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

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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

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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 10.0 mg/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 population 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 analyses 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 animal's behavior concomitantly with neuronal recordings.

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1. 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 psychostimulant's effect on the brain's 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 [14–16].

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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 [17–20]. 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 the same dose elicited behavioral tolerance [17–19]. 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 [16,19,24,25]. Recent studies examined the neurophysiological effect of a single 2.5 mg/kg MPD dose on the NAc, PFC and caudate nucleus (CN) [26–28]. Leading to our second hypothesis, the NAc unit electrophysiological responses recorded from animals exhibiting behavioral sensitization will be different from the 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.

2. Materials and methods

2.1. Subjects

Forty-seven (47) adult male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 150–175 g upon arrival were housed individually for 5 to 7 days in single Plexiglas cages inside a sound-attenuated animal facility room for adaptation. The home cage was utilized as the test cage throughout the 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 ± 20 °C and humidity between 58 and 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.

2.2. 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 intraperitoneally (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 08:00 am and 9:00 am.

2.3. Surgery

On the day of surgery the 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 0.6 mm diameter holes were drilled over the NAc in accordance to the coordinates derived from [33] rat brain atlas (1.70 mm anterior from bregma, 1.2 mm 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 the tips) were secured each to a 1 cm 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 μm 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 [16,19,25–27,34–36]. 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 h, 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.

2.4. 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 g 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 1 h. 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 min. 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 [16,19,20,25–28,32]. 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.

2.5. 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 [15–17,19,20,31,37]. 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

Table 1
Experimental protocol.

Treatment group	Experimental day			
	1	2–6	7–9	10
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

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 1 h 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 individual 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 1 h later following a rechallenge of either saline, 0.6, 2.5 or 10.0 mg/kg MPD administration.

downloaded to a PC in 10 min 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 a 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 min bin increments and evaluated from 60 min 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.

2.6. Analysis of behavioral data

The locomotor activity recorded on ED1 and ED10 was each summed into 10 min bins for 60 min (i.e.6 bins/h). The HA and NOS were 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 elicit 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.

2.7. Electrophysiological recording

2.7.1. 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 BioSystems (Durham, NC, USA) head stage was connected to the electrode pins of the skull cap. The Triangle BioSystems 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.

2.7.2. 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 point 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 that the spike pattern captured on ED1 is the same as ED10.

2.7.3. Analysis of the electrophysiological data

The sorted neuronal activity obtained from the fixed template matching system was converted by the Spike2 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 spread sheet 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 event 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 ($C.R. = (E - C) / (\sqrt{E + C}) = \pm 1.96 = p < 0.05$) 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 the changes of each unit activity induced by the treatment were considered statistically

significant if the firing rate after drug treatment differed by at least 2 standard error (S.E.) from the mean [26,27,33,38,39].

A one way ANOVA was used (per dose) to determine if the NAc neuronal populations recorded from animals exhibiting behavioral sensitization were 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.

2.7.4. Histological verification of electrode placement

At the end of the experimental protocol, rats were deeply anesthetized with sodium pentobarbital. The rats' 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 s 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 cresyl violet. The coordinate position of the electrode tips was established by matching the equivalent locations of the lesion and the Prussian blue spot by using the Rat Brain Atlas by [33] (see Fig. 1).

3. Results

3.1. 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. Fig. 2A 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 Fig. 2Bc, Cc, and Dc. Fig. 2 summarizes behavioral data for those animals expressing behavioral sensitization and Fig. 2Bb, Cb, and Db summarizes the locomotor activity data for those animals expressing no change and/or behavioral tolerance.

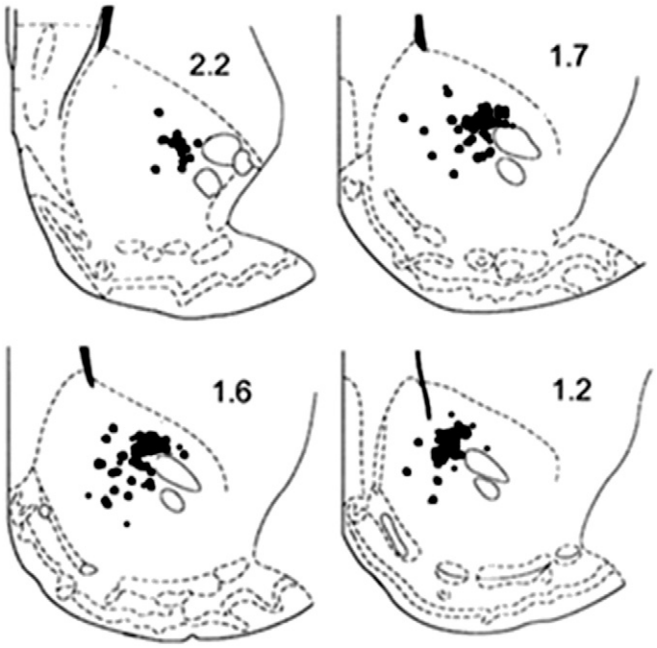

Fig. 1. Histological reconstruction of the electrode tip placement in the NAc. The black dots on the rat atlas plates [33] represent the location of the NAc recording electrodes in serial coronal sections. The number on the top right corner of each section represents the anterior distance (mm) from bregma.

The ten animals exposed to 0.6 mg/kg MPD when statistically analyzed as a group, showed no significant difference (Fig. 2Bc; 0.6 mg/kg MPD inset, 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 [$F1,6 = 5.98$, $p = .02$] following chronic MPD exposure (Fig. 2Ba; 'ED1 vs. ED10 MPD'), while six animals individually exhibited no change in activity and as a group showed also no significant [$F1,10 = 4.96$, $p = .8$] (Fig. 2Bb; '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 (Fig. 2Cc inset upper right). When divided based on the animals' individual analysis, seven animals exhibited significant behavioral sensitization [$F1,7 = 5.18$, $p = .05$] following MPD rechallenge when compared to ED1 MPD acute injection (Fig. 2Ca 'ED10 vs ED1 MPD'), the eight animals that exhibited behavioral tolerance individually, did not exhibit significant [$F1,8 = 5.37$, $p = .3$] differences as a group (Fig. 2Cb; '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 (Fig. 2Dc inset upper right) compared to ED1 acute MPD. When the behavioral data was evaluated based on each animal's individual locomotion; seven animals exhibited significant behavioral sensitization [$F1,10 = 21.57$, $p = .001$] (Fig. 2Da; 'ED10 vs ED1 MPD') and three behavioral tolerance. The three animals expressing behavioral tolerance did not exhibit significant [$F1,6 = 5.98$, $p = 0.14$] changes in activity as a group (Fig. 2Db; '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 elicited behavioral sensitization in some animals 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.

3.2. 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 the 2.5 mg/kg MPD and 75 units from the animals treated with 10.0 mg/kg MPD.

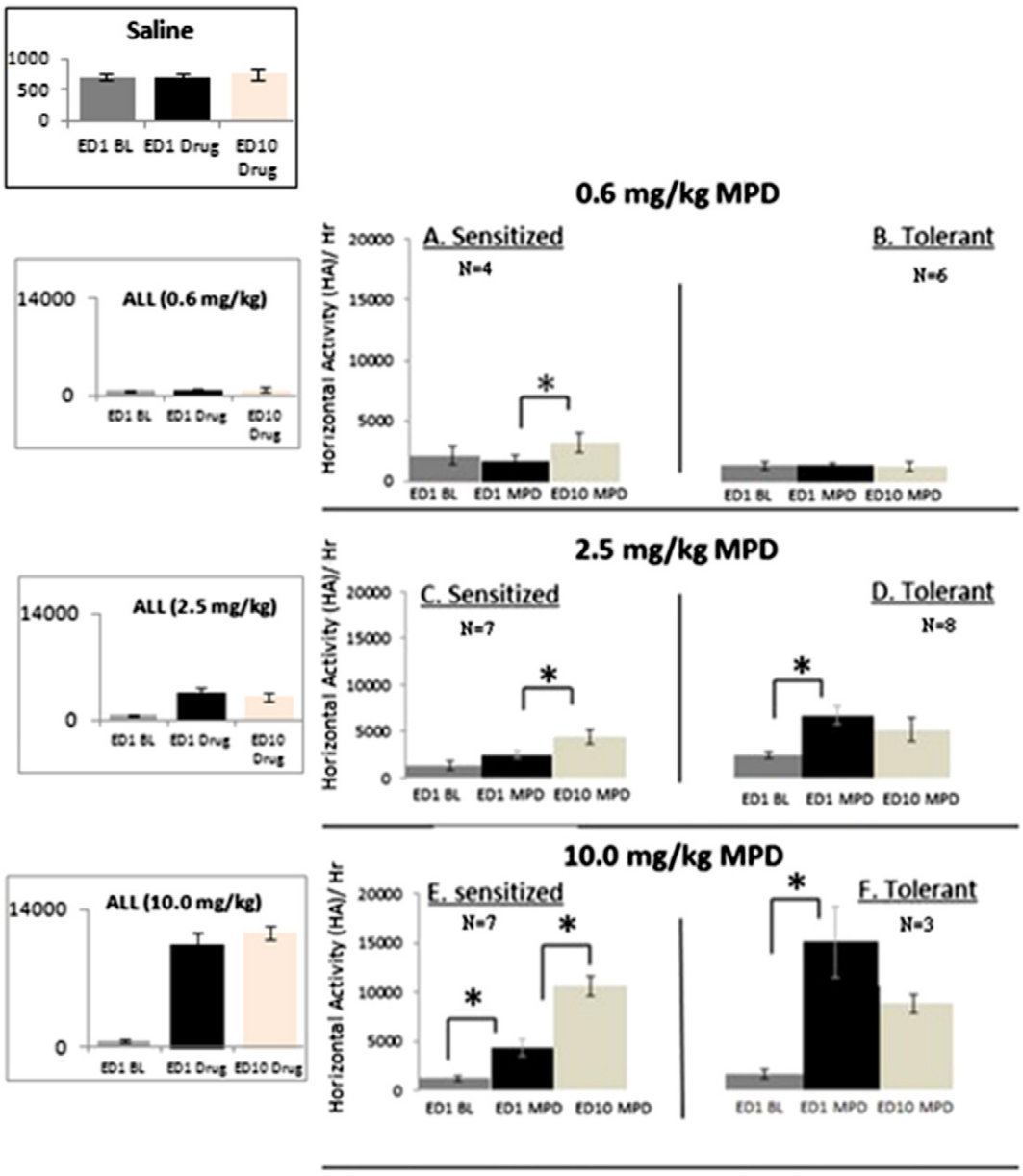

Fig. 2. Summarizes the locomotor behavioral data. The initial histogram (Fig. 2A) shows the saline control group (N = 11). The upper right inset histograms (Fig. 2Bc, Cc, and Dc) show behavioral activity of each MPD dose as a group. 2Ba, Ca, and Da summarize the data obtained from animals expressing behavioral sensitization (N = 4, 7 and 7, respectively) while F, H, and J 2Bb, Cb, and Db summarize 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 $p < 0.05$.

3.3. 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.

3.4. Overall NAc units exposed to MPD

Fig. 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,

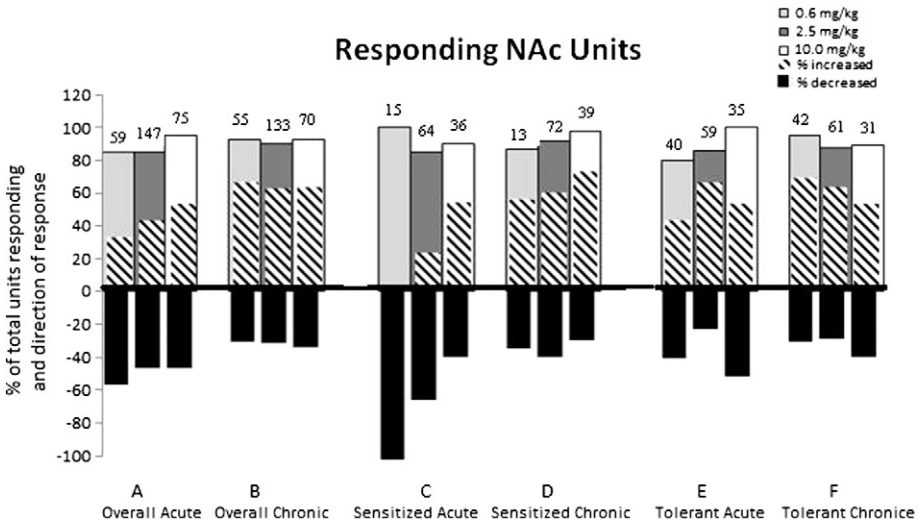

Fig. 3. Summarizes the total responsiveness (outer upward histogram) and the response direction (increase [striped upper histogram]/decrease [solid black negative histograms]) to acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD. The percent of NAc units responding to acute and chronic MPD is broken down into three groups. Histograms A and B summarize the acute ED1 and chronic (ED10 MPD effect compared to ED1) effects of 0.6, 2.5, and 10.0 mg/kg MPD on Nac units from 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) effects 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 and chronic effects of 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 MPD indicates statistically significant differences between the chronic sensitized and chronic tolerant neuronal firing rates. The number centered above each histogram represents the total number of NAc units responding in each group.

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).

3.5. NAc units recorded from animals expressing behavioral sensitization

Fig. 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

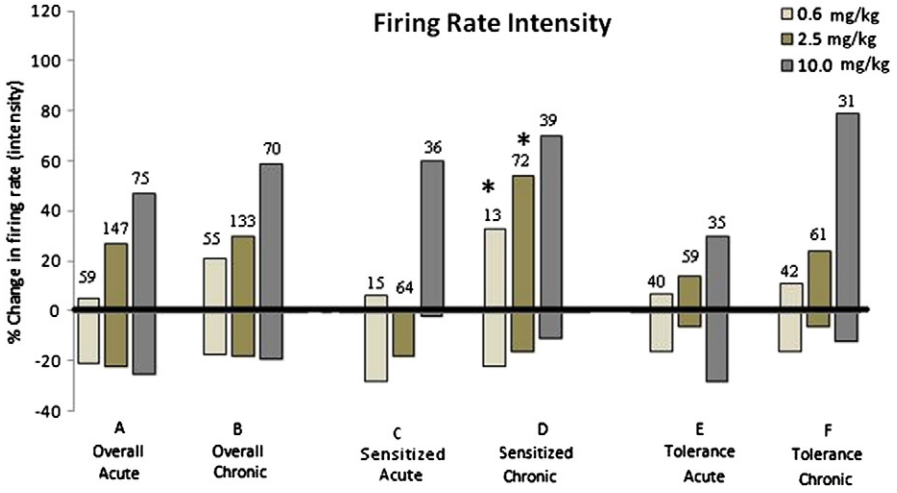

Fig. 4. Summarizes the intensity (percent change in firing pattern — baseline set arbitrarily as 0%) of NAc units following acute and chronic 0.6, 2.5, and 10.0 mg/kg MPD, respectively. The histograms are broken into three groups. Histograms A and B represent the change in firing rate following acute 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.

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).

3.6. NAc units recorded from animals expressing behavioral tolerance

Fig. 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 a 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 10.0 mg/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 in which the NAc units responded to the drug was different between the two groups (sensitized/tolerant).

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

Fig. 4 histogram represents the percentage change in firing rate of NAc units responding to MPD exposure compared to their baseline activity (set arbitrarily as 0%), for all doses. First, acute and chronic neuronal responses 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 effects of MPD on NAc neuronal activity recorded from animals expressing behavioral sensitization (Fig. 4C and D). Lastly, the acute and chronic effects 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.

3.8. 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 5%, 27%, and 48% increases in neuronal activity and those exhibiting significant decreases in firing rates showed −20%, −21% and −24% decreases in firing following initial 0.6, 2.5, and 10.0 mg/kg MPD compared to their baseline activity, respectively (Fig. 4A).

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

3.9. MPD rechallenge at ED10 compared to ED10 baseline

The NAc units recorded from all animals at ED10 following MPD rechallenge when compared to the acute (initial) MPD exposure (set arbitrarily at 0%) [with no correlation to the animals' behavioral response to MPD] are as follows. Of those eliciting increases, a 21%, 30%, and 59% increase was observed while those exhibiting significant decreases showed a −16%, −17% and −18% decrease decreases in neuronal firing rates to 0.6, 2.5 and 10.0 mg/kg MPD, respectively. Those units that expressed an increase in neuronal activity showed a 21%, 30%, and 59% increase, while those exhibiting significant decreases showed −16%, −17% and −18% decreases 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).

Fig. 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 decreases 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).

Fig. 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 11%, 24%, and 79% increases in firing rates, while −15%, −5% and −11% showed decreases in their neuronal firing rates to 0.6, 2.5, and 10.0 mg/kg MPD, respectively. An ANOVA was used to statistical 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 4 F).

3.10. 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 the 0.6 or the 2.5 mg/kg MPD dose is significantly (df 2; χ^2:7.39; $p = 0.0248$ and df 2; χ^2:7.22; $p = 0.0270$) different from the NAc unit 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: χ^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 Fig. 4, there is a significant difference in the change of NAc responsiveness (in percentage) recorded from the 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 ($p = 0.092$, $F = 2.899$).

3.11. 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

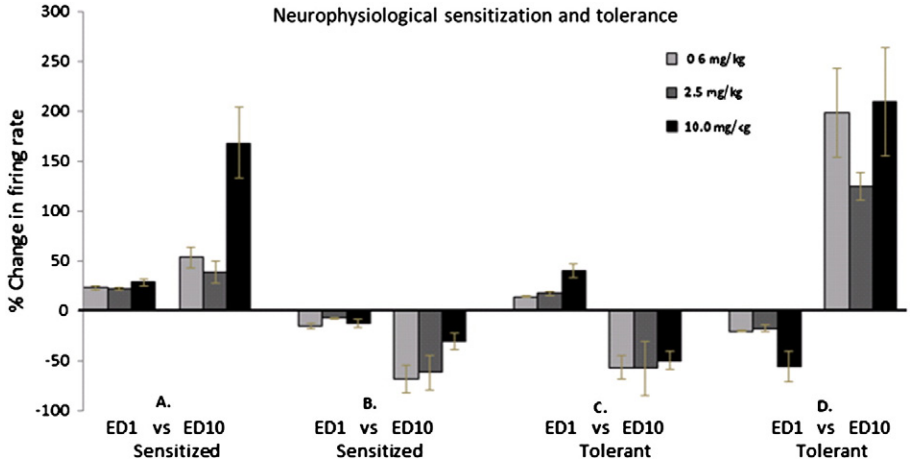

Fig. 5. Histogram represents two of the three types of neurophysiological sensitization and neurophysiological tolerance, respectively. Histogram A represents NAc units that following MPD exposure responded by increasing their neuronal firing at ED1 and further increasing their neuronal activity at ED10 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.

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 53%, 38% and 168% further increases 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 −69%, −62% and −30%

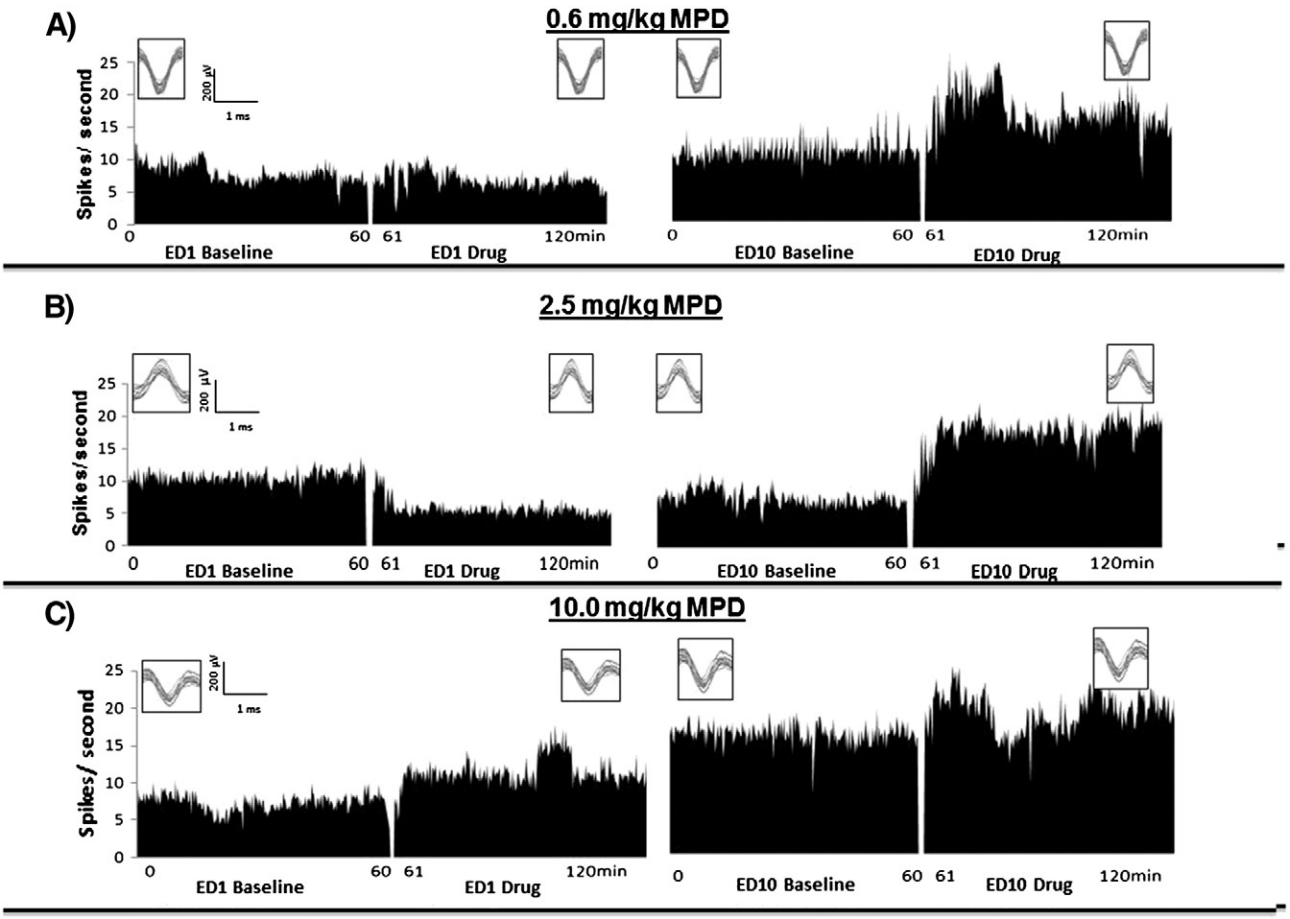

Fig. 6. Shows 3 representative firing rate histograms of NAc units following 0.6, 2.5, and 10.0 mg/kg MPD at ED1 and at ED10. The frame above each histogram shows 20 superimposed spikes following saline and MPD of ED1 and ED10 for the same unit (verifying the same amplitude and waveform). Fig. 6A and B represent units expressing two types of neurophysiological sensitization and Fig. 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 responses at ED10 baseline following six days MPD exposure and three days washout followed by ED10 rechallenge MPD exposure.

further decreases 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 −56%, −58%, and −50% decreases in neuronal activity to 0.6, 2.5, and 10.0 mg/kg MPD, respectively (Fig. 5C). Fig. 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 units showed 198%, 124%, and 209% increases 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.

4. 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 groups 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 the 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 differently 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 that 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 that 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 decreased) 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.

The 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 2.5 mg/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 2.5 mg/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 types of excitatory/inhibitory receptors [47–49]. Dopaminergic VTA neurons ascend to the NAc medium-sized spiny neurons (MSN) to modulate the excitatory glutamatergic input from the PFC [49,50]. 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 having an increase in phosphorylation of the transcription factor cAMP response element binding protein (CREB) in the NAc that mediates the aspects of behavioral tolerance [11,12,51]. Conversely, the same dose of the same psychostimulants in other animals causes an overexpression of ΔFosB 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]. 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 the overexpression of 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 phenotypes and rates 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 regard 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.

Conflict of interest

The authors declare no competing financial interests.

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