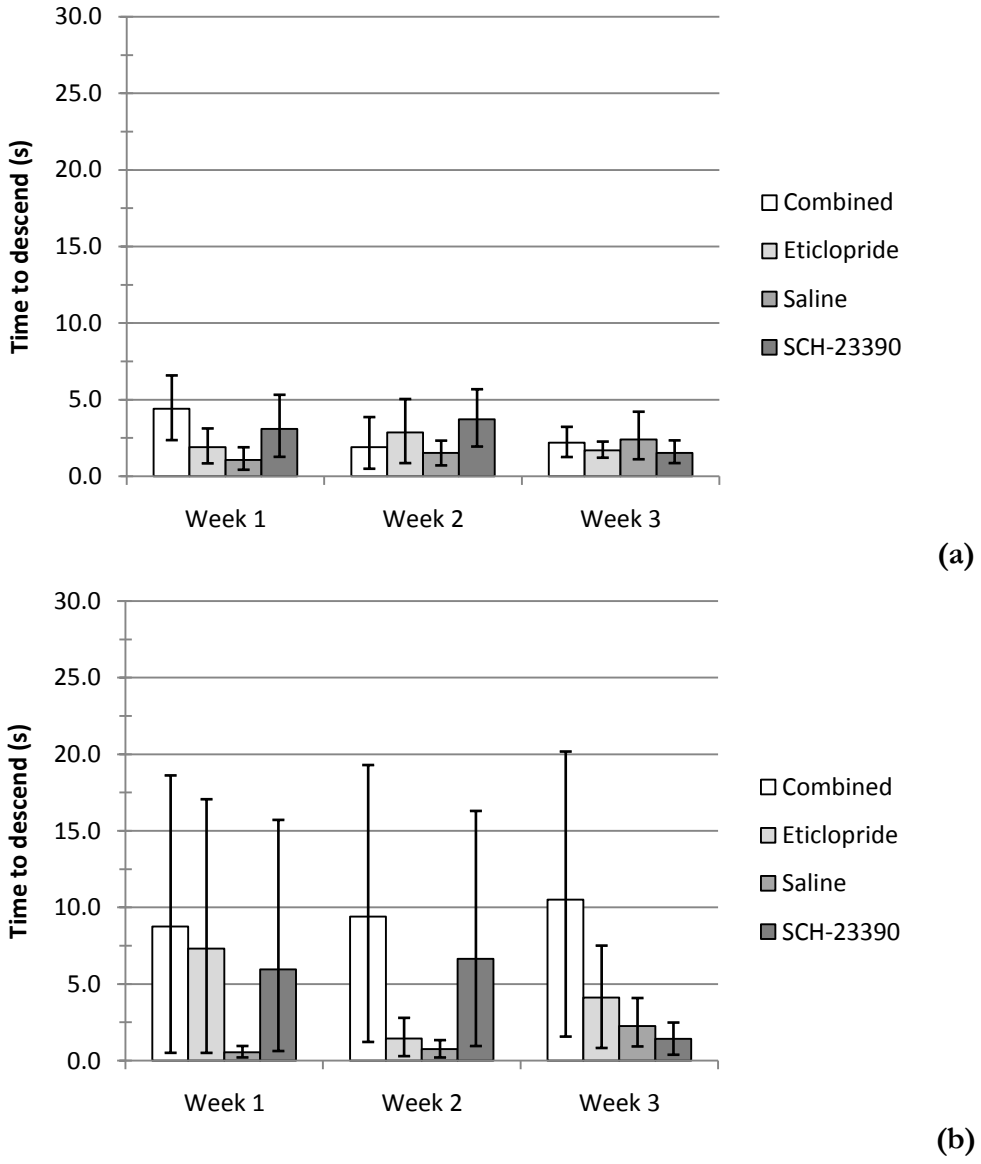
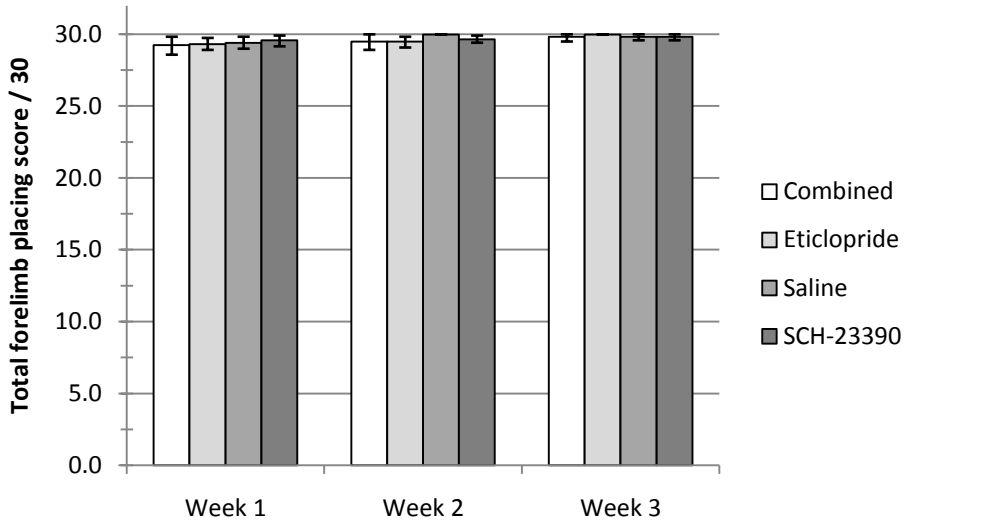
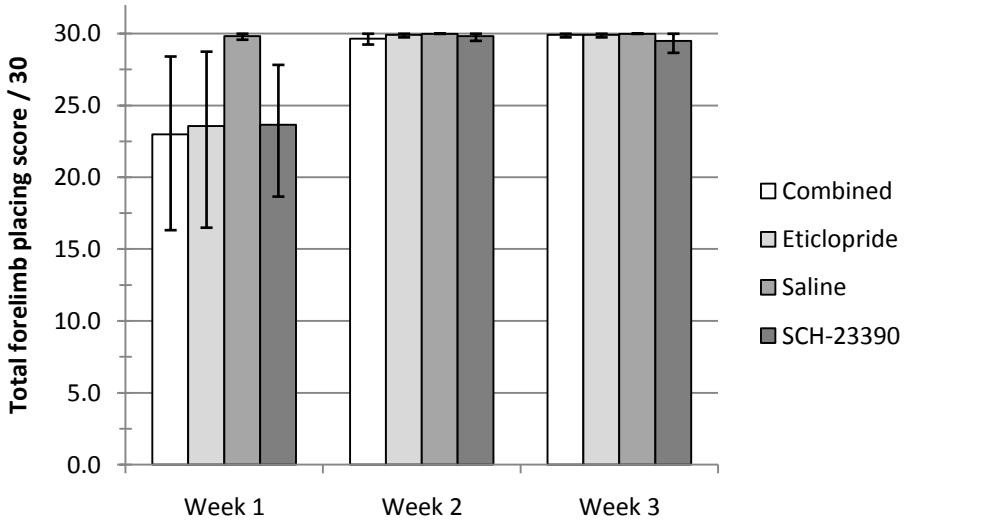


Figure 4.3 The effect of daily administration of dopamine antagonists on catalepsy

Catalepsy was measured as time taken to descend from a raised bar on which the rats' forelimbs were placed. Panel **(a)** shows mean performance just prior to daily drug administration while panel **(b)** shows performance measured 15 min after drug injection. Both antagonists produce catalepsy compared to the pre-drug condition; catalepsy is quite pronounced when the two antagonists are given together. Data are means ± bootstrapped **95%** confidence intervals.



(a)



(b)

Figure 4.4 The effect of daily administration of dopamine antagonists (this time using eticlopride) on performance in the forelimb placing test

Panel **(a)** shows mean performance just prior to daily drug administration while panel **(b)** shows performance measured 15 min after drug injection. Despite the catalepsy produced by these drug treatments (see Figure 4.3), the antagonists, either alone or in combination, do not affect forelimb placing after the first week, a time at which depolarization block would be expected to be in effect. Data are means ± bootstrapped **95%** confidence intervals.

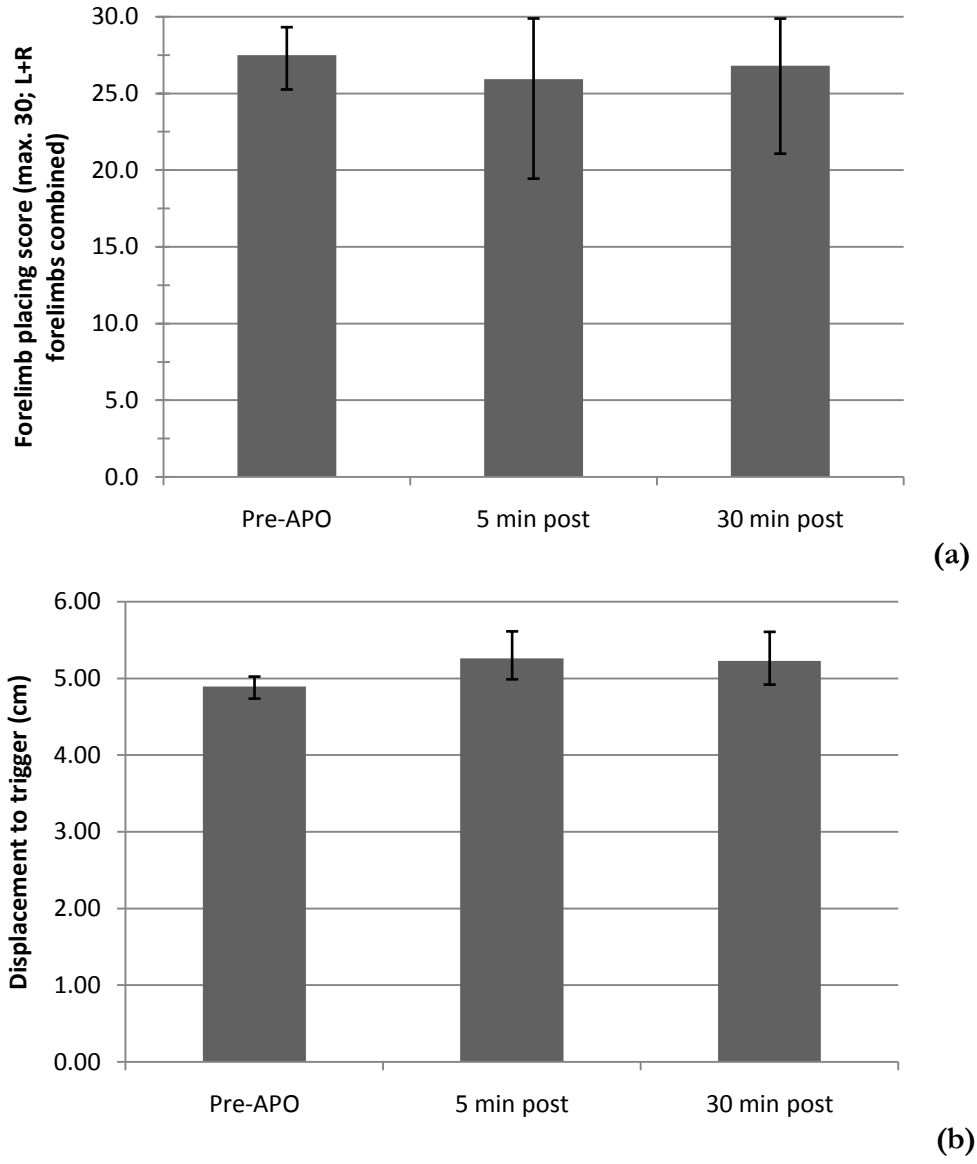


Figure 4.5 Scores from the forelimb placing test **(a)** and PIT test **(b)** for rats treated with low, autoreceptor-preferring doses of the dopamine antagonist drug apomorphine

Despite evidence that low doses such as those used effectively serve to reduce firing of dopamine neurons, no prominent behavioral effects were noted. Data are means ± bootstrapped **95%** confidence intervals.

Results

Effects of haloperidol and/or SCH-23390 on forelimb placing

Performance of rats in the pilot group is plotted in Figure 4.1 for the forelimb placing test. Panel (a) shows performance measured just prior to daily drug administration while panel (b) shows performance 15 min post-injection. In the pre-injection state (i.e., approximately 24 h since the prior injection), placing ability did not differ by treatment group. Fifteen min after the injection, however, placing ability was mildly decremented in all groups. In the second week, a combination of the two antagonists had a strong effect on placing, but this disappeared in the third week. Analysis was carried out using a classical (i.e. non-bootstrapped) 3-way ANOVA with pre- vs. post-injection and week of testing as within-subjects factors, and drug treatment group as a between-subjects factor. The pre- vs. post-injection condition and the week of testing were significant as main effects (pre/post: $F(1,69)=40.7$, $p<.001$; week: $F(2,138)=7.1$, $p=.001$); however, the drug treatment main effect did not reach significance despite the difference between the combination and the singly-administered drugs in the second week ($F(2,69)=2.8$, $p=.07$). All possible interactions were significant at $p<.05$. Considering only week 2 post-injection data, the effects of giving both antagonists together differed from the effects of either drug singly, in both cases with $p<.001$ as determined by bootstrapped unpaired t-tests (combo versus haloperidol, $t(58)=3.74$, $p<.001$; combo versus SCH-23390, $t(58)=3.55$, $p<.001$).

Effects of haloperidol and/or SCH-23390 on the PIT test

Performance of rats in the pilot group is plotted in Figure 4.2 for the PIT test. Panel (a) shows performance measured just prior to daily drug administration while panel (b) shows performance 15 min post-injection. Analysis was carried out using a classical (i.e. non-bootstrapped) 3-way ANOVA with pre- vs. post-injection and week of testing as

within-subjects factors, and drug treatment group as a between-subjects factor. All three factors were statistically significant as main effects (pre/post injection: $F(1,141)=503$, $p<.001$; week: $F(2,282)=62.9$, $p<.001$; drug treatment: $F(2,141)=4.16$, $p=.018$). All possible two-way interactions were also significant at $p<.001$, but the three-way interaction was not ($p=0.27$). The data indicate that the PIT scores worsen across three weeks of continuous antagonist administration, and that they also worsen when the drug is on board, indicating a likely direct antagonist effect rather than an effect of depolarization block. The combination of the two antagonists clearly has a stronger effect in this test than either antagonist given alone, at least in the first two weeks.

Effects of eticlopride and/or SCH-23390 on catalepsy descent times

For rats in the second (larger) study using eticlopride and SCH-23390, catalepsy descent times are graphed in Figure 4.3. Panel (a) shows performance measured just prior to daily drug administration while panel (b) shows performance 15 min post-injection. The antagonists, either alone or in combination, increased catalepsy relative to saline injections as measured on the test in the first week; effects varied in the second and third weeks. Analysis was carried out using a classical (i.e. non-bootstrapped) 3-way ANOVA with pre- vs. post-injection and week of testing as within-subjects factors, and drug treatment group as a between-subjects factor. Only the main effect of pre- versus post-injection condition was significant ($F(1,20)=6.6$, $p=.02$); the effects of drug group and week of administration were not (drug group: $F(3,20)=2.1$, $p=.13$; week: $F(2,40)=0.23$, $p=0.79$). There were no significant interactions.

Effects of eticlopride and/or SCH-23390 on forelimb placing

Forelimb placing data for rats in the second study using eticlopride and SCH-23390 are graphed in Figure 4.4. Panel (a) shows performance measured just prior to daily drug

administration while panel (b) shows performance 15 min post-injection. The dopamine antagonists, given alone or in combination, caused modest disruption of placing ability after administration in the first week, but this effect disappeared in subsequent weeks. Analysis was carried out using a classical (i.e. non-bootstrapped) 3-way ANOVA with pre- vs. post-injection and week of testing as within-subjects factors, and drug treatment group as a between-subjects factor. The pre- vs. post-injection condition and the week of testing were significant as main effects (pre/post: $F(1,44)=10.5$, $p=.002$; week: $F(2,88)=15.4$, $p<.001$); however, the drug treatment main effect did not reach significance ($F(3,44)=2.0$, $p=.12$).

Behavioral effects of low autoreceptor-preferring doses of apomorphine

The effects of low-dose apomorphine are shown in Figure 4.5a for the placing test, and in Figure 4.5b for the PIT test. Within each test, bootstrapped paired-samples t-tests were run for all possible comparisons among the three time points. No statistically significant differences were found, though there did seem to be a trend towards increased displacement needed in the PIT test upon post-injection testing.

Discussion

The data we have collected show that there is a disparity in performance between the forelimb placing and PIT tests. As can be seen in Figures 4.1 and 4.4, neither the D1-selective postsynaptic dopamine receptor antagonist SCH-23390 nor the prototypical dopamine antagonist haloperidol or its more D2-selective cousin eticlopride, even when given in doses high enough to block >95% of postsynaptic receptors, is capable of causing any appreciable decrement in forelimb placing ability in normal rats. Combining these drugs to ensure a broad spectrum of dopamine receptor subtype blockade did not produce an effect either. In all cases this occurred even when the drugs were administered daily for three weeks, a regimen which has been said to produce a cessation of presynaptic dopamine

neuron firing (Moore et al., 1998; Boye and Rompre, 2000) more similar to the state that might be seen following a 6-OHDA lesion.

The drugs did, however, produce marked catalepsy as measured in our descent test (see Figure 4.3), particularly when the drugs were given in combination, though there is some sign that animals receiving either the D1 or D2 antagonist alone may habituate to this effect somewhat across the three weeks of daily administration. Moreover, the antagonists did have a marked effect on performance in the PIT test (Figure 4.2). The contrast of this effect to that seen with forelimb placing suggests that though the PIT and forelimb placing tests both seem at face value to be measuring aspects of postural stability, the physiological underpinnings of these behaviors are obviously different to some extent. In any case forelimb placing does seem to be a more difficult behavior to disrupt, yet 6-OHDA lesions are able to do this readily.

We had no mechanism for confirming the onset of depolarization block in this study, and as this concept is still somewhat controversial (Chrapusta and Egan, 2006) it remains possible that in fact a cessation of presynaptic firing never occurred in our animals. This leaves open the possibility that somehow a presynaptic silencing is necessary to abolish forelimb placing, consistent with results from 6-OHDA lesioned animals. Indeed, results from Experiment 6 will show that reserpine, which exerts its dopamine-disrupting actions presynaptically, is able to disrupt forelimb placing ability to some extent, though still not to such an absolute degree as 6-OHDA lesions can. However, a second method that we employed, attempting to dampen dopamine neuron firing by using low autoreceptor-preferring doses of apomorphine (Skirboll et al., 1979), was also without behavioral effect.

These results may indicate that even high doses of the types of drugs (i.e., competitive antagonists) used in this study can still be temporarily displaced under extraordinary circumstances, leading to dopamine receptor activation and permitting any

behaviors dependent on such. Dopamine antagonists may not be sufficient to create a placing deficit simply because they may be displaced by sufficiently strong dopamine spikes. Future studies may be able to address the possibility by using non-competitive agents, for example fluphenazine-N-mustard (Cross et al., 1983; Weiss-Wunder and Chesselet, 1992), which permanently deactivate postsynaptic dopamine receptors and therefore would not even permit high-concentration dopamine spikes to have a downstream effect.

EXPERIMENT 5: INTERHEMISPHERIC COMPETITION IN UNILATERAL LESION MODELS AND ITS POSSIBLE CONTRIBUTION TO POSTURAL INSTABILITY

Introduction

6-OHDA lesions are commonly given unilaterally so that the animals remain able to care for themselves following the lesion, and so that convenient behavioral tests of motor asymmetry can be used to assess lesion severity, in which the unlesioned side can be used as a within-subject control. Our original observations of the placing deficit were made in unilaterally-lesioned animals, but it is possible that the deficit is an effect of competition between a lesioned and an unlesioned hemisphere (e.g., with the intact hemisphere inhibiting activity in the damaged hemisphere) or other effects which differ in unilateral versus bilateral lesion models (e.g., Salin et al. (1996), disparities which would presumably not be present in animals treated with systemically acting dopamine antagonists such as haloperidol. Detrimental effects in functional recovery after brain lesions have in some cases been linked to interhemispheric competition (Hilgetag et al., 1999; Murase et al., 2004), so we wished to explore this possibility further.

To test the hypothesis that postural stability problems might arise due to interhemispheric competition effects, we used two approaches. First, we lesioned a set of

animals bilaterally with 6-OHDA and tested them as we had the unilaterally-lesioned rats. In bilaterally lesioned animals the amount of dopaminergic tone between the two hemispheres should be roughly equal, thus negating any effects that are hypothetically due to an imbalance between the two sides. Second, we took unilaterally-lesioned rats that showed the expected contralateral forelimb placing deficit and treated them with systemic haloperidol, the same dopamine antagonist which was originally used in Experiments 3 and 4. In theory this would balance dopaminergic signaling between the hemispheres by disrupting the unlesioned side, potentially leading to a paradoxical recovery of forelimb placing ability in the impaired limb if the hypothesis were correct.

Methods

Bilateral 6-OHDA lesions

For this study, 13 adult male Long-Evans rats weighing 500-600 g were used. Rats were housed singly in clear polycarbonate cages. Prior to surgery the rats were acclimated to the PIT and forelimb placing tests. They were then either lesioned bilaterally with 8 μg of 6-OHDA delivered into the MFB of each hemisphere as described in Appendix 2 (n=9), or they received sham infusions of the ascorbic acid vehicle under the same surgical procedures (n=4).

Following surgery, rats were given daily injections of 10 ml/kg saline containing 5% glucose, and were also fed Nutri-Cal paste by mouth to maintain their nutritional status. Palatable treats such as cookies and jelly were also placed in their home cages to encourage eating following the bilateral lesions. Behavioral testing in both the PIT and forelimb placing tests (see Appendix 2) was carried out one week after the lesion.

The effect of haloperidol after unilateral 6-OHDA lesion

Seven adult male Sprague-Dawley rats were selected from among a group lesioned unilaterally with 6-OHDA and confirmed to have a stable post-operative forelimb placing deficit in the contralateral limb (these animals were the same as those used in Experiment 2). Their forelimb placing ability was evaluated in the contralateral limb both before and 15 min after receiving an i.p. injection of 3 mg/ml/kg haloperidol dissolved in 0.3% tartaric acid.

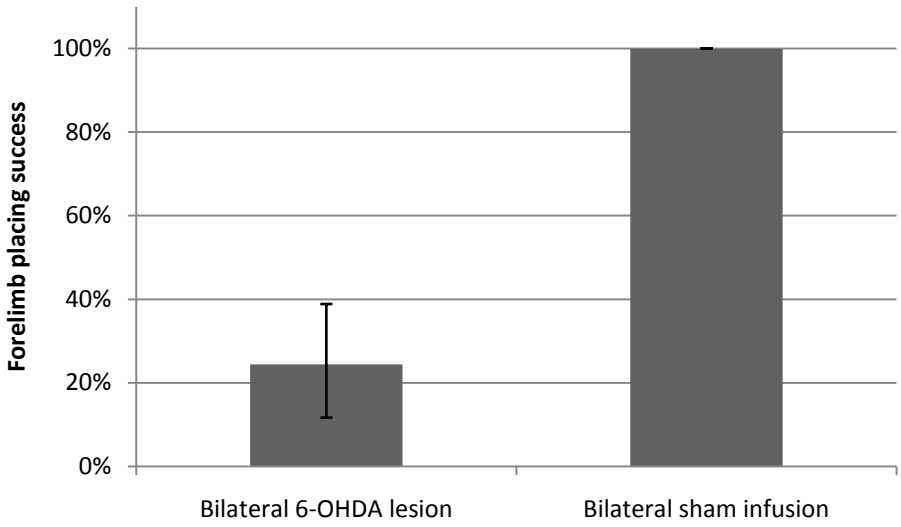


Figure 5.1 Average forelimb placing success across the two forelimbs (i.e., left and right) for animals lesioned bilaterally with 6-OHDA versus those receiving a bilateral sham infusion

Bilateral 6-OHDA lesion produced bilateral placing deficits, with the greatest difference between the two sides being 30% in one animal. Data were collected approximately one week post-lesion and are means ± bootstrapped **95%** confidence intervals.

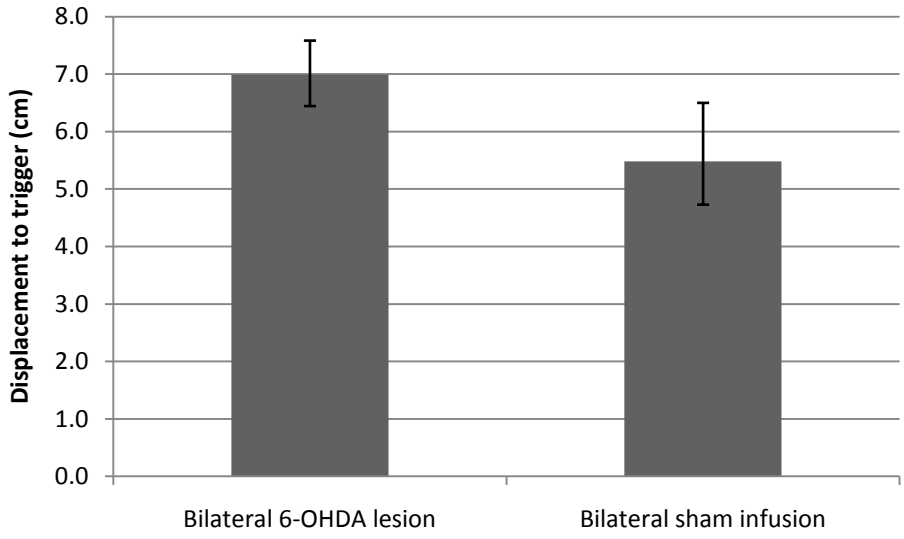


Figure 5.2 Performance in the PIT test following bilateral 6-OHDA lesion versus sham vehicle infusion

The mean left side-to-right side difference for animals in the 6-OHDA group was 0.9 cm and for animals in the sham group, 1.0 cm. The difference is statistically significant at $p<.01$. Data are means ± bootstrapped **95%** confidence intervals.

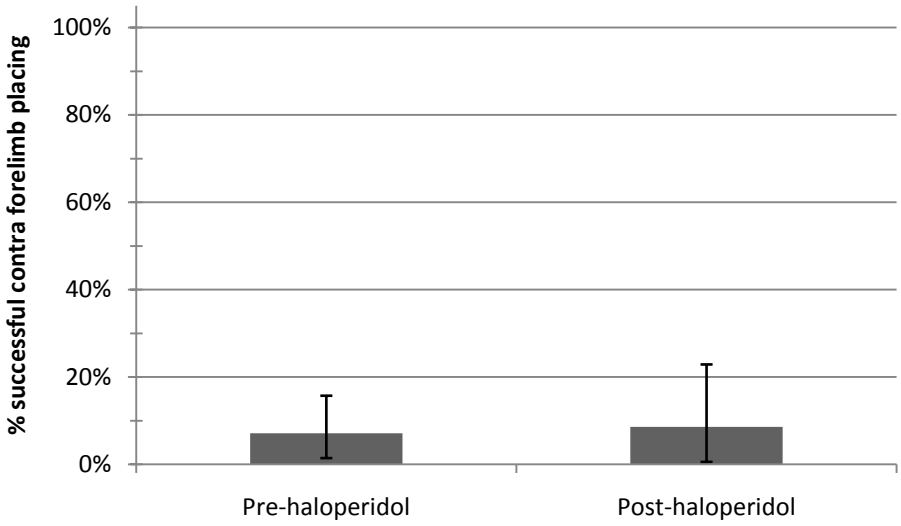


Figure 5.3 No effect on contralateral forelimb placing from administering the dopamine antagonist haloperidol to rats that had previously been lesioned unilaterally with 6-OHDA

Data are means ± bootstrapped **95%** confidence intervals.

Results

Forelimb placing after bilateral 6-OHDA lesion

Figure 5.1 shows the results of applying the forelimb placing test to rats lesioned bilaterally with 6-OHDA versus rats receiving bilateral sham infusions. Most bilaterally lesioned rats showed severe forelimb placing deficits (6 out of 9 rats had an average placing success of <30%, averaging across the two forelimbs). In general, the deficit was symmetrical in the lesioned animals, with the greatest difference in placing success between the two sides being 30% in one animal who showed 60% and 90% placing on the left and right sides, respectively. Sham-infused animals showed no placing deficits despite being infused with the ascorbic acid vehicle into the medial forebrain bundle.

PIT test after bilateral 6-OHDA lesion

Figure 5.2 shows performance in the PIT test. Interestingly, though animals with bilateral lesions did show a bilateral increase in the shift of center of gravity needed to trigger a catch-up step, this increase was not as significant as it typically is in unilaterally-lesioned animals (e.g., a mean of 7.0 cm displacement required in bilaterally lesioned animals, versus 8.5 cm required in the impaired limb of unilaterally lesioned animals at one week post-op in Experiment 2), suggesting that components of this test may be influenced by interhemispheric competition such that the presence of a dopaminergically intact hemisphere following unilateral lesion may have further negative consequences for the forelimb contralateral to the lesion. Nevertheless, lesioned animals did differ from sham-infused ones by an average of 1.5 cm. A bootstrapped two-sample t-test showed the difference to be statistically significant ($t[24]=2.7$, $p<.01$). The mean side-to-side difference in performance for lesioned animals was 0.9 cm; for sham animals, it was 1.0 cm.

Effects on placing of administering haloperidol to unilaterally 6-OHDA-lesioned rats

Figure 5.3 shows that administering the dopamine antagonist haloperidol to rats lesioned unilaterally with 6-OHDA (to, in theory, balance dopaminergic signaling between the two hemispheres) was without effect on forelimb placing in the limb contralateral to the lesion. The treatment also did not affect placing in the forelimb ipsilateral to the lesion.

Discussion

Bilateral 6-OHDA lesions delivered into the MFB produced rats with marked akinesia and aphagia in the week following the lesion. The health of lesioned animals had to be maintained by the researchers via manual feeding and daily saline injections, and spontaneous movement in the home cage was very reduced. Upon testing one week after the lesions, animals were found to have bilateral forelimb placing deficits as well as a bilateral increase in the body weight displacement required to trigger a catch-up step in the PIT test. In addition, administration of the dopamine receptor antagonist haloperidol to rats that had previously been lesioned unilaterally with 6-OHDA did not affect behavioral performance in the forelimb placing test.

These observations indicate that the disruption of placing behavior contralateral to a presynaptic dopamine lesion is not the result of an interhemispheric competition effect, because in both of the experiments in this section, dopaminergic activity between the two hemispheres was roughly equal, yet disruption of postural support behaviors still occurred.

Though these effects seem clear when looking at the forelimb placing test, it is interesting that in the PIT test, the magnitude of the deficit in either limb of bilaterally lesioned animals (mean displacement: 7.0 cm) did not reach that seen in the contralateral limb of unilaterally lesioned animals (mean displacement: 8.5 cm at one week post-op; see Figure 2.2), indicating the possibility that interhemispheric effects due to the presence of an intact "normal" nigrostriatal dopamine system may have detrimental effects on the

behavioral performance of the impaired limb. In a bilaterally lesioned rat, too, there is no opportunity to observe the "enhanced" reactivity seen in the intact limb of unilaterally-lesioned animals. This may indicate that performance in the PIT test is indeed influenced to some extent by interhemispheric interactions; but then the PIT test, unlike the forelimb placing test, is also affected by administration of haloperidol. This indicates that though the PIT and forelimb placing tests are both ostensibly measuring aspects of postural stability behaviors, they likely have different mechanistic underpinnings. This subject will be discussed further in the dissertation conclusions.

In the meantime, the discrepancy in forelimb placing between 6-OHDA-lesioned and dopamine-antagonist treated animals remains. Results gathered after dopamine agonist treatment in Experiment 3 raised the possibility that norepinephrine systems may play a critical role in determining the extent of placing deficit following 6-OHDA lesion. In the next experiment we look into this possibility more closely.

EXPERIMENT 6: THE POSSIBLE CONTRIBUTION OF NOREPINEPHRINE SYSTEMS TO SYMPTOMS OF POSTURAL INSTABILITY

Introduction

The neurotoxin 6-OHDA used in our surgeries is not specific to dopamine. 6-OHDA kills dopamine cells after gaining entry into the cytoplasm via the dopamine reuptake transporter (Ahlskog and Hoebel, 1973); it so happens that the norepinephrine (NE) reuptake transporter (NET) also has a relatively high affinity for 6-OHDA (Schallert and Wilcox, 1985). For this reason, 6-OHDA surgical protocols aiming to produce dopamine-specific degeneration usually include preoperative administration of the drug desipramine, an inhibitor of the NET. This putatively prevents uptake of the toxin into NE terminals, thus

making the lesion specific to dopamine. Rarely has the effectiveness of systemically-administered desipramine in preventing NE damage been measured specifically, however (but see Roberts et al. (1975)).

We discovered in our 6-OHDA treated rats that administration of L-DOPA was able to reverse the placing deficit (see Experiment 3). L-DOPA is the drug most commonly used in treatment of humans with PD; it acts as a synthetic precursor to dopamine and thereby increases levels of DA in depleted tissues. We also tried administering lesioned animals the drug apomorphine, which is a full D2 receptor agonist as well as a weak partial D1 agonist. Unlike L-DOPA, apomorphine was without therapeutic effect, i.e. it did not reverse the placing deficit. Because NE is synthesized from DA, administration of L-DOPA also indirectly increases levels of NE (Dolphin et al., 1976). For this reason we suspected that perhaps partial damage to NE systems due to the 6-OHDA lesion was responsible for creating the placing deficit, an effect which could not be mimicked by administering dopamine-specific receptor antagonists. Additionally, it is known that administration of NE agonists can help relieve some symptoms in cases of advanced PD, the stage where PI symptoms normally appear (Narabayashi et al., 1984; Kondo, 1993; Oribe et al., 1993); and targeted depletion of locus ceruleus norepinephrine has been shown to enhance parkinsonian symptoms in the 6-OHDA rat model (Srinivasan and Schmidt, 2003).

To test our hypothesis we used two approaches. First, we administered the drug L-Threo-DOPS to a set of animals which had previously been lesioned with 6-OHDA, and in which the presence of a forelimb placing deficit had been established. This drug is a synthetic precursor for NE synthesis which is converted directly into NE by the endogenous enzyme aromatic amino acid decarboxylase (AADC), and its administration markedly increases NE levels while having relatively less effect on DA levels (Tohgi et al., 1993). This

compound has also been shown to have beneficial effects in countering haloperidol-induced catalepsy in the rat (Verhagen-Kamerbeek et al., 1993).

Second, in a set of sixteen intact rats, we administered high doses of two drugs in combination: alpha-methyl-para-tyrosine (AMPT), a drug which inhibits the action of the enzyme tyrosine hydroxylase and thus inhibits synthesis of all catecholamines (Sjoerdsma et al., 1965), and reserpine, a drug which prevents packaging of monoamine transmitters into synaptic vesicles, thus interfering with the normal release of not only DA and NE but also 5-HT (Stitzel, 1976). The administration of these two drugs in combination pharmacologically rendered rats effectively devoid of brain catecholamine transmission, and animals so treated were extremely akinetic and cataleptic in addition to having trouble maintaining normothermia (indicating disruption of NE systems). In addition, we took another group of normal rats and treated them for five consecutive days with a lower dose of reserpine (rather than in a "one-shot" trial as above), to rule out the possibility that the disruption produced by the drug needed to be relatively long-lasting before deficits in forelimb placing would emerge.

The recovery of placing ability after administration of L-Threo-DOPS would be taken as a sign that agents which selectively boost NE function could help relieve postural instability symptoms, a possibility suggested by earlier studies in humans (Kondo, 1993; Tohgi et al., 1993), and therefore may indicate that our lesioning protocol does cause unintended damage to NE systems. The use of reserpine, especially when combined with AMPT, produces an animal that is severely deficient in its ability to release both DA and NE from the presynaptic terminal, thus if disruption of either or both of these systems alone is sufficient to elicit problems with postural stability, the symptoms should be evident in animals so treated. Furthermore, in animals treated with reserpine plus AMPT, we administered L-DOPA and apomorphine to determine whether any behavioral deficits

produced by the treatment could be reversed by dopaminergic stimulation, thus allowing us (in the case of apomorphine, at least) to partition out the dopaminergic versus noradrenergic contributions of any behavioral changes observed after the depleting treatment.

Methods

Animals

For the reserpine studies, two groups of adult male Long-Evans rats (n=16 for the acute study with AMPT and reserpine; n=10 for the subchronic reserpine study) from Harlan laboratories were used, weighing 600-800 g at the beginning of the study. For the L-Threo-DOPS study, rats (n=7) were adult male Sprague-Dawleys from a local colony, weighing 400-500 g at the time of the study. All rats were housed in pairs in clear polycarbonate cages with sawdust bedding on a 12:12 h light:dark cycle with food and water available *ad libitum*. Behavioral tests were performed in the dark portion of the light cycle by a tester blind to experimental condition or lesion side.

Drug treatments

For the L-Threo-DOPS studies, a group of 7 animals who had previously been lesioned unilaterally with 6-OHDA (as per Appendix 2), and which were confirmed later to have good lesions based on performance in the limb-use asymmetry, forelimb placing, and PIT tests, were used. L-Threo-DOPS (Sigma) was dissolved at 100 mg/ml in 0.1N HCl and then diluted 9:1 with saline to form a solution of 10 mg/ml L-Threo-DOPS. The animals were injected i.p. with 3 ml/kg of this solution for a total dose of 30 mg/kg L-Threo-DOPS. Behavioral testing (PIT and forelimb placing) commenced 30 min after the injection.

For the acute reserpine with alpha-methyl para tyrosine study, reserpine (MP Biomedicals) was dissolved at 60 mg/ml in glacial acetic acid then diluted 19:1 with saline to a final concentration of 3 mg/ml reserpine. This was then administered once to sixteen rats

via s.c. injection at a volume of 1 ml/kg, for a total dose of 3 mg/kg reserpine. Eighteen hours later, AMPT (Sigma) was dissolved at 200 mg/ml in distilled water and administered to rats at 1 ml/kg for a dose of 200 mg/kg AMPT per rat. One hour after this injection, the first round of behavioral testing commenced. Rats later received s.c. injections of 0.5 mg/kg apomorphine, freshly dissolved in saline, and later L-DOPA, dissolved in 1N HCl and diluted 19:1 then given at a final dose of 50 mg/kg (see section on behavioral testing, below, for the time course used).

For the chronic reserpine study, reserpine was dissolved at 20 mg/ml in 100% DMSO and then administered once daily to six (n=6) rats via i.p. injection at a volume of 0.1 ml/kg, for a dose of 2 mg/kg/day. The treatment was repeated for five days in a row. A control group (n=4) received daily injections of the vehicle, 100% DMSO, at 0.1 ml/kg, for five days.

Behavioral testing

In the acute reserpine with AMPT study, rats were tested on the forelimb placing test prior to receiving any drugs, and then again 19 h and 1 h after receiving their injections of reserpine and AMPT, respectively. All rats showed normal (i.e. 100%) placing in both forelimbs prior to drug administration. Rats were also tested on the PIT and cataleptic descent tests (see Experiment 4 for catalepsy test methods) 1 h after AMPT injection. At 2.5 h after the AMPT injection, animals were administered 0.5 mg/kg apomorphine and tested 15 min after this injection on all three tests. Finally, 1 h 15 min after the apomorphine injection, rats were administered 50 mg/kg L-DOPA and tested 30 min after this final injection, again on all three tests.

In the subchronic reserpine study, rats were tested on the forelimb placing test prior to any injections (as a baseline) and at days 2, 4, 7, 18, and 37 relative to the first injection

day (the injections continued for only 5 days, so the last three testing points were on days when the animals did not receive any drug, to assess the permanence of any effects caused by reserpine). Within two days of the last behavioral test, animals were sacrificed and their striata were analyzed for monoamine contents via HPLC as described in Appendix 2.

In the L-Threo-DOPS studies, the (previously-lesioned) rats were tested before injection on the forelimb placing and PIT tests, and then tested again 30 min after the drug injection.

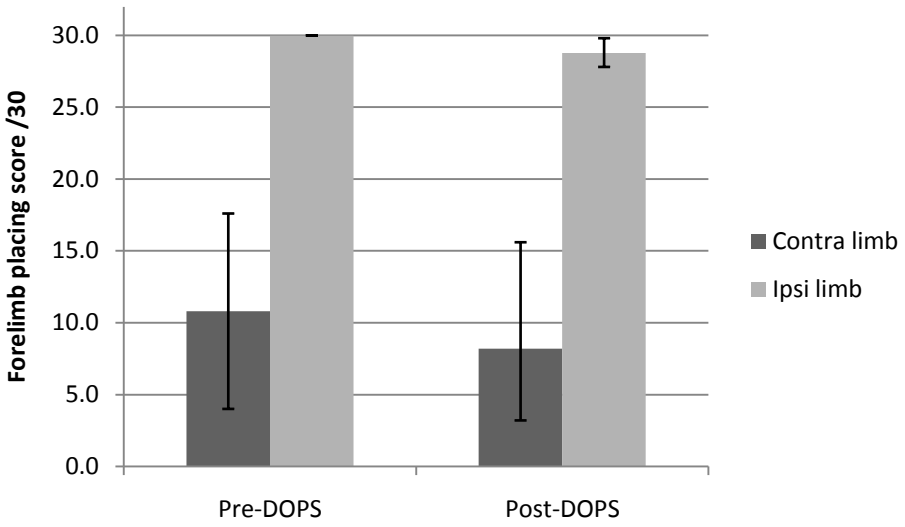


Figure 6.1 6-OHDA-induced forelimb placing deficits are not ameliorated by administration of L-Threo-DOPS

L-Threo-DOPS is a synthetic norepinephrine precursor which elevates brain levels of the neurotransmitter. Data are means ± bootstrapped **95%** confidence intervals.

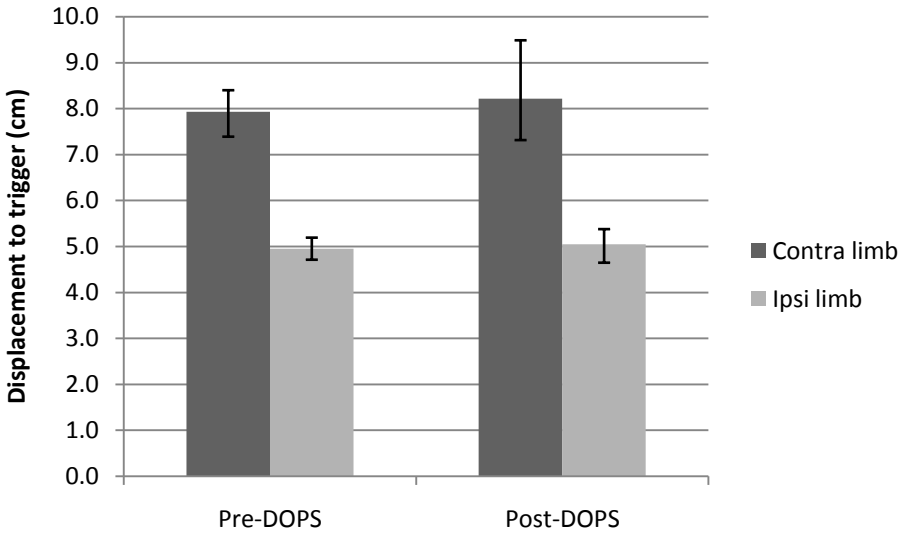


Figure 6.2 Administration of L-Threo-DOPS has no effect on performance in the PIT test in animals previously lesioned with 6-OHDA

Data are means ± bootstrapped **95%** confidence intervals.

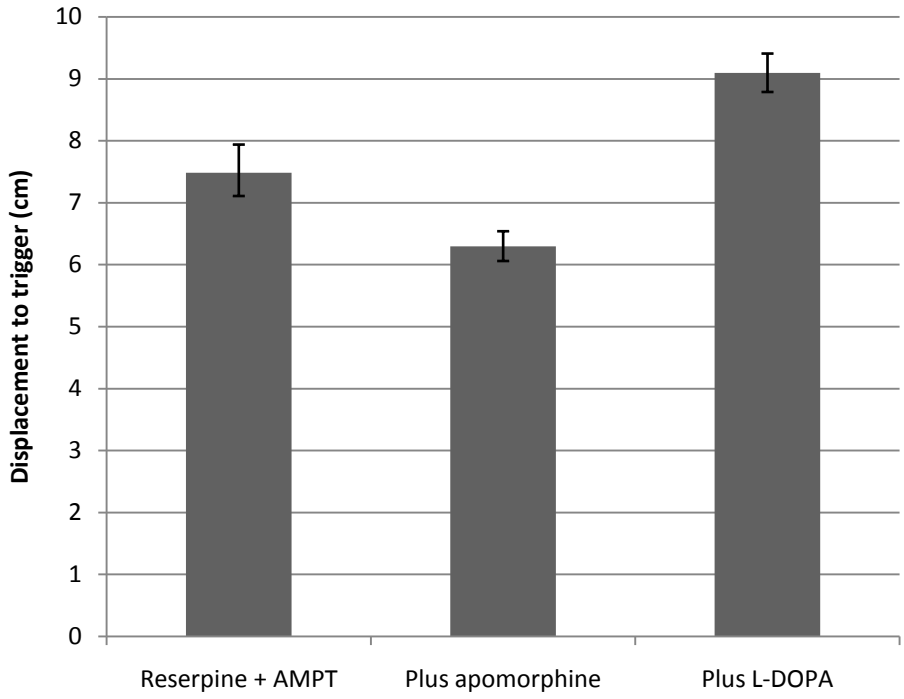


Figure 6.3 Performance on the PIT test following administration of reserpine and AMPT, subsequent administration of apomorphine at 2.5 h after the AMPT injection, and administration of L-DOPA at 1 h 15 min after the apomorphine injection

Apomorphine seems to restore more normal PIT scores in the reserpinized & AMPT-treated rat, while L-DOPA appears to have a paradoxical negative impact in this test. All group differences are statistically significant with $p<.001$. Data are means ± bootstrapped **95%** confidence intervals.

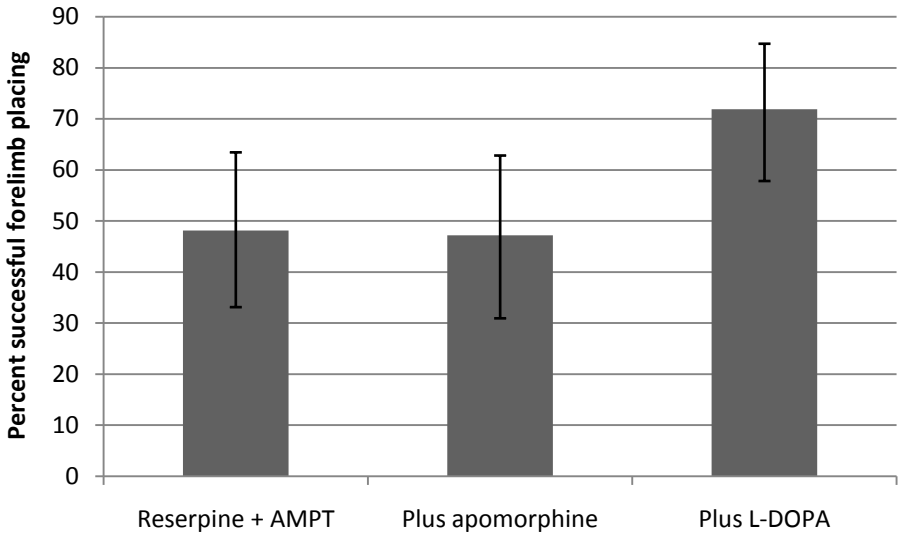


Figure 6.4 Performance on the forelimb placing test following administration of reserpine and AMPT, subsequent administration of apomorphine at 2.5 h after the AMPT injection, and administration of L-DOPA at 1 h 15 min after the apomorphine injection

Apomorphine has no effect on the below-normal placing ability in the reserpinized & AMPT-treated rat, while L-DOPA ameliorates the deficit. All rats placed with 100% success when tested at baseline before any drugs were given. The L-DOPA condition differs from the other two with $p<.05$. Data are means ± bootstrapped **95%** confidence intervals.

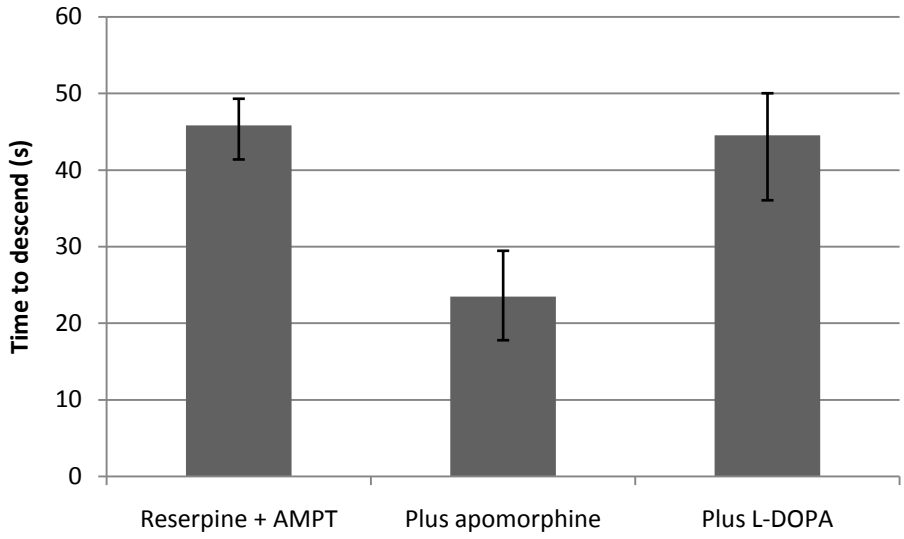


Figure 6.5 Mean time taken for the forelimbs to descend from an elevated bar in the catalepsy test

The test was cut off at a maximum of 50 seconds; several rats reached this cutoff. Times were measured after (left) administration of reserpine and AMPT, (center) subsequent administration of apomorphine at 2.5 h after the AMPT injection, and (right) administration of L-DOPA at 1 h 15 min after the apomorphine injection. Apomorphine seems to restore more normal (faster) descent behavior, while L-DOPA appears to have a paradoxical negative impact in this test. The apomorphine-treated condition differs from the other two with $p<.01$. Data are means ± bootstrapped **95%** confidence intervals.

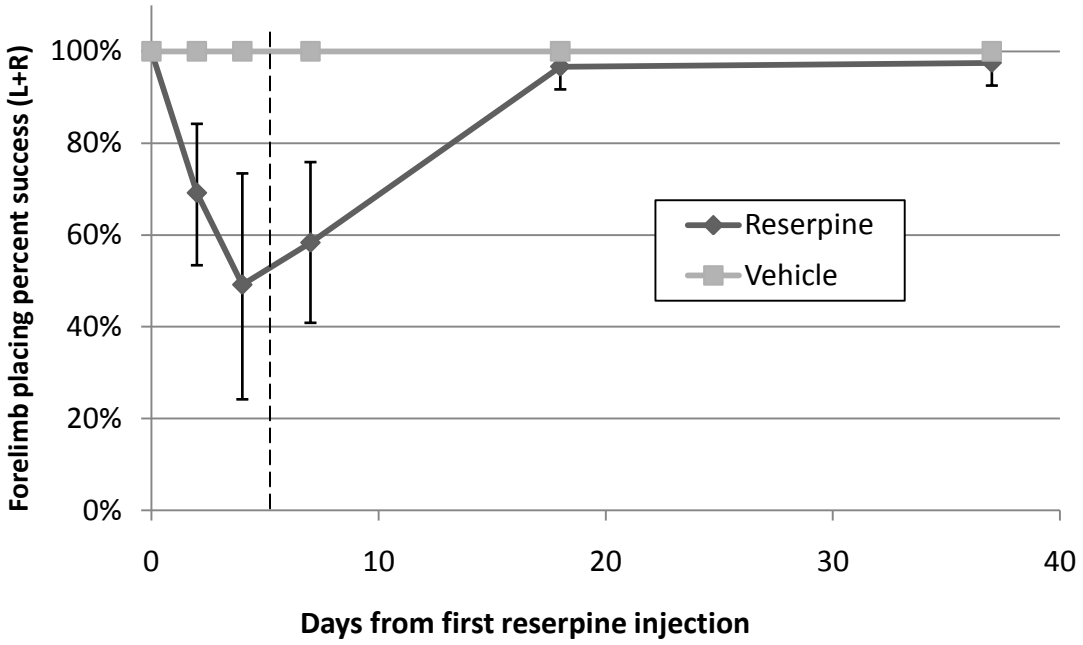


Figure 6.6 The effect of repeated reserpine injections on forelimb placing in normal animals

Animals were injected daily with 2 mg/kg reserpine for 5 days (up to the dotted line on the graph). Reserpine treatment did cause a decrement in forelimb placing ability, though not as severe as that seen with 6-OHDA lesion. Following washout of reserpine, forelimb placing ability returned to normal. Data are means ± bootstrapped **95%** confidence intervals.

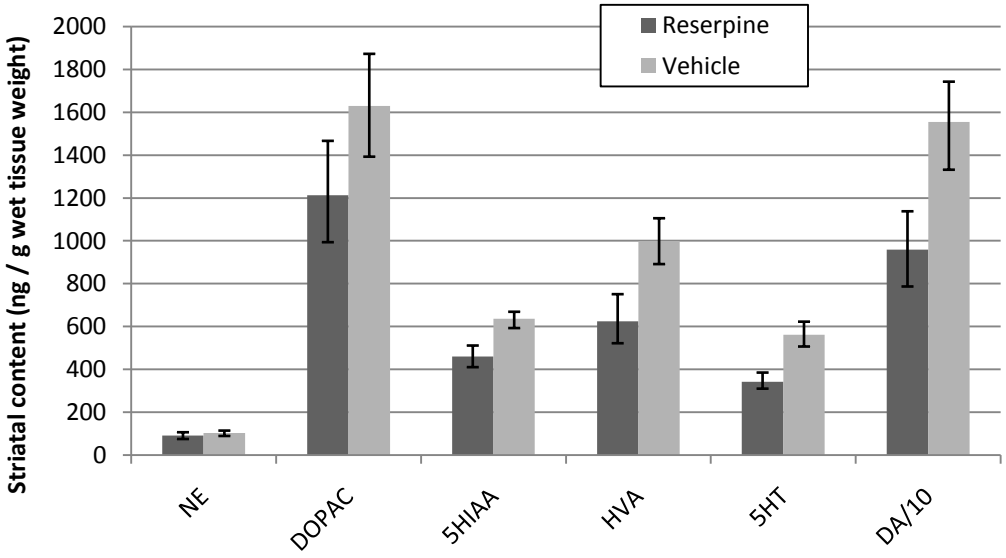


Figure 6.7 Striatal content of monoamines and their metabolites in animals treated with reserpine versus vehicle for 5 days

Animals were sacrificed 5 weeks after the last reserpine/vehicle injection. Subchronic reserpine treatment leads to a lasting partial suppression of the levels of dopamine and serotonin and their metabolites relative to control animals, despite the lack of a lasting forelimb placing deficit. All group differences were significant at $p<.05$ except in the case of NE. Data are means ± bootstrapped **95%** confidence intervals.

Results

The effect of L-Threo-DOPS on postural stability deficits

Data collected before and after administration of L-Threo-DOPS to animals previously lesioned with 6-OHDA are shown in Figure 6.1 (forelimb placing) and Figure 6.2 (PIT test). Administration of L-Threo-DOPS did not ameliorate forelimb placing deficits, nor did it affect scores on the PIT test, suggesting that either norepinephrine systems were unaffected by the original 6-OHDA lesion, or that if they were affected, pharmacological replacement of NE by L-Threo-DOPS was not sufficient to allow recovery of postural stability deficits.

Acute effects of reserpine & AMPT administration and reversal by apomorphine or L-DOPA

The data in Figures 6.3 – 6.5 show the effect of treatment with reserpine followed the next day with AMPT (given 1 hour before testing) on performance in the PIT test (Figure 6.3), the forelimb placing task (Figure 6.4), and time to descend from a raised bar (as a test of catalepsy; Figure 6.5), and also the effects of giving apomorphine and, later, L-DOPA to rats so treated. For each test, three paired-samples bootstrapped t-tests were used to assess each possible comparison. For the PIT test, all conditions differed from each other with $p<.001$. For placing, the L-DOPA treated condition differed from the other two (L-DOPA vs. reserpine: $t(31)=2.1$, $p=.047$; L-DOPA vs. apomorphine: $t(31)=2.2$, $p=.032$), but there was no significant difference from the reserpine & AMPT only condition upon treatment with apomorphine ($t(31)=0.1$, $p=0.92$). For catalepsy, the apomorphine-treated condition differed from both of the other conditions (reserpine vs. apomorphine: $t(31)=5.42$, $p<.001$; apomorphine vs. L-DOPA: $t(31)=3.94$, $p=.001$), but the reserpine-only and L-DOPA treated conditions did not differ ($t(31)=0.16$, $p=0.87$).

Though placing in these animals was decremented significantly, it should be noted that the extent of the placing deficit still does not approach that seen in unilaterally 6-OHDA lesioned rats (see e.g., Figures 3.1a and 3.2a). This is despite the fact that the animals in this study were severely akinetic and cataleptic due to the drug treatments, and generally looked very unwell (in fact, one animal died a few hours after the testing sessions; the rest were sacrificed shortly thereafter). In contrast, animals lesioned unilaterally with 6-OHDA appear quite healthy and normal in their home cage, yet are much worse performers with their contralateral limb in the forelimb placing test.

Once again, as in Experiment 3, placing deficits appear to be treatable with L-DOPA but not apomorphine, while virtually the opposite is true in the PIT test, suggesting that these two behaviors likely have different mechanistic underpinnings. Generally, catalepsy as measured in the descent test appears to more closely follow a pattern similar to the PIT test.

Subchronic reserpine administration: behavioral and neurochemical outcomes

As seen in Figure 6.6, repeated administration of reserpine did cause deficits to appear in the forelimb placing test, though the deficits were not as severe as those previously seen in 6-OHDA lesioned animals. Following cessation of reserpine treatment and its subsequent washout, placing ability returned to normal in treated animals. However, interestingly, when animals were sacrificed approximately 5 weeks after the last reserpine injection, they were found to have depleted levels of striatal monoamines (Figure 6.7). Bootstrapped unpaired t-tests were run to compare the reserpine- versus vehicle-injected groups on all six substances analyzed, and differences were found to be significant at $p<.01$ for 5-HIAA, HVA, 5-HT, and DA, and at $p<.05$ for DOPAC ($t[8]=2.02$, $p=.039$). The NE difference was not significant ($t[8]=0.91$, $p=0.39$). The mean percentage dopamine depletion was 38%. Despite these differences no persistent behavioral differences were

noted in the forelimb placing test. A previous study, however, has shown persistent behavioral effects from even a single dose of reserpine (Palfai and Walsh, 1979; Sussman et al., 1997) which may be consistent with our neurochemical findings.

Discussion

The use of reserpine and/or AMPT in normal animals can shed light on the mechanisms underlying postural stability control for a couple of different reasons. First, unlike the postsynaptic receptor antagonists used in Experiment 4, both of these drugs exert their actions primarily on the presynaptic dopamine terminal. Indeed, though treatment with these drugs did not abolish placing so completely as 6-OHDA lesions do, they did disrupt the behavior to a significantly greater extent than postsynaptic dopamine antagonists. This indicates that the type of dopamine system disruption needed to produce this deficit is primarily presynaptic in nature, consistent with the profound deficit seen in 6-OHDA lesioned animals. The effects of reserpine in particular may also have something to do with its reported ability to alter the morphology and characteristics of corticostriatal dendritic spines (Day et al., 2006; Shen et al., 2007), an idea we will return to in Experiment 9.

Secondly, both of these drugs act not only on dopamine systems but on norepinephrine and (in the case of reserpine) serotonin systems too. Therefore their limited ability to disrupt placing behavior is consistent with the hypothesis that norepinephrine systems may play a role in mediating this behavior. This is in line with the finding from Experiment 3 that administration of L-DOPA can ameliorate placing deficits, since this compound increases NE levels along with its traditionally-recognized use as a booster of DA synthesis (Dolphin et al., 1976). However, our demonstration of the lack of any effect on postural stability deficits due to administration of L-Threo-DOPS, which can be used to synthesize NE but not DA, seems to negate this possibility. Future studies could address

this question further by using various combinations of selective NE receptor agonists to more fully verify whether NE is a major factor in 6-OHDA-induced postural instability

Thus, it does seem most likely that L-DOPA exerts its symptomatic benefit in the forelimb placing test by increasing presynaptic dopamine levels, and that in this light the placing deficit is brought out by a "dopamine plus" degenerative condition, in which not only dopamine disruption but secondary changes in other systems (triggered hypothetically by the severe and chronic loss of DA input initially caused by 6-OHDA lesion) are required—in combination—to produce forelimb placing deficits. This is somewhat supported by our finding that chronic administration of reserpine across five days led to a progressively worsening placing deficit, indicating that in the chronically reserpinized rats, perhaps a dopamine-loss-induced reorganizational process was getting underway similar to that which occurs in 6-OHDA lesioned rats. Future studies can carry along in this vein, using longer-term administration of reserpine and/or AMPT to see whether stronger placing deficits can be produced. Evidence from such a study, however, would still need to be tempered by the finding that even long-term (3 weeks; Experiment 4) administration of dopamine receptor antagonists cannot create this effect, leaving open the question of why presynaptically-targeted manipulations appear to affect placing behavior while postsynaptically-targeted ones do not. We will return to this issue in the concluding remarks of this dissertation.

Again we find that behavior as measured with the forelimb placing versus PIT tests shows interesting differences, specifically with regard to treatment by dopamine agonists. In this study, treatment with reserpine plus AMPT produces deficits in both forelimb placing and the PIT test; however, unlike forelimb placing, deficits in the PIT test are ameliorated by apomorphine but are not helped (and indeed are perhaps even adversely affected) by L-DOPA. Measurements of catalepsy induced by the drug treatment more closely mirror the

pattern seen in the PIT test. Postural instability as it is generally referred to in the context of human PD is particularly resistant to treatment with L-DOPA (Hurtig, 1997), thus indicating that the more L-DOPA resistant behavior measured with the PIT test may be more analogous to the condition in persons with PD. Somewhat paradoxically, however, it may remain the case that placing behavior is the one that has less to do with dopamine, due to the lack of demonstrated effect of the direct dopamine agonist apomorphine on placing in this study.

EXPERIMENT 7: THE EFFECTS OF EXPERIMENTAL LESION LOCATION IN DETERMINING OUTCOMES ON TESTS OF POSTURAL INSTABILITY

Introduction

Our original observations of the placing deficit were made in animals that were lesioned unilaterally with 6-OHDA delivered into the medial forebrain bundle (MFB). This bundle carries dopaminergic axons of the nigrostriatal pathway from the cell bodies in the substantia nigra to the terminal fields in the striatum. In addition, a number of other neural pathways are carried in the MFB, including the mesolimbic dopaminergic fibers originating in the ventral tegmental area as well as various norepinephrine fibers and GABAergic return projections that originate in the striatum and terminate in the substantia nigra *pars reticulata* (Fix, 2001). 6-OHDA lesions are commonly given in the MFB because it represents an easy target in which a single toxin administration can lead to evenly-distributed and thorough destruction of nigrostriatal dopamine neurons, as opposed to the multiple infusions that may be needed if 6-OHDA is delivered directly into the substantia nigra or (especially) the striatum. However, though 6-OHDA is usually presumed to be a catecholamine-specific neurotoxin (and administration of desipramine during the lesioning surgery, as used in our

methods, is supposed to protect norepinephrine neurons from 6-OHDA), the toxin can still have some effect on other neuron types especially if the dose is high enough (Butcher, 1975; Willis et al., 1976). Therefore, infusion of the toxin into brain regions that contain other vulnerable cell types (e.g., norepinephrine axons or neurons) may induce non-dopaminergic damage, in addition to any non-specific damage that could be caused to nearby tissues simply by the physical act of infusing fluid via a cannula inserted into brain tissue.

Since severe deficits in the forelimb placing deficit had to this point only been observed in 6-OHDA lesioned animals, it was important to determine whether the deficit might be arising due to nonspecific damage created by injection of 6-OHDA into the MFB (i.e., damage to fibers other than the nigrostriatal dopaminergic fibers). To address this possibility, we lesioned a group of animals by delivering 6-OHDA directly into the cell body regions of the substantia nigra *pars compacta* instead, and verified their behavioral performance afterwards. This procedure reduces the possibility of widespread damage to other cell populations that share passage through the MFB, and makes a stronger case for the argument that any postural stability deficits seen after 6-OHDA lesion are due to dopaminergic cell loss (or another process triggered by dopaminergic cell loss in particular) specifically. We confirmed the extent of our lesions with post-mortem analysis of striatal dopamine content via HPLC, to ensure that the severity of lesions in this experiment was similar to that obtained in other studies where 6-OHDA was delivered into the MFB.

Methods

Male Long-Evans rats (n=5) from Harlan laboratories were used, and weighed 400-500 g at the time of surgery. They were housed in pairs in clear polycarbonate cages with sawdust bedding on a 12:12 h light:dark cycle with food and water available *ad libitum*.

Behavioral tests were performed in the dark portion of the light cycle by a tester blind to experimental condition or lesion side.

Prior to surgery, the rats were acclimated to both the forelimb placing and PIT tests, and following acclimation were tested twice on each test within the week prior to lesioning surgery to establish a behavioral baseline. They were also tested twice in the limb-use asymmetry ("cylinder") test, and the results of this test were used to assign lesion placement in the hemisphere opposite the preferred limb.

Animals were lesioned with 16 μg of 6-OHDA delivered into the substantia nigra *pars compacta* of one hemisphere. Lesion methods were similar to those described in Appendix 2 but with the following exceptions. Rather than using pentobarbital with chloral hydrate boosters as the anesthetic, a mixture of ketamine 90 mg/kg and xyalzine 10 mg/kg was combined into a single injection and given i.p. to induce anesthesia. Boosters consisting of 30 mg/kg ketamine only were used as needed. Also, the stereotaxic coordinates for the injection differed (in order to target the nigra instead of the MFB) and were AP: -5.4 mm; DV: -7.6 mm (measured from dural surface); and ML: ±2.2 mm.

Following the lesion, rats were allowed to recover and were subsequently tested on the forelimb placing, limb-use asymmetry, and PIT tests at 1, 2, and 5 weeks post-lesion. Following the last testing session rats were sacrificed and their striata analyzed for levels of DA, DOPAC, and HVA via HPLC-ED as described in Appendix 2.

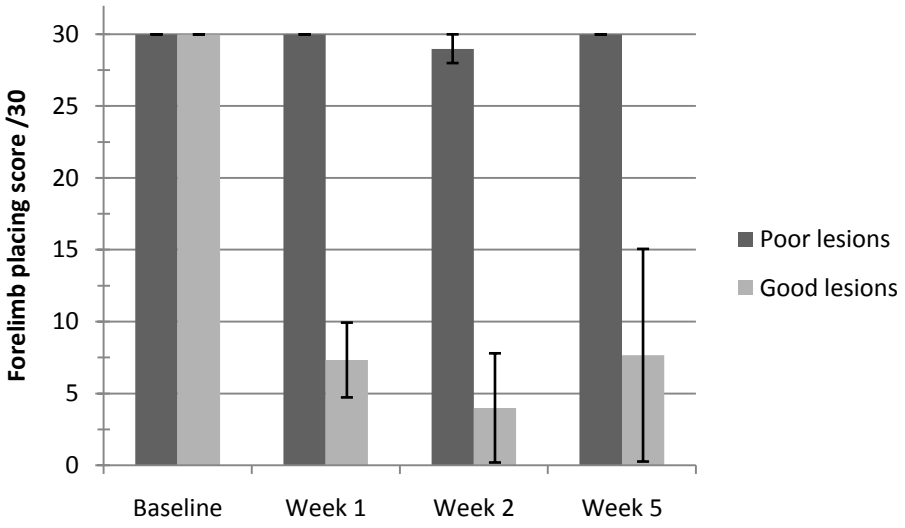
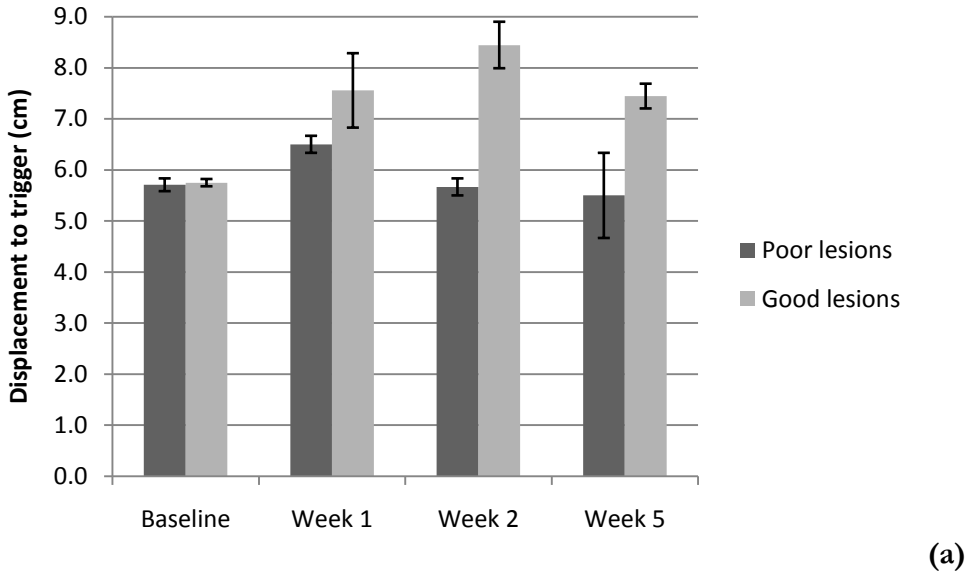
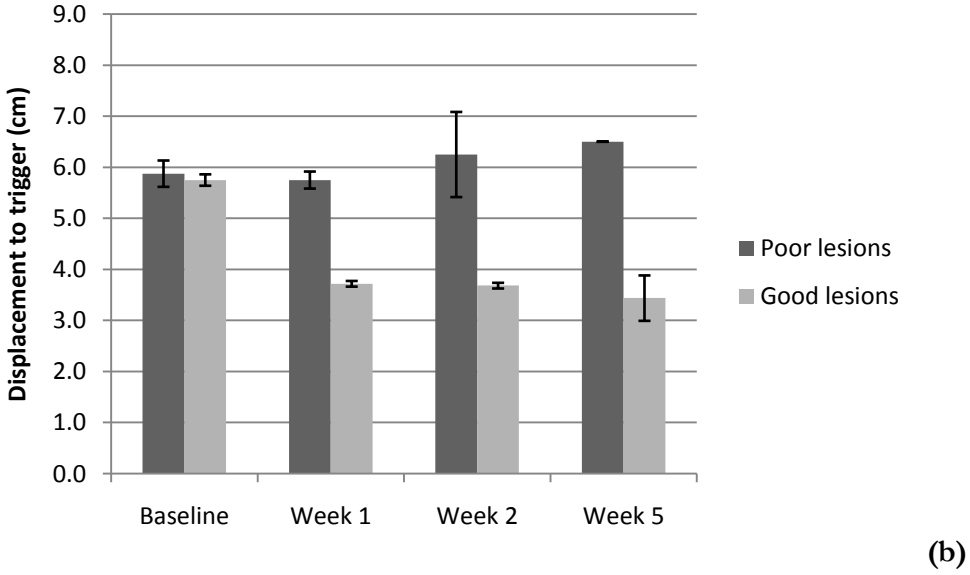


Figure 7.1 Performance in the forelimb placing test for rats lesioned with 6-OHDA in the substantia nigra instead of in the medial forebrain bundle

Delivery of 6-OHDA to the nigra, avoiding the potential nonspecific damage of delivering the toxin to the MFB, still adversely affects forelimb placing ability measured 1, 2, or 5 weeks after the lesion as long as the dopamine depletion is sufficiently severe. Data are means ± SEM.



(a)



(b)

Figure 7.2 Performance in the PIT test after 6-OHDA lesion of the substantia nigra for the contralateral limb **(a)** or the ipsilateral limb **(b)**

Nigral lesions show the same pattern as lesions of the MFB, with a slowing of reaction in the forelimb contralateral to the lesion coupled with enhanced reactivity in the ipsilateral limb. Date are means ± SEM.

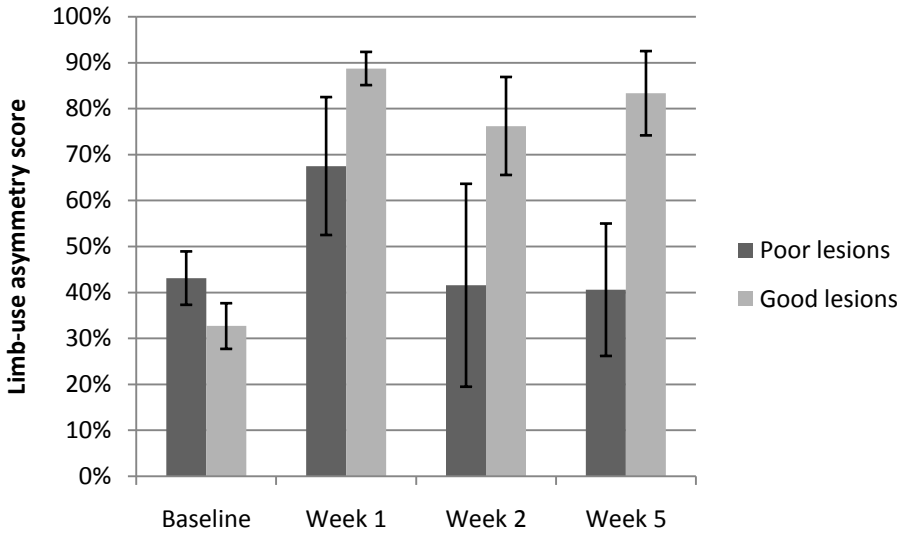


Figure 7.3 Performance in the cylinder test following 6-OHDA lesion of the substantia nigra

A score of 50% reflects symmetrical forelimb use while higher scores denote a greater reliance on the forelimb ipsilateral to the lesion. Provided the dopamine depletion is sufficient, lesions of the nigra create a forelimb use asymmetry similar to that produced by intra-MFB lesions. Data are means ± SEM.

		DA	DOPAC	HVA	Percent depleted
Well-lesioned animals	**Lesioned side**	2186 ± 1055	361 ± 243	273 ± 139	86% ± 6%
	Unlesioned side	15434 ± 1096	1648 ± 182	1085 ± 149	
Poorly-lesioned animals	**Lesioned side**	9194 ± 2683	1094 ± 342	706 ± 213	25% ± 2%
	Unlesioned side	10125 ± 1146	1047 ± 190	754 ± 153	

Table 7.1 Post-mortem striatal content of dopamine and its major metabolites in animals receiving 6-OHDA lesions of the substantia nigra *pars compacta*

Data are ng of substance per g of wet tissue weight, expressed as means ± SEM.

Results

Subgroups

Following HPLC analysis of striatal monoamine contents (results below), 2 of the 5 lesioned rats were found to have dopamine depletions of less than 70% relative to the unlesioned striatum. Thus, results are reported separately for well-lesioned rats (>70% depletion, n=3) and poorly-lesioned rats (<70% depletion, n=2). Because of the small number of animals present in each group, inferential statistics were not applied in this study. The point of this study is simply to show that postural instability symptoms can be elicited by specific lesion of the substantia nigra, so the presence of such symptoms in 3 lesioned animals relative to the extreme rareness of the symptoms in normal animals is taken as sufficient evidence that this occurs.

Behavioral outcomes

Results from the forelimb placing test are graphed in Figure 7.1 for the contralateral forelimb only (performance scores for the ipsilateral forelimb remained above 28 out of 30 for all animals throughout the study and are not shown). In animals with a >70% dopamine depletion, a marked reduction in forelimb placing scores is noted similar to that seen following lesions in the MFB.

Figure 7.2 shows performance in the PIT test after nigral lesion. Results are similar to those obtained in animals lesioned in the MFB, with the contralateral limb requiring more displacement before a step is triggered while the ipsilateral limb shows an enhanced reactivity. Interestingly, the ipsi-limb enhancement effect appears to be even more pronounced in these animals than in previous studies; however, more animals would be needed before one could conclude that this is an effect specific to nigral lesions.

Finally, scores from the limb-use asymmetry test shown in Figure 7.3 show that the lesion also affected performance on a now widely-used index of dopamine depletion which is known to be well-correlated with the extent of depletion (Schallert and Woodlee, 2004). The partial depletions in the "poor lesions" group appear to have caused a transient limb-use asymmetry which recovers by the second week post-lesion.

Striatal dopamine content

Table 7.1 shows the levels of dopamine and its metabolites measured in post-mortem striatal tissue. In animals classified as having "good lesions", the mean dopamine depletion amount was 86%. In MFB-lesioned animals this level of depletion is at the borderline of what would cause forelimb placing deficits to appear, but in all of these animals a placing deficit was noted in the limb contralateral to the lesion.

Discussion

6-OHDA lesions administered into the medial forebrain bundle, on which most of the studies in this dissertation are based, run the risk of inadvertently damaging other neural systems which send axons through the bundle. To eliminate the possibility that such surgically-induced secondary damage might artifactually be producing problems with postural stability, we took a small group of rats and administered the toxin directly into the cell body region of the substantia nigra *pars compacta*, thus reducing the possibility of creating secondary damage.

One interesting finding from this group of animals is the hint that lesions of the SNpc produce a greater "enhanced reactivity" in the PIT test in the forelimb ipsilateral to the lesion, compared to animals lesioned in the MFB. This is coupled with the observation of higher levels of striatal dopamine in the hemisphere contralateral to the lesion (Table 7.1), which may be tied to this behavioral observation. A compensatory increase in dopamine

levels contralateral to a 6-OHDA lesion would constitute a very interesting finding which would go a long way towards explaining our findings of enhanced function in the PIT test (see Experiment 2). Indeed, there are other reports in the literature indicating that these sorts of compensatory dopaminergic changes can occur (Warenycia and McKenzie, 1987; Robinson and Whishaw, 1988), but such results are by no means consistently found. More study may be warranted into why changes in overall dopamine levels in the hemisphere contralateral to a lesion are seen in some studies but not others.

Despite infusing the toxin into this more dopamine-specific region, animals in this study showed the same types of behavioral deficits as those lesioned in the MFB. HPLC analysis confirmed a significant loss of striatal dopamine content in those animals displaying the more prominent behavioral deficits. This evidence partially counters the argument that non-dopaminergic damage caused by the surgical procedure was producing the forelimb placing deficit.

However it is possible that some damage was caused, even in the procedure used in this study, to the GABAergic cells of the substantia nigra *pars reticulata*. In the rat, the pancake-shaped *pars compacta* portion of the nigra, containing the dopaminergic cells, is draped over the thicker, more ovoid shape of the *pars reticulata* (Paxinos and Watson, 1998), thus making it hard to target only the compacta without having some error along the dorsal-ventral axis which might lead to the infusion of some toxin into the reticulata. Indeed, that this occurs may be indicated by the lack of a dopamine depletion in two of the five animals that we infused according to the present surgical procedure. However, 6-OHDA is still putatively specific to catecholaminergic neurons, which the reticulata is practically devoid of. Furthermore, due to the larger size of the reticulata, even a "stereotaxic miss" along the DV axis would still only lead to infusion of 6-OHDA into a relatively small part of the overall structure, meaning that the reticulata would have to be exquisitely sensitive to damage for

such a severe placing deficit to be observed. This remains an interesting possibility, however, because as we will see in the next experiment, changes in the reticulata may in part be tied to the deficits in postural stability seen in the 6-OHDA model.

Perhaps a more likely possibility is that damage to the dopamine neurons themselves, regardless of how it occurs, leads to a change in other systems related to the nigrostriatal pathway, which is more directly related to the observation of postural stability deficits in lesioned animals. In the next chapter, we turn to a search for any such secondary damage that may be occurring as a result of dopamine cell loss, but outside of the nigrostriatal pathway itself.

Chapter Three: Attempts to identify plastic changes resulting from dopamine depletion that may contribute to postural instability

CHAPTER OVERVIEW

The results of the experiments reported in Chapter Two led us to the conclusion that postural instability may be tied to a significant non-dopaminergic change, which likely occurs as a result of the initial 6-OHDA-induced dopamine depletion.

In this chapter, we seek evidence for such changes. In Experiment 8, we will look at neuronal numbers in the striatum and the substantia nigra *pars reticulata* for evidence of cell loss in these regions several weeks post-lesion, and will attempt to determine if any such loss can be related to the problems with postural instability seen in our model. In Experiment 9 we will use pharmacological methods to block a now-established mechanism on non-dopaminergic change following dopamine depletion, namely the loss of dendritic spines on striatopallidal medium spiny neurons, to determine whether postural impairments can be prevented by such treatment.

Because PI remains as one of the most intractably hard-to-manage symptoms of PD, elucidating its underpinnings might lead to therapies very valuable to those in the later stages of the disease. Understanding if and how non-dopaminergic changes give rise to this problem may allow for the opening of new therapeutic avenues which seek to prevent these secondary changes. Importantly, if future work is able to show that such secondary changes do result quite directly from dopamine loss, such results would lend an important new voice to the debate over how early and aggressively dopamine-replacement therapies should be pursued in persons with PD, since it is possible that maintaining dopaminergic tone beginning early in the disease process could delay or prevent detrimental secondary changes.

EXPERIMENT 8: THE EFFECTS OF DOPAMINE DENERVATION ON NEURONAL NUMBERS IN DOWNSTREAM BRAIN REGIONS

Introduction

Based on the results of Chapter Two, we conclude that the postural instability seen in the 6-OHDA rat model of PD may be connected to a substantial non-dopaminergic component. As a result, we decided to undertake this study to determine whether dopamine depletion leads to a secondary loss of non-dopaminergic neurons in two key basal ganglia structures, the striatum and the substantia nigra *pars reticulata* (SNpr).

The finding of outright death of non-dopamine neurons following specific 6-OHDA lesions would be a novel one, though it would follow the findings of Ariano et al. (2005), who noted increases in markers of apoptotic activity in the striatum following partial 6-OHDA lesion. In the present study, the striatum was selected as one site for investigation on the basis of Ariano's work, and because striatal cells are the direct target of the dopamine innervation lost following 6-OHDA lesion. We also investigated neuron number in the substantia nigra *pars reticulata*, as neurons there have been shown to degenerate following lesions to certain cortical areas or to the intrinsic neurons of the striatum, indicating that this site may be a useful proxy marker of secondary degeneration following lesions in corticostriatal motor systems (Saji and Reis, 1987; Schallert et al., 1990; Schallert and Lindner, 1990; DeGiorgio et al., 1998).

In this experiment, stereological methods were used to evaluate neuron numbers in brain structures related to but outside of the nigrostriatal dopamine system, to determine whether 6-OHDA lesions result in the loss of neurons other than those dopamine neurons targeted by (and traditionally assumed only to be affected by) the lesion. If non-dopaminergic degeneration occurs in response to 6-OHDA-induced dopamine depletion, perhaps such secondary degeneration is responsible for the development of postural

instability in our model, and we further investigated this possibility by examining the relationship between neuronal number in the SNpr and behavioral outcomes in our tests of postural instability. If the presence of non-specific damage can be confirmed, future work can focus on the prevention of such secondary damage as an additional endpoint in determining the effectiveness of potential preventive treatments for PD.

Methods

Subjects

Adult male Sprague-Dawley rats (n=24) weighing 400-500 g and obtained from a local colony were used for the studies. They were housed in pairs in clear polycarbonate cages with sawdust bedding and kept on a 12:12 light:dark cycle. All experimental manipulations were performed during the dark phase of their light cycle.

Surgeries and behavioral testing

Prior to surgery, all rats were acclimated to the forelimb placing and PIT tests, and baseline-tested in the limb-use asymmetry ("cylinder") test in order to assign lesion sides opposite the preferred limb (see Appendix 2 for behavioral test methods). All rats were lesioned with 7 μg of 6-OHDA delivered unilaterally into the medial forebrain bundle, following the methods detailed in Appendix 2.

Following surgery, rats were tested on the forelimb placing, PIT, and limb-use asymmetry tests at 2 and 6 weeks post-operatively. Four of 20 rats did not show reliable behavioral deficits consistent with a successful 6-OHDA lesion and were excluded from further study. Rats were sacrificed within one week of the last testing session.

Tissue preparation

Eleven of the 20 remaining rats were deeply anesthetized with pentobarbital (75 mg/kg, i.p.) and transcardially perfused with a 0.1 M phosphate buffer (PB) rinse followed by a solution of 4% w/v paraformaldehyde in 0.1 M PB as fixative. Brains were removed and postfixed at 4ºC for approximately 48 h in the same fixative. Brains were then sectioned coronally at 100 μm on a vibratome, with slice collection starting just anterior to the striatum and ending approximately mid-way through the hippocampus. Every third section was mounted on slides and stained with Toluidine blue (a Nissl stain) to allow visualization of neurons.

The remaining nine rats were anesthetized with halothane and decapitated. The brains were removed and cut along a coronal plane approximately half-way through the hippocampus. From the anterior brain half, the left and right striata were dissected out for subsequent analysis of striatal monoamine contents via HPLC, as detailed in Appendix 2. The posterior brain half was dropped into a solution of 4% w/v paraformaldehyde in 0.1 M PB and allowed to post-fix in a refrigerator at 4ºC for 48-72 h, after which the tissue was sectioned coronally at 60 μm on a vibratome, and slices containing the substantia nigra were collected. Every third section was mounted on slides and stained with Toluidine blue (a Nissl stain) to allow visualization of nigral neurons.

Neuron quantification

Striatum

In slices through the striatum taken from the first set of eleven animals, stereological techniques were used to estimate the volume of and total number of neurons in each striatum (i.e., contralateral and ipsilateral to the 6-OHDA lesion). For the analyses, the first seven slices were used, starting on the anterior end with the first slice that contained a joined corpus callosum. Therefore, since slices were collected every 300 μm, the set of slices used

represented the anterior 2.1 mm of the striatum, corresponding to the region where the borders of the striatum are most easily identifiable and thus readily subject to volume analysis.

In these slices, the volume of the anterior 2.1 mm of each striatum was estimated using a version of the method of Cavalieri (Henery and Mayhew, 1989). Volume was computed with the equation $V = T \times \sum a$, where T is the average distance between slices (300 μm in this case), and a represents the projected surface area of the striatum in each slice. The area of the striatum in each slice was determined by using Nikon's NIS Elements: Basic Research software on digitally-captured photomicrographs of each slice following calibration (via use of a stage micrometer) of the 1x objective used to capture the images, such that the real area of manually-outlined portions of the digital image could be calculated by the software. Using this setup, the striatum of each hemisphere was outlined by the experimenter in each slice, and the computer-calculated area of the outlined striatum was recorded.

Optical dissector techniques (West and Gundersen, 1990; Harding et al., 1994) were then used to estimate the total number of neurons in each striatum. Sampling frames were selected within each striatum by overlaying a uniform grid on the zoomed-out digital photomicrograph of each brain slice. A random number x between 1 and 40 was selected, and (counting only grid intersections that fell within the boundaries of the striatum), intersections of the overlaid grid were counted off from left-right and top-bottom (e.g., "like reading a book") until the x^{th} grid intersection was reached. The area where this grid intersection fell was then centered in the field of view and zoomed in upon by switching to the 100x objective on the microscope. An unbiased sampling frame was then superimposed upon the zoomed-in image. Within the sampling frame (the area of which is known based on software calibration against the microscope objective being used), the total number of

neurons is counted. The sampling frame included two inclusion lines and two exclusion lines for determining whether neurons that touched the edges of the frame would or would not be counted. The top edge of the slice was then brought into focus and neurons "cut off" at this boundary were counted and subtracted from the total number of neurons counted in that frame. Following counting from the first frame, the "zoomed out" slice image was returned to, and grid intersection counting resumed until the 40th grid intersection past the previously-targeted intersection was located, whereupon neurons were again counted at high magnification at the new intersection. If the number of remaining intersections counted within the current slice did not reach 40, the next slice was brought into view and counting resumed on the new slice until reaching the 40th intersection past the intersection where the last sampling frame was taken. Sampling frame selection and counting continued in this manner throughout the seven striatum-containing slices obtained from each animal. On average, 1.2 sampling frames were quantified from each slice.

From this data, neuronal density is then calculated using the equation:

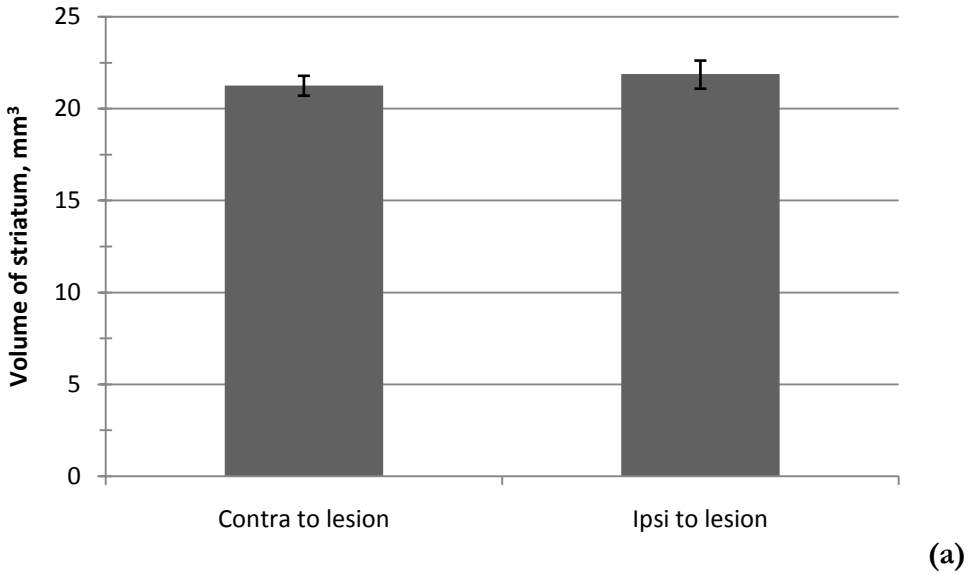
$$Nv_{neurons} = \Sigma Q- / \Sigma v(frame)$$

Where $\Sigma Q-$ represents the total number of neurons counted within the sampling frames for that particular striatum and $\Sigma v(frame)$ is the aggregate volume of all of the sampling frames used, which is calculated as the (known) sampling frame area multiplied by the nominal slice thickness, multiplied by the number of frames used in each striatum (the mean number of frames used within each striatum in this study was 8.6). Given values for the volume (as determined earlier) and neuronal density in each striatum, total neuron number is then estimated with the equation:

$$N_{neurons} = V_{ref} * Nv_{neurons}$$

Substantia nigra *pars reticulata*

In contrast to the striatum, stereological techniques were not used for the SNpr. In this small region, the neurons are large and readily identifiable even at lower magnifications, and it was not difficult to count all of the neurons visible in each slice. Therefore, starting with the most posterior slice containing a well defined substantia nigra *pars compacta* and continuing for three additional slices anterior (i.e., four slices total), the total number of visible neurons in each SNpr was counted. The total number of neurons counted from the four slices in each SNpr was summed for each hemisphere.



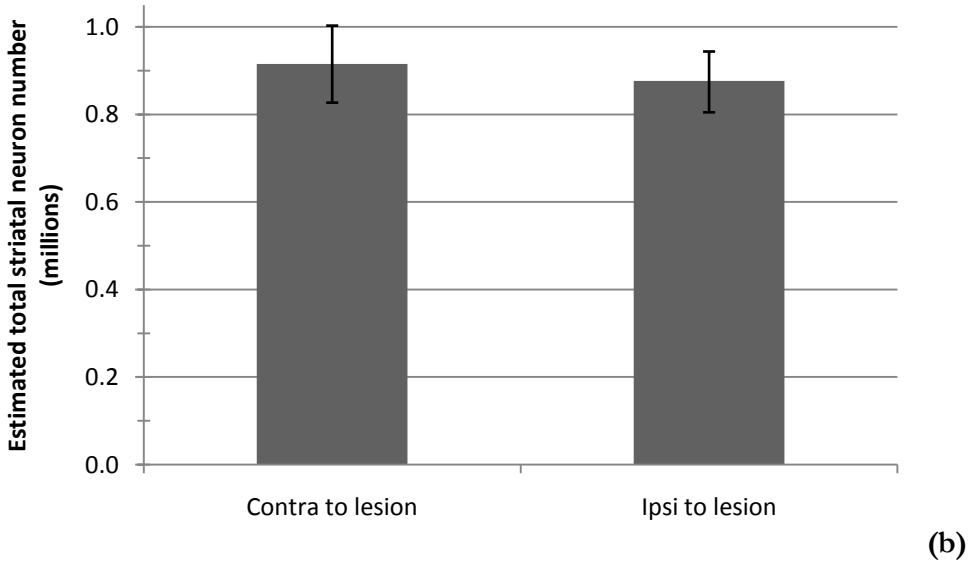


Figure 8.1 Volume of the anterior 2.1 mm of the striatum **(a)** and estimated total number of neurons in that region of the striatum **(b)** following unilateral 6-OHDA lesion of the MFB in animals with confirmed postural stability deficits

Differences are not statistically significant. Data are means ± bootstrapped **95%** confidence intervals.

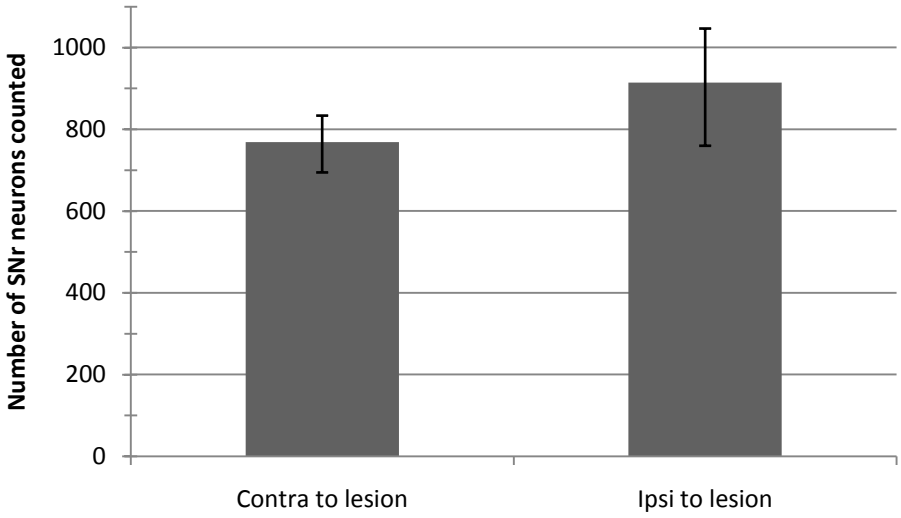


Figure 8.2 Total number of neurons counted in slices through the SNpr

Surprisingly, the apparent number of neurons was *higher* on the lesioned side than on the "normal" side. It is not clear whether this reflects neuronal hypertrophy in the lesioned hemisphere or the loss of neurons opposite the lesion. The difference, however, did not quite reach significance ($p=0.09$ by bootstrapped paired-samples t-test). Data are means ± bootstrapped **95%** confidence intervals.

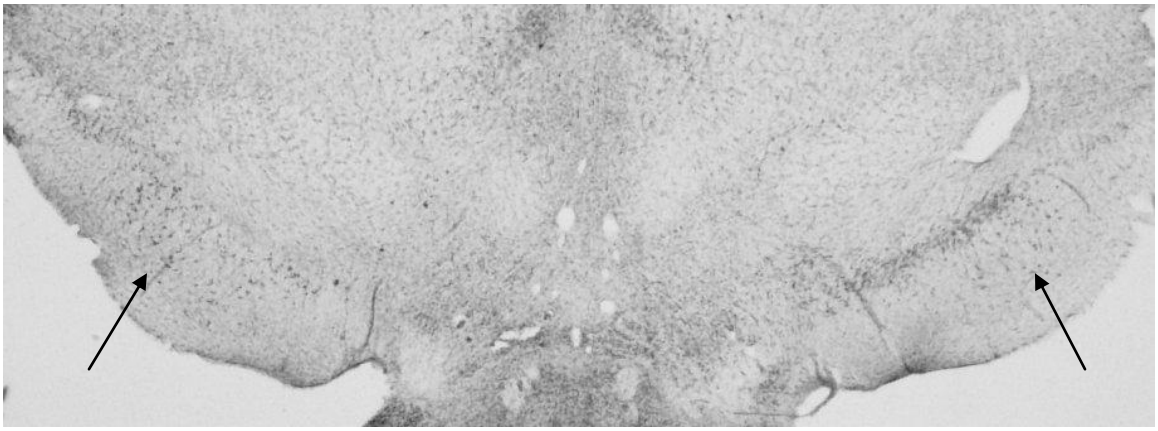


Figure 8.3 Photomicrograph of one of the sections used to quantify neurons in the substantia nigra *pars reticulata*

In this representative section a smaller number of SNpr neurons can be observed underneath the intact, densely-stained dopaminergic neurons of the SNpc on the right side, as compared to the larger number visible under the lesioned SNpc on the left.

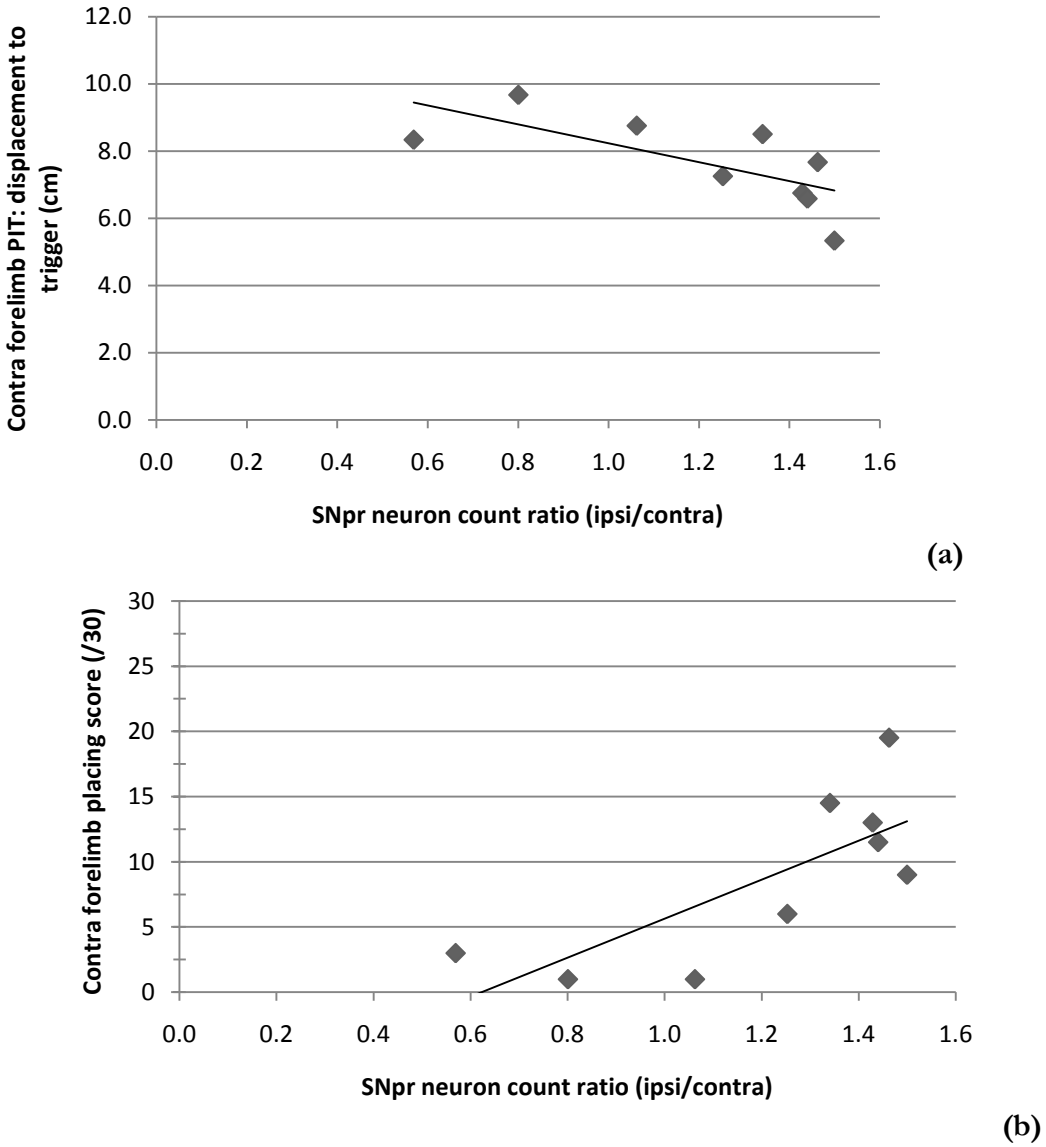


Figure 8.4 Relationship between the ipsi-to-contra hemispheres ratio of SNpr neuron counts and **(a)** performance of the contra limb in the PIT test and **(b)** performance of the contra limb in the forelimb placing test

Note the surprising number of cases where the ipsi/contra neuron count ratio is above 1.0. Both correlations are significant at $p < .05$.

Results

Striatal volume and neuron number

Figure 8.1 shows striatal volumes and the estimated total number of striatal neurons for the anterior 2.1 mm of the striatum in the eleven rats lesioned unilaterally with 6-OHDA and later confirmed to have a contralateral deficit in our tests of postural stability. Neither the volume of the striatum nor the total number of neurons contained therein differed between the lesioned and unlesioned hemispheres of these animals (using paired t-tests—volume: $t(10)=1.34$, $p=0.21$; number: $t(10)=0.78$, $p=0.45$), indicating that postural stability deficits are not tied to the outright loss of the striatal cells which lose their dopamine input as a result of the 6-OHDA lesion.

Striatal dopamine content

In the set of nine rats from which HPLC data were gathered, all rats but one were found to have striatal dopamine depletions of >98% relative to the unlesioned hemisphere. Because of this high-end clustering of lesion severity, the HPLC data was not used further, e.g., for correlations against behavior or SNpr cell counts. The one "outlying" animal had a depletion of 87%, and was roughly in the middle of the pack regarding forelimb placing behavior (scoring 9 out of 30 when averaged across week 2 and week 6 performance), but appeared unimpaired in the PIT test (5.3 cm displacement needed on the contra side). Interestingly, despite this animal's lower depletion, he had the highest ipsi/contra SNpr neuron count ratio (1.50; see below) of all animals studied.

SNpr neuron counts and relation to behavior

Figure 8.2 shows the mean number of neurons counted in the hemispheres ipsilateral or contralateral to the 6-OHDA lesion. Figure 8.3 shows a slice through the substantia nigra representative of those used for counting. Surprisingly, the hemisphere ipsilateral to the

lesion appeared to have more SNpr neurons, but this difference did not quite reach significance (bootstrapped paired-samples t-test: $t(8)=1.80$, $p=0.09$).

Variability in SNpr neuron counts was quite a bit higher in the hemisphere ipsilateral to 6-OHDA lesion, indicating that comparing these values to behavioral performance might yield an interesting correlation. Several correlations were run comparing either ipsilateral SNpr neuron count or the ratio of ipsilateral to contralateral neuron count to behavioral performance in either the ipsi or contra limb in the PIT test, or in the contra limb in the forelimb placing test (the ipsi limb, as usual, remained unimpaired in the placing test). The most interesting relationships are shown in Figure 8.4. The performances of the contralateral limb in the PIT test (panel A) and the forelimb placing test (panel B) were both well-correlated with the ipsi/contra SNpr neuron count ratio (PIT: Pearson's $R=0.692$, $p=0.039$; placing: $R=0.762$, $p=0.017$).

Discussion

In humans with PD, MRI studies have demonstrated atrophy of the putamen beginning in early stages of the disease, with later stages also sometimes producing volume loss in the globus pallidus (Lisanby et al., 1993; Geng et al., 2006). It has not previously been clear whether degeneration in non-dopaminergic subcortical structures is a result of dopamine loss *per se*, or whether it is part of a broader degenerative process seen only in idiopathic human PD (Braak et al., 2003; Braak et al., 2004). Rat models employing dopamine-specific lesions caused by 6-OHDA have an opportunity to shed light on this question, and on the relationship between any such degeneration and the symptoms of postural instability.

In our study we found no evidence of changes in either the volume or estimated neuron number in the ipsilesional striatum of rats lesioned with 6-OHDA, despite the

confirmed presence of postural stability impairments in these animals. Other studies have demonstrated striatal neuron loss due to conditions which elevate dopaminergic tone in the striatum (Jakel and Maragos, 2000; Cyr et al., 2003), but since dopamine was depleted in our study it is perhaps not surprising that we noted no changes in this brain area.

In contrast, the results of neuron counting in the SNpr led to the surprising finding that neuron numbers appear to be somewhat elevated in the nigra ipsilateral to the lesion, compared to the contralateral side. Whether this reflects a loss of neurons contralateral to the lesion or neuronal hypertrophy in the ipsilateral SNpr is not clear. Changes in the SNpr have previously been shown to be a sensitive marker of disruption in other brains areas, particularly in models of cortical or intrinsic striatal damage (Krammer, 1980; Schallert et al., 1990; Schallert and Lindner, 1990; Jones and Schallert, 1992a; Stefanis and Burke, 1996). Others have previously noted increases in markers of SNpr GABAergic activity following dopaminergic denervation (Vila et al., 1996; Vila et al., 1997), but this would seem to be the first observation of a possible increase in neuron numbers in the SNpr in response to dopamine depletion. However, it has been shown that inactivation or lesioning of the SNpr can improve behavioral deficits in PD models, and in particular various types of postural impairments (Wichmann et al., 2001; Henderson et al., 2005), so an increase in relative GABAergic activity in the SNpr ipsilateral to a dopamine depletion may indeed be tied to the development of postural deficits. We found that the variability in neuron number was considerably higher in the hemisphere ipsilateral to the lesion, such that changes in that hemisphere were the primary driver in the wide range of ipsi/contra cell number ratios (see Figure 8.4) that we observed. Though more studies will be needed to show this conclusively, it appears as though this ratio increases well above 1.0 with less-severe lesions, but as the lesion become more complete the ratio decreases back towards and then even below 1.0.

Another possible explanation for our findings, especially given the aforementioned known increase in SNpr neuronal activity following dopamine depletion, is that the cells in the SNpr grew larger on the side ipsilateral to the lesion. Because we did not use an unbiased stereological estimator such as the optical dissector in this case, but rather counted all visible cells, such a discrepancy in soma size between the two sides would artificially increase the count on the side with larger somata (i.e., due to the greater likelihood that cells were counted which were sliced through at either face of the tissue slice under analysis). Future studies should employ unbiased estimators of neuron number to see if the effects found here continue to hold or, perhaps more interestingly, also analyze the size of SNpr neurons to see if this variable may also change in response to dopamine depletion.

We found that this ratio of ipsi/contra SNpr neurons was well-correlated with performance in our two tests of postural instability (Figure 8.4). Those animals with the most severe deficits were those in which the number of neurons ipsi to the lesion was lower than the number contra, especially in the case of the forelimb placing test. This may indicate that in some way an increase in the ipsi/contra ratio is a compensatory mechanism that the brain employs in response to the dopamine depletion, which is ultimately overwhelmed with more severe insults. It is, we think, very interesting indeed that this marker is well-correlated with behavior despite the fact that almost all animals in this study had >98% dopamine depletions as measured via HPLC. Referring back to Figure 3.3 we can see that there is indeed a great deal of variability in postural stability performance even among the most severely dopamine-depleted animals, and further studies may show that this is accounted for by events occurring in the SNpr. Though PI is resistant to therapy with L-DOPA or other dopaminergic drugs in those with PD (Zetusky et al., 1985), newer surgical therapies for the disease do show some efficacy in treating this symptom (Bronte-Stewart et al., 2002; Bakker

et al., 2004; Hamani et al., 2005), possibly reflecting their effect on the SNpr and other connected subcortical non-dopaminergic structures.

Of course, why SNpr dysfunction occurs in response to dopamine depletion is not yet clear, and further studies will need to be done to determine whether this is a result of dopamine depletion in particular, rather than a simple artifact of the experimental lesioning procedures used. It appears as though the SNpr will be a promising target for the study of the neural mechanisms underlying the genesis of postural instability in PD, and because the cell population of this region is primarily GABAergic, future studies employing GABA-based pharmacotherapies might yield interesting outcomes.

EXPERIMENT 9: THE BEHAVIORAL EFFECTS OF PREVENTING LESION-INDUCED STRIATAL DENDRITIC SPINE LOSS USING NIMODIPINE

Introduction

Evidence from Chapter 2 rules out a number of possible explanations for the performance differences between 6-OHDA-lesioned and dopamine antagonist-treated animals on tests of postural stability. A remaining and increasingly well-supported hypothesis is that some behavioral symptoms of PD do not arise as a direct result of the disruption of dopamine systems but instead, such disruption triggers some form of plastic or degenerative change in other brain systems which may be more directly involved in the observed behavioral changes (Ariano et al., 2005; Day et al., 2006; Miklyaeva et al., 2007; Solis et al., 2007). This hypothesis may also help explain the variation in response to dopaminergic therapies seen among the various symptoms of PD in humans (Chaudhuri et al., 2006; Brooks, 2007; Williams-Gray et al., 2007), and secondary non-dopaminergic changes have also been implicated in the pathogenesis of L-DOPA-induced dyskinesias

(Jenner, 2000; Cenci and Lindgren, 2008), which constitute perhaps the biggest challenge in current pharmacotherapy of PD.

One well-documented consequence of some types of brain insult is a change in dendritic morphology and/or in the spines of those dendrites. Jones and Schallert (1992b, 1994) were the first to note this phenomenon in response to damage to sensorimotor areas of the neocortex, following which an apparently compensatory overgrowth and subsequent pruning of dendrites in the contralateral homotopic sensorimotor cortex was observed. Other studies showed that reactive dendritic changes can also occur in PD models. Striatal medium spiny neurons, which are the primary target of the nigral dopamine neurons lost in PD, atrophy in the disease (McNeill et al., 1988; Zaja-Milatovic et al., 2005), and neurons of the substantia nigra *pars compacta* also show dendrite shortening and loss of dendritic spines (Patt et al., 1991). Importantly, significant changes in the striatum are seen with regards to glutamatergic corticostriatal connections (Anglade et al., 1996; Ingham et al., 1998; Stephens et al., 2005; Solis et al., 2007) and not just at spines that receive dopamine input, indicating that plastic processes are not confined only to areas that are directly adjacent to the degenerating nigrostriatal dopamine pathway. Indeed Miklyaeva et al. (2007) found that 6-OHDA lesions can even lead to an increase in dendritic arborization in motor cortical pyramidal cells in the hemisphere contralateral to the lesion. It is not yet clear whether these secondary changes result directly from the dopamine denervation or are themselves a secondary result of increased behavioral demand on the unimpaired limb in unilateral lesion models, but in any case the implication that targeted lesioning of the nigrostriatal pathway can lead to widespread brain structural changes is clear.

In the wake of these findings and amid a general lack of understanding of how exactly dopamine loss leads to the some of the observed behavioral deficits in PD, Day et al. (2006) published results confirming that dopamine depletion leads to a rapid loss of spines

and glutamatergic synapses onto GABAergic striatopallidal medium spiny neurons (see also Ingham et al. (1998)) and, furthermore, it is possible to block this spine loss by administering an L-type calcium channel blocker (nimodipine) during the first couple of weeks after a dopamine lesion is experimentally administered. Using multiphoton imaging they make a convincing case that such spine changes result directly from dopamine system disruption due to either 6-OHDA or administration of reserpine, though their study is lacking an analysis of behavioral outcomes. In this experiment, borrowing from the work and methods of this research group, we set out to similarly block these striatal spine changes using nimodipine, and then to behaviorally assess the development of postural instability symptoms. If changes in striatopallidal dendritic spines do indeed contribute to the development of PI, our hypothesis was that their blockade should spare the animals from developing PI, and possibly other behavioral deficits seen in the 6-OHDA PD model.

Methods

Groups

Animals (n=16 male Sprague-Dawley rats from a local colony, aged 11 months at the time of surgery and housed in pairs on a 12:12 h light cycle with food and water available *ad libitum*) were lesioned unilaterally with 7 μg of 6-OHDA delivered into the MFB as described in Appendix 2. Two days after the lesion surgery, the animals were ranked based on performance in the limb-use asymmetry ("cylinder") test and divided into two groups of eight in a counterbalanced manner based on the results. The two groups were assigned to receive either a subcutaneously-implanted slow-release pellet designed to continuously release 1 mg/kg/day nimodipine (for 21 days) or a control pellet (following the methods of (Day et al., 2006).

Drug treatment

The pellets (nimodipine-containing or vehicle "blanks") were implanted two days after the 6-OHDA lesion (i.e. on the same day that the group-determining limb-use asymmetry test was performed), under brief halothane anesthesia accompanied by long-duration local anesthesia using Marcaine. Pellets were implanted by shaving and then making a small incision in the dorsal neck scruff, gently separating connective tissues underneath, and inserting the pellet into the newly-formed "pocket" before suturing the wound and applying Neosporin.

Behavioral testing

Animals were tested twice in the week prior to surgery on the PIT, forelimb placing, and limb-use asymmetry tests (see Appendix 2 for methods) as a baseline. Performance in these tests was also evaluated at 1, 2, and 6 weeks post-lesion. Eight weeks after the lesion, animals were sacrificed and their striata dissected out for HPLC evaluation of monoamine content as described in Appendix 2. Any observed differences in behavior between the two groups combined with hypothetically similar degrees of dopamine depletion will be interpreted as evidence that the specific plastic changes blocked by nimodipine (i.e., dendritic spine loss in the striatum) contribute to the symptoms of PD reflected by the tests used in this study.

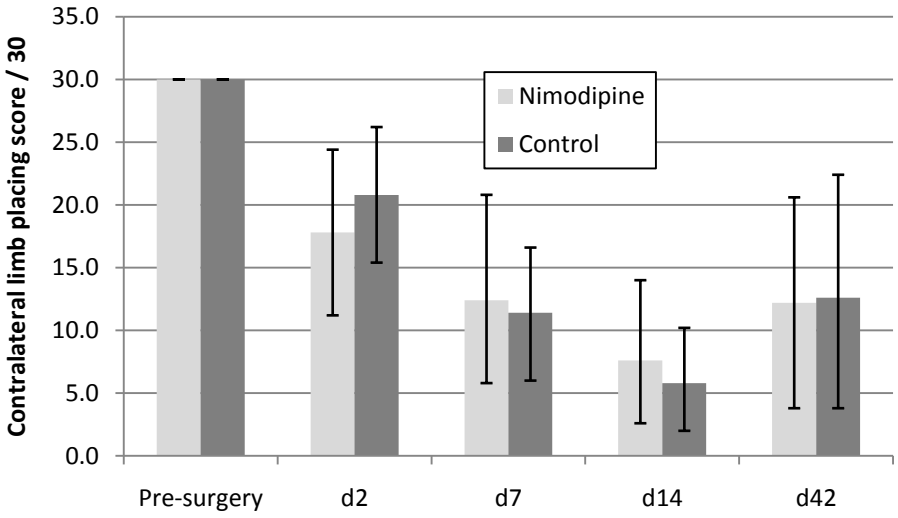


Figure 9.1 Performance of the contralateral limb in the forelimb placing test is not affected by administration of nimodipine

Data is not shown for the ipsilateral limb which, as expected, remained unaffected by the unilateral 6-OHDA lesion used. A typical worsening in the performance of both groups can be seen across the first two weeks post-lesion. Interestingly, both groups recover some limited placing ability between the second and sixth weeks post-lesion. An ANOVA showed no differences between the two groups. Data are means ± bootstrapped **95%** confidence intervals.

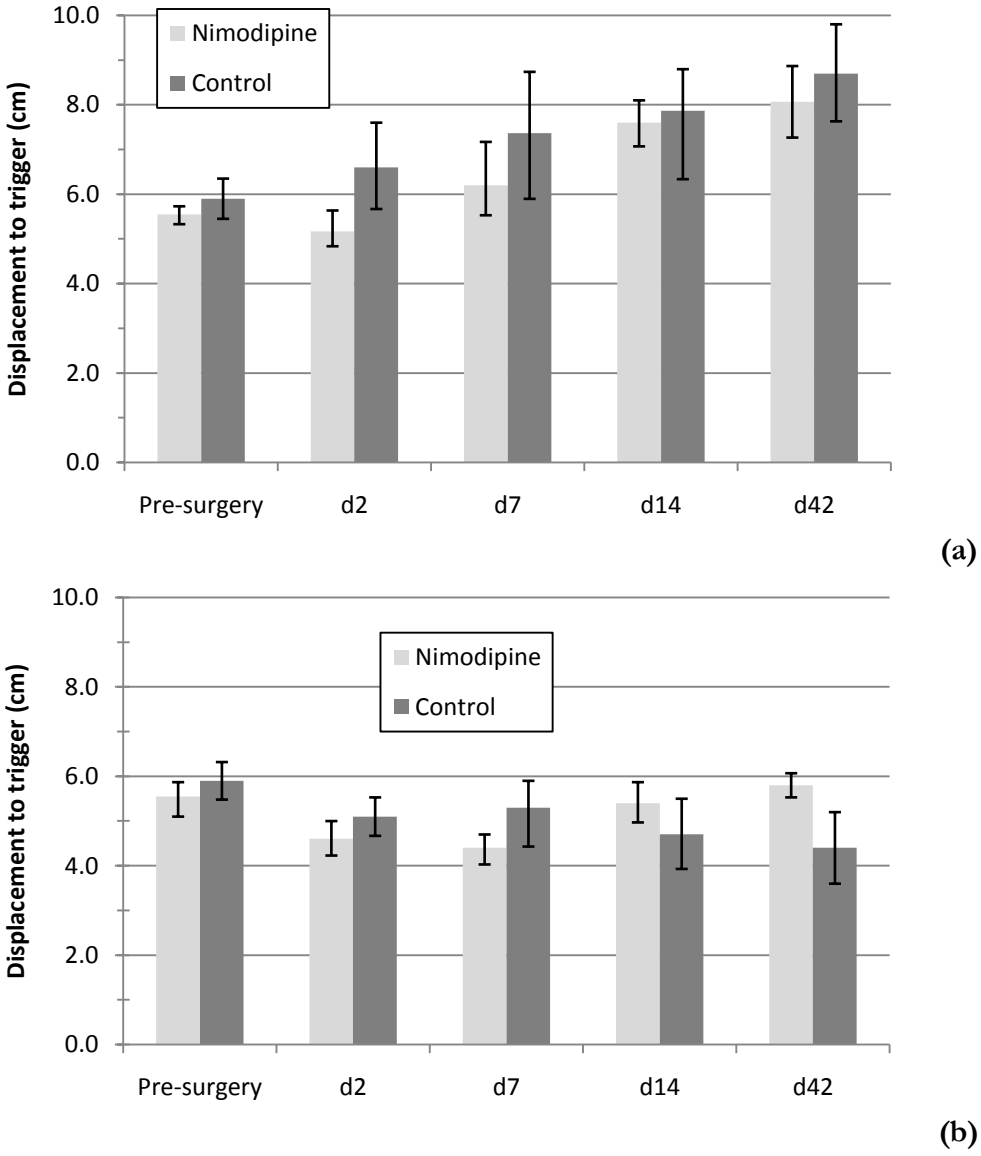


Figure 9.2 Performance of the contralateral **(a)** and ipsilateral **(b)** forelimbs in the PIT task in animals treated with nimodipine versus controls

For the most part, performance of the contralateral forelimb degrades with increasing time post-lesion, except that in nimodipine-treated animals there appears to be a sparing of behavior at earlier time points. The ipsilateral limb shows the expected "enhancement effect" (see Experiment 2) early after the lesion, though this seems to disappear later in the nimodipine-treated group. ANOVAs showed no differences between the groups. Data are means ± bootstrapped **95%** confidence intervals.

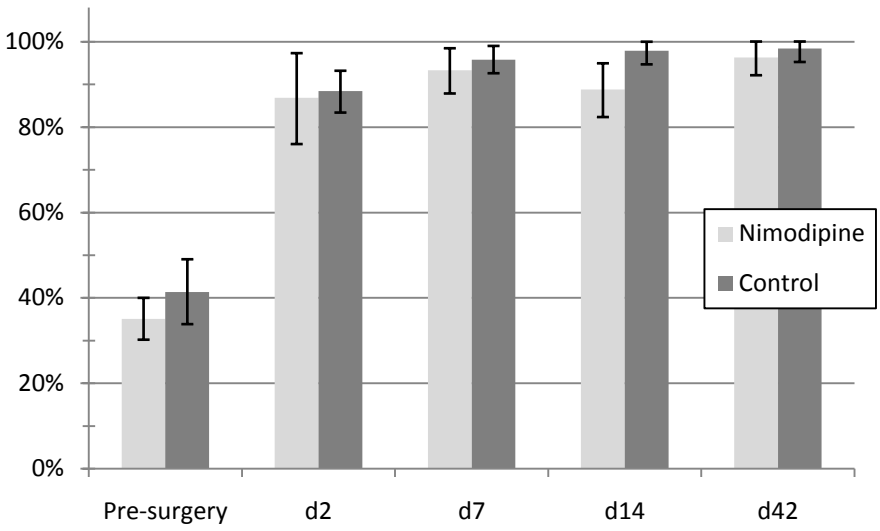


Figure 9.3 Performance in the limb-use asymmetry ("cylinder") test following 6-OHDA lesions in nimodipine- versus control-treated rats

A score of 50% would indicate unbiased use of the two forelimbs while scores nearer 100% indicate greater impairment of the contralateral-to-lesion forelimb. Nimodipine administration does not affect performance on this test. Data are means ± bootstrapped **95%** confidence intervals.

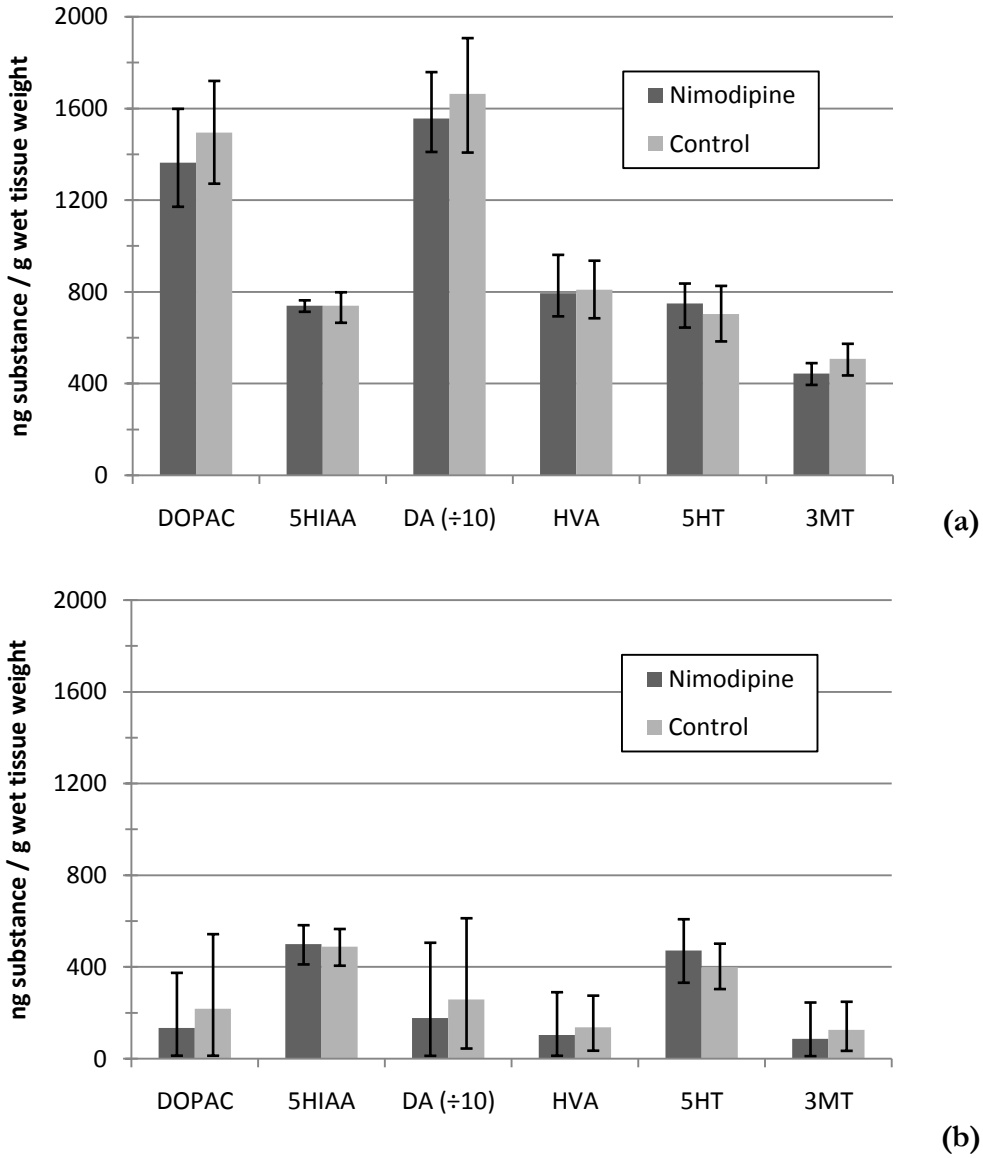


Figure 9.4 Post-mortem concentrations of dopamine, serotonin, and their metabolites in the dorsal striatum as determined by HPLC-ED

Values from the "normal" striatum are in panel **(a)**; panel **(b)** reflects values from the lesioned side. (Dopamine values are divided by 10 to place them on the same scale as the other substances.) Data are means ± bootstrapped **95%** confidence intervals.

Results

Forelimb placing test

Performance of the contralateral forelimbs in the forelimb placing test is charted in Figure 9.1. The forelimb ipsilateral to the lesion displayed normal placing throughout the study in both groups, so data from this limb have been omitted for succinctness. Data for this test and PIT test (below) were analyzed using a 2-way ANOVA with post-op time as a within-subjects repeated measure and treatment group as a between-subjects factor. The main effect of post-op time was significant ($F(4,32)=19.9$, $p<.001$), but there was no main effect of treatment group ($F(1,8)=.001$, $p=0.97$) or an interaction effect ($F(4,32)=0.2$, $p=0.93$). Interestingly, rats appeared to recover a limited amount of forelimb placing ability by the sixth week post-lesion.

Postural instability test

Performance of each forelimb in the postural instability test is graphed in Figure 9.2. The forelimb contralateral to the lesion displayed a steadily worsening impairment in the task (panel A), regardless of group; however, nimodipine-treated rats did show limited sparing of behavioral impairments in this limb at the earlier post-lesion time points examined (e.g., 2 d and 14 d post-lesion). For the contra limb, the ANOVA showed a main effect of post-op time point ($F(4,32)=19.9$, $p<.001$) but no effect of group ($F(1,8)=2.0$, $p=0.19$) nor an interaction ($F(4,32)=0.98$, $p=0.43$). The ipsilateral forelimb showed the enhanced post-lesion reactivity first explored in Experiment 2 in both groups (panel B); however, by the end of the experiment the enhanced reactivity disappeared in the nimodipine-treated group. For the ipsi limb, the ANOVA also showed a main effect of post-op time point ($F(4,32)=8.4$, $p<.001$) but no effect of group ($F(1,8)=.002$, $p=0.97$) nor an interaction ($F(4,32)=0.94$, $p=0.45$).

Limb-use asymmetry test

Asymmetry scores from the limb-use asymmetry test are plotted in Figure 9.3. Both groups displayed immediate and severe asymmetry in this test (which is expected, as work in our lab has shown this test to be among the most sensitive to even partial depletions of striatal dopamine (Schallert and Tillerson, 2000; Tillerson et al., 2001). Treatment with nimodipine did not affect performance relative to control animals.

Post-mortem monoamine contents in the dorsal striatum

Figure 9.4 shows dorsal striatal levels of dopamine, serotonin, and selected metabolites of these neurotransmitters assessed at eight weeks after the lesion. Bootstrapped two-sample t-tests revealed no group differences in the levels of any of the substances analyzed, regardless of hemisphere.

Discussion

Nimodipine is a Cav-1.3 L-type calcium channel antagonist, the administration of which, following the same protocol as used in this experiment, has been previously shown to block the loss of spines and glutamatergic synapses on striatopallidal medium spiny neurons which normally occurs following the loss of dopamine input onto those neurons (Day et al., 2006). In addition to the Day study, others have shown that nimodipine has little to no effect on the primary dopaminergic lesion when administered in 6-OHDA studies (Sautter et al., 1997). In this study we measured the behavioral consequences of nimodipine treatment following 6-OHDA lesion, hypothesizing that blockade of spine loss might lead to enduring sparing of behavioral symptoms, and in particular the postural instability symptoms which previous experiments in this dissertation have shown are not clearly dopamine-linked.

We found that administration of nimodipine generally had no major impact on behavioral outcomes in this model, either on more accepted tests of dopamine depletion

severity (the limb-use asymmetry test) or in our tests of postural instability. Nimodipine did seem to have a mild beneficial effect on postural instability as measured in the PIT test, but this effect was transient, indicating that any such effect is likely only symptomatic and not reflective of the consequences of preventing long-term spine loss on medium spiny neurons. In agreement with the Day study, nimodipine administration was also found to have no significant effect on neurochemical outcomes upon measurement of post-mortem striatal monoamine contents via HPLC.

Because our study did not measure spine density in the striatum, further replications will be necessary before it can be satisfactorily concluded that striatal spine changes are not tied to the behaviors we examined. Though our protocol of nimodipine administration mimicked that used in the previous study demonstrating spine sparing, there were differences in the lesion used (notably, our lesion infused 6-OHDA into the MFB, as opposed to their lesions of the nigra) which could have affected the outcomes with regards to striatal spines. Until it can be shown that spine sparing coincides with a lack of behavioral effect in the same animals, this door remains open.

Nevertheless, our results indicate that it may be more fruitful to search elsewhere for the neural changes which might underlie the development of postural instability in lesioned animals. Data from the previous experiment (Experiment 8) suggest that the substantia nigra *pars reticulata* may be a promising site of investigation in this regard.

However, even if postural instability does not result from striatal spine changes, It would be interesting to find out if there are behavioral correlates to this now well-established (Anglade et al., 1996; Ingham et al., 1998; Stephens et al., 2005; Day et al., 2006; Solis et al., 2007) anatomical event. These changes occur mostly on spines receiving corticostriatal glutamatergic input, and indeed it has been shown that drugs acting on glutamate systems can have interesting effects on the symptoms of PD in rat models (Breysse et al., 2003;

Ossowska et al., 2003). It therefore remains possible that such changes may have more to do with the progression of PD symptomology than is currently recognized, especially given the relative ineffectiveness of dopaminergic drugs in relieving symptoms later in the course of the disease (Lang and Obeso, 2004). Future studies examining a broader range of behaviors in animals where such anatomical changes are blocked pharmacologically may prove fruitful.

GENERAL DISCUSSION

Appreciation of the substantial non-dopaminergic components contributing to many of the most troubling symptoms of Parkinson's disease has grown considerably among researchers in recent years. Increased recognition of poor symptom management by dopaminergic therapies, coupled with renewed enthusiasm over the effectiveness of surgical ablative or deep-brain stimulation techniques in treating the disease, have highlighted the complexity of the neural changes occurring among several subcortical structures in PD and have heightened awareness that more effective treatments for the disease in the 21st century must begin to tackle the dynamic function of these complex, largely non-dopaminergic networks.

Postural instability is one such dopamine-treatment-resistant symptom that is relatively common in the disease, and is among the more debilitating symptoms because of the increased risk of personal injury that results from frequent falling. In this dissertation we have provided further evidence that this particular behavioral deficit likely has substantial non-dopaminergic components, and have indicated possible avenues for further studying the genesis and possible treatment of this symptom using rodent models. Though the deficit does seem most directly related to non-dopaminergic events, it does seem likely that these events are initially triggered by dopamine loss, suggesting that it may be possible to develop treatments that effectively slow the progression of PD enough so that PI symptoms might not appear, even in individuals that have already received their diagnosis.

In Chapter one of the dissertation, we illustrate the utility of two new behavioral tests for assessing PI in rodents. The forelimb placing test reflects animals' ability to regain support from a precariously unsupported position. In addition to its use as a test of PI, we also demonstrated interesting uses of cross-midline variants of the test in studying the

interhemispheric events that can occur after certain types of unilateral brain injury, as occurs in stroke. We also introduced a new test of animals' ability to adjust their center of gravity in response to experiment-imposed weight shifts, and showed how performance in this test responds to perturbations of the nigrostriatal dopamine system. We were also able to use this test to demonstrate a compensatory enhancement of function in the "good" limb following unilateral dopamine depletion, extending a line of research from this lab which illustrates the remarkable neural and behavioral compensatory mechanisms that the nervous system employs in response to a variety of insults.

Chapter two of this dissertation introduces the initial evidence, based on work with dopamine antagonist drugs, that some aspects of PI as measured with our behavioral tests are not dependent on brain dopamine systems. There are hints from these experiments that norepinephrine may play some role in the development of PI, and avenues of exploring this possibility in future studies were discussed. However, it appears that a more significant explanation for the observed behavioral deficits may be that dopamine depletion leads to a plastic reorganization of other brain systems which leads to the observed behavioral changes.

Chapter three addresses two possible forms of non-dopaminergic changes which might be tied to the development of PI, and provides evidence for one of these possibilities (i.e., that changes in the SNpr may be linked to the behavioral deficits), while generally leaning against another (that the loss of dendritic spines in the striatum may affect motor behavior in animals PD models). The finding of changes in the SNpr which are well-correlated with the PI deficits observed in our tests on experimental rodents opens up the possibility for a number of potentially interesting further studies on the behavioral correlates of changes in the SNpr and related subcortical nuclei, and provides an avenue for explaining the puzzling disconnect that is sometimes noted between markers of dopaminergic sparing

and behavioral outcomes in experimental models of potential PD therapies, in particular exercise-based therapies (Howells et al., 2005; Poulton and Muir, 2005; O'Dell et al., 2007).

At a number of points in this dissertation, it was noted that there are significant differences in the outcomes of, and probable mechanisms underlying, performance in the PIT and forelimb placing tests. These two tests appear in various ways to be connected to the symptoms of PI as seen in (and as tested in) humans with PD. Outwardly, the PIT test appears more like the "push-pull" tests often used clinically in persons with PD to assess their ability to catch themselves following shifts in their center of gravity. However, unlike the forelimb placing test, the PIT test is relatively amenable to treatment with dopaminergic therapies (and disruption by dopaminergic antagonists), unlike the largely dopamine-insensitive symptom of PI as seen in PD. This may indicate that the PIT test is in fact testing something more like movement initiation (which of course is also impaired in PD), while the placing test is targeting those behavioral problems that lead to falling in what is typically referred to as "postural instability" in PD patients. In any case, for the purposes of studying the non-dopaminergic pathology underlying the treatment-resistant aspects of PI in PD, the forelimb placing test does seem to be a more useful tool. The PIT test certainly remains useful as a test of dopamine-related motor deficits, as well as being useful in assessing "hyper-normal" function in the intact side. It should also be noted that even the PIT test appeared sensitive to asymmetries in SNpr neuron number (Experiment 8), despite the fact that the subjects had uniformly severe dopamine depletions, indicating that the integrity of many systems may influence final outcomes in this test.

The finding that L-DOPA can restore function in the forelimb placing test points to two possibilities. First, the presentation of PI may be tied to a "dopamine plus" condition, such that dopamine systems must be disrupted alongside the occurrence of other non-dopaminergic changes in order for the deficit to manifest. We did a small pilot study (not

otherwise reported in this dissertation) in which we took animals that had received sham-infusions into the MFB and administered haloperidol to them, on the premise that perhaps the sham infusion caused non-specific damage which, when combined with dopamine disruption by haloperidol, would create a placing deficit. However, no such deficit was observed, suggesting that if the "dopamine plus" hypothesis is correct, the secondary damage could not be caused simply by sham infusion and therefore is likely a results of the dopamine loss specifically. Second, L-DOPA's effect may be reflective of a restoration of norepinephrine signaling in other brain regions (e.g. in areas targeted by neurons of the locus ceruleus), a possibility that is in line with previous findings of norepinephrine agonists' effects on PD symptoms (as cited in Experiment 6), and with our own findings of disrupted postural support behaviors in rats treated with reserpine and/or AMPT. This indicates that further studies on the effects of noradrenergic drugs in patients with PD might show some benefit, especially in patients that have developed L-DOPA resistant symptoms.

Results from Experiment 3 (Figure 3.3) show that even severe dopamine depletion can leave rats able to perform the forelimb placing test. It is therefore possible that some animals are resistant for some reason to the putative secondary changes which cause PI to appear, and it would be interesting to explore why this might be the case. For this reason it is not clear that the "critical" secondary changes are actually a result of dopamine loss. Finding out what they may instead result from will be important, because the notion that reduced dopaminergic tone might lead to other detrimental brain changes has implications for current practice in the administration of pharmacological dopamine replacement therapies to PD patients. Controversy exists over whether L-DOPA administration in particular may hasten the progression of PD (Fahn, 2005), but if dopamine replacement prevents other changes from occurring this may not be such a concern, and indeed the study cited above provides evidence that though L-DOPA administration may indeed worsen PD

when measured with neuroanatomical markers, it improves clinically-assessed behavioral features of the disease even after drug washout. L-DOPA may therefore prevent other changes from occurring in the brains of PD patients, and determining whether and under what conditions this is true would be important indeed. Now that we have developed rat models for assessing specific behavioral sequelae that might be affected by such secondary sparing, opportunities exist for applying various types and intensities of dopamine-replacement therapies in rat models of PD to elucidate the particulars of how this effect might work, while avoiding the ethical problems implicit in withholding potentially disease-ameliorating therapies in human studies.

One possibility raised by the behavioral discrepancy in 6-OHDA-treated versus dopamine antagonist-treated animals is that forelimb placing is a behavior that is dependent specifically on phasic (i.e., transient, high-concentration spikes caused by dopamine neuron firing) rather than tonic (sustained background release not necessarily mediated by neuron firing) modes of dopaminergic signaling (Grace, 1991). In animals treated with competitive dopamine antagonists, it may be possible that high-concentration phasic dopamine spikes are sufficient to momentarily displace the antagonist from postsynaptic dopamine receptors, allowing the dopamine signal to flow through and permitting the placing response (though admittedly the extent to which this occurs is not likely to be major given the considerably greater affinity of haloperidol for dopamine receptors, relative to dopamine itself). This could also explain the discrepancy in the effects of L-DOPA and apomorphine on forelimb placing in lesioned animals: L-DOPA, by providing the presynaptic terminal with more dopamine, would presumably have effects on both phasic and tonic modes of dopamine signaling, while direct receptor agonists such as apomorphine exert their actions in a tonic mode which may not be as critical for "fast-action" behaviors like placing. In addition, directly-acting dopamine agonists (and antagonists) display a symmetry insofar as they have

opposing effects on overall dopamine signaling via their concurrent actions on both presynaptic autoreceptors and postsynaptic heteroreceptors. This may all play into the difference, mentioned several times in this dissertation, between presynaptically-acting and postsynaptically-acting manipulations with regards to postural support behaviors in general, with the former affecting the (possibly critical) phasic signaling mode while the latter, by acting on receptors in a steady-state manner, have "tonic-like" effects only. Thus forelimb placing and other behaviors which are differently affected by pre- versus post-synaptic manipulations may prove a useful tool in teasing apart the behavioral correlates of these two modes of dopaminergic signaling.

Results from the studies in Experiments 2 and 3 show that dopamine disruption alone does not cause PI, while Experiment 1 shows that placing can recover after lesions of the sensorimotor cortex or even the loss of much larger cortical territory following MCAo. Experiments 4, 5, and 7 indicate that our findings are not simply an idiosyncratic result of the experimental methods we used but are instead a real effect resulting from an initial dopamine depletion. Experiments 8 and 9 suggest that the integrity of and spinal morphology within the striatum does not underlie PI. And finally, Experiments 6 and 8 suggest interesting new avenues for where to look for the neural systems (and detrimental neural events) that participate in producing PI in experimental models of PD and therefore, probably in PD itself. It is hoped that this work can serve as a springboard for more thorough investigations of PI and the several other treatment-resistant symptoms of PD whose importance in the disease is now gaining increased appreciation in the PD research community.

Appendix 1: Abbreviations index

3-MT	3-methoxytyramine, a dopamine metabolite
5-HIAA	5-hydroxyindoleacetic acid, a serotonin metabolite
5-HT	serotonin
6-OHDA	6-hydroxydopamine, a catecholamine neurotoxin
AADC	aromatic amino acid decarboxylase
AMPT	alpha-methyl para-tyrosine, an antagonist of tyrosine hydroxylase
ANOVA	analysis of variance
AP	anterior-posterior (used for stereotaxic measurement)
CCA	common carotid artery
DA	dopamine
DHBA	3,4-dihydroxybenzylamine, used as internal standard in HPLC
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acetic, a dopamine metabolite
DV	dorsal-ventral (axis used in stereotaxic measurement)
ECA	external cervical artery
EDTA	ethylenediaminetetraacetic acid
GABA	gamma-amino butyric acid
HPLC-ED	high-pressure liquid chromatography with electrochemical detection
HVA	homovanillic acid, a dopamine metabolite
ICA	internal cervical artery
L-DOPA	levodopa, a drug used in the treatment of Parkinson's
L-Threo-DOPS	L-Threo-3,4-dihydroxyphenylserine
MCA	middle cerebral artery
MCAo	middle cerebral artery occlusion
MFB	medial forebrain bundle
MHPG	3-methoxy-4-hydroxyphenylglycol, a norepinephrine metabolite
ML	medial-lateral (used for stereotaxic measurement)
NE	norepinephrine

NET	norepinephrine [reuptake] transporter
PB	phosphate buffer
PBS	phosphate-buffered saline
PD	Parkinson's disease
PET	positron emission tomography
PI	postural instability
PIT	postural instability test, a test developed in this work
SEM	standard error of the mean
SNpc	substantia nigra *pars compacta*
SNpr	substantia nigra *pars reticulata*
TH	tyrosine hydroxylase, an enzyme involved in the synthesis of dopamine
UPDRS	unified Parkinson's disease rating scale

Appendix 2: Detailed methods common to several experiments

STATISTICS

Unless indicated otherwise, statistical analyses for all studies in this dissertation were performed using R version 2.6.1, available free online at http://www.r-project.org. Data in the charts and graphs are expressed as mean values ± 95% confidence intervals as determined using bootstrapping methods with 10,000 replicates per data point. When appropriate, bootstrapped versions of various tests of significance were also applied to the data (e.g., bootstrapped t-tests or ANOVAs). In these cases, p-values were determined by comparing the calculated statistic against an empirical sampling distribution generated from 10,000 bootstrap replicates, rather than using idealized (i.e., normality-assuming) distributions. For more information on bootstrap methods and their applications, see Davison and Hinkley (1997) and Efron and Tibshirani (1993). When multiple bootstrapped t-tests were applied to a given dataset, Bonferroni corrections were used to account for the multiple comparisons.

DOPAMINE DEPLETION VIA LESIONING WITH 6-OHDA

On the day of surgery animals were pretreated with 0.1 mg/kg atropine sulfate (s.c.) to dry respiratory secretions and 0.05 mg/kg buprenorphine (s.c.) for trans-operative analgesia, followed 10 min later by anesthesia induction with 40 mg/kg (i.p.) pentobarbital sodium, plus treatment with 20 mg/kg (i.p.) desipramine to block uptake of 6-OHDA by norepinephrine terminals. When necessary, anesthesia was boosted with injections of 80 mg/kg chloral hydrate (i.p.), given no closer than 15 min apart. Upon anesthesia the rats were placed in a stereotaxic apparatus and their skull exposed and leveled in the dorsal-ventral plane. Body temperature (as measured with a digital rectal thermometer) was maintained between 36.5 and 38.5ºC by use of a heating pad. A small burr hole was drilled through the skull at -4.3 mm AP and ±1.5 mm ML relative to the bregma juncture. The sterilized needle of a 2 µl Hamilton gastight syringe was then slowly lowered (at approximately 1 mm / 15 s) through the center of the burr hole to a depth of 8.0 mm below the dural surface, to target the medial forebrain bundle. Via this needle, a solution of 7 to 10 µg (free base weight; the exact dose given varied by experiment and is noted separately in

each experiment) of 6-OHDA hydrobromide dissolved in 2 µl of artificial cerebrospinal fluid containing 0.05% (w/v) ascorbic acid was infused at a rate of 0.2 µl/min for 10 min. At the end of the 10 min infusion, the needle was left in place for 2 min before being slowly retracted. The burr hole was sealed with bone wax and the scalp incision sutured, and the animals were then allowed to recover in a humidified incubator before being returned to their home cages. Sham-operated animals received the same treatment except that no solution was infused via the Hamilton needle, and the needle was removed immediately following its lowering into the medial forebrain bundle. All lesions used in this dissertation were administered unilaterally except for those noted otherwise in Experiment 5.

ANALYSIS OF TISSUE MONOAMINE CONTENTS USING HIGH PRESSURE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION (HPLC-ED)

Rats were rapidly decapitated without anesthesia and their brains removed and rinsed in saline. The brain was then buried in water ice for a few minutes, after which a 2-mm thick coronal slice through the striatum was then manually obtained with the help of a brain block. From this slice, the dorsal half of each striatum (excluding the nucleus accumbens) was dissected, blotted free of excess moisture, and accurately weighed to the nearest 0.1 mg. The striatal tissue samples were then placed in a 1.5 ml centrifuge tube and suspended in 40 µl of mobile phase solution (see below) per mg of tissue weight obtained. The samples were then homogenized by probe sonication for 3-4 s and briefly vortexed. They were then centrifuged at 15,000 RPM for 15 minutes and, following this, the supernatants were drawn off for use in the HPLC assay.

The mobile phase for HPLC consisted of a 15 mM sodium acetate / 20 mM citric acid buffer (yielding a final pH of 3.7) containing 70 µM disodium EDTA, 0.04% (w/v) sodium 1-heptanesulfonate, and 10% (v/v) methanol in water (based on the work of Saito et al. (1992)). This mobile phase was pumped at a flow rate of 0.9 ml/min through a stationary phase consisting of a BDS Hypersil C18 column, dimensions 100 x 3 mm, with a 3 µm particle size. Ten µl of the sample supernatants were injected onto the column by a ESA Model 540 autosampler and column eluates were analyzed by an ESA Model 5011A dual coulometric screening / amperometric detection electrode connected to an ESA Coulochem

II detector unit. Cell potentials were set at 0 mV (screening) and +400 mV (detecting), and in addition a guard cell set at +450 mV was placed between the pump and sample injector to reduce electrochemical interference from mobile phase impurities. This setup was capable of detecting the following compounds in tissue samples: 3-methoxy-4-hydroxyphenylglycol (MHPG, a norepinephrine metabolite), norepinephrine (NE), 3,4-dihydroxyphenylacetic acetic (DOPAC, a dopamine metabolite), 5-hydroxyindoleacetic acid (5-HIAA, a serotonin metabolite), dopamine (DA), homovanillic acid (HVA, a dopamine metabolite), 3-methoxytyramine (3-MT, a dopamine metabolite), and serotonin (5-HT). In addition all samples and standards were spiked with a fixed concentration of 3,4-dihydroxybenzylamine (DHBA, an exogenous catecholamine) as an internal standard to ensure consistent operation of the system. Peaks were recorded and integrated (i.e., their height measured) by computer and compared to the peaks generated by injection of known amounts of the aforementioned substances (obtained from Sigma and diluted appropriately in mobile phase). From this the quantities of each substance in the tissue samples was determined. Though data on all the aforementioned substances was gathered, in general, only the data for DA, DOPAC, and HVA was dealt with in these studies.

VIBRISSAE-ELICITED FORELIMB PLACING TEST

In this test (Barth et al., 1990a; Barth et al., 1990b; Woodlee et al., 2005b), rats are gently held aloft by the torso in such a way that all four of the animals' limbs are suspended, i.e., they have no stable support. The animals are then moved towards the edge of a tabletop such that their vibrissae brush against the table's edge. Upon detecting the presence of a solid surface with their vibrissae, normal animals will reach out to place their forelimb on the table to regain support (see Figure A2.1, left). This test therefore evaluates the rat's ability to use an ethologically pertinent sensory modality to guide it in regaining postural support from an unstable state.

Typically, ten trials are performed on each forelimb. Trials in which the animal struggles are not counted. Animals are pre-trained on the placing test before surgery or other manipulations are done, so that struggling and other artifacts of the rats' acclimatization to the experimenter and the handling necessary for testing are virtually eliminated prior to the

critical post-manipulation testing sessions. Pre-testing also allows for exclusion of rare anomalous rats that do not perform the test flawlessly prior to experimental manipulation.

In some early experiments using the test, we also devised and used a novel cross-midline test of forelimb placing. In the cross-midline placing test, animals are again held gently by the torso, but are turned sideways so that the vibrissae are perpendicular to the surface of the table (Figure A2.1, right). The now downwardly-oriented limb is gently restrained against the rat's body by the experimenter as the downwardly-oriented vibrissae are brushed against a table edge. Rats will then attempt to place the top limb (i.e., contralateral to the vibrissae being stimulated), and again normal rats can perform this variation with near 100% success. This variation is useful for evaluation of sensorimotor integration across the midline, and its application yielded especially interesting results when used in stroke models as detailed in Experiment 1. When this method is included, four "variants" of the test are therefore used: ipsilesional or contralesional vibrissae eliciting placing in either the ipsilesional or contralesional limb. It should be noted that visual information does not affect test scores, since identical data are obtained when the experimenter occludes the eyes.

Results from applying this test to animals with stroke-like damage and 6-OHDA lesions are presented in the form of a published paper (Woodlee et al., 2005b) and as Experiment 1 in this dissertation. In this paper and some of the earlier studies in this dissertation, forelimb placing ability was evaluated as the percent successful placing out of ten trials on this test. However, we have recently developed a newer rating scale which is more appropriate to the kinds of behavior seen in dopamine-depleted rats specifically. This test uses a rating scale of 0-3 applied to ten trials for each forelimb, for a maximum ("best") score of 30 per forelimb, determined as follows:

0: no detectable movement of the forelimb upon vibrissae stimulation

1: movement of the forelimb but no contact with the table (i.e. a "twitch")

2: weak or slow limb placement or placement on only the side of the table

3: full normal forelimb placement on the top surface of the table

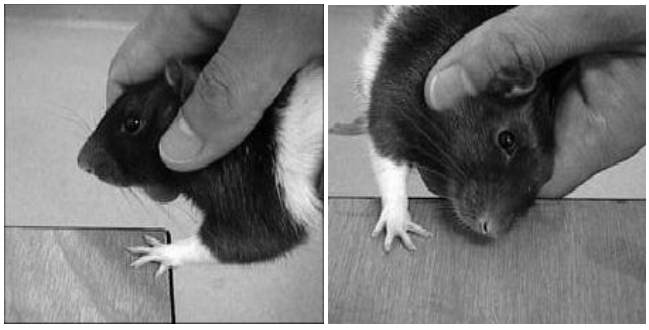


Figure A2.1 A rat performing successful forelimb placements in the vibrissae-elicited forelimb placing test

Left: Same-side placing. *Right*: Cross-midline placing.

POSTURAL INSTABILITY TEST ("PIT TEST")

In this test, rats were held almost vertically upside-down (in a "wheelbarrow"-like position) over a sandpaper-covered surface alongside a ruler (see Figure A2.2), by a tester who was blind to the experimental condition of the rats. This rough surface material induced stepping, rather than dragging or bracing, in response to imposed weight shifts. Viewed from above, the tip of the rat's nose was aligned with the zero line of the ruler, and one forelimb was gently restrained against the animal's torso by the experimenter while the animal was moved forward over the single planted forelimb until making a "catch-up" step to regain its center of gravity. The new position of the nose tip indicated the displacement of the body needed to trigger a catch-up step in the unrestrained supporting forelimb. We examined each forelimb independently while slowly shifting the center of gravity during weight support, and quantified the size of the adjusting response used to regain center of gravity. We performed three trials on each forelimb on a given day of testing. This is a new test that is a refinement of an earlier test variously referred to as the stepping test, adjusting-steps test, or bracing test (Schallert et al., 1979; Schallert et al., 1992; Olsson et al., 1995; Schallert and Tillerson, 2000) which, in contrast to the present test, measured number of steps taken across a set distance of displacement (in 6-OHDA-lesioned rats these tests showed that the number of contralateral forelimb steps were reduced compared to the number of ipsilateral steps, due to bracing reactions on a smooth surface). Earlier pilot work with the PIT test in our lab and others (J. Mithyantha and P. A. Garris, unpublished data) also measured subsequent multiple steps (i.e., continuing to move the rat forward after the first catch-up step until an additional step or two had been made), as well as sideways stepping (measuring lateral displacement required in either direction to trigger a step), but found that these measures were not more sensitive or qualitatively different in dopamine depleted rats than the easier-to-perform version of the test described above. In testing a large group of intact rats we also found the results of this test to be dependent upon body weight, size, and/or age, as presented in the results (see Figure 2.1 in Experiment 2), as expected based on the physics of maintaining center of gravity.

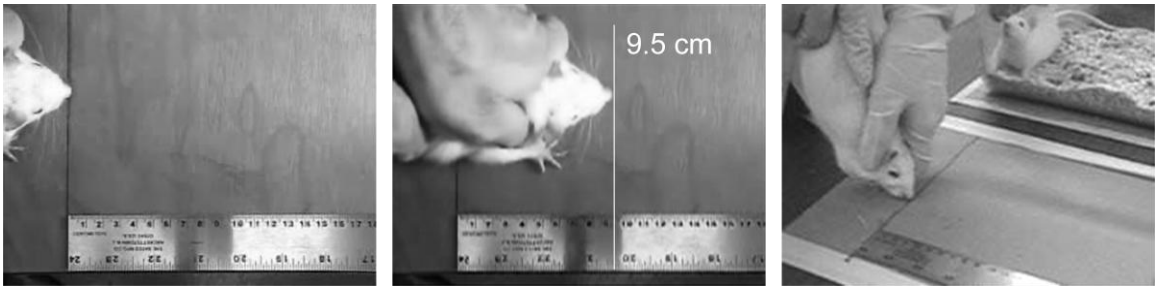


Figure A2.2 Performing the PIT test

Left: a rat at the zero-line before being moved forward. *Center*: a rat having made a catch-up step after 9.5 cm of displacement. *Right*: a sideways view showing the angle at which the rat is held.

LIMB-USE ASYMMETRY ("CYLINDER") TEST

In the limb-use asymmetry test (Schallert et al., 2000), rats are placed in a clear acrylic cylinder (30 cm tall by 20 cm diameter). The cylinder is high enough to prevent rats from jumping out, and wide enough to allow a small (1 cm) gap between the base of the tail and the cylinder wall when the rat is on all fours. Rats placed in the cylinder engage in exploratory behavior in which they rear and contact the wall of the cylinder with their forepaws. The number of wall contacts made using the ipsilateral (unimpaired), the contralateral (impaired), and both (simultaneously) limbs is recorded, and an asymmetry score calculated as the number of "ipsi" observations plus 1/2 the number of "both" observations, divided by the total number of observations (ipsi plus contra plus both). In this overall asymmetry percentage score, 50% indicates an animal that explores symmetrically with both limbs, higher scores (>50%) indicate a greater reliance on the ipsilateral limb, and lower scores (<50%) indicate a greater reliance on the contralateral limb (see Schallert and Woodlee (2004) for more details and tips on performing the test). On each day of testing we recorded 20 limb uses; the time required to generate this number of observations varied from animal to animal.

In addition to this standard version of the test, in some experiments we also measured "serial stepping" behavior in each forelimb as an additional measure of adaptation in the "intact" limb. Whenever the animal placed one forelimb independently onto the inner wall of the cylinder, it would sometimes subsequently proceed to make several rapid lateral weight-shifting steps with the same limb, without using the other limb. We observed this behavior to be relatively rare in intact rats but common in the ipsilateral forelimb of 6-OHDA-lesioned animals. Thus, "ipsi-step" or "contra-step" was recorded for any forelimb placement made in this way following the initial independent forelimb use, as long as the chain of lateral steps was continuous and not broken by long pauses or the animal pushing back to a standing rear in which no forelimbs were in contact with the cylinder wall. In calculating the asymmetry percentage score (as described above), these "step" behaviors were treated as ordinary ipsi- or contra-limb use events. However, in the results these behaviors are also analyzed independently as percentage "step" behaviors out of the total number of independent forelimb uses for each forelimb.

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Vita

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