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Nucleus accumbens neuronal activity correlates to the animal's behavioral response to acute and chronic methylphenidate

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Abstract

Acute and chronic Methylphenidate (MPD) exposure was recorded simultaneously for the rat's locomotor activity and the nucleus accumbens (NAc) neuronal activity. The evaluation of the neuronal events was based on the animal's behavior response to chronic MPD administration: 1) Animals exhibiting behavioral sensitization 2) Animals exhibiting behavioral tolerance. The experiment lasted for 10 days with four groups of animals; saline, 0.6, 2.5, and 10.0mg/kg MPD. For the main behavioral findings, about half of the animals exhibited behavioral sensitization or behavioral tolerance to 0.6, 2.5, and/or 10 mg/kg MPD respectively. Three hundred and forty one NAc neuronal units were evaluated. Approximately 80% of NAc units responded to 0.6, 2.5, and 10.0 mg/kg MPD. When the neuronal activity was analyzed based on the animals behavioral response to chronic MPD exposure, significant differences were seen between the neuronal populations responses recorded from animals that expressed behavioral sensitization when compared to the NAc neuronal responses recorded from animals exhibiting behavioral tolerance. Three types of neurophysiological sensitization and neurophysiological tolerance can be recognized following chronic MPD administration to the neuronal populations.

Collectively, these findings show that the same dose of chronic MPD can elicit either behavioral tolerance or behavioral sensitization. Differential statistical analysis were used to verify our hypothesis that the neuronal activity recorded from animals exhibiting behavioral sensitization will respond differently to MPD compared to those animals exhibiting behavioral tolerance. Thus, suggesting that it is essential to record the animals behavior concomitantly with neuronal recordings.

Keywords

Ritalin; nucleus Accumbens; neuronal activity; behavior; sensitization; tolerance

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Introduction

Recent reports indicate the increased use of methylphenidate (MPD) for behavioral disorders, cognitive enhancing effects, and for recreation [1,2]. MPD exerts its effects by altering the dopamine (DA) system. The exact role of MPD on the DA system is unclear; however MPD has been shown to have a chemical structure similar to cocaine, a drug with a high probability of abuse [3,4].

The nucleus accumbens (NAc) plays a key function in the neural circuitry underlying psychostimulant action and the constructs of reward [5]. The NAc mediates reward behavior through dense dopaminergic projections from the ventral tegmental area (VTA) [6]. The VTA interacts with the pre-frontal cortex (PFC) glutamatergic transmission facilitating the rewarding actions of psychostimulants [7,8]. The NAc also receives excitatory glutamatergic inputs from the thalamus, hippocampus, and amygdala [9]. Glutamatergic inputs to the NAc form synapses onto the densely populated medium spiny neurons (MSN). MPD has also been shown to inhibit the norepinephrine (NE) transporter, thus increasing the levels of NE in the NAc [10]. Behavioral sensitization and tolerance are linked to the process of neuronal plasticity of drug-induced cellular and molecular adaptations [11,12]. The propensity for a drug to elicit behavioral sensitization or tolerance is linked to the psychostimulants effect on the brains mesolimbic DA and NE system, which includes the NAc [13,14]. Behavioral sensitization is defined as an increased behavioral response to psychostimulant exposure following repetitive administration [14,15,16].

The majority of investigations study the property of MPD's effects on animal behavior as a group using behavioral assays before and after different means of drug administration; lesioning or neurochemical/molecular approaches [17,18,19,20]. Current literature reports conflicting results within the same drug dose [17,19,21]. Some report that the same dose of MPD elicits behavioral sensitization, while other studies report that same dose elicited behavioral tolerance [17,18,19]. Thus leading to initial hypothesis; the same repetitive dose of MPD can elicit either behavioral sensitization or behavioral tolerance.

Previous studies have explored the properties of psychostimulants either in-vitro [22], in-vivo under anesthesia [22,23], or on sensory evoked responses [16,19,25]. Recent studies examined the neurophysiological effect of a single 2.5 mg/kg MPD dose on the NAc, PFC and caudate nucleus (CN) [26,27,28]. Leading to our second hypothesis; the NAc units electrophysiological responses recorded from animals exhibiting behavioral sensitization will be different from those NAc units recorded from animals exhibiting behavioral tolerance. To verify this hypothesis, we recorded simultaneously the behavioral and the neuronal activity from freely behaving rats following an MPD dose response protocol to acute and chronic MPD administration, our two hypotheses were confirmed.

Materials and Methods

Subjects

Forty- seven (47) adult male Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) weighing 150–175 grams upon arrival were housed individually for 5 to 7 days in single Plexiglas

cages inside a sound-attenuated animal facility room for adaptation. The homepage was utilized as the test cage throughout experiment. The room was maintained on a 12-h light/dark cycle (lights on 06:00). The room was maintained at an ambient temperature of $21 \pm 20^{\circ}\text{C}$ and humidity between 58–62%. Rats were supplied food and water ad libitum for the entire duration of the study. All experiments were 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.

Drug

Methylphenidate hydrochloride (MPD) was obtained from Mallinckrodt Inc. (St. Louis, MO, USA). Based on previous dose response experiments (from 0.1 to 40 mg/kg i.p. MPD), the doses of 0.6, 2.5, and 10.0 mg/kg MPD administered intraperitoneal (i.p.) in the morning were selected since these doses elicited behavioral sensitization or tolerance [16,17,19,20,29,30,32,32]. MPD was dissolved in 0.9% saline (NaCl) solution and the dose was calculated as free base. All injections were equalized to 0.8 ml with saline and were administered between 08:00am and 9:00am.

Surgery

On the day of surgery rats were weighed and anesthetized with 50 mg/kg i.p. pentobarbital. The top of the rat's head was shaved to expose the skin and coated with a thin layer of 2% Lidocaine Hydrochloride Jelly (Akorn, Inc.). The animal was then placed in a stereotaxic instrument. A one inch incision was made and the muscle and connective tissue was removed to expose the skull. A single hole was made above the frontal sinus for the reference electrode and two bilateral 0.6mm diameter holes were drilled over the NAc in accordance to the coordinates derived from [33] rat brain atlas (1.70mm anterior from bregma, 1.2mm lateral from midline). Prior to electrode placement, 6 anchor screws were put in vacant areas of the skull to secure the skull-cap with dental acrylic. Two twisted Nickel-Chromium, Diamel coated; 60 micron diameter wire electrodes (fully insulated except at tips) were secured each to a 1cm copper connector pin made prior to surgery. The reference electrode was placed in the frontal sinus and two twisted recording electrodes were implanted in the NAc as follows: One twisted electrode (i.e., two electrodes together) was inserted into the drilled hole at an initial depth of 6.8 mm. Unit activity was monitored during placement of electrodes by using a Grass emitter Hi Z Probe connected to a Grass P511 series pre-amplifier. Electrodes were fixed to the skull only when spike activity exhibited at least a 3:1 signal to noise ratio in both electrodes. If the activity did not match the 3:1 spike to noise ratio criteria, the electrode was moved down in approximate increments of 10 microns until they displayed a proper signal to noise ratio of neuronal activity. Once a sufficient signal was obtained, the electrode was fixed in the skull with Webglue, cyanoacrylate surgical adhesive (Webster Veterinary). The secondary twisted electrode was implanted using identical procedures in the other hemisphere [16,19,25,26,27,34,35,36]. The electrode connector pins were inserted into Amphenol plugs which were positioned on the skull and secured to the skull with dental acrylic cement. Rats were allowed to recover from the surgical procedure for approximately 4 to 7 days. During this recovery period, every day for 2 hours, the rat, with his home cage, was placed in the experimental behavioral apparatus and connected to the wireless (telemetric) head stage

transmitter (Triangle BioSystems, Inc; Durham, NC, USA) for daily acclimation to the recording systems.

Experimental protocol

Animals were randomly assigned into four groups; saline, 0.6, 2.5, or 10.0 mg/kg MPD. Experimentation began 4 to 7 days post-surgery when animals were approximately 200 to 220 grams and lasted for 10 days. On experimental day 1 (ED1) prior to the start of the recording session, animals were again allowed to acclimate to the recording system for one hour. During this time, the recording parameters were organized in order to properly record the neuronal activity and save the files. Immediately post saline (0.8 ml of 0.9%) injection, a 60 min baseline of neuronal and behavioral activity was recorded simultaneously. Next, a saline, 0.6, 2.5, or 10.0 mg/kg MPD injection (depending on the group) was administered and recordings were resumed for another 60 minutes. From ED2 to ED6 rats were injected once daily with the same MPD concentration and at the same time as on ED1. All injections were done in their home cages (which were also their test cage), ED7 to ED9 were washout days where no injections were given. On ED10, identical experimental protocol as ED1 was followed; neuronal and behavioral baseline activity was recorded for 60 min following a saline injection, as well as an additional 60 min neuronal and behavioral recording after a saline or MPD rechallenge injection (See table 1). The saline injection group was used as a control for handling, injection, and injection volume [16,19,20,25,26,27,28,32]. To eliminate the environmental contribution to the drug effect, such as novel conditions, all the recordings and the MPD injections were carried out in the animal's home cage. This arrangement warranted that any change from baseline activity is due to the drug (MPD) effect.

Behavioral Apparatus

Locomotor activity was recorded using an open field computerized animal activity system (Opto-M3, Columbus Instruments, Columbus, OH). The animals were housed in clear acrylic cages which fit into the recording apparatus thus allowing us to record the animal's behavior in their home cages. The Columbus open field system comprised of infrared beam sensors that run 40 cm in length, by 20 cm in width with 16 by 8 infrared beams respectively, and their sensors set 5 cm above the floor of the cage. The open field assay has been previously described in detail [15,16,17,19,20,31,37]. In short, the activity monitoring system records each of the sensor interruptions at a 100 Hz frequency to determine when a beam was interrupted. Any interruptions were subsequently recorded by software and downloaded to a PC in 10 minute bins. The program organized the data from the beam interruptions into locomotor movement indices, horizontal activity (HA) which records the overall locomotor activity and the number of stereotypy (NOS) activity which counts the number of repetitive movement episodes with at least one second interval before the beginning of another episode of movements (data not shown). The beam break count was compiled and downloaded to a PC in 10 minute bin increments and evaluated from 60 minute post injection for both the saline (baseline) and MPD administration on ED1 and at ED10. The objective of the behavioral recoding was to distinguish animals that expressed behavioral sensitization from animals that expressed behavioral tolerance following repeated

MPD exposure. This grouping would be used as the basis for analyzing the neurophysiological data.

Analysis of behavioral data

The locomotor activity recorded on ED1 and ED10 was summed each into 10 minute bins for 60 minute (i.e. 6 bins/hr). The HA and NOS was then analyzed for each individual rat using a paired t test with significance set at $P < 0.05$. Three comparisons were made: (1) ED1 baseline compared to ED1 post MPD administration to determine the acute effect of the drug (see Table 1); (2) ED10 baseline compared to ED1 baseline to evaluate whether the six daily MPD exposures and the three washout days elicits changes on ED 10 baseline compared to ED1 baseline activity (3) ED10 MPD rechallenge compared to ED1 MPD acute exposure to determine if behavioral sensitization or tolerance was expressed (Table 1). Based on the third comparison, the animals were divided into two groups of either behaviorally sensitized or no change/tolerant. As a group the rats were then analyzed (for all three comparisons) using an ANOVA with repeated measures with adjustments for correlation among measurements. Bonferroni Post ad hoc comparisons were used to estimate changes between days within groups.

Electrophysiological recording

Data acquisition—On the experimental recording day, the rat was placed with his home cage in a Faraday testing box to reduce noise during signal transmission. The wireless Triangle Bio Systems (Durham, NC, USA) head stage was connected to the electrode pins of the skull cap. The Triangle BioSystem head stage sent neuronal activity signals through a receiver that connects to a Cambridge Electronic Design (CED) analog –to- digital converter (Micro1401-3; Cambridge, England) which collected and stored the recorded data onto a PC. Spike 2.7 software (CED) was used off-line to sort for identical spike amplitude and waveforms by examining the single unit spike activity that exhibited similar amplitude and wave form patterns before and after MPD administration for ED1 and ED10 (See section 2.7.2 on spike sorting for more details). This activity was used to produce a sequential frequency histogram and to calculate the firing rate in spikes per second. Approximately one to two spikes (units) were analyzed per electrode.

Spike sorting—For spike sorting we used the Spike2 version 7 software (Cambridge Electronics Design- CED). The raw recording (sampling rates up to 200 kHz) was captured by the program and processed using low and high pass filters (0.3–3 kHz). Two window levels were set, one for positive-going spikes and one for negative-going spikes. Spikes with peak amplitudes that were triggered by the window were used to create templates. One 1000 waveform data points was used to define a spike. The spikes were extracted when the input signal enters an amplitude window. Spikes with peak amplitude outside these limits were rejected. The algorithm that was used to capture a spike allowed the extraction of templates that provide high-dimensional reference points that can be used to perform accurate spike sorting, despite the influence of noise, spurious threshold crossing and waveform overlap. All temporally displaced templates were compared with the selected spike event to find the best fitting template that yields the minimum residue variance. Secondly, a template matching procedure is then performed; when the distance between the template and

waveform exceeds some threshold (80%) the waveforms were rejected. That means that the spike sorting accuracy in the reconstructed data is about 95%. All these parameters of spike sorting for each electrode were sorted and used for the activity recorded in experimental day 1 (ED1) and in ED10 i.e., we use identical criteria to sort spikes in ED1 and ED10 to ensure the spike pattern captured on ED1 is the same as ED10.

Analysis of the electrophysiological data—The sorted neuronal activity obtained from the fixed template matching system was converted by the spike 2 version 7 software (CED) into their firing rates (spikes per second) for the baseline control recording and for the activity following MPD administration. These firing rates were exported into a spreadsheet format displaying the rat's number, experimental day, MPD dose and channel (to distinguish hemisphere). Firing rates were evaluated for normality assumptions to determine parametric or non-parametric methods to evaluate differences in neuronal events activity before and after MPD treatments. The firing rates were determined to not hold normality assumptions, so we assessed differences in mean firing rates by using the critical ratio (CR) test. This test was used to determine whether acute and chronic MPD treatments altered NAc unit activity ($C.R. = (E - C) / (E + C) = \pm 1.96 = p < 0.05$) when comparing the effect of the initial (acute) MPD exposure, C – represents the activity following saline and E – the activity post MPD injection; when comparing the effect of six daily MPD exposures and three washout days on ED10 baseline C – represents the ED1 baseline, and E – represents the ED10 baseline activity; when comparing the effects of MPD rechallenge at ED10 to ED1; E represents the effects of MPD at ED10, and C represents the ED1 neuronal activity post MPD injection. In addition changes of each unit activity induced by the treatment was considered statistically significant if the firing rate after drug treatment differed by at least 2 standard error (S.E.) from the mean [26,27,33,38].

A one way ANOVA was used (per dose) to determine if the NAc neuronal populations recorded from animals exhibiting behavioral sensitization was significantly different from those exhibiting behavioral tolerance. A log linear model with a chi square value was used next to control for MPD dose when comparing the overall activity (acute, baseline and chronic) between the two groups (behaviorally tolerant and sensitized) to determine if there was a significant difference between dose behavior and firing patterns for each group (0.6, 2.5, and 10.0 mg/kg). P-values of < 0.05 obtained from the log linear model were considered as significant.

A two way ANOVA was used to determine if there was a statistically significant difference between the acute ED1 responses of neuronal populations in comparison to the chronic ED10 response for all doses, considering the two groups as two separate populations.

Histological verification of electrode placement—At the end of the experimental protocol, rats were deeply anesthetized with sodium pentobarbital. The rat's brain was transcardially perfused with 10% formalin solution containing 3% potassium ferrocyanide. A 2 mA DC current was passed through the electrode connector pin for 40 seconds to produce a small lesion. The brain was then excised and stored in 10% formalin for subsequent histological processing. Placements of the electrodes were verified in 60 micron thick coronal sections that were stained with cresylviolet. Coordinate position of the

electrode tips were established by matching equivalent locations of the lesion and the prussian blue spot by using the Rat Brain Atlas by [33]. (See figure 1)

Results

Locomotor behavior

Forty-seven (46) rats met the histological verification of electrode location placement within the NAc and the neurophysiological requirements of exhibiting similar spike amplitude and pattern at ED1 and at ED10 in-order to be included in the study (saline N=11; 0.6 mg/kg N = 10; 2.5 mg/kg N = 15; 10.0 mg/kg MPD N = 10 animals, respectively). The saline control group showed no effect on behavioral activity following acute and multiple injections of saline. Figure 2 summarizes the behavioral data following MPD exposure; the insert histogram in the upper right corner shows all animals with no correlation to their individual behavioral response to MPD. Figure 2A, C, E summarizes behavioral data for those animals expressing behavioral sensitization and Figure 2B, D, and F summarizes the locomotor activity data for those animals expressing no change and/or behavioral tolerance.

The ten animals exposed to 0.6 mg/kg MPD when statistically analyzed as a group, showed no significant difference (Figure 2; 0.6 mg/kg MPD insert upper right histogram) in locomotor activity following acute or chronic MPD exposure. When each animal was individually analyzed and grouped; four individuals exhibited significant behavioral sensitization [$F_{1,6}=5.98$, $P=.02$] following chronic MPD exposure (Figure 2A; 'ED1 vs. ED10 MPD'), while six animals individually exhibited no change in activity and as a group showed also no significant [$F_{1,10}=4.96$, $P=.8$] (Figure 2B; 'ED10 vs. ED1 MPD') changes in activity. Moreover, when the activity at ED1 acute MPD exposure was analyzed using an ANOVA, the animals expressing behavioral tolerance did not show a significant difference in locomotor activity compared to those expressing behavioral sensitization ($P=0.4$).

The 2.5 mg/kg MPD group showed a significant increase in locomotor activity at ED1 to acute MPD treatment and no significant effect following ED10 MPD rechallenge (Figure 2D insert upper right). When divided based on the animals individual analysis, seven animals exhibited significant behavioral sensitization [$F_{1,7}=5.18$, $P=.05$] following MPD rechallenge when compared to ED1 MPD acute injection (Figure 'ED10 vs ED1 MPD'), the eight animals that exhibited behavioral tolerance individually, did not exhibit significant [$F_{1,8}=5.37$, $P=.3$] differences as a group (Figure 2D; 'ED10 vs ED1 MPD'). When the acute ED1 MPD locomotor activity from animals expressing behavioral sensitization was statistically compared to the acute MPD activity recorded from animals expressing behavioral tolerance, a significant ($P=0.003$) difference was observed.

The 10.0 mg/kg MPD group showed significantly increased activity at ED1 following MPD acute exposure and no significant differences at ED10 following MPD rechallenge (Figure 2F insert upper right) compared to ED1 acute MPD. When the behavioral data was evaluated based on each animals individual locomotion; seven animals exhibited significant behavioral sensitization [$F_{1,10}=21.57$, $P=.001$] (Figure 2E; 'ED10 vs ED1 MPD') and three behavioral tolerance. The three animals expressing behavioral tolerance did not exhibit significant [$F_{1,6}=5.98$, $P=0.14$] changes in activity as a group (Figure 2F; 'ED10 vs ED1

MPD'). When the acute MPD effect at ED1 of animals expressing behavioral sensitization was statistically compared to the acute MPD effect on locomotor activity recorded from animals expressing behavioral tolerance, a significant ($P=0.007$) difference was shown. These data shows that the same dose of chronic MPD in some animal's elicited behavioral sensitization and in others behavioral tolerance. Moreover, the animals that expressed behavioral tolerance showed a significantly higher increase in acute activity following MPD compared to the animals expressing behavioral sensitization.

Electrophysiological Responses

A total of 341 NAc units were recorded from 188 electrodes (4 electrodes per animal) with identical spike wave form and amplitude on experimental day 1 (ED1) and on ED10. Approximately 1 to 2 units were evaluated per electrode: 60 units were recorded and evaluated from the saline group, 59 units from the 0.6 mg/kg MPD group, 147 units from 2.5 mg/kg MPD and 75 units from animals treated with 10.0 mg/kg MPD.

NAc units exposed to saline only (control)

Sixty NAc units were recorded from 11 rats injected with saline only (Table 1). In general these NAc units exhibited similar neuronal firing activity at ED1 following the second saline injection compared to the initial saline injection. Their baseline activity at ED10 following six daily saline injections and three washout days compared to the activity at ED1 showed that the units exhibited similar baseline neuronal activity at ED1 and at ED10. The second saline injection at ED10 in general did not cause changes in the NAc unit firing rates. This observation in the saline injection (control) shows that daily handling and injection volume did not modulate the NAc unit's neuronal firing rates.

Overall NAc units exposed to MPD

Figure 3A and B summarizes in percentage the number of NAc units exhibiting significant ($p<0.05$) change in firing rates and how many of them responded by increasing or decreasing their NAc neuronal firing rates following acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD with no regard to the animal's behavioral activity.

The acute 0.6, 2.5, and 10.0 mg/kg MPD exposure altered significantly, ($p<0.05$) the firing rate of; 50/59 (85%); 125/147 (85%) and 71/75 (95%) NAc units, respectively (Fig 3A). From the units responding to MPD exposure, 33/50 (56%); 67/125 (46%) and 34/75 (46%) of them exhibited a decrease in their neuronal activity following 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig 4A black column histograms). 0.6 mg/kg MPD elicited mainly a decrease in NAc neuronal activity, however when MPD doses were increased, more NAc units increased their neuronal activity from 17/50 (29%) to 58/125 (39%) and to 37/55 (49%) following 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig 3A overall acute striped histogram).

MPD rechallenge at ED10 when statistically compared to ED1 resulted in 55/59 (93%); 133/147 (90%) and 70/75 (93%) of the NAc units significantly ($p<0.05$) altering their neuronal activity following 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig 3B overall chronic). At ED10, of those units that responded the majority of NAc units elicited a

significant ($P<0.05$) increase in neuronal activity: 37/55 (62%); 85/133 (53%) and 44/70 (59%), after 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig 3B striped histogram).

NAc units recorded from animals expressing behavioral sensitization

Figure 3C and D summarizes the percentage of neuronal units responding to acute ED1 exposure compared to ED1 baseline and rechallenge ED10 MPD administration compared to ED1 acute MPD exposure to 0.6, 2.5, and 10.0 mg/kg MPD recorded from animals that exhibited behavioral sensitization.

Acute 0.6, 2.5, and 10.0 mg/kg MPD exposure recorded from animals expressing behavioral sensitization resulted in significantly, ($p<0.05$), altering the NAc neuronal activity of 15/15 (100%); 66/78 (85%) and 36/40 (90%), respectively (Fig 3C). From the responding units, the majority responded by decreasing their firing rates; 15/15 (100%); 50/66 (65%) and 16/36 (40%), following 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig 3C sensitized acute, black column histogram). The lower MPD dose elicits in all the NAc units decreased neuronal activity while the higher MPD doses (10.0 mg/kg) caused the majority of NAc units to respond with increases in their neuronal activity (Fig 3C sensitized acute, striped histogram).

Rechallenge MPD (0.6, 2.5, or 10.0 mg/kg) exposure following six daily injections and three days of washout resulted in 13/15 (87%); 72/78 (92%) and 39/40 (98%) of the NAc units to respond with significant ($P<0.05$) changes in their neuronal activity, respectively. The majority of those units responded to MPD by increasing their neuronal activity; 9/15 (52%); 44/72 (56%) and 27/39 (67%), while less units, 4/15 (40%); 28/72 (36%) and 12/39 (30%), exhibited decreased firing rates (Fig 3D).

NAc units recorded from animals expressing behavioral tolerance

Figure 3E and F summarizes the percentage of neuronal units responding by changing their firing rates to acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD recorded from animals exhibiting behavioral tolerance.

Acute MPD exposure recorded from animals expressing behavioral tolerance resulted in significant, ($p<0.05$), alteration of the neuronal activity for: 35/44 (80%); 59/69 (86%) and 35/35 (100%) of the NAc units, respectively. From the responding NAc units, the majority exhibited increases in their neuronal firing rates; (17/35 (39%); 42/59 (62%) and 17/35 (50%), following 0.6, 2.5, and 10.0 mg/kg MPD respectively (Fig 3E tolerant acute).

MPD exposure following six daily injections and three days of washout resulted in 42/44 (95%); 61/69 (88%) and 31/35 (89%) of the NAc units responded significantly ($P<0.05$) by changing their firing rates to 0.6, 2.5, or 10.0 mg/kg MPD, respectively. 14/44 (31%); 20/61 (29%) and 14/31 (40%) of the NAc neuronal units responded with a decrease in their neuronal firing rates, while 28/44 (64%); 41/61 (59%) and 17/31 (49%) exhibited significant ($p<0.05$) increases in their neuronal activity to 0.6, 2.5, and 10.0mg/kg MPD, respectively (Fig 3F tolerant chronic).

In summary the total responsiveness to 0.6, 2.5, and 10.0 mg/kg MPD was approximately the same in all the above comparisons (more than 80%) however the direction the NAc units responded to the drug was different between the two groups (sensitized/tolerant).

Comparing the percentage change (intensity) in firing rate following MPD exposure

Figure 4 histogram represents the percentage change in firing rate of NAc units responding to MPD exposure compared to their baseline activity (set arbitrary as 0%), for all doses. First, acute and chronic neuronal response with no correlation to behavior (Fig 4A and B) exposed to 0.6, 2.5, and 10.0 mg/kg MPD, respectively. Next, the acute and chronic effect of MPD on NAc neuronal activity recorded from animals expressing behavioral sensitization (Fig 4C and D). Lastly, the acute and chronic effect of MPD on NAc neuronal activity recorded from animals expressing behavioral tolerance (Fig 4E and F) to 0.6, 2.5 and 10.0 mg/kg MPD, respectively.

MPD exposure at ED1 compared to ED1 baseline

The NAc units that increased their neuronal firing rates (in percentage) recorded from all animals with no correlation to behavior exhibited a 5%, 27%, and 48% increase in neuronal activity and those exhibiting significant decreases in firing rates showed a -20%, -21% and -24% decrease in firing following initial 0.6, 2.5, and 10.0 mg/kg MPD compared to their baseline activity, respectively (Fig 4A).

Figure 4C makes similar comparisons as above, however this activity is evaluated for the NAc units recorded from animals exhibiting behavioral sensitization. A 6%, 0%, and 60% increase in NAc firing rates following acute 0.6, 2.5 and 10.0 mg/kg MPD respectively, while -27%, -17% and -1% decreased their neuronal NAc neuronal firing rates. Figure 4E makes similar comparisons as above to NAc units recorded from animals exhibiting behavioral tolerance. A 7%, 14%, and 30% increase in neuronal firing rates following acute MPD compared to the baseline activity (set as 0%), while a -15%, -5% and -27% decreased their neuronal firing rates to 0.6, 2.5, and 10.0 mg/kg MPD, respectively.

MPD rechallenge at ED10 compared to ED10 baseline

The NAc units recorded from all the animals at ED10 following MPD rechallenge compared to the acute (initial) MPD exposure (set arbitrarily at 0%) with no correlation to the animals behavioral response to MPD. Those units that expressed an increase in neuronal activity showed a 21%, 30%, and 59% increase, while those exhibiting significant decreases showed a -16%, -17% and -18% decrease in neuronal firing rates to 0.6, 2.5 and 10.0 mg/kg MPD, respectively. An ANOVA was used to statistically compare the overall MPD acute effect at ED1 with the effect of MPD at ED10, a statistically significant ($p < .011$, $F = 12.54$) difference was observed (Fig 4A compared to Fig 4B).

Figure 4D makes similar comparisons at ED10 MPD rechallenge compared to ED1 acute MPD exposure (set arbitrarily as 0%) for those NAc units recorded from animals exhibiting behavioral sensitization. 33%, 54%, and 70% of these NAc units exhibited increases in their neuronal firing rates, while -21%, -15% and -10% exhibited a decrease in their neuronal firing rates to 0.6, 2.5, and 10.0 mg/kg MPD, respectively. An ANOVA was used to

statistically compare the overall MPD acute effect at ED1 with the effect of MPD at ED10, no statistically significant ($p < 0.312$, $F = 1.17$) difference was observed (Fig 4C compared to Fig 4D).

Figure 4F summarizes similar comparisons as above for those NAc units recorded from animals exhibiting behavioral tolerance. NAc units exhibiting increased activity at ED10 rechallenge compared to ED1 acute MPD showed a 11%, 24%, and 79% increase in firing rates, while -15%, -5% and -11% showed a decreased in their neuronal firing rates to 0.6, 2.5, and 10.0 mg/kg MPD, respectively. An ANOVA was used to statistically compare the response to acute ED1 MPD exposure with the response to MPD rechallenge ED10, a statistically significantly ($P < 0.003$, $F = 5.81$) difference was observed (Fig 4E compared to 4F).

Statistical comparison between the groups controlling for dose

A log linear model was used to determine the relationship between dose, behavior and firing patterns for each group (0.6, 2.5, and 10.0 mg/kg MPD) with significance set at ($P < 0.05$). The calculation shows that the response pattern of NAc units recorded from animals that exhibited behavioral sensitization to either 0.6 or 2.5 mg/kg MPD dose are significantly (df 2; X^2 : 7.39; $p = 0.0248$ and (df 2; X^2 : 7.22; $p = 0.0270$) different from the NAc units firing patterns recorded from animals that exhibited behavioral tolerance, respectively. The response of NAc units to MPD recorded from animals that exhibited behavioral sensitization following 10.0 mg/kg MPD were not significantly (df 2; X^2 : 1.84; $p = 0.3977$) different from the NAc units which were recorded from animals exhibiting behavioral tolerance.

To determine if the actual percentage change in NAc firing pattern following MPD was significant, an ANOVA was performed. Based on the data obtained in figure 4, there is a significant difference in the change of NAc responsiveness (in percentage) recorded from animals exhibiting behavioral sensitization to those exhibiting behavioral tolerant. Doses 0.6 and 2.5 showed significant ($p = 0.027$, $F = 5.094$; $p = 0.003$, $F = 9.095$, respectively), while the 10.0 mg/kg MPD dose showed no significant differences ($p = 0.092$, $F = 2.899$).

Neurophysiological sensitization and neurophysiological tolerance

By recording the NAc units responding to MPD at ED10 with the neuronal responses to MPD at ED1 we were able to classify three types of neurophysiological sensitization and three types of tolerance (Fig 5).

The NAc units that responded to the initial (acute) ED1 MPD exposure by increasing their neuronal firing rates and at ED10 following rechallenge with MPD exhibited a further increase in their neuronal firing rates were classified as a unit expressing neurophysiological sensitization (Fig 6C). Those units showed a 53%, 38% and 168% further increase in their neuronal activity to 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig 5A). The NAc units that exhibited decreased neuronal activity at ED1 MPD and a further decrease in firing rates at ED10 following MPD rechallenge were classified also as expressing neurophysiological sensitization. Those units showed a -69%, -62% and -30% further decrease in neuronal firing rates to 0.6, 2.5 and 10.0 mg/kg MPD (Fig 5B). Some NAc units did not respond at ED1 to the initial MPD exposure, however they did respond with either increased or

decreased activity at ED10 rechallenge (data not shown), these units were considered to also be expressing neurophysiological sensitization, (Fig 6A).

The NAc units that responded to the initial (acute) MPD exposure at ED1 with an increase in neuronal firing rates and then at ED10 MPD rechallenge expressed an opposite response by significantly decreasing their activity were classified as expressing neurophysiological tolerance. Those NAc units showed a -56%, -58%, and -50% decreases in neuronal activity to 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig 5C). Figure 6B shows a representative NAc unit histogram following acute and chronic 2.5 mg/kg MPD exposure expressing neurophysiological tolerance. The NAc units that showed a decrease in activity at ED1 after acute MPD followed by a significant increase in activity at ED10 after MPD rechallenge were classified as expressing neurophysiological tolerance. Those unit showed a 198%, 124%, and 209% increase in their ED10 firing rates to 0.6, 2.5, and 10.0 mg/kg MPD (Fig 5D). Some NAc units responded at ED1 initial drug exposure, however they did not respond at ED10 rechallenge (data not shown), these units were also considered to be expressing neurophysiological tolerance.

An ANOVA was performed to determine (for each dose) whether the neuronal activity from animals expressing neurophysiological tolerance was different from those NAc recorded from animals expressing neurophysiological tolerance. For 0.6 and 2.5 mg/kg a statistically significant ($p < 0.03$, $F = 5.09$; $p < 0.003$, $F = 9.10$) difference was observed. For 10.0 mg/kg a no significant differences ($p < 0.09$, $F = 2.9$) was observed.

Discussion

The NAc acts as a functional interface between the limbic and motor system [40], also mediating the rewarding effects of psychostimulants [41]. Repetitive administration of psychostimulants into the NAc elicits behavioral sensitization while NAc lesioning prevents behavioral sensitization [42,43].

The main behavioral findings of this study show that within the same dose some animals elicited behavioral tolerance and others behavioral sensitization. Furthermore the 0.6 mg/kg MPD dose did not elicit significant changes in the rat behavioral activities at ED1 or at ED10 MPD rechallenge. However the 2.5 and 10.0 mg/kg group did show a significant increase in activity at ED1, however no significant differences were observed at ED10. Once separated, the sensitized animals for all doses showed a significant increase in activity at ED10 compared to ED1 drug. The tolerant animals for the higher doses (2.5 and 10.0 mg/kg MPD) showed a more robust locomotor response to acute MPD exposure compared to animals expressing behavioral sensitization.

The behavioral data confirms our hypothesis that the same dose of MPD can elicit different behavioral responses. Clinical and imaging studies using MPD in ADHD patients and in normal control subjects reported individual differences in their responses to MPD [20]. The physiological effect of MPD on the DA system is mediated by a family of G protein coupled D1 and D2 DA receptors based on cAMP and ligand binding [43]. There are differences in the D1 and D2 DA receptor density between subjects and different genotypes between

subjects [20] which play an important role in the response to MPD. Forty-seven animals were used in this study, and no more than four animals came from the same shipment, therefore it can be anticipated that there would be individual variation of responses to MPD.

The main electrophysiological findings of this study show that saline had no effect on the behavioral or on the NAc neuronal firing rates. More than 80% of the NAc units responded to acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD exposure (Fig 3). Animals expressing behavioral sensitization responded to MPD exposure by decreasing their neuronal firing rates while animals expressing behavioral tolerance exhibited increases in their NAc firing rates in response to MPD exposure (Fig 4). NAc units recorded from animals exhibiting behavioral sensitization responded significantly different to 0.6 and 2.5 mg/kg MPD. The neuronal response to 10.0 mg/kg MPD between the groups of animals showed no significant differences. 10.0 mg/kg MPD i.p. is considered a high dose therefore it is possible to assume a ceiling effect for the neuronal responses may have been observed resulting in no differences in neuronal activity [45,46].

The baseline activity recorded from animals exhibiting behavioral sensitization, showed mainly a decrease in ED10 when compared to ED1. For those animals exhibiting behavioral tolerance, the opposite response was observed. It is possible to assume that the change in baseline neuronal activity at ED10 determined how these units will respond to the MPD rechallenge at ED10, and/or these changes in baseline activity at ED10 are expressing withdrawal.

Based on the NAc unit's responses to MPD, it is possible to classify three types of neurophysiological sensitization and tolerance. The NAc units exhibiting increased (or decrease) neuronal activity at ED1 MPD exposure and a further increase (or further decrease) in neuronal activity at ED10 MPD rechallenge can be interpreted as neurophysiological sensitization. Some of the NAc did not respond to acute MPD at ED1, but after repetitive MPD exposure responded to the rechallenge MPD at ED10, an expression of neurophysiological sensitization. Similarly, three types of electrophysiological tolerance were observed; MPD rechallenge at ED10 exerts no effect when compared to ED1 acute exposure. Also, some NAc units at ED10 MPD elicited the opposite effects from their response at ED1 MPD exposure.

Correlations between the animal's behavior and their NAc neuronal activity were noticed. The majority of NAc units that responded to acute 0.6 and 2.5mg/kg MPD administration by attenuation of their neuronal activity were recorded from animals exhibiting behavioral sensitization and the majority of NAc units that responded to 2.5mg/kg MPD by excitation were recorded from animals expressing behavioral tolerance. Animals expressing behavioral sensitization or tolerance showed a significant likelihood to reduce or increase their baseline neuronal activity at ED10, respectively.

The NAc is comprised of a heterogeneous population, with different type of excitatory/inhibitory receptors [47,48,49]. Dopaminergic VTA neurons ascend to the NAc medium-sized spiny neurons (MSN) to modulate the excitatory glutamatergic input from the PFC [49]. Chronic psychostimulant exposure has previously been shown to increase the dendritic

branch points and spines of MSNs in the NAc [47]. The NAc neurons contain D1 and D2 like DA receptors, each exerting different outcomes when activated. MPD has been shown to cause increased spine formation in MSN-D1, however this was not observed for MSN-D2 spines [47]. The activation of the D1 like DA receptors results in excitation while the activation of the D2 DA like receptors results in attenuated activity [44]. The electrodes that were recorded from neurons expressing D1 like DA receptors will respond to MPD by increasing their firing rate, while those units that have mainly D2 like DA receptor will respond to the drug by decreasing their NAc neuronal firing rate.

Using other psychostimulants it was reported that the same dose of the drug can result in some animals an increase in phosphorylation of the transcription factor cAMP response element binding protein (CREB) in the NAc that mediates aspects of behavioral tolerance [11,12,51]. Conversely, the same dose of the same psychostimulants in other animals causes an overexpression of FosB which contributes to sensitized responses [12,17,51]. It was reported that chronic psychostimulant exposure results in alterations of several neuronal cell types in the NAc [47,55]. In some animals the chronic exposure of psychostimulants results in an increase in the density of dendrite spines in medium spiny neurons (MSN) that express DA D1 receptors [47]. The same dose of psychostimulant can also result in overexpressing DA D2 receptors [47] and decreases in the density of the dendritic spine of the MSN in another animal [52]. [53,54] reported individual differences in response to stimulants due to different phenotype and rate of drug metabolism which can explain the variety of responses to chronic MPD observed in this study.

In conclusion, this study provides evidence that when completing any type of assay in regards to chronic psychostimulant exposure, the animal needs to be grouped and characterized based on their individual behavioral responses. Understanding that two animals given the same dose of MPD can respond in different ways (sensitization/tolerance) is imperative to keep in mind when performing any type of assay.

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Highlights

- Animals expressed different behaviors to the same chronic MPD dose
- Tolerant animals showed a more robust acute increase in behavior.
- NAc units from sensitized animals responded differently than tolerant animals

Figure 1.

Histological reconstruction of the electrode tip placement in the NAc. The black dots on the rat atlas plates (Paxinos and Watson, 1986) represent the location of the NAc recording electrodes in serial coronal sections the number on the top right corner of each section represents the anterior distance (mm) from bregma.

Figure 2.

summarizes the locomotor behavioral data. The initial histogram shows the saline control group (N=11). The histograms to the left are the data obtained from all the animals. A,C and E summarize the data obtained from animals expressing behavioral sensitization (N=4, 7 and 7, respectively) while B, D and F summarizes the data obtained from individual animals expressing behavioral tolerance (N= 6, 8, and 3, respectively) to 0.6, 2.5, and 10.0 mg/kg MPD respectively. ED1 – Experimental Day 1; BL – baseline; MPD – Methylphenidate; significance was set at $p < 0.05$.

Figure 3.

summarizes the total responsiveness and the response direction (increase/decrease) to acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD. The percent of NAc units responding to acute and chronic MPD is broken down into three groups. Histograms A and B summarize the acute (N=59, 147, 75) and chronic (N=55, 133, 70) effect of 0.6, 2.5, and 10.0 mg/kg MPD for all the animals without regard to their individual behavior response to chronic MPD exposure. Histograms C and D summarize the acute (15, 64, 36) and chronic (13, 72, 39) effect of 0.6, 2.5, and 10.0 mg/kg MPD NAc units recorded from animals expressing behavioral sensitization. Finally, histograms E and F summarize the acute (N= 40, 59, 35) and chronic (N= 42, 61, 31) effect 0.6, 2.5 and 10.0 mg/kg MPD NAc units recorded from animals expressing behavioral tolerance. * above the 0.6 and 2.5 mg/kg indicates statistically significant differences between the chronic sensitized and chronic tolerant neuronal firing rates.

Figure 4.

summarizes the intensity (percent change in firing pattern – baseline set arbitrarily as 0%) of NAc units following acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD, respectively. The histograms are broken into three groups. Histogram A and B represents the change in firing rate following acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD exposure from all animals with no regard to their individual behavioral response to chronic MPD. Histograms C and D represent the change in firing rate following acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD, respectively recorded from animals expressing behavioral sensitization. Histograms E and F represent the change in firing rate following acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD, respectively recorded from animals expressing behavioral tolerance. Acute summarizes the data following the initial MPD exposure; Chronic – summarizes the ED10 firing rate following MPD exposure compared to the initial ED1 firing following MPD exposure. * above the 0.6 and 2.5 mg/kg indicates statistically significant differences between the chronic sensitized and chronic tolerant neuronal firing rates.

Figure 5.

histogram represents two of the three types of neurophysiological sensitization and neurophysiological tolerance, respectively. Histogram A represents NAc units that following MPD exposure responded by increasing their neuronal firing at ED1 and further increasing their neuronal activity at ED10 MPD rechallenge to 0.6, 2.5, and 10.0 mg/kg MPD respectively, classified as neurophysiological sensitization. Histogram B represents NAc units that decreased their neuronal firing at ED1 following MPD exposure and further decreased their activity at ED10 following MPD rechallenge to 0.6, 2.5, and 10.0 mg/kg MPD respectively, classified as expressing neurophysiological sensitization. Histogram C represents NAc units that increased their neuronal firing at ED1 following MPD exposure and oppositely decreased their activity at ED10 following MPD rechallenge to 0.6, 2.5, and 10.0 mg/kg MPD respectively expressing neurophysiological tolerance. Histogram D represents NAc units that decreased their neuronal firing at ED1 and oppositely increased their activity at ED10 MPD rechallenge to express neurophysiological tolerance to 0.6, 2.5, and 10.0 mg/kg MPD respectively.

Figure 6. shows 3 representative firing rate histograms of NAc units following 0.6, 2.5, and 10.0 mg/kg MPD at ED1 and at ED10. The frame above each histogram shows 20 superimposed spikes following saline and MPD of ED1 and ED10 for the same unit (verifying same amplitude and waveform). Figure 6A and B represents units expressing two types of neurophysiological sensitization and figure 6C represents NAc units expressing neurophysiological tolerance. The histograms on the right show the initial baseline ED1 NAc response followed by the ED1 initial MPD exposure response. The histograms to the left show NAc unit's responses at ED10 baseline following six days MPD exposure and three days washout followed by ED10 rechallenge MPD exposure.

Table 1

summarizes the experimental protocol. There were four groups (saline, 0.6, 2.5 and 10.0 mg/kg MPD). On experimental day 1 (ED1) the neuronal recording for one hour was obtained following a saline injection and for another hour preceding initial saline, 0.6, 2.5 or 10.0 mg/kg methylphenidate (MPD) administration. On ED2 through ED7 a single saline, 0.6, 2.5 or 10.0 mg/kg MPD injections were given without neuronal activity recordings. Animals went through a washout stage on ED7 through ED9 in which no injections were given, recording was resumed at ED10 following a saline injection and one hour later following a rechallenge of either saline, 0.6, 2.5 or 10.0 mg/kg MPD administration.

Experimental Day	1	2–6	7–9	10
<u>Treatment Group:</u>				
1) Saline (N=11)	saline/saline	saline	Washout	saline/saline
2) 0.6 mg/kg (N=10)	saline/0.6 mg/kg	0.6 mg/kg	Washout	saline/0.6 mg/kg
3) 2.5 mg/kg (N=15)	saline/2.5 mg/kg	2.5 mg/kg	Washout	saline/2.5 mg/kg
4) 10.0 mg/kg (N=10)	saline/10.0 mg/kg	10.0 mg/kg	Washout	saline/10.0 mg/kg