

# Glutamatergic Signaling in the Nucleus Accumbens Modulates the Behavioral Response to Acute and Chronic Methylphenidate

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## Abstract

Methylphenidate (MPD) is a psychostimulant that is widely used to treat attention deficit hyperactivity disorder, and is being increasingly misused as a recreational drug and cognitive enhancer. MPD acts on the reward system of the CNS through specific signaling pathways to produce its effects on behavior, including tolerance, withdrawal, and sensitization. The nucleus accumbens (NAc) is one of the predominant components of this system, however the role of the NAc's glutamatergic system in the behavioral response to MPD has not been studied. The objective of this study was to assess the role of glutamatergic signaling and the response to acute and chronic MPD exposure as measured by three different locomotive behaviors following selective bilateral lesions. Three groups of  $n=8$  rats were used: control, sham NAc lesions, and glutamatergic-specific (ibotenic acid toxin) NAc lesions. On experimental day (ED) 1, all groups received saline injections to establish a baseline. On ED 2, NAc surgeries took place, followed by a 5-day recovery period (ED 3-7). On ED 8 saline was given to obtain a post-surgical baseline. Groups then received six daily MPD 2.5 mg/kg injections (ED 9-14) to produce a chronic effect, behavioral sensitization in this study, then three days of washout with no injection (ED 15-17), followed by a re-challenge with the previous 2.5 mg/kg MPD dose on ED 18. Locomotive activity was recorded for 60 minutes after each injection by a computerized animal activity monitoring system. All groups showed an increase in behavioral activity following acute MPD exposure, and developed behavioral sensitization following chronic MPD exposure that was maintained after three days of MPD washout. Compared to controls and sham lesions, the horizontal activity response was significantly ( $P<0.05$ ) attenuated both acutely and chronically following glutamatergic selective ibotenic acid lesions to the NAc however the other indices showed no change. This indicates that glutamatergic signaling in the NAc plays a role in modulating the response to acute and chronic MPD.

**Keywords:** Methylphenidate, Ritalin, Nucleus accumbens, Lesion, Sensitization, Glutamate

## Introduction

Methylphenidate (MPD), more commonly known as Ritalin® or Concerta®, is a psychostimulant that is prescribed to treat behavioral disorders such as attention deficit hyperactivity disorder (ADHD) but is increasingly being misused and abused as a cognitive performance enhancer or recreational stimulant in normal individuals [1-5]. This has been driven by the rapid increase in patients diagnosed with Attention Deficit Hyperactivity Disorder (ADHD) for which MPD is the drug of choice [6-10], as well as the rise in non-prescription use of MPD for academic enhancement and recreation [7,11-15].

This is of concern as MPD shares pharmacologic characteristics with other addictive psychostimulants such as amphetamine and cocaine, and could thus share similar addictive potential [6,16-20]. MPD, like amphetamine and cocaine, acts as an indirect dopamine agonist by inhibiting the dopamine reuptake at the pre-synaptic terminal, leading to increased dopamine within the synaptic cleft [21-23]. Acute MPD exposure produces an increase in behavioral locomotor activity; chronic use elicits sensitization, tolerance, and/or withdrawal which are behavioral markers indicating a substance has the potential to elicit dependence [17,24-29]. Sensitization is a sustained increase in behavioral activity beyond the acute

effect following chronic administration of a substance [30,31].

The central nervous system's (CNS) reward system is known to participate in the long-term changes associated with substance abuse [32-37]. The circuit consists of multiple CNS structures, however the core pathway is the mesolimbic pathway in which dopaminergic neurons from the ventral tegmental area (VTA) project to the nucleus accumbens (NAc) and the ventral striatum, then onwards to the prefrontal cortex (PFC). The Nucleus Accumbens (NAc) is a reward circuit structure that is critical for motivation, emotion, limbic functions, and motor execution [30,38-43]. Non-specific and dopaminergic specific lesions to the NAc have shown it to be critical to regulating the response to MPD [44,45], however the role of the glutamatergic system remains uninvestigated. Glutamatergic signaling has been shown to modulate the long-term response between other reward/motive circuit nuclei [26,28,29,36,44,46-60], and it known to participate in inputs to the NAc, however its role in the acute and chronic response to MPD is unknown.

This study set out to determine if the glutamatergic system of the NAc participates in the response to MPD. To do this, 3 groups of animals were used: NAc intact controls, sham lesions, and specific glutamatergic chemical lesions. Animals were exposed to acute and chronic (repetitive) MPD and the response was monitored with a computerized monitoring system in an open field assay.

## Methods

### Animals

Twenty-four male Sprague-Dawley rats weighing 170-180g were obtained from Harlan Labs (Indianapolis, IN, USA). Animals were individually placed in plexiglass cages (40.5x40.5x31.5 cm in dimension) in a soundproof room without disturbance to the experimental environment for

4-5 days to acclimate prior to experimentation. These cages served as the home and test cage. Animals were maintained on a 12-hour light/dark cycle that began at 06:00. Food and water were provided ad libitum throughout the experiment, and the temperature was kept at  $21 \pm 2^\circ\text{C}$  with a relative humidity of 37-42%. At the beginning of the experimental phase, the rats were weighed and randomly divided into three groups: NAc-intact controls (n=8), sham operation (n=8), and ibotenic acid chemical ablation of the glutamatergic system (n=8). This protocol was approved by our Animal Welfare Committee and carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

### Experimental procedure (Table 1)

Rats were given 4-5 days to acclimate in their home cage before experimentation. On experimental day 1 (ED 1-Sal) animals were weighed and 0.8 mL of 0.9% saline was administered intra-peritoneal (ip). All animals weighed 200-220 g at that time. Locomotive behavioral activity was recorded for 120 minutes post-injection to establish a baseline prior to surgical manipulation. On experimental day 2 (ED 2), the lesion and sham groups underwent surgery and were then allowed to recover for approximately 5 days (ED 3-7). On experimental day 8, saline was re-administered (ED 8-Sal) and post-surgical locomotor activity was recorded for 120 minutes to compare with the pre-surgical baseline (ED 1-Sal). Starting on experimental day 9 (ED 9-MPD), daily injections of 2.5 mg/kg MPD (Mallinckrodt, Hazelwood MO) dissolved in 0.8 mL of 0.9% saline were administered for 6 consecutive days (ED 9-MPD to ED 14-MPD), and activity recorded for 120 minutes post-injection. This dose of 2.5 mg/kg MPD has been shown to be sufficient to elicit behavioral sensitization in rats in previous dose-response experiments [27-29,48,51,61-68]. For the next 3 days (ED 15-17), animals received no injections (the washout period). After the washout period (ED 18-MPD), the rats were re-challenged with MPD at the previous dose

Group	Experimental Schedule						
	ED 1*	ED 2	ED 3-7	ED 8*	ED 9-14*	ED 15-17	ED 18*
Control	Saline			Saline	MPD	Washout	MPD re-challenge
Sham	Saline	Surgery	Recovery	Saline	MPD	Washout	MPD re-challenge
Ibotenic acid lesion	Saline	Surgery	Recovery	Saline	MPD	Washout	MPD re-challenge

**Table 1: Methylphenidate administration schedule.** The table shows the experimental treatment protocol for the 5 groups of rats used. Each group consisted of N=8 rats. Displayed are the experimental days (ED's) either normal saline or methylphenidate (MPD) 2.5 mg/kg ip was administered according to injection protocol, in a standardized volume of 0.8 ml at 07:30. \* indicates day rats were behaviorally recorded post-injection. The experiment lasted 18 experimental days. The experimental schedule began after several days of acclimatization of the rats to their home/experimental cages.

of 2.5 mg/kg and behavioral activity was observed for 60 minutes (the expression phase). All boluses were given at approximately 07:30 in the morning in 0.8 mL volumes.

### Surgical Procedure (ED 2)

On ED 2, the sham operation group, the electrolytic lesion group, the 6-OHDA group, and the ibotenic acid group animals were anaesthetized with 60 mg/kg pentobarbital and placed in the stereotaxic apparatus. An incision was to expose the skull. For surgery, holes were drilled in the skull 1.7 mm anterior from the bregma and 1.6 mm lateral to the midline bilaterally based on the co-ordinates derived from Paxinos and Watson Rat Brain Atlas [69].

**Sham operation:** For the sham group, the animal was anesthetized, the skin opened, holes drilled in the skull, and a 27G cannula was inserted bilaterally to a depth of 6.8 mm but no agent administered. The cannulas were then removed, and the incision closed with wound staples.

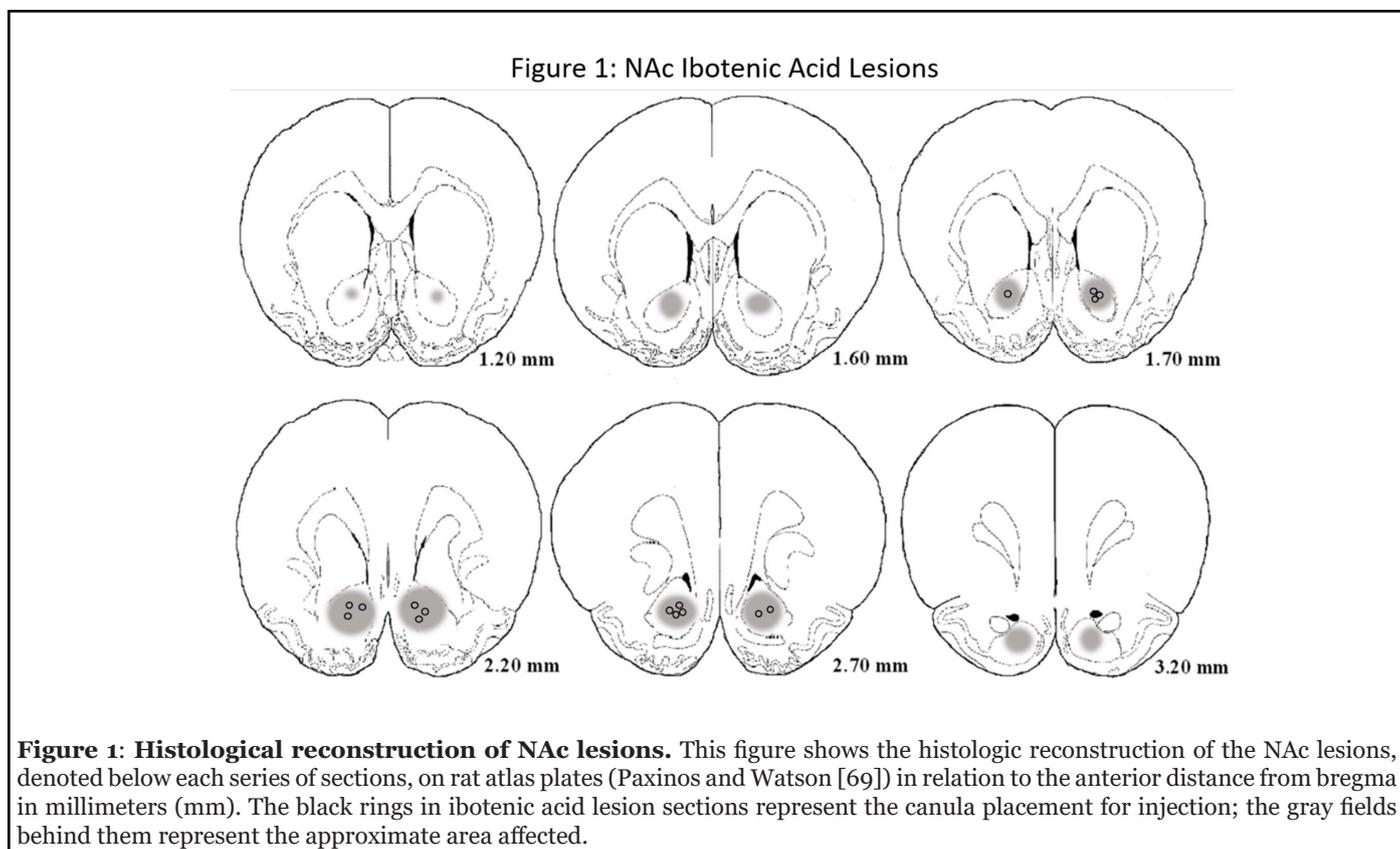
**NAc Glutamatergic system ablation:** For the glutamatergic ablation group, ibotenic acid, a glutamatergic toxin, was employed [70-74]. A 27G cannula was inserted bilaterally to a depth of 6.8 mm. 5 µg of ibotenic acid was dissolved in 5 µl of 0.9% normal saline was slowly infused then the cannula left in place for 6 minutes to allow for full diffusion. The cannulas were then removed, and the incision closed with wound staples.

### Apparatus

Behavioral locomotive activity was recorded using the open field computerized animal activity monitoring system (CAAM, AccuScan Instruments, Inc., Columbus OH). The CAAM system consists of 2 arrays of 16 infrared light beams with sensors on the opposite side, spaced every 2.5 cm that cross orthogonally through the plexiglass cage. Sensor polling frequency was set at 100 Hz. Movement of the rats interrupted the infrared light beams, and each beam-break detected by a sensor was collected as an event by the AccuScan Analyzer and transferred to a computer. Events over a 5-minute period were summed, giving 12 5-minute bins for each hour of observation. These bins were transferred to the OASIS data collecting software and three indices of behavioral locomotion were compiled for each collection period: total travelling distance (TD)- all forward locomotion in cm, horizontal activity (HA)- the overall movement in the lower level of the cage, and the number of stereotypic movements (NOS)- episodes of purposeless, repetitive movement in the upper level of the sensors separated by at least 1 second.

### Histology (Figure 1)

At the conclusion of the experiment, animals were overdosed with sodium pentobarbital and perfused with 10% formaldehyde. The brains were removed stored in 10% formaldehyde. 60 µm thickness coronal sections were



cut, stained, and scanned with a high-resolution scanner to identify lesion size and location correlated to the NAC using the Paxinos and Watson rat brain atlas [69] (Figure 1).

### Data analysis

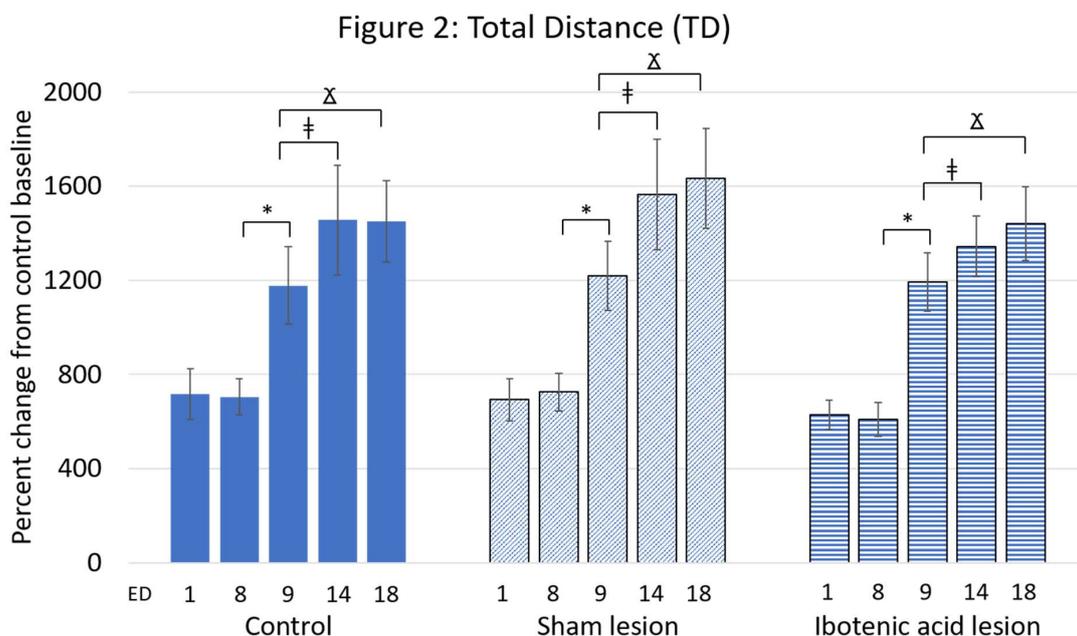
Rat behavioral locomotive activity was quantified by three compiled indices of movement (HA, TD, NOS) obtained in twelve 5-minute bins collected the hour after injections for each rat were averaged across each experimental group based on the experimental day to allow for comparisons. Post-surgical manipulation effects on baseline behavioral locomotor activity were determined by comparing the animal's activity after a saline injection before and after the surgical intervention (ED 8-Sal vs. ED 1-Sal). The acute effects of MPD were determined by comparing the first day of MPD administration to the post-surgical baseline (ED 9-MPD vs. ED 8-Sal). The effects of repetitive (chronic) MPD exposure over 6 consecutive days on behavioral locomotor activity were determined by comparing the final day of administration to the first, i.e. the induction phase (ED 14-MPD vs. ED 9-MPD). The effects of chronic MPD exposure following a washout period on behavioral locomotor activity were determined by comparing MPD re-challenge to the initial administration, i.e the expression phase (ED 18-MPD vs. ED 9-MPD) (See Table 1). Significance of change among these within-group comparisons was determined by ANOVA, with

repeated measures with adjustments for correlation among measurements within each animal. Post ad hoc comparisons were used to estimate changes between days within groups. A p-value<0.05 was considered statistically significant. The effects of the ibotenic acid lesion were determined by comparing the ibotenic acid lesion group to both the control and sham groups on each of the recording days (ED 1-Sal, ED 8-Sal, ED 9-MPD, ED 14-MPD, and ED 18-MPD). Significance of change among the between-group comparisons was determined with Turkey-Kramer Honest Significant Difference (HSD) post hoc test. A p-value<0.05 was considered statistically significant.

## Results

### Effect of MPD on activity (Figure 2)

Figure 2 shows the effect of the MPD administration on total distance (TD) traveled on the five recording days (ED 1-Sal, ED 8-Sal, ED 9-MPD, ED 14-MPD, and ED 18-MPD) for the NAc control, sham, and ibotenic acid lesion groups. Surgery with or without chemical intervention to the NAc (ED 8-Sal vs. ED 1-Sal) did not lead to a statistically significant change in TD for the sham and ibotenic acid lesion groups as compared to the control group (Figure 2). Similar results were seen in horizontal activity (HA) and number of stereotypic movements (NOS). This observation indicates that animal handling, injection volume, and injection procedure were consistent, and



**Figure 2: Total Distance within groups.** This figure shows the mean total distance (TD) traveled and standard error for each of the groups on experimental day (ED) 1, 8, 9, 14, and 18. Each group consists of n=8 rats. \* indicates statistically significant (p<0.05) difference between ED 8 baseline and ED 9 MPD. † indicates statistically significant (p<0.05) difference between ED 9 and ED 14. Δ indicates statistically significant (p<0.05) difference between ED 9 MPD and ED 18 MPD.

that the surgical intervention did not modulate baseline activity. The administration of 2.5 mg/kg MPD yielded a statistically significant ( $* p < 0.05$ ) increase in TD following MPD exposure for all groups relative to their post-surgical baseline (ED 9-MPD vs. ED 8-Sal) (Figure 2). Similar results were seen in HA and NOS. Administration of a repetitive 2.5 mg/kg MPD dose for an additional five consecutive days resulted in a further statistically significant ( $\ddagger p < 0.05$ ) increase in TD beyond the acute effect of MPD for all groups (ED 14-MPD vs. ED 9-MPD) (Figure 2). Similar results were seen in HA and NOS. This further augmentation in locomotive behavior following repeated exposure to MPD confirms that 2.5 mg/kg MPD induces behavioral sensitization. Re-challenge with the same 2.5 mg/kg MPD dose after a three-day washout period following chronic MPD exposure (six days of MPD administration) caused all groups to again show a further statistically significant ( $\text{X} p < 0.05$ ) increase in TD as compared to acute MPD administration (ED 18-MPD vs. ED 14-MPD) (Figure 2). Similar results were seen in HA and NOS. This continued augmentation of the response to MPD even after drug washout is the continued expression of sensitization to chronic psychostimulant use, i.e. the expression phase.

### Effect of ibotenic acid lesion on TD (Figure 3)

Figure 3 shows the effects of ibotenic acid lesions to the NAc on TD by comparing each group (control, sham,

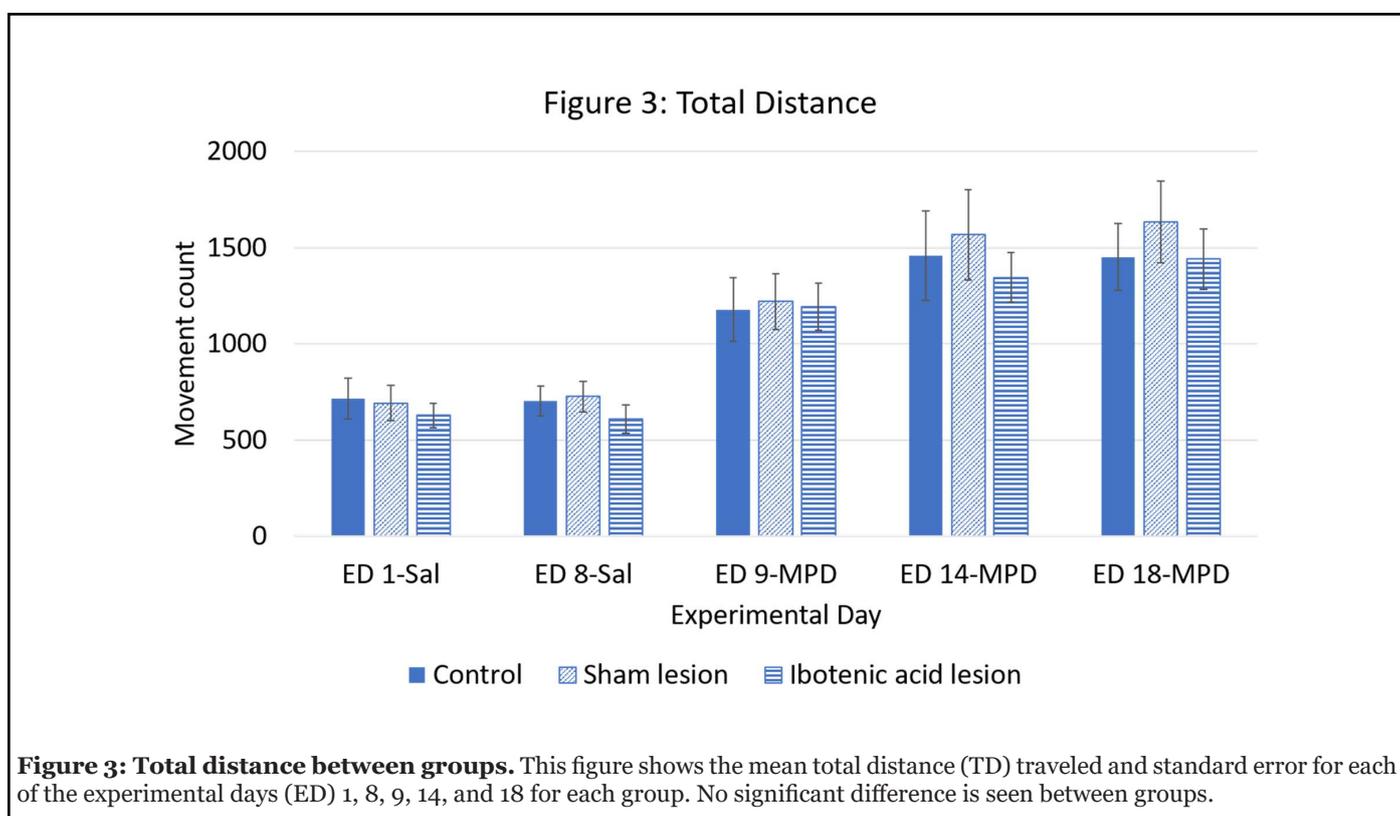
and ibotenic acid lesion) to the other two groups on each experimental day. No significant difference was seen between any of the groups on any of the experimental days, i.e. all groups behaved similarly in response to MPD exposure.

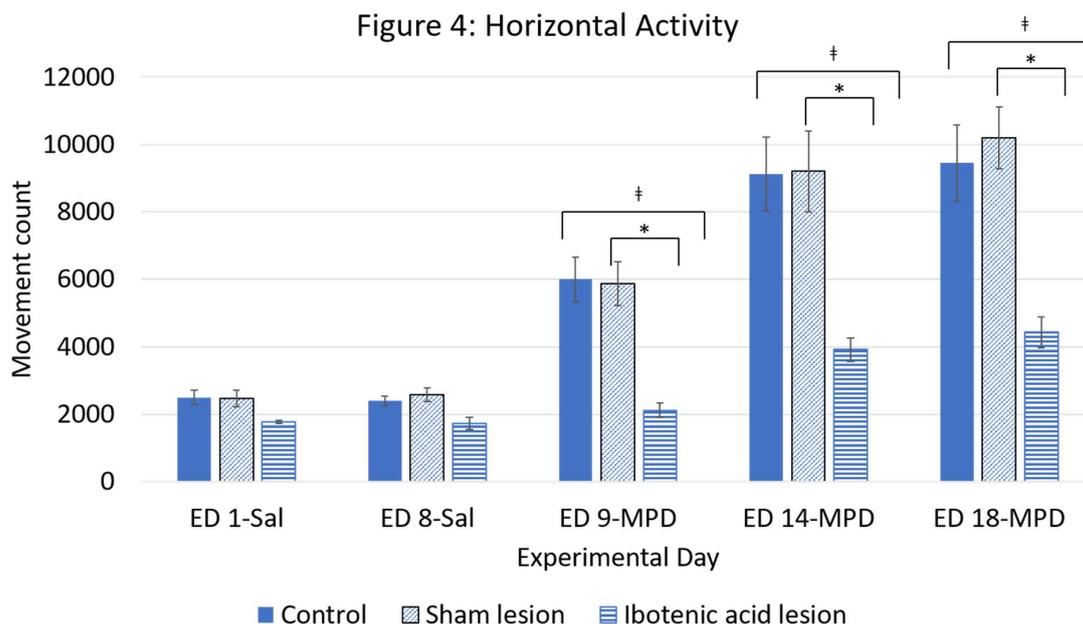
### Effect of ibotenic acid lesion on HA (Figure 4)

Figure 4 shows the effects of ibotenic acid lesions to the NAc on HA by comparing each group (control, sham, and ibotenic acid lesion) to the other two groups on each experimental day. Compared to the control and sham groups, the group that received lesions to the NAc showed a significant difference between the control ( $\ddagger p < 0.05$ ) and the sham ( $* p < 0.05$ ) groups in response to MPD both acutely (ED 9-MPD) and chronically (ED 14-MPD and ED 18-MPD).

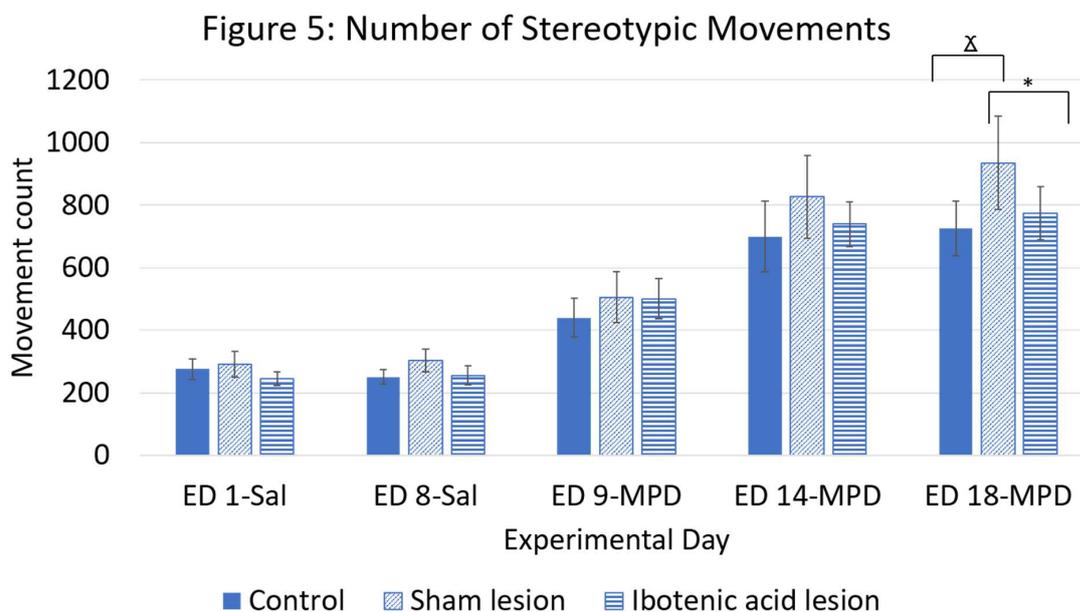
### Effect of ibotenic acid lesion on NOS (Figure 5)

Figure 5 shows the effects of ibotenic acid lesions to the NAc on NOS by comparing each group (control, sham, and ibotenic acid lesion) to the other two groups on each experimental day. No significant difference was seen between any of the groups on ED 9-MPD or ED 14-MPD. On ED 18-MPD, a significant difference was seen between the sham lesion group and both the NAc intact control ( $\text{X} p < 0.05$ ) and the ibotenic acid lesion groups ( $* p < 0.05$ ). No significant difference was seen between the control and ibotenic acid lesion group.





**Figure 4: Horizontal activity between groups.** This figure shows the mean horizontal activity (HA) and standard error for each of the experimental days (ED) 1, 8, 9, 14, and 18 for each group. † indicates statistically significant ( $p < 0.05$ ) difference between the control group and the ibotenic acid lesion group. \* indicates statistically significant ( $p < 0.05$ ) difference between the sham lesion and the ibotenic acid lesion group. No difference is seen between the control and sham groups.



**Figure 5: Number of stereotypic movements between groups.** This figure shows the mean number of stereotypic movements (NOS) and standard error for each of the experimental days (ED) 1, 8, 9, 14, and 18 for each group. \* indicates statistically significant ( $p < 0.05$ ) difference between the sham lesion and the ibotenic acid lesion group. X indicates statistically significant ( $p < 0.05$ ) difference between the sham lesion and the control group. No difference is seen between the control and ibotenic acid lesion groups.

## Discussion

This experiment was conducted to determine the role of glutamatergic signaling in the nucleus accumbens (NAc) in the response to acute and chronic methylphenidate (MPD). The findings of this work show that in NAc intact animals, 2.5 mg/kg MPD results in an acute increase in activity in all locomotor indices studied (TD, HA, NOS, Figure 2), and that chronic repetitive exposure results in behavioral sensitization- the further significant increase above the acute effect (Figure 2). This effect is clearly modulated following a specific bilateral glutamatergic lesion to the NAc with ibotenic acid, with HA specifically showing a consistent significant difference from the NAc intact control and sham groups following both acute and chronic 2.5mg/kg MPD exposure (Figure 4). This difference was absent in the other indices (TD and NOS, Figures 3 and 5 respectively). The sham lesion alone shows a difference from the control and ibotenic lesion groups, which appears to be a statistical artifact as the magnitude is similar to the NAc intact controls and the ibotenic acid lesion group and the difference is not seen on ED 14-MPD when the chronic effect of MPD is also manifested.

The NAc is a structure located near the anterior commissure that is critical for the motivation and reward-seeking behavior. It is composed primarily of dopaminergic medium spiny neurons (MSN's) and is divided into a shell and a core that mediate different functions [75-80]. The NAc receives input primarily from the VTA, in addition to inputs from the substantia nigra, the amygdala, the hippocampus, and the PFC. The NAc outputs ascend to various basal ganglia and midbrain structures including the substantia nigra, the VTA, the ventral pallidum, the thalamus, the subpallidus, and the stria terminalis [78,81-84].

Previously reported lesions to the NAc have confirmed its role in mediating the behavioral response to MPD. Psychostimulants such as MPD cause an increase in dopaminergic transmission from the VTA to the NAc, and increased dopamine within the NAc leads to increased locomotion [85-87]. Direct chronic microinjection of dopaminergic agonists such as amphetamine, cocaine, or morphine into the NAc can induce sensitization [38,88-96], suggesting that the NAc is involved in the induction of behavioral sensitization. Non-specific lesions to the NAc have been shown to lead to an enhanced acute effect of MPD, but absent long-term behavioral changes such as sensitization following chronic exposure [44]. This is also seen with amphetamine, cocaine, and nicotine [97-103]. Dopaminergic lesions to the NAc have produced more complex behavioral changes, with some animals exhibiting no increase in locomotor activity acute MPD exposure and others showing a significantly elevated locomotor activity following MPD exposure [45]. Animals that responded

to MPD acutely did not develop behavioral sensitization, while those that showed no behavioral change following the dopaminergic lesion did show behavioral sensitization [45]. This work was noted to not determine lesion accuracy which could explain the dichotomy of animal responses, however it still indicated that accumbal dopaminergic signaling is critical for the response to psychostimulants.

Glutamatergic signaling in the NAc has been unexplored till this present study, but has been shown to be critical in other reward circuit nuclei [26,28,29,36,44,46-60]. This study found that following specific glutamatergic ablation of the NAc by ibotenic acid, animals showed the same general response to acute and chronic MPD exposure as the control and sham NAc lesion groups, with an acute increase in behavioral activity following MPD and then further augmentation with chronic exposure (Figure 2). However, when the different behavioral expressions (HA, TD, NOS) to MPD exposure were compared between groups, a significant attenuation in the behavioral activity comprising forward motion as measured by HA was seen following glutamatergic-specific lesions to the NAc (Figures 3-5). This attenuation of HA indicates that glutamatergic signaling in the NAc is critical in modulating behavior and influences signaling pathways differently. This fits with the current knowledge that glutamatergic inputs to the NAc come from other reward circuit nuclei [104,105], and with other work showing that glutamatergic signaling is responsible for modulating the core effect of MPD at other reward circuit nuclei [26,28,29,36,44,46-60]. It also seems to indicate that different subcortical circuits govern different behavioral responses, as animals with glutamatergic lesions to the NAc, HA exhibited significantly less behavioral activity in response to both acute and chronic MPD exposure. HA is a measure of forward motion, and can be regarded as a goal-directed behavior (although in this experiment the open field assay is not designed to stimulate this mode of activity) which would seem to imply that glutamatergic signaling specifically modulates motivational circuits versus generalized motor modulation, which would involve both TD and NOS.

Previous work initially determined the NAc shell to be critical for the excitatory response to psychostimulants, as it showed the greatest response in response to their administration [90,95,106,107]. However, it is increasingly being recognized that the NAc core also participates in the response to psychostimulants [108-111], and that both play a role in motivation and behavioral actions [112-114]. The results seen here agree with emerging work showing that while the NAc core and shell are anatomically distinct, distinct circuits between them govern different behavioral responses [108-114]. Targeting a spherical shell structure with a chemical lesion presents a substantial technical problem and further interrogation of these distinctions will require further work. The strength of the paper

is the using of local injection of specific neurotoxin to eliminate the glutaminergic system in the NAc. Additional experiments are needed to use specific neurotoxin to study other neurotransmitters signaling.

## Conclusion

The nucleus accumbens (NAc) is a rewards circuit structure that is critical for the response to MPD. It is divided into a shell and core that serve distinct roles in the response to psychostimulants such as MPD. Three different locomotive behaviors were studied, and it was found that lesions to the glutaminergic signaling pathways of the NAc result in significant attenuation of forward motion HA compared to control and sham groups. This difference was not seen in the other behaviors (TD and NOS), indicating that different NAc circuits govern specific behavioral expressions to acute and chronic MPD.

## Acknowledgements

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