Journal of Neural Transmission (2022) 129:1061–1076
https://doi.org/10.1007/s00702-022-02526-0

PSYCHIATRY AND PRECLINICAL PSYCHIATRIC STUDIES - ORIGINAL ARTICLE

Age differences to methylphenidate-NAc neuronal and behavioral recordings from freely behaving animals

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Received: 14 February 2022 / Accepted: 21 June 2022 / Published online: 16 July 2022

Abstract

Methylphenidate (MPD) is a psychostimulant that is widely prescribed to treat attention deficit-hyperactivity disorder, but it is abused recreationally as well. The nucleus accumbens (NAc) is part of the motivation circuit implicated in drug-seeking behaviors. The NAc neuronal activity was recorded alongside the behavioral activity from young and adult rats to determine if there are significant differences in the response to MPD. The same dose of MPD elicits behavioral sensitization in some animals and behavioral tolerance in others. In adult animals, higher doses of MPD resulted in a greater ratio of tolerance/sensitization. Animals who responded to chronic MPD with behavioral sensitization usually exhibited further increases in their NAc neuronal firing rates as well. Different upregulations of transcription factors (ΔFOSB/CREB), variable proportions of D1/D2 dopamine receptors, and modulation from other brain areas may predispose certain animals to express behavioral and neuronal sensitization versus tolerance to MPD.

Keywords Ritalin · NAc · Adolescent · Adult · Neuronal and behavioral recording

Introduction

Psychostimulants, such as methylphenidate (MPD) and amphetamine, have been increasingly prescribed worldwide for behavioral disorders, with MPD now being the most widely prescribed psychostimulant used to treat attention deficit hyperactivity disorder (ADHD) (Lee et al. 2012). In addition to treating behavioral disorders, MPD is growing in illicit use for cognitive enhancement and recreationally purposes amongst ordinary subjects, being coined as a "study drug" or "smart drug" (Arria and Wish 2006; Kim et al. 2009). For example, there has been an estimated 20% increase in the use of unprescribed MPD and/or other stimulants by college students, as well as a growing market for the illicit exchange of these medications (Harris et al. 2008; Stix 2009; Swanson et al. 2011; Dietz et al. 2013; Kim et al. 2020). Therefore, it is important to study the effect of MPD on primary animal models. Many previous studies have addressed the safety and efficacy of MPD in the ADHD population, but questions remain about the potential for abuse in non-ADHD patients (Kollins et al. 2001; Kim et al. 2020).

Since the FDA approved the use of MPD in 1955 there has been a multitude of studies examining the similarities between MPD and cocaine, as well as other drugs of abuse, which found that MPD competes with the same binding sites as cocaine in the striatum (Kollins et al. 2001; Volkow et al. 1995). Similarly to amphetamine and cocaine, MPD binds to the dopamine transporters (DAT) and increases the amount of dopamine (DA) available to stimulate the postsynaptic receptors by preventing the reuptake of DA from the synaptic cleft (Patrick and Markowitz 1997; Gatley et al. 1999). Illicit use of MPD may lead to undesirable behavioral changes in the brain manifested by depression, the potential for addiction and withdrawal, dependence, and even death. The increasing usage of MPD in both adolescents and ordinary adult populations has sparked an interest in investigating its effects in ordinary adults compared to younger individuals, as their brains are still in developmental stages, including synaptic changes and pruning (Marco et al. 2011; Imbert et al. 2014; Brenhouse and Anderson 2011). It is, therefore, important to study the properties of MPD in adolescents when their brain is still going through development changes, as compared to adults.

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A collection of mesolimbicocortic nuclei, termed the motive or reward circuit, is believed to contribute to both drug-seeking behavior as well as the expression of behavioral sensitization and behavioral tolerance following repetitive chronic psychostimulant consumption (Florence et al. 2020; King et al. 2019; Ming and Dafny 2021). The nucleus accumbens (NAc) is one of the nuclei that belongs to this circuit, and it is connected to other motive neuronal nuclei that participate in reward-related behavior (Wise 1996). Furthermore, the NAc receives excitatory, glutaminergic projections from the prefrontal cortex (PFC), thalamus, hippocampus, and amygdala, facilitating interaction with the limbic system (Kita and Kitai 1990; Ikemoto et al. 2015; Reppucci and Petrovich 2016; Venkataraman et al. 2019, 2020). Similar to its effect on DA via the DAT, MPD has been shown to bind to the norepinephrine (NE) transporter in the NAc, inhibiting NE reuptake and strengthening NE's effect on post-synaptic receptors (Weikop et al. 2007; Ming and Dafny 2021). Past studies on the NAc have confirmed its role in the expression of behavioral sensitization in response to chronic 0.6, 2.5 and 10.0 mg/kg MPD use (Tu et al. 2019; Podet et al. 2010; Gaytan et al. 1997; Ming and Dafny 2021).

There is a debate as to whether MPD exposure during ontogeny may modulate some CNS function (Dafny and Yang 2006). Since the adolescent brain is still in a developing state, MPD can modulate this process and elicit paramount changes, as reported by the use of psychostimulants in non-ADHD adolescents (ages 13–19) and young adults (20's) (Jernigan et al. 1999; Blakemore et al. 2010; Safer 2016). Impulsive behaviors that characterize the adolescent period are thought to result as an imbalance between limbic and cortical control systems in the brain, highlighted by earlier maturation and stronger activation of the NAc in adolescent relative to the PFC (Casey et al. 2008; Galvan et al. 2006). Additionally, it was reported that rats exposed to MPD during childhood had long-lasting behavioral changes that persisted into adulthood (Andersen et al. 2002; Brandon et al. 2001). Further clarification is needed as to the different effect's stimulants like MPD have on adolescent and adult brains.

This study investigates the dose-response effect of acute and chronic MPD on NAc neuronal activity in freely behaving adolescent and adult male, concomitantly using the open field assay as means for the determination of the locomotor behavioral MPD effect. Free behavior is a cornerstone of this study, as many adverse interactions between MPD and anesthesia have been documented (Ririe et al. 1997; Solt et al. 2011; Chemali et al. 2012). Prior studies have shown that the same chronic dose of MPD (0.6, 2.5 and 10.0 mg/kg) may induce tolerance, behavioral sensitization, and/or withdrawal, which are used as one of the experimental biomarkers to determine MPD's ability to cause dependence (Podet et al. 2010; Claussen et al. 2014, 2015; Frolov et al. 2015; Venkataraman et al. 2017, 2019, 2020). We hypothesize that there will be a significant difference between the age groups in the ratio of how many rats express sensitization versus tolerance to repetitive (chronic) MPD. Our secondary hypothesis is that these ratios will be dependent on the doses of MPD that is administered, with higher doses leading to tolerance more often than sensitization. A portion of the recordings from adolescent only and from adult animals only, which use identical experimental protocols and MPD doses, have been previously published in preliminary studies (Claussen et al. 2014; Frolov et al. 2015).

Methods

Animals

One hundred and sixty-one adolescent and 141 adult male Sprague Dawley (SD) rats were purchased from Harlan (Indianapolis, IN, USA) at P-30 and P-50, respectively. Upon arrival, the rats were housed individually in enriched Plexiglas cages and kept on a 12-h light–dark schedule, with lights on at 6:00 A.M. The room was maintained at a temperature of 21 ± 2 °C and a humidity of 58–62%. After several days (3–5) of acclimation, four neuronal recording electrodes were implanted bilaterally in the NAc (two on each side). Five to six recovery days were allowed prior to the initiation of recordings. All recordings and injections took place in the animal's home cage, beginning around 8:00 A.M. Animals remain all the time in their home cage that was also used as the testing cage, to eliminate environmental stimulation. The recordings were obtained on the experimental day (ED) 1 and 10 as shown in Table 1. The age of the adolescent rats start on experimental day 1 (ED1) was kept constant at P-40 and lasted until P-50. For the adult group and started at P-60 and lasted until P-70. These ages are based on correlations between the laboratory rat and human lifespans (Yang et al. 2007). Rats were supplied food and water ad libitum for the entire duration of the study. All experiments were approved by the University of Texas Medical School at Houston Animal Welfare Committee and carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

Drugs

Methylphenidate hydrochloride (MPD) was donated by Mallinckrodt (St. Louis, MO, USA). Administration of the drug during the experiment was as a solution dissolved in saline (0.9% NaCl). Our past dose-response experiments have used MPD doses ranging from 0.1 to 40 mg/kg intraperitoneally (i.p.) (Askenasy et al. 2007; Dafny and Yang 2006; Gaytan et al. 1997). Neuronal and behavioral changes

Table 1 Four animal groups for the adolescent and for the adult animals, and the MPD dose protocol that was followed for each group

Treatment groups	Experimental days (ED)			
	ED 1*	ED 2–6	ED 7–9	ED 10*
1. Saline	Saline/Saline	Saline	Washout	Saline/saline
2. 0.6 mg/kg MPD	Saline/0.6 mg/kg MPD	0.6 mg/kg MPD	Washout	Saline/0.6 mg/kg MPD
3. 2.5 mg/kg MPD	Saline/2.5 mg/kg MPD	2.5 mg/kg MPD	Washout	Saline/2.5 mg/kg MPD
4. 10.0 mg/kg MPD	Saline/10.0 mg/kg MPD	10.0 mg/kg MPD	Washout	Saline/10.0 mg/kg MPD

Four groups of animals were used for each age group: saline, 0.6, 2.5 and 10.0 mg/kg MPD. On experimental day 1 (ED1), animals are given an initial dose of saline and recordings were taken for one hour followed by 2nd injection one h later by the four designated injections of saline, group 1; group 2 0.6 mg/kg MPD; group 3 2.5 mg/kg MPD and group4 10.0 mg/kg of MPD. Recordings were resumed for an additional hour post-injection. On ED 2–6, the animals are given an injection each morning of the designated dose. ED 7–9 are washout days where the animal gets no injection of any kind. On ED10, the animals are given another dose of saline to obtain BL on ED10 for one hour (ED10 BL) followed by the designated MPD dose for one hour and recordings were taken, identical to that given on ED1 (ED10 MPD). *Indicates the recording day

were observed from 0.6 mg/kg i.p and above, Therefor the MPD doses used for this study was 0.6, 2.5, and 10 mg/kg MPD to represent low, medium and high MPD dose. The same three doses were used in past studies with similar protocols and (Broussard et al. 2019; Kharas et al. 2017; Venkataraman et al. 2017, 2019, 2020; Frolov et al. 2015; Claussen et al. 2014). The brain blood levels of similar doses of MPD were compared between oral, i.p., and i.v. administration and it was concluded that the doses used in this study are clinically relevant to human use. The drug was measured as a free base prior to dissolving in saline. All injection volumes were equalized to 0.8 mL by adding the difference in saline for all MPD doses and took place between 8:00 and 9:00 a.m.

Surgery

After the initial 3–5-day acclimation period, the surgeries were performed following protocols established in previous studies (Broussard et al. 2019; Kharas et al. 2017; Venkataraman et al. 2017, 2019, 2020; Frolov et al. 2015; Chong et al. 2012; Claussen and Dafny 2011; Claussen et al. 2014). Rats were anesthetized using i.p. pentobarbital, 30 mg/kg for adolescent and 50 mg/kg for adult. The rat's head was shaved to expose the skin, which was then coated with a layer of 2% Lidocaine Hydrochloride Jelly (Akorn, Inc.). The rat was then placed in a stereotaxic instrument. For the adult group, the Paxinos and Watson rat brain atlas (1986) served as a guide to drill one hole in front of the frontal sinus for the ground electrode and bilateral 0.6 mm diameter holes above the NAc for the recording electrodes (2 mm anterior to the bregma and 1.6 mm lateral from midline; Paxinos and Watson 1986). Similarly, for the adolescent group, the Sherwood and Tiramas developing rat brain atlas was used to drill a hole above the frontal sinus, and bilateral 0.6 mm diameter holes above the NAc (1.7 mm anterior to the bregma and 1.2 mm lateral from midline; Sherwood and Tiramas 1970). Additionally, six anchor screws were placed in vacant areas of the skull to secure the skullcap with dental acrylic. One reference electrode was placed in front of the frontal sinus. Two recording electrodes were created, each consisting of two nickel–chromium, Teflon coated 60 µm diameter wire electrodes (fully insulated except at the tips) twisted together. The ends of each of the twisted electrodes were secured to a 1 cm copper connector pin. Each twisted recording electrode was inserted to a depth of 5.8 and 6.8 mm in each hole above the adolescent and adult NAc, respectively. Electrical activity was monitored during electrode placement using a Grass emitter Hi Z Probe connected to a Grass P511 series amplifier. When there was at least a 3:1 signal-to-noise ratio in each electrode, the electrodes were affixed to the skull with Webglue, a cyanoacrylate surgical adhesive (Webster Veterinary). If the 3:1 ratio was not achieved, the electrode was inserted deeper into the hole in increments of 10 µm. Upon successful implantation of the electrodes, the connector copper pins were inserted into Amphenol plugs, which were fastened to the skull with dental acrylic cement. The rats were allowed 5–7 days to recover from the surgery. During each of these recovery days, the rats, in their home cages, were placed in the experimental behavioral apparatus and connected to the wireless headstage transmitter (~4 g in weight; Triangle BioSystems, Durham, NC, USA) for 2 h to get the rats acclimated to the recording system. Recording started after the recovery from surgery at P-40 for adolescent animals and P-60 for adult animals.

Experiment

Table 1 shows the 10-day experimental protocol for each age group and the recording days (marked *). Rats of each age group were randomly assigned to four subgroups: saline,

0.6, 2.5, and 10 mg/kg MPD. The saline injection group was used as a control for the handling, injection, and injection volume. On experiment day 1 (ED1) and experimental day 10 (ED10), the animals were allowed to acclimate to the recording apparatus for 20–30 min prior to obtaining a baseline. All animals were then injected with 0.8 mL of saline intraperitoneally and a 60-min baseline (BL) of both NAc neuronal and behavioral activity was recorded concomitantly on an experimental day 1 (ED1 BL). This was followed by another injection of either saline (Fig. 1) or 0.6, 2.5, or 10 mg/kg MPD (Fig. 2). Neuronal and behavioral activity recordings were resumed for an additional 60 min immediately following injection. On ED2-6, rats were injected into their home cage according to their assigned group (saline, 0.6, 2.5 or 10.0 mg/kg MPD). ED7-9 were washout days, with no injections or recordings. ED10 was identical to ED1 (injections and recordings), with a saline injection for all groups followed by 60 min of recording to establish an ED10 baseline (ED10 BL) followed by an injection of either saline or MPD (0.6, 2.5 or 10.0.0 mg/kg MPD) rechallenge (ED10 MPD) with another 60 min of recording, similar to previously done on ED1 (Table 1) (Brousard et al. 2019; Kharas et al. 2017; Chong et al. 2012; Claussen and Dafny 2011; Venkataraman et al. 2017, 2019, 2020). Since all injections took place in the animal's home cage, any change from baseline activity was attributed to the drug effect.

Behavioral apparatus

Locomotive activity was recorded using an open field computerized animal activity monitoring system (CAAMS, Opto-M3, Columbus Instruments, Columbus, OH, USA).

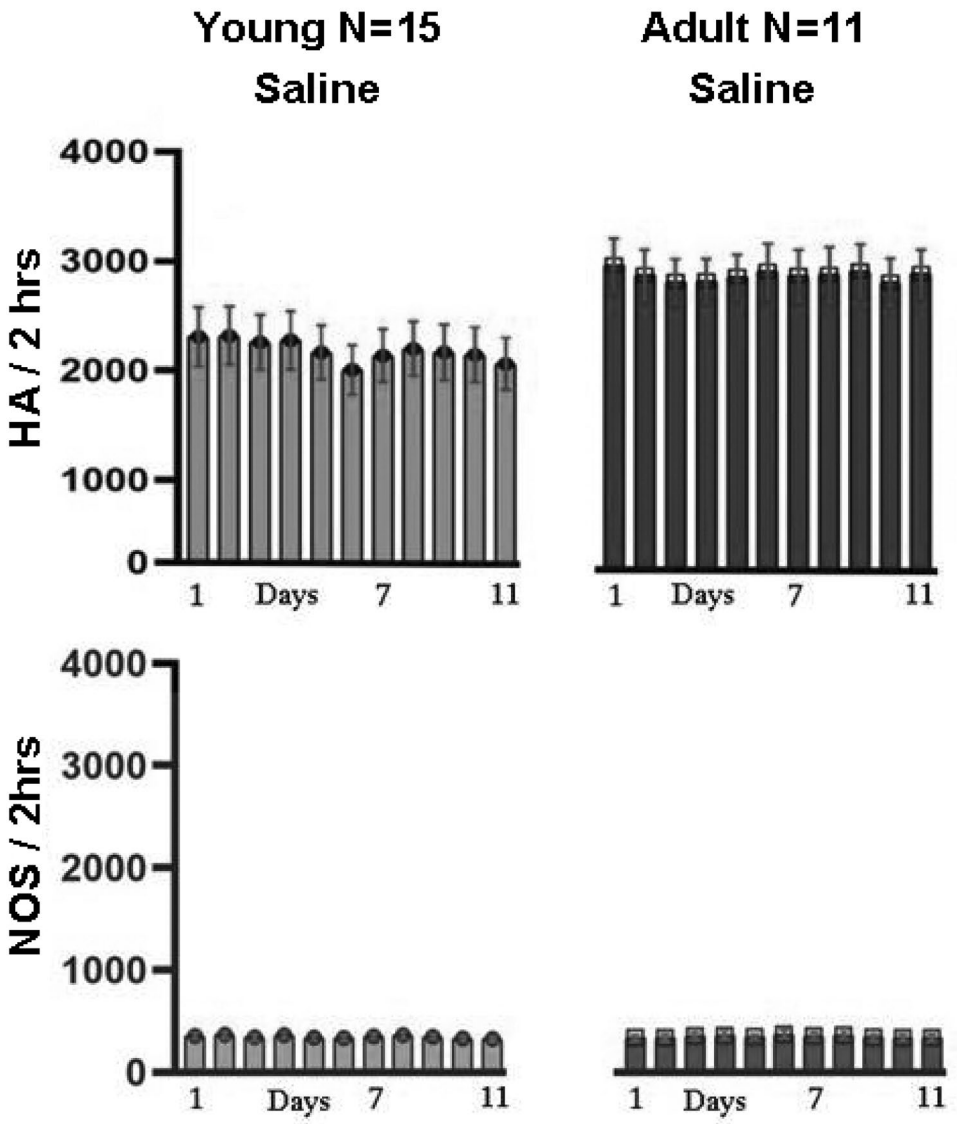

Fig. 1 The horizontal activity (HA) and number of stereotypic movements (NOS) activity of the 11 experimental days (ED) of time control and the effect repetitive 11 daily saline injections (saline control). Recordings were obtained for two hours from 08:00 to 10:00 a.m. each day, demonstrating the locomotor activity with minor non-significant fluctuation during the 11 experimental recording days following saline injections

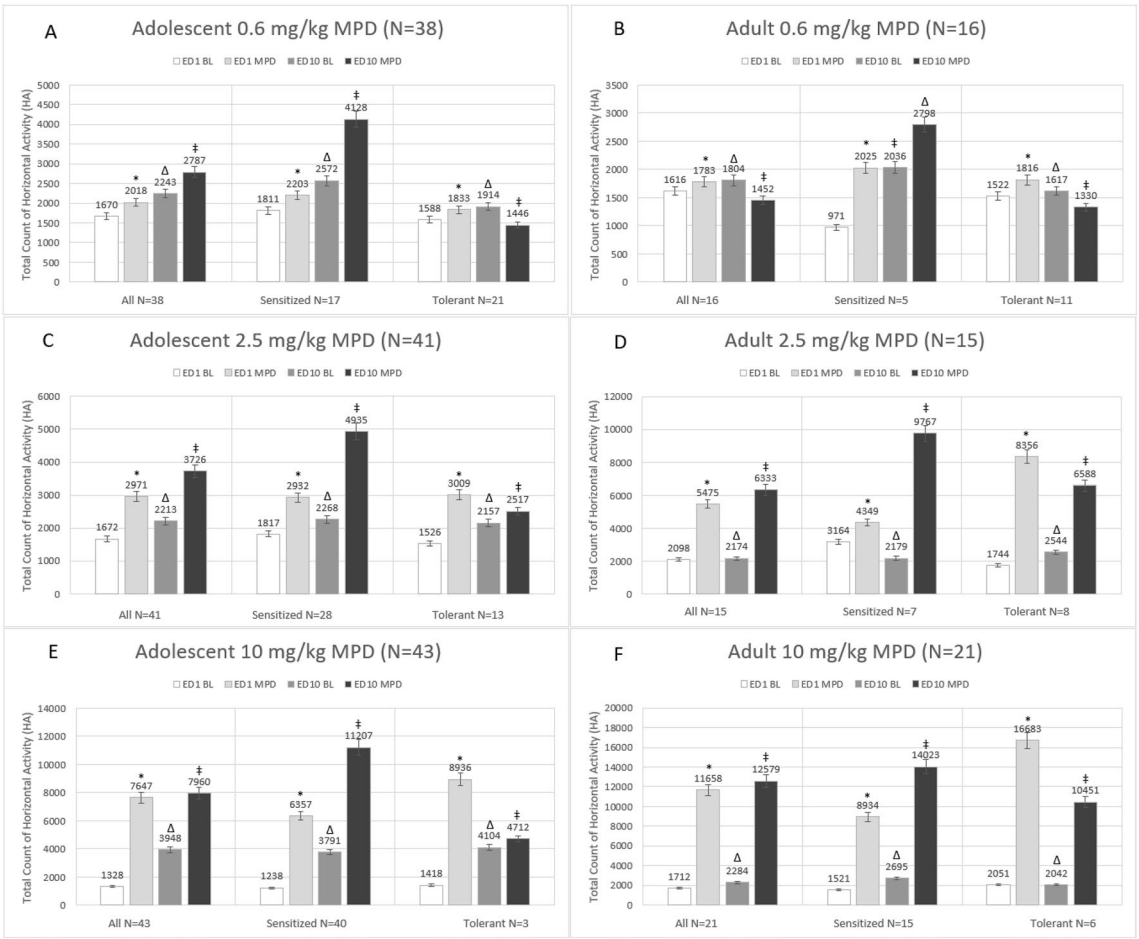

Fig. 2 All the behavioral data for adult and young rat groups as well as for each experimental MPD dose (0.6 mg/kg, 2.5 mg/kg, and 10.0 mg/kg MPD). Each square contains three groups of histograms. *N* represents the number of animals in each group. The animals for each experimental dose group were divided into three subgroups; (a) total, (b) behaviorally sensitized and (c) behaviorally tolerant animals. "Total" group summarizes all the animals for a particular MPD dose. "Sensitized" sub-group summarizes only animals that expressed behavioral sensitization to chronic MPD, and "Tolerant" subgroup summarizes only animals that expressed behavioral tolerance to chronic MPD at ED10 after six daily MPD exposures (0.6, 2.5 and 10.0 mg/kg) and three washout days (ED7, 8, 9) as compared to the initial MPD exposure at ED1, respectively. Each histogram contains four columns as follows; experimental day 1 baseline (ED1 BL); ED1 MPD; ED10 BL; ED10 MPD. The left histograms summarize all the adult animals tested following 0.6, 2.5 and 10.0 mg/kg MPD, and the right histograms summarize all the young animals. For each group, the horizontal activity (HA) of ED1 MPD is compared to the HA of ED1 BL to obtain the acute MPD effect. The ED10 BL was compared to the ED1 BL (ED10BL/ED1BL) to obtain whether six daily MPD exposures and three washout days modulate ED 10 BL. The HA of ED10 MPD is compared to the HA of ED1 MPD (ED10 MPD/ED1 MPD) to obtain the chronic MPD effect. The HA of adolescent ED1 MPD is compared to the HA of adult ED1 MPD to examine the differences between the acute response for adolescents and adults; and the HA of adolescent ED10 MPD is compared to the HA of adult ED10 MPD to examine the difference in chronic MPD response between adolescent and adult animals. Above each column are the standard deviation (SD). *Indicates significant ($p < 0.05$) differences from ED1 BL (ED1 MPD/ED1 BL). $^{\Delta}$Indicates significant ($p < 0.05$) differences from ED1 BL ED10 BL/ED1 BL). $^{\pm}$Indicates significant ($p < 0.05$) differences from ED 1 MPD (ED10 MPD/ED1 MPD)

The home cages, made of clear acrylic, fit into the recording apparatus, which allowed for recording in the home cage. The Columbus open field system consists of 16 by 8 infrared beams with sensors on the opposite side, creating a field that runs 40 cm in length and 20 cm in width at a height of 5 cm above the floor of the cage. Movement across any of the infrared beams causes a beam break and the sensors count the interruptions at a 100 Hz frequency to see if any breaks have occurred. Any breaks are recorded by software and transmitted to a PC in increments of 10 min, with six bins constituting the 60 min recording period. The program converts the breaks into several locomotor activities indices. Based on previous experiments, two locomotor behaviors were selected for this study: horizontal activity (HA) and a number of stereotypic movements (NOS) (Fig. 1). HA is a measurement of overall locomotive activity by looking at the number of beam breaks from one beam to the next. NOS counts the number of repetitive movements that result in the

interruption of the same beam repeatedly, with at least a 1 s gap between breaks (Claussen et al. 2012; Eckermann et al. 2001; Gaytan et al. 2000; Podet et al. 2010; Venkataraman et al. 2020). Therefore, any movement would lead to beam breaks and was counted. The counted activity of each session was stored on the PC for each 60 min segments post-injection of saline and another 60 min post-injection of MPD on both ED1 and ED10 (Table 1). The animal's behavioral and electrophysiological response was statistically evaluated offline.

Histological verification of electrode placement

Upon completion of the recording on ED10, the rats were deeply anesthetized with sodium pentobarbital and transcardially perfused with 10% formalin solution with 3% potassium ferrocyanide. Next, a 2-mA DC current was passed through the electrodes for 20 s to create a small lesion at the electrode tips. The brain was removed and stored in 10% formalin. Several days later, the brain was histologically cut into 60 µm thick coronal sections and stained with cresyl violet. The locations of the lesions were confirmed using the Rat Brain Atlas (Paxinos and Watson 1986). Behavioral and NAc electrophysiological data from each rat was only evaluated and included if the electrode was found to be in the NAc and exhibited identical spike amplitude and waveforms at ED1 and ED10 (Fig. 3).

Data acquisition

On the recording days (ED1 and ED10), the rat and his home cage were placed in a Faraday testing cage to minimize noise during signal transmission. The wireless headstage Triangle BioSystems (Durham, NC, USA) was connected to the electrode pins of the skull cap. The headstage sent neuronal activity signals to the receiver that connects to a Cambridge Electronic Design (CED) analog-to-digital converter (Micro1401-3; Cambridge, England) which digitized the electrical events and stored the recorded data onto a PC for offline evaluation. Only single spike activity that was histologically confirmed to be recorded from the NAc and exhibiting similar waveforms and amplitudes from both recording days before and after MPD injection (ED1 and ED10) were analyzed (Figs. 2, 3).

Analysis of behavioral data

The data recorded by the CAAMS was analyzed for each dosage group using an ANOVA test with a significance set at $p < 0.05$. The following comparisons were made: (a) The locomotor and neuronal recordings at ED1 MPD were compared to ED1 BL to obtain the acute MPD effect, (b) The locomotor and neuronal recordings at ED10 BL were compared to ED1 BL to observe whether the ED10 BL was changed after six consecutive days of MPD exposure and three washout days. Any change in ED10 BL from ED1BL was interpreted as an expression of withdrawal, (c) and the locomotor and neuronal recordings at ED10 MPD were compared to ED1 MPD to obtain the chronic effect of MPD i.e. whether behavioral sensitization or tolerance was expressed.

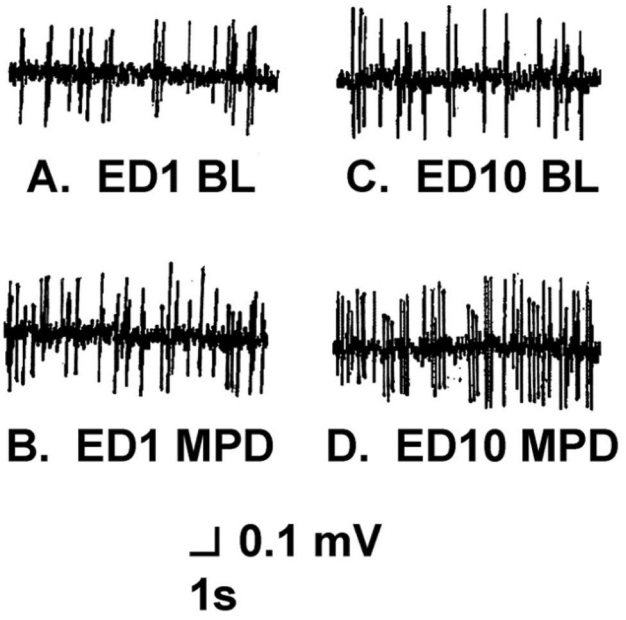

Fig. 3 Representative analog of neuronal activity recorded from the adult NAc. **A** represents the spike activity following the saline injection on an experimental day 1 (ED1 BL). **B** represents the spike activity after acute 2.5 mg/kg MPD injection at experimental day 1 (ED1 MPD). **C** represents the baseline spike activity after six daily 2.5 mg/kg MPD injections and three washout days (ED10 BL). **D** represents the spike activity after MPD rechallenge on experimental day 10 (ED10 MPD). The recordings were obtained 15 min after each injection. In this analog activity, the acute MPD exposures (ED1 MPD) results in increased NAc firing rates compared to those recorded for the ED1 BL. The baseline neuronal activity recorded on ED10 (ED10 BL) after six daily injections and three washout days also displayed increased firing rates compared to ED1BL. Finally, the MPD rechallenge on experimental day 10 (ED10 MPD) demonstrated further increases in NAc firing rates compared to the NAc activity recorded after ED1 MPD. These further increases in firing rates are examples of neurophysiological sensitization

Next, the data of each rat was also analyzed individually using a paired t-test with a significance set at $p < 0.05$. The same three comparisons were used (ED 1 MPD/ED1 BL; ED10 BL/ED1 BL; and ED 10 MPD/ED1 MPD) and based on the third comparison, the rats were divided into whether or not they expressed behavioral sensitization, behavioral tolerance, or no change. If the ED10 MPD behavioral activity was significantly increased compared to ED1 MPD (ED10 MPD/ED 1 MPD), the rat was classified as expressing behavioral sensitization. Conversely, if ED10 MPD behavioral activity was significantly less than the ED1 MPD

activity, the animal was expressing behavioral tolerance. All the data from the animals expressing behavioral sensitization to chronic MPD were summed in one group, and the data from animals expressing behavioral tolerance to another, i.e. three groups of data were used. Data obtained from all the animals, data obtained only from animals expressing behavioral sensitization, and data obtained from only animals expressing behavioral tolerance to ED10 MPD as compared to ED1 MPD. Bonferroni post ad hoc comparisons were used to estimate changes between days for each MPD dose group depending on whether the animal expressed behavioral tolerance or sensitization.

Neuronal spike sorting

Spikes were sorted and processed in-line similar to our previous studies (Broussard et al. 2019; Chong et al. 2012; Claussen et al. 2012, 2014, 2020; Kharas et al. 2017; Salek et al. 2012; Venkataraman et al. 2017, 2019). Spike 2.7 software (CED) was used offline to sort for identical spike amplitude and waveforms at sampling rates of up to 200 kHz and run through low and high-pass filters (0.3–3 kHz) with two window discriminatory levels for positive-going and negative-going spikes. Spikes with peak amplitudes within the window were used to create templates by tracing each spike into 1000 discrete waveform data points (Figs. 4, 5). The spikes were extracted when the input signal entered the amplitude window, so spikes with peak amplitudes outside the windows were rejected. The algorithm that was used to capture spikes provided high-dimensional reference points that were used for accurate spike sorting, taking into account background noise, waveform overlap, and false threshold crossing. All templates were compared with the selected spike event to find the best fitting template with the minimum variance. Furthermore, a template matching procedure is performed that excludes waveforms if the distance between template and waveform exceeds a threshold value (80%). In sum, the accuracy of the reconstructed data was estimated to be 95%. The same parameters for spike sorting were used on ED1 and ED10 for each electrode to ensure the captured spike patterns were identical on ED1 and ED10.

Analysis of electrophysiological data

The sorted neuronal activity obtained from the template matching system was converted by the Spike2 version 7 software (CED) into their firing rates for 60 min of baseline control recording and for 60 min post-MPD administration on ED1 and ED10 (see Table 1). The firing rates of each unit were exported into an Excel spreadsheet along with an identifier for the rat number, the electrode, the experimental day, and the MPD dose. The NAc neuronal activities were found to not hold normality assumptions, therefore, to determine parametric or nonparametric

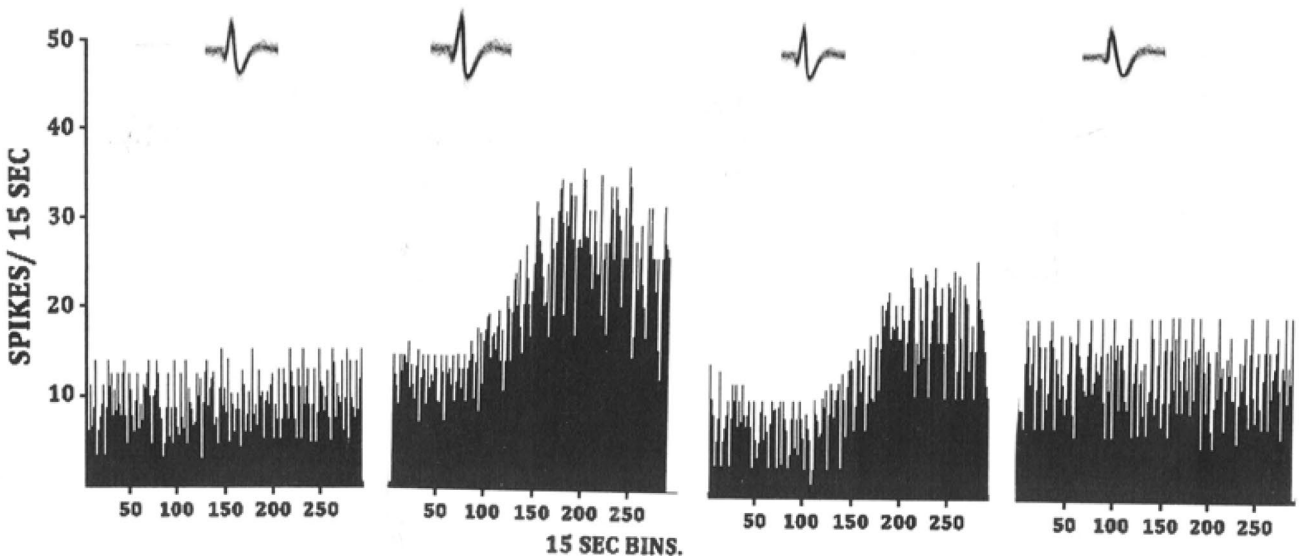

Fig. 4 A histogram of NAc units recorded from adult animals summarizing 60-min sequential neuronal firing rates/15-s following acute 2.5 mg/kg MPD exposure. The first section on the left shows the NAc unit activity recorded at baseline on ED1 (ED1 BL), the second to the left shows the NAc unit activity recorded following acute MPD exposure (ED1 MPD), the second from the right shows the baseline firing rates on an experimental day 10 after six daily MPD exposures and three washout days (ED10 BL). Increased activity of ED10 BL compared to ED1 BL (ED10 BL/ED1 BL) indicates withdrawal activity. Finally, the furthest right shows the unit activity recorded from the NAc following repetitive (chronic) MPD exposure (ED10 MPD). These histograms show excitation at ED1 following acute MPD injection, and on ED10 the increase in firing rate after chronic MPD is less than that on ED1, indicating neuronal tolerance

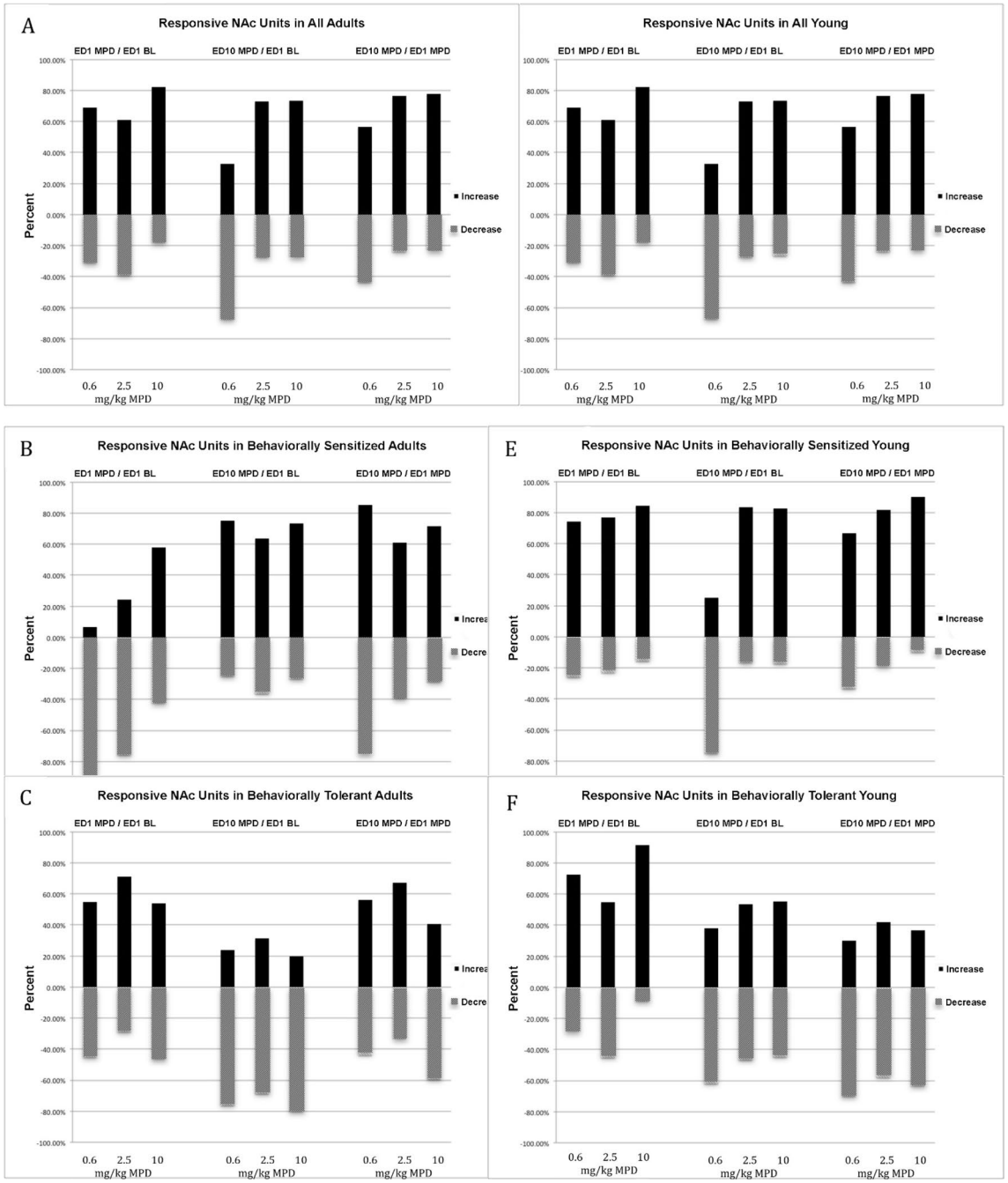

Fig. 5 The responsiveness direction (increase or decrease) in % of how many NAc neurons respond significantly to acute and chronic MPD doses. Each segment has three columns and three sections showing in percentage how many NAc neurons respond significantly by either increasing or decreasing firing rates in response to acute MPD (ED1 MPD/ED1 BL), the BL change of ED10 compared to ED1 after six daily MPD exposures and three washout days (ED10 BL/ED1 BL), and the chronic effect of the drug on ED10 (ED10 MPD/ED1 MPD). In **A**, **D** are the NAc units recorded from all the animals. In **B**, **E** are the NAc neurons recorded only from behaviorally sensitized animals, and in **C**, **F** are the NAc units recorded from only the behaviorally tolerant adult and adolescent animals, respectively

methods to evaluate differences in neuronal activity before and after MPD treatments, the critical ratio (CR) test was used. $\mathrm{CR} = \frac{E-C}{\sqrt{E+C}} \pm 1.96 = P < 0.05$ (C = control, E = activity after treatment). CR values greater than + 1.96 indicate a significant increase in neuronal activity, whereas values less than − 1.96 indicate a significant decrease in neuronal

activity (Chong et al. 2012; Claussen and Dafny 2011; Salek et al. 2012; Broussard et al. 2019; Kharas et al. 2017; Venkataraman et al. 2017, 2019, 2020). The same three comparisons as before (in the behavioral analysis) were made: acute MPD effect (ED1 MPD/ED1 BL), (ED10 BL/ED1 BL), and chronic MPD effect (ED10 MPD/ED1 MPD). When the activity (neuronal and behavioral) at ED10 MPD/ED1 MPD exhibits a significant different increase indicating sensitization, and when the significant difference at ED10 MPD/ED1 MPD was decreasing it indicates tolerance.

Further statistical analysis was done to test the hypotheses. A one-way ANOVA was used to determine if the NAc neuronal activity recordings from animals expressing behavioral sensitization were significantly different from the NAc neuronal activity recordings from those expressing behavioral tolerance for each dosage group. Additionally, a log-linear model with a chi-square value was used to control for dose when comparing the overall activity between the behaviorally tolerant and sensitized groups to again see if there was a difference between dose behavior and firing patterns for each dose group. P values of < 0.05 obtained for the Chi square test and the log-linear model were considered significant. Lastly, a two-way ANOVA was used to determine if there was a statistically significant difference between the acute ED1 MPD response and the chronic ED10 MPD response for all doses.

Results

Locomotor behavioral expression

A total of 302 male SD rats were evaluated; 141 adult and 161 adolescent animals (after exclusion of rats with incorrect electrode placement). Eleven, 42, 36 and 52 adult and 15, 51, 41, and 54 adolescent animals were used following saline, 0.6, 2.5 and 10.0 mg/kg MPD, respectively.

Animals of both ages in the control (saline) group (Fig. 1, saline) had no change in behavioral activity following single and multiple injections of saline as compared to the initial injection, demonstrating that the handling, injection procedure, and environment had no effect on the animal's behavior during the 10 experimental days.

Effect of acute and chronic 0.6 mg/kg MPD on HA (Fig. 2A, B)

Figure 2 summarizes the effect of 0.6, 2.5 and 10.0 mg/kg MPD on HA of all three groups (all, sensitized, tolerant).

All groups acute 0.6 mg/kg MPD

Acute 0.6 mg/kg MPD elicits significant [$p < 0.05$; F (2, 42) = 9.13] increases in HA in both ages (Fig. 2, all). The adolescent animals exhibit more increase in activity (excitation) as compared to the adult animals following acute 0.6 mg/kg MPD [$p < 0.05$; F (2, 42) = 5.21] (Fig. 2A compared to Fig. 2B). Comparing ED10 BL to ED1 BL (ED10 BL/ED 1BL) after six daily 0.6 mg/kg MPD in both age groups exhibited significant [$p < 0.05$; F (2, 42) = 4.58] increases in HA; i.e. this change is interpreted as withdrawal behavior. The level of increased activity (ED10 BL/ED1 BL) in adolescent animals was significantly [$p < 0.05$; F (2, 42) = 4.82] higher than their adult counterparts.

All groups chronic 0.6 mg/kg MPD

The chronic effects of 0.6 mg/kg MPD (ED10 MPD/ED1 MPD) resulted in behavioral tolerance in the adult group and further excitation (i.e. behavioral sensitization) in the adolescent group (Fig. 2A, B, all adult and adolescent).

Behaviorally sensitized groups 0.6 mg/kg MPD

The behaviorally sensitized group exhibited significant [$p < 0.05$; F (2, 42) = 4.93] differences in response to 0.6 mg/kg MPD as compared to all groups. The acute 0.6 mg/kg MPD elicited significant [$p < 0.05$; F (2, 42) = 4.84] attenuation in the adult and excitation in the adolescent group, respectively (Fig. 2A, B, sensitized). The ED10 BL/ED1 BL were about the same in both age groups, exhibiting withdrawal behavior i.e., increase in HA. Similar differences were observed comparing ED10 MPD/ED1 MPD. The adolescent group exhibited significant [$p < 0.05$; F (2, 42) = 6.42] further excitation (Fig. 2A, B, sensitized).

Behaviorally Tolerant groups 0.6 mg/kg MPD

The behaviorally tolerant animals for both age groups responded to 0.6 mg/kg MPD about the same (Fig. 2A, B, tolerant). There are significant [$p < 0.05$; F (2, 42) = 8.03] differences in response to 0.6 mg/kg MPD between adults compared to adolescent animals, i.e. adolescent groups respond to MPD with more HA compared between the three groups (all, sensitized, tolerant).

Sensitized/tolerant 0.6 mg/kg MPD

The ratio of how many animals expressed behavioral sensitization versus behavioral tolerance to chronic 0.6 mg/kg MPD between the two age groups was significantly ($p < 0.05$) different using the chi-square test. It was observed that 0.6 mg/kg MPD in adult animals elicited higher ratios

of behavioral sensitization compared to behavioral tolerance (26:16 compared to 27:24, relatively) (Fig. 2, 0.6 mg/kg MPD).

Effect of acute and chronic 2.5 mg/kg MPD on HA (Fig. 2C, D)

The acute effects of 2.5 mg/kg MPD on HA of all three groups (all, sensitized and tolerant) elicit significant [$p < 0.05$; F (2, 42) = 4.64] increases in behavioral locomotion in response to 2.5 mg/kg MPD.

All groups 2.5 mg/kg MPD

The adult group responded to acute 2.5 mg/kg MPD with higher HA compared to the adolescent group (Fig. 2C, D). ED10 BL/ED 1BL HA in the adult group was the same, i.e. no significant differences, while the adolescent group exhibited significantly [$p < 0.05$; F (2, 42) = 4.83] higher increases in HA, i.e. exhibiting withdrawal. Chronic 2.5 mg/kg MPD caused further significant [$p < 0.05$; F (2, 42) = 5.74] increases compared to the initial effects (ED10 MPD/ED1 MPD). The adult group exhibited significant [$p < 0.05$; F (2, 42) = 4.94] increases in HA than their adolescent counterparts (Fig. 2C, D, all).

Behaviorally sensitized 2.5 mg/kg MPD

Following acute 2.5 mg/kg MPD the behaviorally sensitized animals of both ages responded by excitation while the adult group responded with significantly [$p < 0.05$; F (2, 42) = 5.03] higher increases in HA as compared to the adolescent animals (Fig. 2C, D, sensitized). The ED10 BL in the adult group was significantly [$p < 0.05$; F (2, 42) = 5.48] attenuated while in the adolescent the opposite was observed, i.e. significant [$p < 0.05$; F (2, 42) = 4.68] increases. The chronic 2.5 mg/kg MPD elicits significant [p < 0.05; F (2, 42) = 4.67] further increases in HA in both age groups, but the adult group exhibited significantly [$p < 0.05$; F (2, 42) = 5.22] more intense excitation as compared to the adolescents.

Behaviorally tolerant 2.5 mg/kg MPD

The behaviorally tolerant animals for both age groups responded to acute 2.5 mg/kg MPD significantly [$p < 0.05$; F (2, 42) = 8.04] differently, i.e. the adult group HA was more than double the adolescent HA counts. The ED10 BL/ED1 BL was about the same. However, the ED10 MPD/ED1 MPD was significantly [$p < 0.05$; F (2, 42) = 5.36] different between the two age groups. In the adult as compared to the adolescent, the HA counts were more than double but the ED10 MPD was significantly lower [$p < 0.05$; F (2, 42) = 9.76] as compared to ED1 MPD, and still significantly higher [$p < 0.05$; F (2, 42) = 7.61] than ED1 BL, i.e. behavioral tolerance was observed (Fig. 2C, D, tolerant).

Sensitized/tolerant animals 2.5 mg/kg MPD

The ratio of how many animals expressed behavioral sensitization versus behavioral tolerance to chronic 2.5 mg/kg MPD was about the same, 25:11 in the adult group compared to 28:13 in the adolescent group (Fig. 2C, D).

Effect of acute and chronic 10.0 mg/kg MPD on HA (Fig. 2E, F)

Figure 2E, F 10.0 mg/kg MPD summarizes the acute and chronic effects of 10.0 mg/kg MPD on HA of all three groups (all, sensitized, tolerant).

All groups 10.0 mg/kg MPD

The figure shows that in response to the highest MPD dose (10.0 mg/kg) the adult animals responded with significantly [$p < 0.05$; F (2, 42) = 8.34] higher HA than previous MPD doses, and that the adults responded with significantly [$p < 0.05$; F (2, 42) = 9.06] higher HA intensity than the adolescent animals. Comparing ED10 BL to ED1 BL, the adolescent animals expressed significantly higher counts [$p < 0.05$; F (2, 42) = 8.08] HA than the adult group. Changes in ED10 BL/ED1 BL represent withdrawal activity i.e., the adolescent expressed more severe withdrawal. Following chronic MPD exposure, further increases in HA were observed in both age groups, while the adult groups HA were significantly [$p < 0.05$; F (2, 42) = 6.58] higher than the adolescent group (Fig. 2E, F).

Behaviorally sensitized groups 10.0 mg/kg MPD

The acute MPD (ED1 MPD/ED1 BL) and the ED10 BL/ED1 BL as well as the ED10 MPD/ED1 MPD were significantly [$p < 0.05$; F (2, 42) = 8.26] increased in both age groups. However, the differences observed between the age groups were that the adult HA was significantly [$p < 0.05$; F (2, 42) = 8.53] higher than the adolescent animals. Additionally, the ED10 BL/ED1 BL was significantly [$p < 0.05$; F (2, 42) = 7.67] higher in the adolescent animals as compared to the adult animals. The NOS activity responded to all the MPD treatments similar to those observed in the HA, therefore additional figures were not shown.

Behaviorally tolerant animals 10.0 mg/kg MPD

The behaviorally tolerant animals for both age groups responded to acute 10.0 mg/kg MPD by excitation, but

significantly [$p < 0.05$; F (2, 42) = 8.02] differently, i.e. the adult HA counts were significantly [$p < 0.05$; F (2, 42) = 9.34] higher than the HA counts in adolescent animals. The ED10 BL/ED1 BL was significantly [$p < 0.05$; F (2, 42) = 4.83] different, whereas the adolescent ED10 BL after six daily MPD exposures and three washout days were more than double the adult group. Following ED10 MPD compared to acute MPD (ED10MPD/ED1MPD), significant ($p < 0.05$; F (2, 42) = 4.76) decreases were observed but the HA counts at ED10 MPD/ED1 MPD were significantly [$p < 0.05$; F (2, 42) = 4.90] higher than the HA count at ED1 MPD, i.e. tolerance was observed.

Sensitized/tolerant 10.0 mg/kg MPD

The ratio of how many animals expressed behavioral sensitization versus behavioral tolerance was 19/32 in adult animals compared to 40/14 in adolescent animals, respectively (Fig. 2E, F), and they exhibited significant ($p < 0.05$) differences using the chi test.

Electrophysiological responses

A total of 985 NAc units were recorded and evaluated, 482 from adult rats and 503 from adolescent rats, on both ED1 and ED10, respectively. Table 2A, B summarize the NAc neuronal units and their responses for the adult and adolescent animals, respectively. Figure 4 shows the typical analog activity on ED1 BL (A), ED1 MPD (B), ED10 BL (C), and ED10 MPD (D), while Fig. 5 shows the histogram of 60-min NAc units recorded from adolescent animals at ED1 BL, ED1 MPD, ED10 BL, ED10 MPD.

Table 2 The number of NAc units recorded from adult and adolescent animals, respectively

A. Units recorded from all adult animals													
		ED1 MPD/ ED1 BL				ED10 BL/ ED1 BL				ED10 MPD/ ED1 MPD			
Dose	N	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive
Sal	60	3/60 (5%)	2/60 (3%)	1/60 (2%)	57/60 (95%)	3/60 (5%)	1/60 (2%)	2/60 (3%)	57/60 (95%)	2/60 (3%)	1/60 (2%)	1/60 (2%)	58/60 (97%)
0.6	142	108/142 (76%	41/142 (29%)	67/142 (47%)	34/142 (24%)	21/142 (15%)	15/142 (11%)	6/142 (4%)	121/142 (85%	119/142 (84%	88/142 (62%)	31/142 (22%)	23/142 (16%)
2.5	147	125/147 (85%	58/147 (39%)	67/147 (46%)	22/147 (15%)	43/147 (29%)	29/147 (20%)	14/147 (10%)	104/147 (71%	133/147 (90%	85/147 (58%)	48/147 (33%)	14/147 (10%)
10	133	129/133 (97%	76/133 (57%)	53/133 (40%)	4/133 (3%)	35/133 (27%)	22/133 (17%)	13/133 (10%)	98/133 (73%)	126/133 (95%	67/133 (50%)	59/133 (44%)	7/133 (5%)
B. Units recorded from behaviorally sensitized animals													
		ED1 MPD/ ED1 BL				ED10 BL/ ED1 BL				ED10 MPD/ ED1 MPD			
Dose	N	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive
0.6	49	45/49 (92%)	3/49 (6%)	42/49 (86%)	4/49 (8%)	4/49 (8%)	3/49 (6%)	1/49 (2%)	45/49 (92%)	41/49 (84%)	35/49 (71%)	6/49 (12%)	8/49 (16%)
2.5	78	66/78 (85%)	16/78 (21%)	50/78 (64%)	12/78 (15%)	11/78 (14%)	7/78 (9%)	4/78 (5%)	67/78 (86%)	72/78 (92%)	44/78 (56%)	28/78 (36%)	6/78 (8%)
10	57	57/57 (100%)	33/57 (58%)	24/57 (42%)	0/57 (0%)	15/57 (26%)	11/57 (19%)	4/57 (7%)	42/57 (74%)	56/57 (98%)	40/57 (70%)	16/57 (28%)	1/57 (2%)
C. Units recorded from behaviorally tolerant animals													
		ED1 MPD/ ED1 BL				ED10 BL/ ED1 BL				ED10 MPD/ ED1 MPD			
Dose	N	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive
0.6	93	71/93 (76%)	39/93 (42%)	32/93 (34%)	22/93 (24%)	42/93 (45%)	10/93 (11%)	32/93 (34%)	51/93 (55%)	89/93 (96%)	50/93 (54%)	39/93 (42%)	4/93 (4%)
2.5	78	59/78 (76%)	42/78 (54%)	17/78 (22%)	19/78 (14%)	16/78 (20%)	5/78 (6%)	11/78 (14%)	62/78 (80%)	61/78 (78%)	41/78 (53%)	20/78 (26%)	17/78 (22%)
10	76	74/76 (97%)	40/76 (53%)	34/76 (45%)	2/76 (3%)	5/76 (7%)	1/76 (1%)	4/76 (5%)	71/76 (93%)	69/76 (91%)	28/76 (37%)	41/76 (54%)	7/76 (9%)

Table 2 (continued)

A. Units recorded from all adolescent animals													
		ED1 MPD/ ED1 BL				ED10 BL/ ED1 BL				ED10 MPD/ ED1 MPD			
Dose	N	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive
Sal	41	6/41 (15%)	2/41 (5%)	4/41 (10%)	35/41 (85%)	5/41 (12%)	2/41 (5%)	3/41 (7%)	35/41 (88%)	4/41 (10%)	1/41 (2%)	3/41 (7%)	37/41 (90%)
0.6	152	55/152 (36%)	38/152 (25%)	17/152 (11%)	97/152 (64%)	64/152 (42%)	21/152 (14%)	43/152 (28%)	88/152 (58%)	78/152 (51%)	44/152 (29%)	34/152 (22%)	74/152 (49%)
2.5	143	90/143 (63%)	55/143 (38%)	35/143 (25%)	53/143 (37%)	99/143 (69%)	72/143 (50%)	27/143 (19%)	44/143 (31%)	101/143 (71%	77/143 (54%)	24/143 (17%)	42/143 (29%)
10	167	142/167 (85%	117/167 (70%	25/167 (15%)	25/167 (15%)	128/167 (76%	94/167 (56%)	34/167 (20%)	39/167 (24%)	157/167 (94%	133/167 (73%	35/167 (21%)	10/167 (6%)
B. Units recorded from behaviorally sensitized animals													
		ED1 MPD/ ED1 BL				ED10 BL/ ED1 BL				ED10 MPD/ ED1 MPD			
Dose	N	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive
0.6	51	27/51 (53%)	20/51 (39%)	7/51 (14%)	24/51 (47%)	28/51 (55%)	7/51 (14%)	21/51 (41%)	23/51 (45%)	42/51 (82%)	28/51 (55%)	14/51 (27%)	9/51 (18%)
2.5	76	61/76 (80%)	47/76 (62%)	14/76 (18%)	15/76 (20%)	61/76 (80%)	51/76 (67%)	10/76 (13%)	15/76 (20%)	66/76 (87%)	54/76 (71%)	12/76 (16%)	10/76 (13%)
10	106	90/106 (85%)	76/106 (72%)	14/106 (13%)	16/106 (15%)	93/106 (88%)	77/106 (73%)	16/106 (15%)	13/106 (12%)	102/106 (96%	92/106 (87%)	10/106 (9%)	4/106 (4%)
C. Units recorded from behaviorally tolerant animals													
		ED1 MPD/ ED1 BL				ED10 BL/ ED1 BL				ED10 MPD/ ED1 MPD			
Dose	N	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive	Responsive	Increase	Decrease	Unresponsive
0.6	101	29/101 (29%)	21/101 (21%)	8/101 (8%)	72/101 (71%)	42/101 (42%)	16/101 (16%)	26/101 (26%)	59/101 (58%)	50/101 (50%)	15/101 (15%)	35/101 (35%)	51/101 (50%)
2.5	67	31/67 (46%)	17/67 (25%)	14/67 (21%)	36/67 (54%)	30/67 (45%)	16/67 (24%)	14/67 (21%)	37/67 (55%)	38/67 (57%)	16/67 (24%)	22/67 (33%)	29/67 (43%)
10	61	58/61 (95%)	53/61 (87%)	5/61 (8%)	3/61 (5%)	38/61 (62%)	21/61 (34%)	17/61 (28%)	23/61 (38%)	57/61 (93%)	21/57 (34%)	36/61 (59%)	4/61 (7%)

Table A, B are section into A, B and C. Section 2A.A and 2B.A summarizes all the neuronal recordings for each dose from all the animals. Sections 2A.B and 2B.B summarize only the units recorded from behaviorally sensitized animals, and Sections 2A.C and 2B.C summarizes the recordings from only behaviorally tolerant animals. Additionally, they are sectioned into the acute (ED1 MPD/ED1 BL), baseline (ED10 BL/ ED1 BL), and chronic (ED10 MPD/ED1 MPD) changes, with either increases, decreases or unresponsive in NAc firing rates

NAc units exposed to saline

Ninety-five percent and 85% of the NAc units recorded from adult and adolescent animals showed no changes in neuronal activity following repeated saline injection on ED1 and ED10 compared to the first saline injection on ED1. This shows that the animal handling, volume injection, and behavioral apparatus do not significantly affect NAc neuronal activity.

NAc unit responses to MPD from all animals (Table 2A, B)

Tables 2A (adult) and 2B (adolescent) summarize the effect of MPD on NAc electrophysiological data by comparing the NAc unit activity of ED1 MPD to ED1 BL, ED10 BL to ED1 BL, and ED10 MPD to ED1 MPD (i.e. ED10MPD/ED1MPD). Table 2A, B have three sub-sections, one depicting all animals (Table 2A.A, B.A). The middle section of each table summarizes the NAc units that were recorded from behaviorally sensitized animals (Table 2A.B, B.B), and the lower section of each table summarizes the NAc units that were recorded from behaviorally tolerant animals (Table 2A.C, B.C), respectively. A dose-dependent increase in the NAc neuronal response to MPD exposure on both ED1 and ED10 was observed in both age group. However, the total responsiveness of the NAc units to acute MPD (ED1MPD/ED1BL) recorded from adult animals was significantly [$p < 0.05$; F (2, 42) = 5.56] different from the NAc units recorded from adolescent animals following the same MPD doses (Table 2A compared to Table 2B).

NAc units recorded from animals expressing behavioral sensitization (Table 2A, B)

Comparisons were made between how many NAc units and their percentages recorded from behaviorally sensitized animals responded by significantly ($p < 0.05$) increasing or decreasing their firing rates. In the adult behaviorally sensitized groups, following *acute* MPD (ED1 MPD/ED1 BL), 92%, 85% and 100% of NAc units responded significantly [$p < 0.0$; F (2, 42) = 5.06] to 0.6, 2.5 and 10.0 mg/kg MPD in adult and 53%, 80% and 85% responded in adolescent animals, respectively, i.e., there were significant [$p < 0.05$; F (2, 42) = 4.83] differences in the response to acute MPD between the age groups (Table 2A.B, B.B under sensitized animals). Comparing ED10BL/ED1BL, 8% 14% and 26% showed significant changes in the recordings from adults' animals and 55%, 80% and 88% of the recordings from adolescent animals., i.e. significant [$p < 0.05$; F (2, 42) = 5.66] differences between the two are groups. In the behaviorally sensitized groups, following *chronic* MPD (ED10MPD/ED1MPD), 84%, 92% and 98%, and 82% 87% and 96% of NAc units responded significantly [$p < 0.05$; F (2, 42) = 4.84] to 0.6, 2.5 and 10.0 mg/kg MPD in adult and adolescent animals.

NAc units recorded from animals expressing behavioral tolerance (Table 2A, B)

Seventy- six percent, 76%, 97% and 29%, 46% and 95% of adult and adolescent animals responded significantly [$p < 0.05$; F (2, 42) = 8.46] to acute dose–response MPD (ED1MPD/ED1BL), respectively (Table 2A.C, B.C under tolerant animals), i.e. significant [$p < 0.05$; F (2, 42) = 8.82] differences were observed to acute MPD exposure between the two age groups. Comparing ED10BL/ED1BL between adult and adolescent 45%, 20% and 7% adult animals changed their baseline at ED10 after six daily MPD and three washout days, compared to 42%, 45% and 62% in adolescent animals following 0.6, 2.5 and 10.0 mg/kg MPD, respectively (Table 2A, B), i.e. significant [$p < 0.05$; F (2, 42) = 7.24] differences between the two age groups. Comparing ED10MPD/ED1MPD 96%, 78% and 91% of the NAc units changed their firing rates in the recordings from adult animals, while 50%, 57% and 93% of the NAc units recorded from adolescent animals changed their firing rates (Table 2A, B). Significant [$p < 0.05$; F (2, 42) = 6.21] differences were also seen between the two age groups following chronic MPD exposure.

NAc unit's response direction (increase or decrease) to MPD (Fig. 5)

Figure 5 is composed of histograms that show in percentages how many units recorded from *adult* animals responded to each MPD dose by increasing or decreasing their neuronal activity (left side of Fig. 5A) and *adolescent* animals (right side of Fig. 5D).

In Fig. 5A, D are the percentages of units that responded significantly [$p < 0.05$; F (2, 42) = 6.04] to acute 0.6, 2.5 and or 10.0 mg/kg MPD (Fig. 5A, D, left column). In the middle, three columns are the changes in ED10 BL compared to ED1 BL (ED10 BL/ED1 BL), and in the right column of each of Fig. 5A–F are the comparisons between ED10 MPD and ED1 MPD.

Comparing within each age, the Fig. 5A–C shows significant [$p < 0.05$; F (3, 9) = 34.72] differences between the three adult groups (all, sensitized and tolerant), as well as between the MPD doses (0.6, 2.5 and 10.0 mg/kg MPD) respectively, i.e., by dividing the "all" group to those NAc units recorded from behaviorally sensitized animals or behaviorally tolerant animals it becomes evident that the neuronal responses to MPD recorded from behaviorally sensitized animals respond to MPD significantly [$p < 0.05$; F(93, 21) = 20.14] differently from those recorded from behaviorally tolerant

animals. Similar observations are seen in the adolescent animal groups (Fig. 5D–F).

Comparing adult to adolescent all groups (Fig. 5A, D) no significant difference in response direction is seen between the ages. However, when comparing the recording obtained from animals expressing behavioral sensitization to MPD (Fig. 5B, E) to those expressing behavioral tolerance (Fig. 5C, F) significant [$p < 0.05$; F (3, 21) = 22.57] differences between the age group response directions (increase or decrease) to 0.6, 2.5 and 10.0 mg/kg MPD are observed. These observations indicate that to get accurate information on the effect of MPD, it is imperative to evaluate the neuronal recording based on the animal's behavioral response to repetitive (chronic) drug exposure.

NOS activity responded similarly to HA with no exceptions.

Discussion

The nucleus accumbens (NAc) is a vital part of the neural circuitry underlying the reward pathway and contains a large amount of DA receptors; it mediates input from dopaminergic projections of the ventral tegmental area (VTA), which itself receives a glutamatergic transmission from the prefrontal cortex (PFC; Kalivas 2009; Wise 1996; Wanchoo et al. 2009; Venkataraman et al. 2020). Additionally, the NAc receives glutamatergic signaling from other structures such as the thalamus, amygdala and hippocampus (Kita and Kitai 1990). The culmination of these inputs to the NAc synpase onto medium spiny neurons (MSN), of which different plasticities and molecular adaptations allow for the expression of behavioral sensitization or behavioral tolerance (Chao and Nestler 2004; Nestler 2012). Therefore, the NAc was selected to be studied following acute and chronic MPD exposure.

The main findings of this study are as follows: (a) the same dose of MPD in some adolescent and adult animals elicits behavioral sensitization, while in others behavioral tolerance; (b) behavioral activities recorded from both adolescent and adult animals after MPD administration responded in a dose-dependent manner, with increasing MPD doses greater activities were observed; (c) significant differences were seen between adolescent and adult animals in their behavioral responses to MPD and how many animals expressed behavioral sensitization/behavioral tolerance (d) when the animals were evaluated separately, based on the behavioral response to chronic MPD, significant differences were observed between the three groups (all, sensitized and tolerant) within each age group and between the two age groups; (e) when the NAc neuronal responses recorded were evaluated based on their behavioral response to chronic MPD, i.e. NAc units recorded from behaviorally sensitized animals as a group compared to those NAc units recorded from behaviorally tolerant animals, significant differences were observed between the two age groups; 6) in the 2.5 and 10.0 mg/kg MPD groups, adolescent animals responded with significantly more animals expressing behavioral sensitization as compared to adults; (f) significant differences were seen between adolescent and adult animals in response percentage and response direction (increase or decrease) of NAc neurons to acute and chronic MPD.

Previous behavioral studies in adult animals have observed similar findings with MPD dose–response protocols in intact and NAc destruction animals (King et al. 2019). This study sought to focus not only on the behavioral changes in response to MPD, as in the previous study, but also on how the NAc neuronal activities change, and the relationship, if any, between the behavioral and electrophysiological changes. It was observed that there were significant differences in the NAc electrophysiological changes following MPD exposure between adolescent and adult animals, as well as within each age group between the neuronal recordings from behaviorally sensitized compared to behaviorally tolerant animals. The NAc neuronal recordings from behaviorally sensitized animals tended to show mostly further significant increases in excitation as compared to the initial MPD effect, while the NAc units recorded from behaviorally tolerant groups tended to show mostly electrophysiologic attenuation following repetitive (chronic) MPD exposure as compared to the initial MPD effect. This suggests that there is a direct relationship between the activity in the NAc and the behavioral response of the animals, similar to findings from previous studies in the PFC but different from findings in the VTA (Broussard et al. 2019; Venkataraman et al. 2019).

In our study the same MPD dose that results in the expression of behavioral sensitization elicits in the majority of the NAc units excitation, however, some NAc units responded by attenuation after chronic exposure to the drug. Our observations are that the drug elicits in some NAc units excitation and in others attenuation, i.e. there is a push–pull arrangement which makes it possible to regulate and adjust the behavioral expression to the drug. Similarly, MPD doses that elicit behavioral tolerance also elicit mostly attenuation from the NAc units, while a minority of the NAc units respond with excitation. These push–pull arrangements are the mechanisms which determine the intensity of the behavioral expression to chronic MPD exposure, whether it be tolerance or sensitization. To obtain this fine adjustment it is essential to have mechanisms of both excitation and inhibition (push/pull) to the same stimulus, as the NAc units expressed in this study.

Why it is that some animals respond to the same chronic MPD with behavioral sensitization and others with behavioral tolerance can be explained by observations from other

studies reporting that the same dose of the drug elicits in some animal's upregulations of transcription factors ΔFosB while in other animals the same dose elicits an upregulation of CREB, respectively, as a result of chronic drug abuse treatment (Chao and Nestler 2004; Nestler 2012). An upregulation of ΔFosB is suggested to underlie the expression of behavioral sensitization, while upregulation of CREB is thought to underlie the expression of behavioral tolerance (Hyman and Malenka 2001; McClung et al. 2005; Kim et al. 2009). The above publication reported that some animals to the same dose of the psychostimulant exhibits an upregulation of ΔFosB and others an upregulation of CREB (Chao and Nestler 2004; Ruffle 2014). The varying patterns and densities of these transcription factors between animals and within the VTA, PFC, NAc and other CNS structures may explain the expression of behavioral and neurophysiological sensitization and tolerance between different animals (Jones and Dafny 2014; Venkataraman et al. 2019, 2020). Additionally, the difference in the rates of behavioral sensitization versus behavioral tolerance between adolescent and adult animals may be due to discrepancies in these transcription factor densities, metabolism of the drug, or differences in synaptic pruning of the neurons. Regardless of the mechanism, the disparity between rates of sensitization and tolerance is an important clinical consideration, as many patients are beginning to take the drug at earlier ages.

For the 0.6 mg/kg MPD groups, the adolescent animals seemed to have greater responsiveness in their NAc units than did their adult counterparts. However, in the 2.5 and 10.0 mg/kg MPD groups, the adult animals showed greater responsiveness, i.e., more NAc units responding to MPD than the adolescent animals. These findings are significant because they show that the NAc neurons recorded from adolescent and adult animals respond differently to MPD when the MPD dose elicits behavioral sensitization or behavioral tolerance.

It has been suggested that the differences in neurophysiological excitation and attenuation between the age groups are due to differences in the distribution of D1 and D2-like dopamine (DA) receptors within the NAc. DA receptors are divided into these two categories, which result in either excitatory or inhibitory effects, respectively (Greengard 2001). Both receptors are found in high quantities in the NAc, and differing proportions between adolescent and adult animals as part of normal development may explain the differences between excitation and attenuation. It is also possible that some electrodes were recorded from areas of DA D1 density receptors, while in other animals the electrodes were recorded from DA D2 density receptors. Additionally, it has been proposed that excitation and attenuation responses to MPD maybe be influenced by remote structures that provide glutamatergic innervations to the NAc, such as the prefrontal cortex, thalamus and hippocampus (Kelley et al. 1982; Kita and Kitai 1990). These inputs and connections may contribute to the differences seen not only between adolescent and adult animals but also between behaviorally sensitized and behaviorally tolerant animals.

Overall, the findings from this study show that there are significant differences in response to MPD between adolescent and adult animals and highlights the importance of assessing these two age groups separately when examining the effects of drugs such as MPD. Additionally, the finding that in response to chronic MPD some animals demonstrate behavioral sensitization and others behavioral tolerance also suggests that animals need to be assessed individually for their response to the chronic effect of the drug (sensitization or tolerance), as well as confirms that MPD has the potential to cause dependence, addiction and be abused by ADHD and ordinary subjects using it as cognitive enhancement or for recreational purposes.

Acknowledgements The author like to appreciate John Concha for the figures preparation and NIH NIDA RO1 DA027222 Grant.

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