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Research Article

Glutaminergic Signaling in the Nucleus Accumbens Modulates the Behavioral Response to Acute and Chronic Methylphenidate -

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SUMMARY

Methylphenidate (MPD) is a psychostimulant that acts on the CNS to produce behavioral effects. The Nucleus Accumbens (NAc) is involved in this, however the role of the NAc's glutaminergic system in the behavioral response to MPD has not been studied. Three groups of animals were used: control, sham NAc lesions, and glutaminergic-specific (ibotenic acid toxin) NAc lesion groups. On Experimental Day (ED) 1, all groups received saline. 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 base line. Groups then received six daily MPD 2.5 mg/kg injections (ED 9-14) to produce a chronic effect of MPD exposure such as behaviora sensitization, then three days of washout (ED 15-17), followed by a re-challenge with 2.5 mg/kg MPD on ED 18. Locomotive activity was recorded for 60 minutes after each injection. All groups showed an increase in behavioral activity following acute MPD exposure, and developed behavioral sensitization following chronic MPD exposure that was maintained after washout. Compared to NAc intact controls and sham lesions, glutaminergic selective ibotenic acid lesions to the NAc significantly ($P<0.05$) attenuated the horizontal activity response to both acute and chronic MPD. Glutaminergic selective ibotenic acid lesions to the NAc also resulted in further significant ($P<0.05$) augmentation of stereotypic activity above the control group. The glutaminergic lesion failed to modulate total distance traveled. This indicates that glutaminergic signaling in the NAc modulates behavioral activity circuits in the NAc differently, and suggests a role in the volitional response to MPD.

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 [17,26-29]. 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 [31-37]. 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 glutaminergic system remains uninvestigated. Glutaminergic 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 glutaminergic system of the NAc participates in the response to MPD. To do this, 3 groups of

animals were used: NAc intact controls, NAc sham lesion, and NAc specific glutaminergic 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.

MATERIALS AND 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 glutaminergic 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 200-220g 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 [27-29,49,52,61-68]. For the next 3 days (ED 15-17), animals received no injections (the

Table 1: Methylphenidate administration schedule. The table shows the experimental treatment protocol for the 3 groups of rats used. Each group consisted of N=8 rats. Displayed are the Experimental Days (ED's) and the intervention performed (surgery, washout, normal saline or methylphenidate (MPD) 2.5 mg/kg ip injection in 0.8 ml at 07:30).

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

* indicates day rat behaviors were immediately 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

washout period). After the washout period (ED 18-MPD), the rats were re-challenged with MPD at the previous dose 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, 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 Glutamnergic system ablation: For the glutamnergic ablation group, ibotenic acid, a glutamnergic 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.

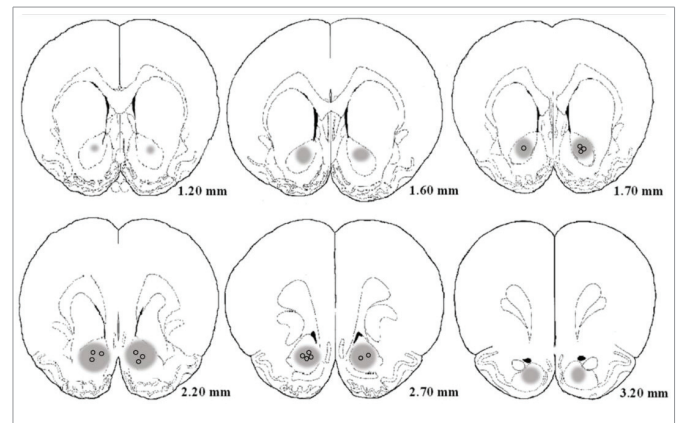


Figure 1: Histological reconstruction of NAc lesions. This figure shows the histologic reconstruction of the NAc lesions, denoted below each series of sections, on rat atlas plates (Paxinos and Watson, 1986) in relation to the anterior distance from bregma in millimeters (mm). The black rings in ibotenic acid lesion sections represent the cannula placement for injection; the gray fields behind them represent the approximate area affected.

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). For these between-group comparison, rat locomotive activity was interpreted as the percent change from baseline for each of the indices. Baseline activity was defined as the average movement of an experimental group on the first experimental day post saline injection (ED1-Sal) for each locomotive index and thus experimental day 1 (ED1-Sal) had no percent change. Each of the locomotive behavior indices for each study-relevant day were calculated as a percent change from that baseline. Significance of change among the between-group comparisons was determined with the Critical Ratio (C.R.) test. A C.R. of greater than 1.96 or less than -1.96, corresponding to $p < 0.05$, was considered statistically significant [64,75-77]

RESULTS

Overall 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 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 ($p < 0.05$) 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 ($p < 0.05$) increase in TD as compared to acute MPD administration (ED 18-MPD vs. ED 9-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 total distance traveled (Figure 3)

Figure 3 shows the percent change in behavioral activity as measured by Total Distance (TD) traveled following both ibotenic acid lesions to the NAc and acute and chronic MPD exposure, and compares each group (control, sham, and ibotenic acid lesion) to the other two groups on each experimental day. A statistically significant ($p < 0.05$) difference is seen between the ibotenic acid lesion group

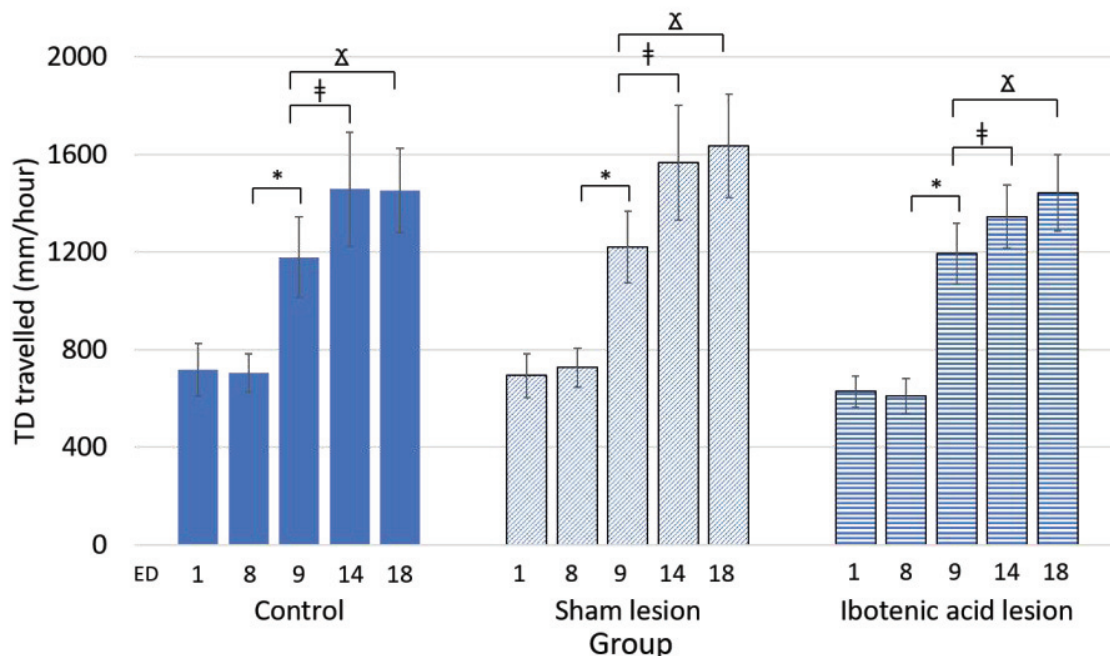


Figure 2: Total Distance traveled (activity count). This figure shows the mean Total Distance (TD) traveled and standard error in mm/hour for each of the groups on experimental day (ED) 1, 8, 9, 14, and 18. Each group consists of $n=8$ rats. ED's within each group were compared using ANOVA. * indicates statistically significant ($p < 0.05$) difference between ED 9-MPD and ED 8-Sal. † indicates statistically significant ($p < 0.05$) difference between ED 14-MPD and ED 9-MPD. ‡ indicates statistically significant ($p < 0.05$) difference between ED 18-MPD and ED 9-MPD.

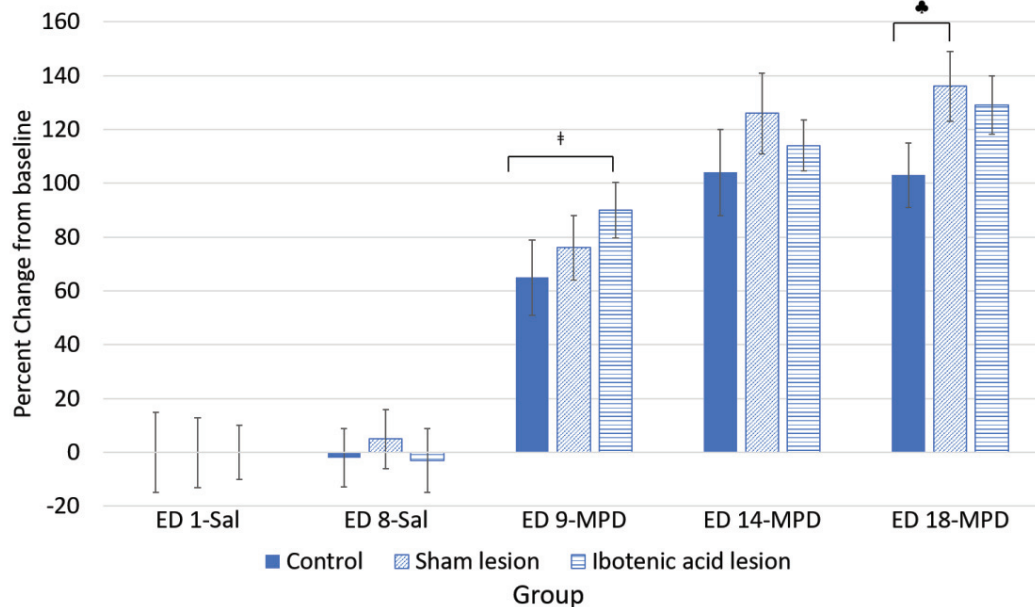


Figure 3: Total distance traveled (percent change). This figure shows the mean Total Distance (TD) traveled and standard error for each of the experimental days (ED) 1, 8, 9, 14, and 18 for each group as a percent change from the ED 1-Sal baseline. Experimental groups were compared across a given ED using the CR test. # indicates a statistically significant ($p < 0.05$) difference between the ibotenic acid lesion group and the control group. ♣ indicates a statistically significant ($p < 0.05$) difference between the sham lesion group and the control group.

and the control group only on ED 9-MPD following acute MPD exposure. A statistically significant ($p < 0.05$) difference is also seen between the sham lesion group and the control group only on ED 18-MPD following MPD re-challenge after a 3-day washout period. No significant difference was seen between the ibotenic acid lesion group and the sham lesion group. The similar overall response to MPD and the inconsistent differences between the ibotenic acid lesion, sham, and control groups indicates that glutaminergic signaling does not participate in the TD traveled in response to MPD.

Effect of ibotenic acid lesion on horizontal activity (Figure 4)

Figure 4 shows the percent change in behavioral activity as measured by forward motion traveled, i.e. horizontal activity (HA), following ibotenic acid lesions to the NAc and acute and chronic MPD exposure, and compares 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 bilateral ibotenic acid lesions to the NAc showed a significant difference between the control ($p < 0.05$) 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). This significant decrease in forward locomotion following glutaminergic lesion to the NAc indicates that this circuit facilitates the excitatory effect of MPD on HA and forward movement behavior.

Effect of ibotenic acid lesion on stereotypic behavior (number of stereotypic movements, NOS) (Figure 5)

Figure 5 shows the percent change in behavioral activity as measured by stereotypic behavior, i.e. the number of stereotypic (NOS) movements, following ibotenic acid lesions to the NAc and

acute and chronic MPD exposure, and compares each group (control, sham, and ibotenic acid lesion) to the other two groups on each experimental day. The group that received bilateral ibotenic acid lesions to the NAc showed a significant difference ($p < 0.05$) between the control group following both acute (ED 9-MPD) and chronic (ED 14-MPD and ED 18-MPD) MPD exposure. The ibotenic acid lesion group also showed a significant difference ($* p < 0.05$) between the sham lesion group only following acute MPD exposure on ED 9-MPD. A significant difference ($p < 0.05$) was seen between the sham lesion and control groups only on ED 18-MPD. This consistent increase in NOS between the control and lesion group suggests that glutaminergic signaling in the NAc plays an inhibitory role in the NOS behavioral response.

DISCUSSION

This experiment was conducted to determine the role of glutaminergic 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 glutaminergic lesion to the NAc with ibotenic acid, with HA 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), and NOS showing a consistent significant difference from the NAc intact control group following both acute and chronic 2.5mg/kg MPD exposure (Figure 5). Inconsistent differences were seen in TD traveled (Figure 3). These findings indicate that distinct glutaminergic circuits in the NAc modulate different behavioral responses to MPD.

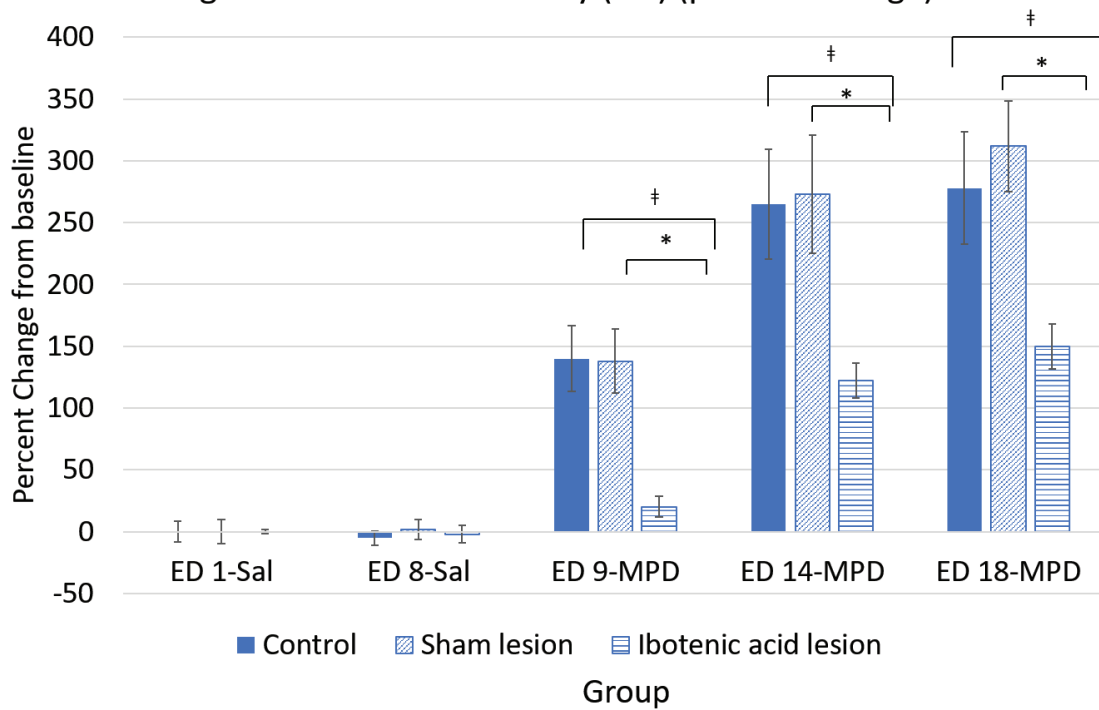


Figure 4: Horizontal activity traveled (percent change). This figure shows the mean horizontal activity (HA) traveled and standard error for each of the experimental days (ED) 1, 8, 9, 14, and 18 for each group as a percent change from the ED 1-Sal baseline. Experimental groups were compared across a given ED using the CR test. † indicates a statistically significant ($p < 0.05$) difference between the ibotenic acid lesion group and the control group.

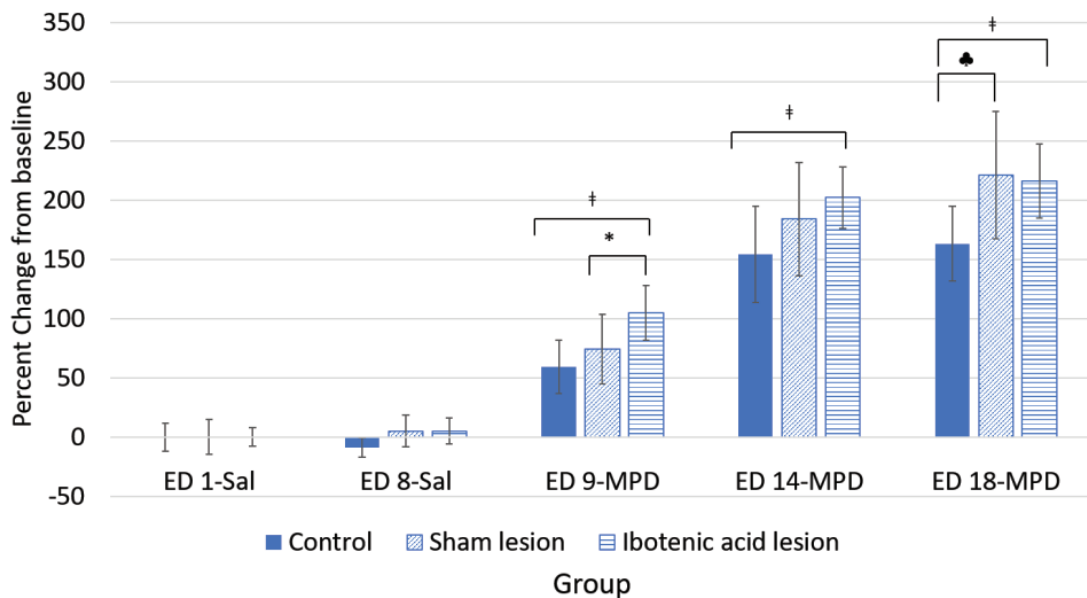


Figure 5: Number of stereotypic behaviors (percent change). This figure shows the mean number of stereotypic movements (NOS) traveled and standard error for each of the Experimental Days (ED) 1, 8, 9, 14, and 18 for each group as a percent change from the ED 1-Sal baseline. Experimental groups were compared across a given ED using the CR test. † indicates a statistically significant ($p < 0.05$) difference between the ibotenic acid lesion group and the control group. * indicates statistically significant ($p < 0.05$) difference between the ibotenic acid lesion group and the sham lesion group. No difference is seen between the control and sham groups. ♣ indicates a statistically significant ($p < 0.05$) difference between the sham lesion group and the control group.



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 [78-83]. 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 [81,84-87].

Previously reported lesions to the NAc have confirmed its role in mediating the behavioral response to MPD [44,45]. 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 [88-90]. Direct chronic microinjection of other addictive substances such as amphetamine, cocaine, or morphine into the NAc can induce behavioral sensitization [38,91-99], 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 [100-106]. Dopaminergic lesions to the NAc have produced more complex behavioral changes, with some animals exhibiting no increase in locomotor activity following 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 in general showed the same characteristic 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 significant 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, while a significant augmentation is seen in stereotypic behavior as measured by NOS (Figures 3, 4, & 5). This attenuation of HA and augmentation of NOS indicates that glutamatergic signaling in the NAc is critical in modulating behavior and plays differing roles in different behavioral signaling pathways. This fits with the current knowledge that glutamatergic inputs to the NAc come from other reward circuit nuclei [107,108], 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 which would

seem to imply that glutamatergic signaling specifically modulates motivational circuits versus generalized motor modulation, which would involve TD. This fits with the observation that purposeless stereotypic movements as measured by NOS are increased following glutamatergic lesions to the NAc, further confirming the loss of volitional behavioral activity. Further work to explore this volitional role of glutamatergic signaling in the NAc could utilize other behavioral assays of goal-directed behavior such as lever pulling or maze running.

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 [93,98,109,110]. However it is increasingly being recognized that the NAc core also participates in the response to psychostimulants [111-114], and that both play a role in motivation and behavioral actions [115-117]. 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 [111-117]. Targeting a spherical shell structure with a chemical lesion presents a substantial technical problem and further interrogation of these distinctions will require further work.

In conclusion, the NAc is a component of the rewards circuit 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 glutamatergic signaling pathways of the NAc resulted in significant attenuation of forward motion HA compared to control and sham groups while significant augmentation was seen in stereotypic movement, NOS, as compared to controls. This difference indicates that different NAc circuits govern specific behavioral expressions to acute and chronic MPD and the glutamatergic circuit likely modulates volitional responses to psychostimulants.

FUNDING AND DISCLOSURES

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AUTHOR CONTRIBUTIONS

N. King assisted in data preparation, statistical analysis, and was the principle drafter of the manuscript. T. Ming performed the experiment and assisted in statistical analysis. N. Dafny designed the experimental protocol, assisted in conducting the experiment, assisted in manuscript preparation, and approved the final draft seen here. The help of N. Kharas is appreciated.

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