FINAL REPORT

6-Hydroxydopamine (6-OHDA) Rat Model: A Model of Hemi-Parkinsonism

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1.0 ABSTRACT

The goal of the study presented here was to demonstrate the functionality of the newly acquired rotometer system at RTI and standardize the methodology for the demonstration of mid-brain dopamine lesions induced by 6-hydroxydopmine (6-OHDA). Sprague-Dawley rats were purchased from Charles River Laboratories (CRL) with apomorphine-verified lesioning of the substantia nigra (SN) by focal administration of 6-OHDA. These animals received *d*-amphetamine and apomorphine challenges from 2 to 4 weeks following 6-OHDA administration and their behavior was assessed in standard rotometers. *d*-Amphetamine and apomorphine produced asymmetrical rotations in all test animals indicating a successful lesion of SN projections to the striatum. This study demonstrates that the rotometer system is fully functional and that RTI can move forward to determine the best locus of lesion (striatum vs. SN) for the assessment of potential neuroprotective compounds.

2.0 INTRODUCTION AND OBJECTIVE

The neurotoxic effect of 6-OHDA provides an excellent and specific model of the in vivo effects of central catecholamine cell loss. In fact, this model was one of the first animal models of Parkinson's disease. The specificity of the 6-OHDA effect is achieved through two key characteristics of 6-OHDA. 6-OHDA cannot cross the blood brain barrier and thus, when administered either via the cerebral ventricular system or focally to key brain regions, all effects of 6-OHDA are limited to the CNS. Secondly, 6-OHDA does not penetrate cells well and is a specific substrate for the dopamine and norepinephrine transport systems, thus 6-OHDA neurotoxicity is predominantly observed in dopaminergic and noradrenergic neuronal systems. In the typical Parkinson's disease model, 6-OHDA is delivered unilaterally by stereotactic injection into the SN or the striatum. If injected into the SN or the nigrostriatal tract, dopaminergic neurons begin to degenerate within 24 hours and striatal dopamine is depleted 2 to 3 days later. If injected into the striatum, 6-OHDA produces a less severe and relatively slow degeneration over a period of weeks. This model is particularly interesting since striatal 6-OHDA injection induces cell death retrogradely and provides a more prolonged window of time for the potential agent to act, thereby increasing the chances of detecting neuroprotective effects.

In both cases, the degree of damage is reported to be dose dependent, and each paradigm produces unique behavioral changes.

While numerous behavioral endpoints have been explored in MPTP mouse models, the primary endpoint for the unilateral 6-OHDA model is rotation, as assessed in a rotometer. Rotation results from an imbalance in functionality of dopamine systems on the left and right sides of the brain following the unilateral lesion. The tendency to rotate and the direction of rotation (i.e., ipsilateral or contralateral to the lesion) is affected by pharmacological agents that either simulate or block dopamine pathways. One week following injection, subjects can be administered 2.5 mg/kg ip of *d*-amphetamine, and the net rotations (i.e., rotational asymmetry) over a 90-minute trial recorded. Alternatively, 3 weeks post-lesioning, 0.25 mg/kg of apomorphine can be administered, and rotational asymmetry monitored over 40 minutes. *d*-Amphetamine acts by releasing dopamine stored in the remaining nerve terminals, whereas apomorphine is an agonist for both D1/D2 receptors. Both drugs are used to identify lesioning success. Although this behavioral model quantifies motor deficits through the use of standardized equipment, there are no clinical correlates to rotating behavior (i.e., neurological damage in Parkinson's patients is bilateral), and the procedure is insensitive to small changes in striatal dopamine (Kirik et al., 1998; Emborg, 2004).

Although 6-OHDA can produce toxicity in many models, rats are the most commonly used species because the results are both replicable and stable. In addition, stereotactic techniques are well established in rats. The injections are typically unilateral (i.e., one hemisphere), leaving the other hemisphere to serve as an internal control. The behavioral manifestation is asymmetric circling motor behavior following administration of dopaminergic drugs. Rotation behavior, a quantifiable motor deficit, provides a major advantage to using this paradigm as a model of Parkinson's disease; however, the model does not mimic all of the clinical signs and pathological features. In short, administration of 6-OHDA injected into the nigrostriatal pathway does not alter brain regions such as the locus coeruleus, nor does it result in the formation of Lewy bodies (cytoplasmic inclusions).

The aims of this study were to establish the techniques for the behavioral assessment of rats with lesions of the SN in newly acquired rotometers. The basic study design was focused on being able to display differential effects between control and treated rats following dosing with *d*-amphetamine or apomorphine. In order to keep animal numbers low, animals received

d-amphetamine followed in a later session by apomorphine. This study was neither designed nor powered to investigate the effects of sequential dosing of *d*-amphetamine and apomorphine, but simply to demonstrate that the equipment was functioning correctly and to assist in the determination of group sizes for future studies.

3.0 MATERIALS AND METHODS

3.1 Test Substances

Name:	R-(-)-Apomorphine hydrochloride hemihydrate
Synonym:	Apomorphine
Empirical Formula:	$C_{17}H_{17}NO_2 \cdot HCl \cdot 1/2H_20$
CAS No.:	41372-20-7
Safety Precautions:	A Material Safety Data Sheet (MSDS) will be maintained in the study file.
Purity:	Certified ≥ 98.5% pure by Sigma-Aldrich
Supplier:	Sigma-Aldrich, Inc.
Storage Conditions:	Room temp
Safety Precautions:	None / standard laboratory PPE
Vehicle:	Sterile saline
Disposition:	Appropriately disposed of following study completion

Name:	*d*-Amphetamine sulfate; alpha-methylphenethylamine sulfate
Synonym:	Amphetamine
Empirical Formula:	$(C_9H_{13}N)_2\ H_2SO_4$
Batch Number:	Lot #068K1069
CAS No.:	51-63-8
Supplier:	Sigma-Aldrich, Inc.
Purity:	Certified ≥ 98.5% pure by Sigma-Aldrich
Storage Conditions:	Room temp
Safety Precautions:	None / standard laboratory PPE
Vehicle:	Sterile saline
Disposition:	Appropriately disposed of following study completion

d-Amphetamine sulfate is a DEA Schedule II controlled substance and all use and disposal was documented in the Use Log File maintained at RTI by J. Henson. Standard laboratory safety equipment was used when preparing dose solution (gloves, lab coat, and safety glasses).

3.2 Test System

Species. The test animals were male Sprague Dawley rats (8–10 weeks, 230–250 g) that received unilateral lesions of the substantia nigra (left hemisphere – performed at Charles River Laboratories) (*Surgical Capabilities Reference Paper*, Vol. 13, No. 1, 2005) with 6-OHDA or sham surgery including probe placement but no injection.

Supplier. Charles River Laboratories, Inc., Raleigh, NC (CRL)

Test System Justification. Sprague Dawley rats are the strain of choice in most 6-OHDA publications. Both young adult males and females can be used, but males were used in this study.

Identification. After allocation to treatment groups, animals were identified by an individual study number. All data generated during the course of the study were tracked by the individual study number.

3.3 Animal Care, Housing, and Environmental Conditions

Quarantine and Acclimation. Animals were quarantined for approximately 6 days and accepted for use on study in accordance with facility SOPs.

Animal Housing Conditions. All animals were individually housed in solid-bottom polycarbonate caging (8” x 19” x 10.5”) with stainless steel wire lids (Laboratory Products, Rochelle Park, NJ). Hardwood Sani-Chip® cage litter (P.J. Murphy Forest Products, Montville, NJ) was used as cage bedding.

Environmental Conditions. Environmental conditions were continuously monitored, controlled, and recorded (Siebe/Barber-Colman Network 8000 System with Revision 4.4.1 for Signal® Software, Siebe Environmental Controls, [SEC]/Barber-Colman Company, Loves Park, IL). Animal rooms used for this study were maintained on a 12:12 hour light:dark cycle. Target conditions for temperature and humidity in the animal rooms were 64–77 °F (22 ± 3 °C) and 30-70% (NRC Guide, 1996).

Feed. Pelleted Purina Certified Rodent Diet® (No. 5002) (PMI, Inc., St. Louis, MO) was available *ad libitum.* All feed was provided through the cage lid hopper. Batch numbers and expiration dates for each bag of chow were recorded. Certified analysis of each feed lot used during the study was provided by the manufacturer and maintained in the facility records to confirm that no contaminants were present in concentrations that would affect the outcome of the study.

Water. Tap water from the Durham, NC, municipal water system was available *ad libitum* and delivered via polycarbonate or polypropylene water bottles with rubber stoppers and stainless steel sipper tubes. The city of Durham provides annual analyses of the drinking water for potential contaminants. Copies of the analyses were maintained in the facility records to confirm that no contaminants were present in concentrations that would affect the outcome of the study.

3.4 Experimental Design

Group Assignment. Animals were assigned to 6-OHDA treated and control groups by CRL at the time of surgery. All animals that received 6-OHDA were tested with apomorphine 1 week after treatment to verify a successful dopaminergic lesion (data provided by CRL is maintained in the study records).

In-life Design. All animals in all groups received a single ip dose of *d*-amphetamine 1 week after arrival at the facility, approximately 14 days following 6-OHDA administration (10 days following apomorphine challenge at CRL). Animals were acclimated to the rotometers for 30 minutes prior to receiving an ip dose of 2.5 mg/kg *d*-amphetamine. Immediately following dosing animals were returned to the same rotometer for a 90-minute test session in which full body rotations were recorded in each direction (clockwise and anticlockwise). Two weeks following the *d*-amphetamine challenge (4 weeks after lesioning) animals were again acclimated to the rotometers and then received a 0.25 mg/kg ip dose of apomorphine immediately prior to a 4-minute test session. Two weeks later, all 6-OHDA lesioned animals received a second apomorphine challenge to confirm the effects of apomorphine. An additional group of normal controls (no sham surgery) were administered *d*-amphetamine in parallel with a second *d*-amphetamine test for the sham control group (5 weeks after the first *d*-amphetamine challenge) in order to compare the responses of the sham animals to that of normal animals.

Data was expressed as both absolute rotations in each direction and group mean net counter-clockwise bias (number of counterclockwise rotations minus the number of clockwise rotations).

Postmortem Design. After euthanasia, rats were decapitated and the brains removed. Striatal and substantia nigral tissue was removed from the unfixed brains of all mice over ice, weighed, placed in individual cryovials, and then drop-frozen in liquid nitrogen. Each animal provided two samples (left and right hemisphere) of striatal and substantia nigral tissue for analysis. Following striatal dissections, samples were stored at -70 °C until sample homogenation and analysis.

Brain tissue samples were weighed and placed in a chilled glass homogenation tube. 0.1 M perchloric acid and 1 mM EDTA solution containing 1 mg/L DHBA (3,4-DiHydroxyBenzylAmine - Internal Standard) were added at 20 µL/mg, and the tissue was homogenized by hand using a Teflon® pestle. Homogenate was transferred to a microcentrifuge tube and centrifuged for 5 minutes at 12,000 rpm, the supernatant was then transferred to an ultrafree-MC centrifugal filter tube and centrifuged for 5 minutes at 12,000 rpm. Supernatant was transferred to HPLC vials and analyzed in duplicate by 20 µL injection volumes when sufficient sample was available. If sample volume was limited, a single injection or smaller injection volumes were used. Samples were quantified against the internal standard DHBA.

Data was expressed as the ratio of transmitter levels in the left hemisphere sample to the right hemisphere sample.

3.5 Euthanasia

Scheduled Euthanasia. At the time of euthanasia, animals were euthanized using CO_2 asphyxiation. Carcasses were discarded following biohazard waste practices for animal waste.

3.6 Compliance with AAALAC Regulations

The RTI ARF is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Thus, this study was conducted in compliance with the AAALAC accreditation standards.

4.0 RESULTS

The primary aim of this study was to demonstrate that the rotational behavior could be recorded in rats that were lesioned at CRL and challenged at RTI with *d*-amphetamine and

apomorphine. This primary aim was met with great success. Clear, asymmetric, rotational behavior was demonstrated in 6-OHDA lesioned animals. Sham control animals showed a mean net counterclockwise bias (average count of counterclockwise rotations – clockwise rotations) of 45.4 ± 58 rotations in response to *d*-amphetamine and lesioned animals showed a mean net counterclockwise bias of 177.9 ± 60 rotations. Normal animals showed a mean net counterclockwise bias of 72.6 ± 51 rotations, however, one of the 5 animals demonstrated 246 rotations counterclockwise, greatly skewing the group mean results (Table 1).

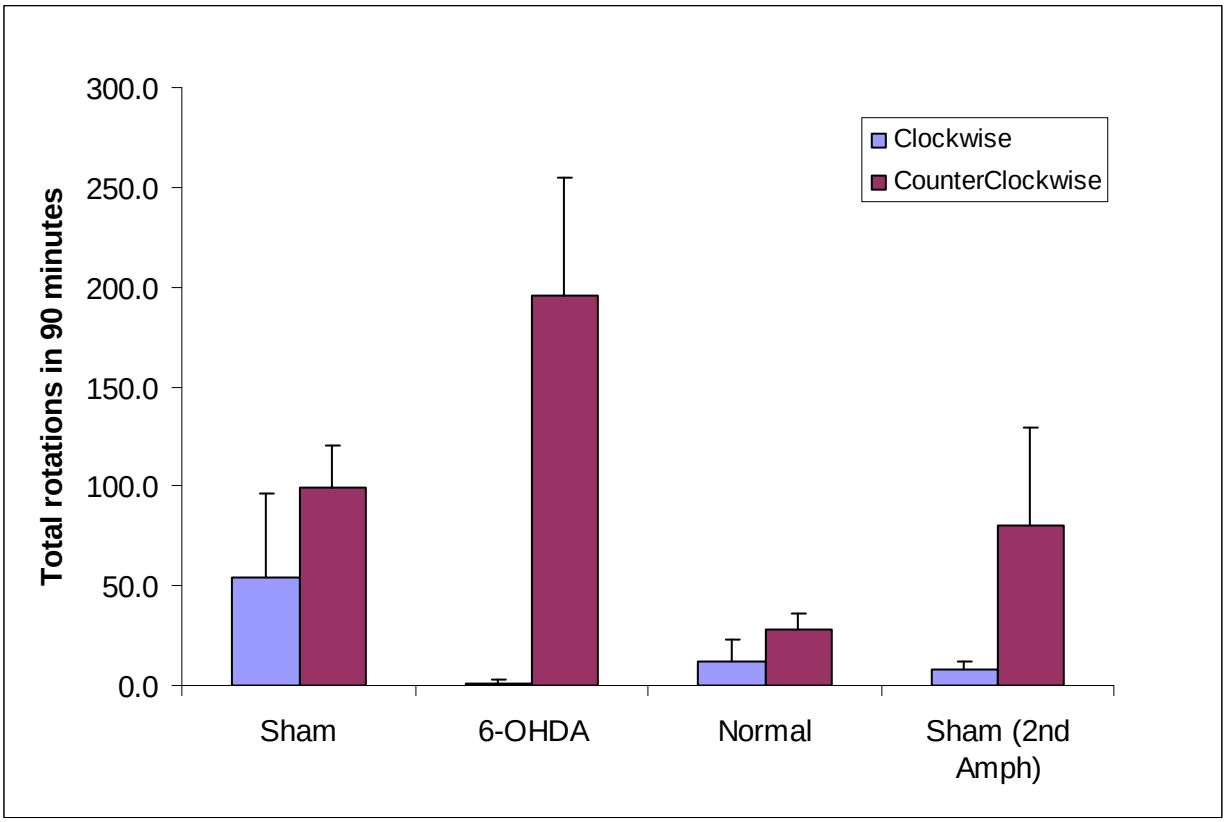
Table 1: The Mean Net Counterclockwise Bias Following Various Challenge Doses

	Challenge Dose			
MEAN ± s.e.m.	Amphetamine	Apomorphine	Apomorphine	Amphetamine
Sham	45.4 ± 58	5.2 ± 4		72.6 ± 52
6-OHDA	177.9 ± 60	-80.4 ± 38	-221.1 ± 35	
Normal				16.7 ± 14

Apomorphine dosing showed a similar magnitude of difference between test and control but with a negative mean net counterclockwise number of rotations (i.e., greater clockwise rotations than counterclockwise; Table 1). However, complexity was introduced into the study design by the repeated dosing of animals with dopaminergic challenge agents: apomorphine at CRL prior to delivery; *d*-amphetamine 1 week after delivery; apomorphine 2 weeks after *d*-amphetamine; a second apomorphine 2 weeks later. The effects of these repeated doses in 6-OHDA lesioned animals, along with the effects on sham operated and normal animals are presented in Figures 1 (*d*-amphetamine) and 2 (apomorphine).

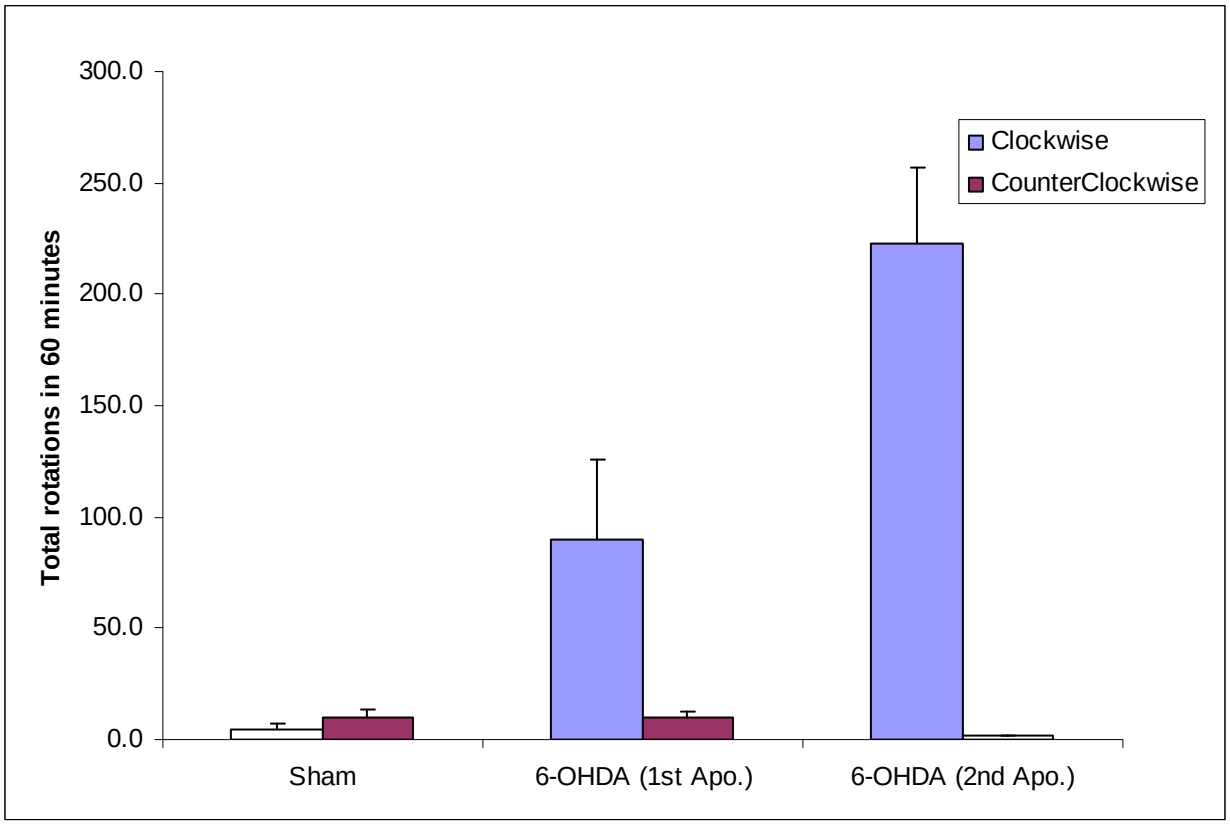
There is a clear rotational asymmetry in lesioned animals following *d*-amphetamine and apomorphine treatment. Challenge with apomorphine showed a far greater difference between sham and test animals and the asymmetry seen in test animals greatly increased with the second challenge test (Figure 2). As mentioned previously, this study was neither designed nor powered to investigate the potential effects of previous experience with dopaminergic agents on rotational behavior elicited by a dopaminergic challenge and as such analysis of covariance or interpretation of this interaction have been performed.

Figure 1: The Effect of Amphetamine on Clockwise and Counterclockwise Rotations



Interestingly, sham operated animals appeared to show a rotational asymmetry following the *d*-amphetamine challenges (Figure 1) which was not seen with normal animals (a generalized increase in rotations would be expected with a psychostimulant such as *d*-amphetamine but not asymmetric rotation), however, with just 5 sham animals and a large variation between animals, whether this asymmetry is statistically significant is unclear. What is clear is that the rotational asymmetry is considerably greater in 6-OHDA lesioned animals.

Figure 2: The Effect of Apomorphine on Clockwise and Counterclockwise Rotations



The brains were collected post-mortem from the animals used in the study, and dopamine, DOPAC, and HVA levels will be assessed using HPLC. Upon completion of this analysis the data will be reported as a separate study report.

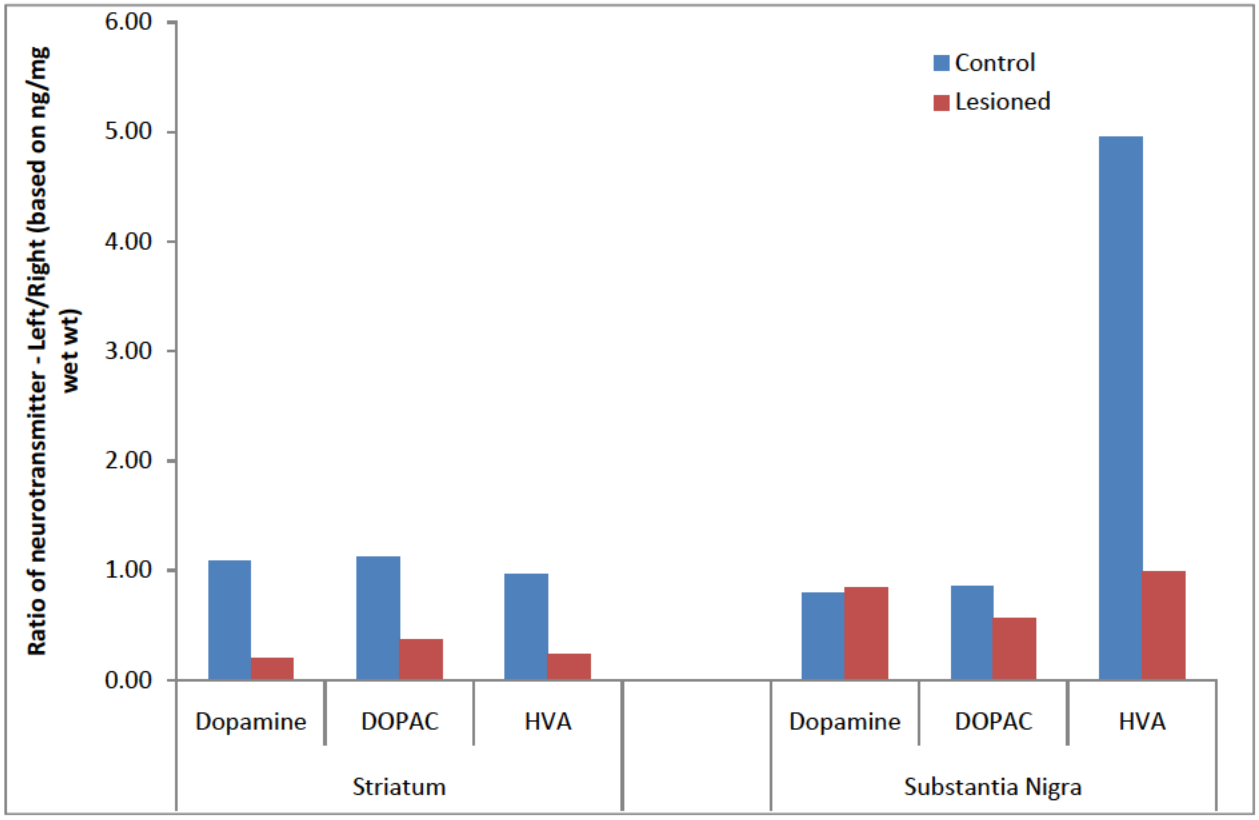
Treatment with 6-OHDA produced a clear loss of dopamine and metabolites in the striatal samples from the left hemisphere (Table 2 and Figure 3). Striatal tissue samples showed a distinctly clearer effect of 6-OHDA dosing than the substantia nigra samples. It is thought that this is related to the relative ease of dissecting the striatum in rats as compared to the substantia nigra – leading to much wider variation in the substantia nigra tissue weight recovered, and may also be a function of the considerably lower signal level in sham treated animals. This lower level of dopamine and metabolites compared to the nerve terminal region of the striatum might be expected but it adds greater burden to the precision of both the tissue sampling and the HPLC assay in order to detect reliable changes. Owing to the difficulty of acquiring clear substantia

nigral data (e.g., HVA levels in control animals in this study), this region will not be used to confirm successful 6-OHDA-induced lesions in future studies.

Table 2: The Effect of 6-OHDA Lesions on Levels of Dopamine and Metabolites in the Striatum and Substantia Nigra

	Dopamine (ng/mL wet weight)				DOPAC (ng/mL wet weight)				HVA (ng/mL wet weight)			
	Left		Right		Left		Right		Left		Right	
STRIATUM	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem	Mean	sem
Control	16.11	0.4	14.8	0.9	1.58	0.1	1.41	0.1	0.59	0.1	0.61	0.1
Lesion	2.91	2.0	14.2	1.4	0.46	0.2	1.26	0.1	0.13	0.1	0.54	0.1
SUBSTANTIA NIGRA												
Control	1.09	0.2	1.38	0.1	0.21	0.02	0.24	0.01	0.92	0.6	0.19	0.02
Lesion	0.73	0.2	0.87	0.2	0.11	0.02	0.21	0.03	0.54	0.1	0.55	0.3

Figure 3: The Effect of 6-OHDA Lesions on the Ratio of Mean Levels of Dopamine and Metabolites between the Left and Right Striatum and Substantia Nigra



5.0 DISCUSSION AND CONCLUSIONS

The effect of 6-OHDA lesioning of the SN on rotational behavior in Sprague Dawley rats was clearly and effectively demonstrated. While no direct conclusions can be drawn, the data presented here also indicate that increased time between either the *d*-amphetamine challenge or the original lesion and the apomorphine challenge may enhance the effect of apomorphine in lesioned animals. The primary objective of this study was met, namely demonstration of effective equipment setup and the ability to discern 6-OHDA induced lesions with behavioral and neurochemical approaches. Further to this it can be surmised that test group sizes of 10 animals is sufficient to see 6-OHDA effects in this model and that it is advantageous to space challenge doses by multiple weeks or use parallel test groups for apomorphine and *d*-amphetamine challenges.

The utility of the 6-OHDA model in the identification of factors involved in Parkinson's Disease is well accepted, and refinements to the standard method, such as delivery of the 6-OHDA to the striatum rather than the SN have been suggested. The next steps for this program of research are to demonstrate a reproducible induction of rotational behavior and loss of striatal dopamine and nigral dopamine cells following striatal administration of 6-OHDA.

6.0 REFERENCES

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