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Research Article

Methylphenidate (Ritalin) affects serotonin signaling differently in young compared to adults. Concomitant behavioral and neuronal recording from dorsal raphe in freely behaving rats

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Abstract

Currently, methylphenidate (MPD) is one of the most commonly prescribed psychostimulants for management and treatment of attention deficit hyperactivity disorder (ADHD). A rise in the consumption of MPD by "ordinary" youth and adults prompted concern regarding the ontogeny effects of acute and chronic MPD exposure. The objective of this study is to concomitantly record behavioral and neuronal activity from the dorsal raphe (DR) nucleus, a major source of serotonergic innervation in the mammalian brain before and following different doses of acute and chronic administration of MPD in freely behaving adolescent (young) and adult rats previously implanted with electrodes in the DR. A wireless recording system over 10 consecutive experimental days was used. Four experimental groups were used: saline, 0.6, 2.5, and 10.0 mg/kg MPD for young and similar groups for adult rats. Animals received one daily MPD injection on experimental days 1-6, followed by three washout days, and then drug rechallenge on experimental day 10 (ED10). 860 DR units were recorded, 356 from adult rats and 504 from young rats. The study provides experimental evidence that the responses to acute and chronic MPD were significantly different between the two age groups. Moreover, the study implies that it is essential to evaluate the electrophysiological responses to a drug based on the animal's behavioral response to chronic drug exposure and that the DR and serotonin signaling has a significant role in the response to MPD as well as a different role in young as compared to adult rats.

Keywords: Methylphenidate, Ritalin, Psychostimulant, Dorsal raphe, Neuronal activity, Behavior

Introduction

Attention deficit hyperactivity disorder (ADHD) is a complex behavioral disorder characterized by hyperactivity, increased impulsivity, and inattention [1-3]. Methylphenidate (MPD) is the most commonly prescribed psychostimulant for management and treatment of ADHD [3-7]. In the last decade more and more "ordinary" young and adults use MPD as cognitive enhancement to improve their academic performance in a present-day competitive society as well as abusing it for recreation [5,8,9]. The number of fatalities from snorting MPD is greater than from using cocaine or amphetamines [10]. A rapid rise in MPD consumption in the healthy population by youths and adults has prompted concern regarding the effects of acute and chronic MPD exposure on ordinary subject models [11-13] though the neurophysiological mechanism of its action remains unclear [14-19].

Research on ADHD has focused primarily on the role of dopamine (DA) and norepinephrine (NE) signaling in its pathophysiology [15,16,19-21]. However, serotonergic transmission has been implicated in behavioral disorders [22], and it suggests that serotonin (5-HT) transmission may play a role in the symptoms of ADHD [23-25]. Behavioral and histochemical evidence suggests that MPD affects serotonin levels as well [26]. The dorsal raphe nuclei (DR) have been recognized as one of the major sources of 5-HT in the mammalian brain [14,27,28]. DR neuronal signaling correlates towards a diverse set of behaviors, including reward and behavior reinforcement [14,29]. The efficacy of MPD has been implicated in part due to its effect on the DR and 5-HT systems [30]. MPD exposure alters both 5-HT transporter and 5-HT levels [31,32], resulting in a calming effect for ADHD patients. Therefore, the DR was selected as the target of this study.

It has been reported that the psychostimulant cocaine elicits dose-dependent inhibition of DR 5-HT neurons in anesthetized rats [33], while long-term exposure of amphetamine has been shown to increase spontaneous firing rates in DR neurons in urethane anesthetized rats [34]. However, electrophysiological studies concerning acute and chronic MPD exposure on DR neurons in unanesthetized, freely behaving rats without anesthesia interfering is limited. Therefore, unanesthetized, freely behaving rats implanted with permanent electrodes bilaterally in the DR were used in this study. Repetitive exposure to MPD has been shown to elicit dose dependent behavioral withdrawal, sensitization, or tolerance [14,18,35-38]. Behavioral sensitization or tolerance are phenomena characterized by an increased or decreased response to a repeated drug exposure when it is compared to the initial effect, respectively [14,18,36,39]. It was reported that young and adult rats differ in their behavioral response to acute and chronic psychostimulant exposure, with chronic periadolescent exposure leading to long term depression-like behavior in adults [40-43]. However, it was also shown that the use of psychostimulants during adolescence produces no significant changes in the cortical mantle, and that ADHD patients using MPD exhibit functional attenuation similar to those of non-MPD users [44,45]. Despite its extensive use, the long-term effects of repetitive (chronic) MPD exposure on young brain development remain poorly understood [46-48]. While the dopaminergic and the glutaminergic signaling system have definitive roles in the neuroplasticity underling psychostimulant action [20,49,50], there are limited studies on the serotonergic signaling participating in the neurophysiological mechanism underlying MPD and other psychostimulants [51,52].

MPD has been shown to induce both behavioral and DR neuronal changes in separate studies using adult and adolescent rats [53-55]. To our knowledge, no studies exist comparing the DR neuronal activity response to acute and chronic MPD exposure in the two age populations. The hypothesis of this study is that the same MPD dose in some adolescent and adult rats will elicit behavioral sensitization and in others behavioral tolerance, but the ratio of how many young rats express sensitization or tolerance will be significantly different from adult rats. Moreover, that DR neuronal activity recorded from behaviorally sensitized rats will significantly differ from the neuronal activities recorded from behaviorally tolerant rats and there will be significant age differences in the number of young rats expressing sensitization or tolerance as compared to adult rats. The aim of this study is to use an acute and chronic dose response protocol of MPD to investigate if there are age differences between behavioral and DR neuronal properties in young rats as compared to adult rats.

Materials and Methods

Animals

Young male Sprague-Dawley rats at post-natal day 30 and adult male rats at post-natal day 50 were purchased (Harlan, Indianapolis, IN, USA) and allowed 3-5 days of acclimation prior to DR recording electrode implantation. Food and water were given ad libitum. Room temperature was maintained at 21 ± 2°C with a relative humidity of 37-45% under 12h:12h alternating light-dark cycle with lights on at 6:00. After electrode implantation, animals were returned to their home cage that were also used as the test cages for the duration of the electrophysiological and behavioral recordings for an additional 5-7 days prior to recording session days. All experimental procedures were approved by the University of Texas Health Science Center Animal Welfare Committee and in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

Surgery

Young animals were anesthetized with a 30 mg/kg pentobarbital intraperitoneal (i.p) injection, and the adults with a 50 mg/kg pentobarbital i.p injection. Each rat's head was shaved, and lidocaine hydrochloride topical gel was applied to the shaved area for local anesthetic. The animal was then placed in a stereotaxic head holder instrument and an incision was made to expose the skull. Bilateral holes were drilled on the skull above the DR at 7.0 mm in adolescents, and in adults, at 7.8 mm posterior to the bregma and 0.2 mm lateral to the midline using the Sherwood and Timiras adolescent rat brain atlas [56] for young rats and using the Paxinos and Watson Rat Brain Atlas [57] for adult rats, with an additional hole in front of the frontal sinus for the reference electrode. Six anchor screws were inserted in the vacant spots of the skull to secure the skull cap with dental acrylic cement. Two twisted Nickel-Chromium (insulated except at tips) wires measuring 60 µm in diameter were secured each to 1 cm copper connector pins and individually inserted into the DR in each hemisphere, such that each animal had four total recording electrodes. During placement of the electrodes, the neuronal unit activity was monitored. The electrodes were inserted at a depth of 6 mm and, if sufficient neuronal activity was observed, were secured to the anchor screws and to the skull by dental acrylic cement. Electrodes failing to detect satisfactory activity were lowered in increments of 5 to 10 µm until satisfactory spike activity demonstrated a 3:1 signal to noise ratio [53-55]. Animals were allowed to recover from the surgical procedure for 5 to 7 days. During recovery, animals were placed within their home cage in the experimental room for two hours each day, and connected to the wireless head stage (Triangle Biosystems Inc,. TBSI, Durham, NC, USA) to adapt and acclimate to neuronal and behavioral recording systems.

Drug

Methylphenidate hydrochloride (MPD) was obtained from NIDA. Previous experiments using dose response MPD protocols from 0.1-40.0 mg/kg i.p have found that behavioral effects of MPD were observed from 0.6 mg/kg doses MPD and higher [37,58,59]. As such, MPD was administered at 0.6, 2.5, and 10.0 mg/kg corresponding to low, moderate, and high experimental dosages, respectively. MPD was dissolved in a 0.9% isotonic saline solution for i.p injection. Control subjects received injections of 0.8 ml isotonic saline solution (0.9% NaCl). All MPD injections were titrated to a volume of 0.8 ml with 0.9% saline to equalize injection volumes for all animals.

Experimental protocol and data acquisition

The DR neuronal and locomotor activity of the animals starting jointly at age P-40 for young and P-60 for adult (**Table 1**) using a wireless neuronal recording system (TBSI, Durham, NC, USA) and an open field computerized animal activity system (Accuscan, Columbus, OH, USA). The TBSI head stage was connected to the rat head cap containing the electrode pins and sent electrical signals through a transmitter to a remote receiver connected to an analog-to-digital converter (Micro 1401-3; Cambridge Electronic Design (CED)). The neuronal activity from each electrode was collected and stored on a PC using the CED Spike 2.7 software.

The open field system consists of a 40x40x32 cm cage with 16x16 infrared beams, which albino rats do not recognize red, and their sensors placed 5 & 8 cm above the floor of the cage. This setup has been previously described in detail [53-55,58,59]. Interruption of the infrared beams by animal movement was detected by the monitoring system at 100 Hz frequency and the red beams interruptions were compiled by the Oasis software (Accuscan, Columbus, OH, USA) and downloaded to a PC every 10 minutes. The software categorized beam interruptions into three different locomotive behaviors: number of movements (NOM), total distance (TD) traveled in cm, and number of stereotypic movements (NOS), which is a count of repetitive movement with at least one second intervals between movements. Data was recorded for 60 minutes post-injection of either saline or MPD on experimental day (ED) 1 and ED 10. The behavioral recording was to provide a way to distinguish between animals that exhibited behavioral sensitization following repetitive MPD exposure from animals that exhibited behavioral tolerance as compared to the initial MPD effect respectively [18,60]. The behavioral locomotive data was used as the basis for analysis of neuronal recordings. Since in both age groups some animals exhibit behavioral sensitization and others behavioral tolerance to each of the 0.6, 2.5, and 10.0 mg/kg doses of MPD groups, in the data evaluation we divided the animals into three groups: 1) data obtained from all animals – all groups; 2) data obtained only from animals expressing behavioral sensitization- sensitized group; and, 3) data obtained only from animals expressing behavioral tolerance to - tolerant group.

Recording for adolescent rats started at age post-natal 40 days (P-40) and for adults at age P-60 and lasted for 10 days (**Table 1**). The rats of each age were randomly subdivided into four groups: saline (control), 0.6, 2.5, and 10.0 mg/kg MPD treatment groups. On experimental day 1 (ED1), rats were placed within their home cage in a Faraday testing cage to reduce background noise. The wireless head stage was connected to the electrode pins of the skull cap, and animals were allowed to acclimate for an additional 20 to 30 minutes prior to the recording session. On ED1, neuronal and behavioral activity was recorded concomitantly for one hour following an initial injection of 0.8 ml saline; this data serves as a baseline activity (ED1 BL). The animals then received a second injection of either saline (saline/saline group), 0.6, 2.5, or 10.0 mg/kg MPD and neuronal and locomotive behavior was recorded for one additional hour (ED1 Sal or ED1 MPD respectively). From ED2 through ED6, animals received either saline or MPD injections in their home cage without behavioral or neuronal recordings in order to induce chronic MPD effects. From ED7 through ED9, the animals underwent a three-day washout period where no injections were given. On ED10, animals were given a 0.8 ml saline injection, and on ED 10 baseline (ED10 BL) neuronal and behavioral activity was recorded for one hour. The animals were then rechallenged with either saline, 0.6, 2.5, or 10.0 mg/kg MPD (ED10 Sal or ED10 MPD respectively) and an additional hour of recording was done as on ED1 (**Table 1**, [18,36,38,53-55,60]).

Behavioral data analysis

The data acquired was used to make three comparisons: **1)** locomotor activity after acute MPD administration on ED1 was compared to the baseline recording post-saline administration on ED1 (ED1 MPD/ED1 BL) in order to determine the acute effects of MPD; **2)** locomotor activity post-saline injection on ED10 was compared to locomotor activity post-saline injection on ED1 (ED10 BL/ED1 BL) in order to determine any significant change in baseline locomotor activity after six daily repetitive (chronic) MPD exposure and three washout days and **3)** locomotor activity after MPD administration on ED10 was compared to locomotor activity after MPD administration on ED1 (ED10 MPD/ED1 MPD) in order to determine the chronic effect of MPD and to find out whether the animal displayed behavioral sensitization or tolerance. Animals displaying a significantly increased locomotor activity after MPD rechallenge on ED10 as compared to MPD

Table 1. Displays the 4 animal groups and the MPD dose protocol that was followed for each group of the adolescent animals. 4 similar groups were used for the adult animals. The four groups of animals that were used are for each age are: 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 to obtain baseline (BL)l followed by one of the four designated injections of saline, 0.6, 2.5 or 10.0 mg/kg of MPD and 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 ED10BL after six daily injections of either saline or MPD; for one hour followed by the designated MPD dose for one hour and recordings were taken, identical to that given on ED1. * - indicates the behavioral and neuronal recording day.

		Experimental Days (ED)			
Treatment Groups		**ED 1***	**ED 2-6**	**ED 7-9**	**ED10***
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

administration on ED1 (ED10 MPD/ED1 MPD) were considered to display behavioral sensitization, while those exhibiting a significant decrease in locomotor activity following MPD rechallenge on ED10 as compared to MPD administration on ED1 were considered to display behavioral tolerance. The student t-test and the Critical Ratio (CR) test were used to determine the effect of MPD on individual animals.

A C.R. test value above +1.96 indicated that the activity showed a significant increase, while a C.R. value below -1.96 indicated that the activity showed a significant decrease after MPD administration. For acute effect of MPD, E represents activity count after MPD injection on ED1 and C represents the ED 1 activity after saline (control) injection (ED1 MPD/ED1 BL). For multiple injections (chronic) effect, E represents activity following Sal or MPD injection on ED10 and C represents activity after Sal or MPD dose on ED1 (ED10 BL/ED1 BL and ED10 MPD/ED1 MPD). Individual rats were categorized as exhibiting either behavioral sensitization or tolerance and sorted based on their classification. A significant difference among the groups was determined using a two-way ANOVA where $p<0.05$ was accepted as the minimal level of significance.

Neuronal data analysis

CED spike 2.7 software was used for spike sorting and statistical analysis of the sorted neuronal data. The data was captured by the program and processed using low and high pass filters (0.3-3.3 kHz). There are two window discriminator levels, one for positive-going spikes and one for negative-going spikes (**Figure 1**). The spikes were extracted when the input signal enters the previously determined amplitude window. Selected spikes with peak amplitudes that

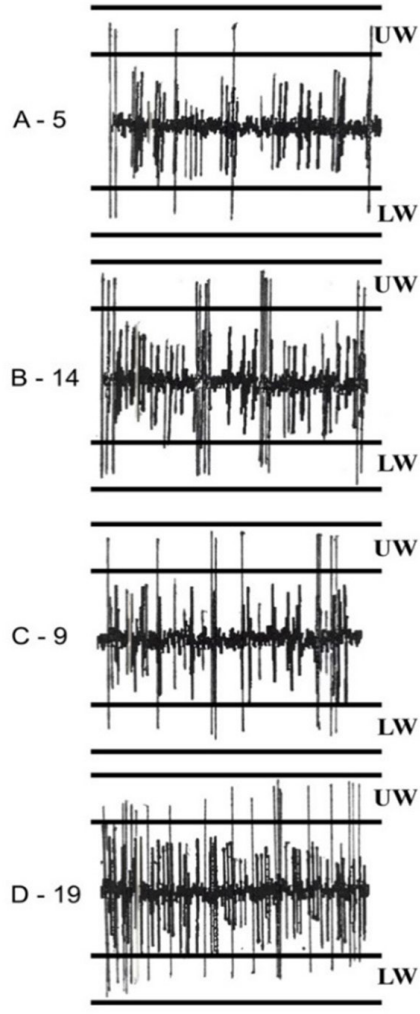

Figure 1. The figure shows 4 traces of PFC analog recordings. **A.** Baseline recordings at experimental day 1 (ED1 BL). **B.** Recordings following 2.5 mg/kg MPD on ED1 showing that MPD elicits excitation as compared to ED1 BL. **C.** Recording following repeated 2.5 mg/kg MPD on ED10 showing MPD tolerance as compared to ED1 MPD. **B** and **D**: Recording ED10 BL compared to ED1 BL showing withdrawal activity (ED10 BL/ED1 BL). The figure shows typical recordings, and the upper (UW) and lower (LW) windows that use the first stage of spike discrimination. The number on the left of each trace summarizes the number of spikes in the trace.

were triggered by the window and exhibit duration of 0.8-1.2 msec were used to create a template. One thousand waveform data points were used to define the selected spike. The algorithm used to capture a spike allows the extraction of templates that provide high-dimensional reference points that can be used to discriminate consistent and accurate spike sorting despite the influence of some noise, false threshold crossing, and waveform overlap. Incoming spikes were compared with all temporal templates to find the best fitting template that yields the minimum residue variance. When the distance between the spike waveform and the template exceeds threshold (80%), the waveforms are rejected. This means that the spike sorting accuracy in the reconstructed data was approximately 95%. The same parameters and template used to sort and count ED1 neuronal activity were loaded onto the ED10 file of the same electrode to sort and count the ED10 activity, ensuring that the waveform sorting parameters used on ED1 and ED10 were identical.

Once spike sorting was completed, the data was exported into a spreadsheet which calculated the average neuronal firing rates for each treatment and produced a sequential firing rates histogram (**Figure 2**). Statistical comparisons were made for each DR neuronal activity as follows: **1)** DR neuronal activity after the initial MPD exposure was compared to DR unit activity following saline administration (baseline activity) on ED1 (ED 1 MPD/ED 1 BL) to obtain the MPD acute effect. **2)** DR neuronal activity BL on ED10 was compared with the DR neuronal activity on ED1 (ED 10 BL/ED 1 BL) to obtain whether withdrawal after six daily MPD exposure is expressed. **3)** DR neuronal activity after MPD administration on ED10 was compared to the activity following initial MPD on ED1 (ED 10MPD/ED 1MPD) to obtain the chronic effect of the drug (sensitization or tolerance). Significant changes in neuronal firing rate and direction of change (increase or decrease) for each DR unit was determined by the student t-test and the Critical Ratio (C.R.) test. A C.R. test value above +1.96 indicated that the neuron showed a significant increase in its activity, while a C.R. value below -1.96 indicated that the neuron showed a significant decrease in its activity after MPD administration.

In addition, the above neurophysiological data analysis was summarized into three subgroups matched on the basis of the animal's behavioral response to each chronic dose of MPD treatment as follows: 1) electrophysiological data recorded from all the animals i.e. the all group; 2) electrophysiological data recorded from animals exhibiting behavioral sensitization i.e. the sensitized group; and 3) electrophysiological data recorded from animals exhibiting behavioral tolerance i.e. the tolerance group (**Tables 2 and 3**). The significance of differences in firing rates of DR neuronal units between these three subgroups (all, sensitized, tolerance) was analyzed using the Chi-square test where $p<0.05$ was considered as significant.

Histological verification of electrode placement

An overdose of sodium phenobarbital was administered upon completion of recording on ED 11. Animals were perfused intracardially with 10% formaldehyde solution containing 3% potassium ferrocyanide and a 20 μA current was passed through the electrode pin for 20 seconds to produce a small lesion at the recording sites in the DR. The brain was removed and preserved in 10% formalin for histological processing. The position of the electrodes in the DR was confirmed by the location of the lesion and Prussian blue spot using the Young Rat Brain Atlas [56] for young rats and using the Rat Brain Atlas [57] for adult rats.

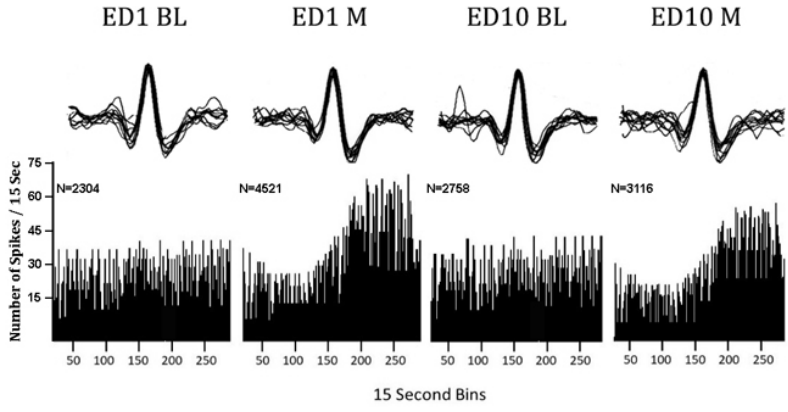

Figure 2. A histogram of DR neuronal units recorded from adult rats summarizing 60-minute sequential neuronal firing rates following acute and chronic 2.5 mg/kg MPD exposure. N is the total number of spikes produced by the units over 60 minutes. The first panel, ED1 BL shows the DR unit activity recorded at baseline on ED1. The second panel, ED M1 (ED1 MPD) shows the DR unit activity recorded after acute 2.5mg/kg MPD exposure. The third panel, ED10 BL shows the DR unit activity recorded after previous exposure to six daily MPD and 3 washout days to determine if there is a withdrawal response. The fourth panel, ED10 M (ED10 MPD) shows DR unit activity after chronic MPD administration to determine whether sensitization or tolerance occurred. Above each histogram are 20 superimposed spikes sorted to produce the histograms, aiming to demonstrate that the same spike unit pattern was counted during each 60-minute recording session. The numbers above each histogram are the total number of spikes /60 min.

Table 2. The table summarizes the DR neuronal responses following 0.6, 2.5 and 10.0 mg/kg MPD recorded from Adult animals. In A, B and C are the summary from the DR neuronal responses recorded from All the animals (2A), from animals expressing behavioral sensitization (2B) and from animals expressing behavioral tolerance (2C) respectively. Under Acute, Baseline and Chronic are how many DR neuronal units responded significantly ($p<0.05$) and their % (in bracket) responded to each MPD dose (0.6. 2.5 or 10.0 mg/kg) by excitation (arrow up) attenuation (arrow down) and the number and % neuronal DR neurons that did not respond to MPD following acute MPD (ED1 MPD/ED1 BL), The baseline (BL) activity of ED10 compares to ED1 BL (ED10 BL/ED1 BL) as well the MPD chronic effect of each MPD dose (ED10 MPD/ED1 MPD). In part D is the summary of total DR neuronal units recorded and how many were recorded from All the animals as well summarizes the number of DR neurons responding significantly ($p<0.05$) to MPD (all doses) and their percentage.

Table 2A: DR Neurons- Adult ALL group.

All Animals

Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic ED 10 MPD/ED 1 MPD)		
		Ý	ß	Û	Ý	ß	Û	Ý	ß	Û
Saline	27	0	0	27 (100%)	1 (4%)	2 (7%)	24 (89%)	1 (4%)	1 (4%)	25 (92%)
0.6 mg/kg	122	39 (32%)	19 (16%)	64 (52%)	51 (42%)	57 (47%)	14 (11%)	48 (39%)	58 (48%)	16 (13%)
2.5 mg/kg	94	49 (52%)	5 (5%)	40 (43%)	35 (37%)	32 (35%)	27 (28%)	45 (48%)	29 (31%)	20 (21%)
10.0 mg/kg	113	72 (64%)	11 (10%)	30 (26%)	37 (33%)	61 (54%)	15 (13%)	39 (35%)	61 (54%)	13 (11%)
Total	356									

2B: DR Neurons - Adult Sensitized group.

Sensitized

Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
		Ý	ß	Û	Ý	ß	Û	Ý	ß	Û
0.6 mg/kg	27	5 (19%)	6 (19%)	16 (59%)	11 (41%)	9 (33%)	7 (26%)	10 (37%)	5 (19%)	12 (44%)
2.5 mg/kg	42	17 (40%)	1 (3%)	24 (57%)	13 (31%)	22 (53%)	7 (16%)	20 (48%)	17 (40%)	5 (12%)
10.0 mg/kg	20	15 (75%)	0 (0.0%)	5 (25%)	4 (20%)	15 (75%)	1 (5%)	3 (15%)	16 (80%)	1 (5%)
Total	89									

2C: DR Neurons - Adult Tolerance group.

Tolerance

Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
		Ý	ß	Û	Ý	ß	Û	Ý	ß	Û
0.6 mg/kg	95	33 (35%)	13 (14%)	49 (51%)	38 (40%)	49 (52%)	8 (8%)	39 (41%)	54 (57%)	2 (2%)
2.5 mg/kg	52	33 (64%)	4 (8%)	15 (28%)	21 (40%)	11 (21%)	20 (39%)	23 (44%)	14 (27%)	15 (29%)
10.0 mg/kg	93	59 (63%)	10 (11%)	24 (26%)	32 (34%)	46 (50%)	15 (16%)	36 (39%)	45 (48%)	12 (13%)
Total	240									

Table 2D: DR Neurons Adult Responsiveness.

DR Unit Adult	**N**	**%**
All Total	356	
Responsive Total	329	92
Tolerance Total	240	73
Sensitized Total	89	27

Table 3. The table summarizes the DR neuronal responses following 0.6, 2.5 and 10.0 mg/kg MPD recorded from Adolescent (Young) animals. In A, B and C are the summary from the DR neuronal responses recorded from All the animals (3A), from animals expressing behavioral sensitization (3B) and from animals expressing behavioral tolerance (3C) respectively. Under Acute, Baseline and Chronic are how many DR neuronal units responded significantly ($p<0.05$) and their % (in bracket) responded to each MPD dose (0.6. 2.5 or 10.0 mg/kg) by excitation (arrow up) attenuation (arrow down) and the number and % neuronal DR neurons that did not respond to MPD following acute MPD (ED1 MPD/ED1 BL), The baseline (BL) activity of ED10 compares to ED1 BL (ED10 BL/ED1 BL) as well the MPD chronic effect of each MPD dose (ED10 MPD/ED1 MPD). In part D is the summary of total DR neuronal units recorded and how many were recorded from All the animals as well summarizes the number of DR neurons responding significantly ($p<0.05$) to MPD (all doses) and their percentage.

Table 3A: DR Neurons - Young All group.

All Animals

Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
		Ý	ß	Û	Ý	ß	Û	Ý	ß	Û
Saline	57	1 (1.8%)	1 (1.8%)	55 96.5%)	3 (5.3%)	2 (3.5%)	52 91.2%)	2 (3.5%)	1 (1.7%)	54 (94.7%)
0.6 mg/kg	137	35 (26%)	32 (23%)	70 (51%)	39 (29%)	26 (19%)	72 (52%)	26 (19%)	40 (29%)	71 (52%)
2.5 mg/kg	142	53 (37%)	45 (32%)	44 (31%)	49 (35%)	36 (25%)	57 (40%)	48 (34%)	48 (34%)	46 (32%)
10.0 mg/kg	168	80 (48%)	65 (39%)	23 (13%)	72 (43%)	63 (38%)	33 (19%)	73 (44%)	66 (39%)	29 (17%)
Total	504									

3B: DR Neurons -- Young Sensitized group.

Sensitized

Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
		Ý	ß	Û	Ý	ß	Û	Ý	ß	Û
0.6 mg/kg	70	43 (61%)	20 (29%)	7 (10%)	33 (47%)	27 (39%)	10 (14%)	42 (60%)	22 (31%)	6 (9%)
2.5 mg/kg	62	49 (79%)	9 (15%)	4 (6%)	44 (71%)	11 (18%)	7 (11%)	48 (77%)	11 (18%)	3 (5%)
10.0 mg/kg	109	73 (67%)	30 (28%)	6 (5%)	53 (49%)	37 (34%)	19 (17%)	67 (62%)	35 (32%)	7 (6%)
Total	241									

3C: DR Neurons - Young Tolerance group.

Tolerance

Dose	N	Acute (ED 1 MPD/ED 1 BL)			Baseline (ED 10 BL/ED 1 BL)			Chronic (ED 10 MPD/ED 1 MPD)		
		Ý	ß	Û	Ý	ß	Û	Ý	ß	Û
0.6 mg/kg	67	14 (21%)	17 (25%)	36 (54%)	15 (23%)	13 (19%)	39 (58%)	10 (15%)	28 (42%)	29 (43%)
2.5 mg/kg	80	14 (17%)	36 (45%)	30 (38%)	19 (24%)	22 (28%)	39 (48%)	12 (15%)	34 (46%)	31 (39%)
10.0 mg/kg	59	11 (19%)	34 (58%)	14 (23%)	22 (37%)	18 (31%)	19 (32%)	6 (10%)	28 (48%)	25 (42%)
Total	206									

3D: DR Neurons - Young Responsiveness.

DR Unit Young	N	%
All Total	504	
Responsive Total	447	87
Tolerance Total	206	46
Sensitized Total	241	54

Results

Locomotor Behavior (Figures 3 and 4)

Time and Saline (Control) (**Figures 3, 4A, and 4B**): In order to determine the effects of time (growing days), injection volume and animal handling on locomotor activity, in each age two groups of rats were used i.e., time control (N=8) and saline (Injection and handling) control N=15 young and N=13 adult (**Figure 3**). Eleven days of consecutive locomotor activity in un-injected groups were recorded from young and adult rats for the time control. All the eleven recording days exhibit similar level of locomotor activities (**Figure 3** Time control). Similar observations were observed in the saline control group. Locomotor activity (NOM, TD and NOS) readings showed non-significant differences with minor non-significant fluctuations between the recording days and following acute and repetitive administration of saline. In addition, following three washout days (ED8-10) at ED10 BL there was no significant change in locomotor activity when compared to initial baseline (ED1 BL), as well as no significant change in locomotor activity upon rechallenge with saline at ED 11 with saline compared to ED1 BL (**Figures 3, 4A, and 4B**). This indicates that the locomotor behavioral activities (NOM, TD and NOS) are not affected by time and the injection process, animal handling, or laboratory conditions. Because there was no significant difference in locomotor behavioral activity between the daily activity (**Figure 3**) and following the first saline injection and the activity following the daily saline injection (**Figures 4A and 4B**), the locomotor activities ED1 saline was used as a control (ED 1 BL) for comparison against MPD injection groups. Therefore, any significant changes in locomotor activity from the ED1 BL activity are due to the effects of MPD exposure.

A total of 324 male SD rats were used to record simultaneously and evaluate their behavioral expression and their DR neuronal units. Of these 324 rats 146 were adults and 178 were young. Of the adult rats, 13, 45, 41, and 47 received acute and repetitive injections of saline, 0.6, 2.5, and 10.0 mg/kg MPD respectively. Of the young rats, 15, 55, 51, 57 received acute and chronic injections of saline, 0.6, 2.5, and 10.0 mg/kg MPD respectively.

Behavioral response to acute and chronic 0.6 mg/kg MPD All group (Figure 4C and 4D, All): Forty-five adult and fifty-five young rats received acute and chronic injections of 0.6 mg/kg MPD. Both age groups of rats demonstrated a significant increase in locomotive behavioral activity ($p<0.05$) following acute administration of 0.6 mg/kg MPD on ED1 (ED1 MPD/ED1 BL) (**Figure 4C and 4D**, All). Only young rats demonstrated significant change ($p<0.05$) in ED10 BL activity after six daily MPD exposures and three washout days when compared to ED1 BL (ED10 BL/ED1 BL). Adult rats demonstrated less significant change as compared

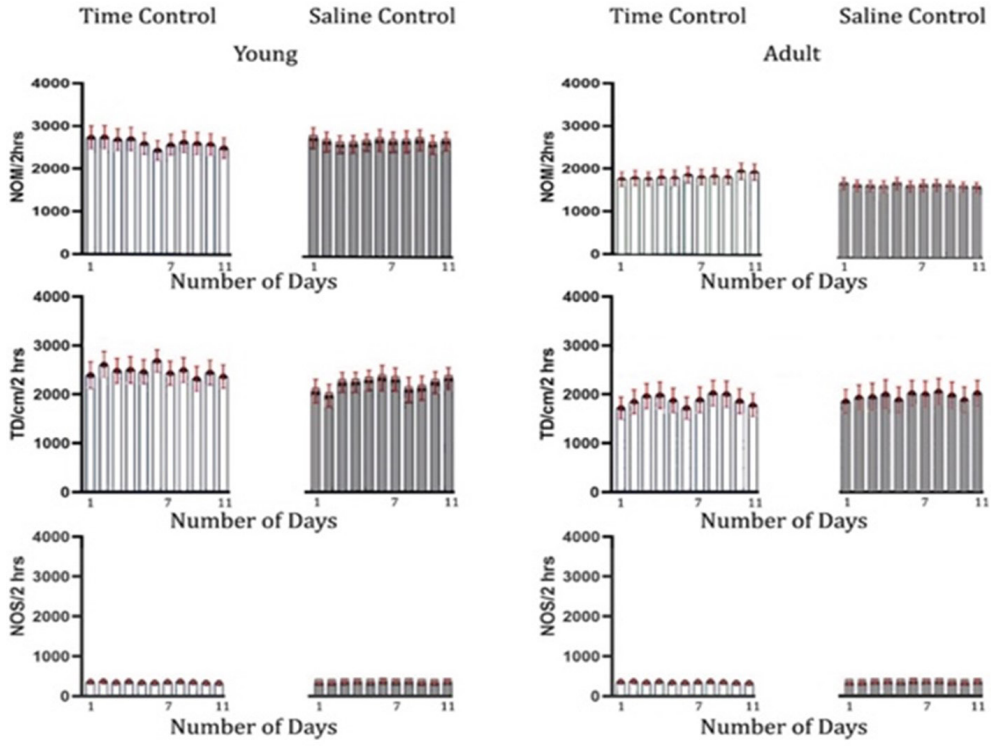

Figure 3. Time and saline control groups of three different locomotor activities of behavioral expression recorded for one hr/day for sequential 11 experimental days. Number of Movement (NOM) summarizes the total number of movements activities. Number of Stereotypic (NOS) movements summarizes repetitive movements recorded from the same sensor with at least one sec. interval between the movements. Total Distance (TD) traveling summarizes in cm the TD traveling in cm/1 hr. During the eleven recording days both animal groups exhibit similar activities with no significant minor fluctuation, indicating that animals handling and injection during the 11 experimental days did not modify the above three locomotor activities expression.

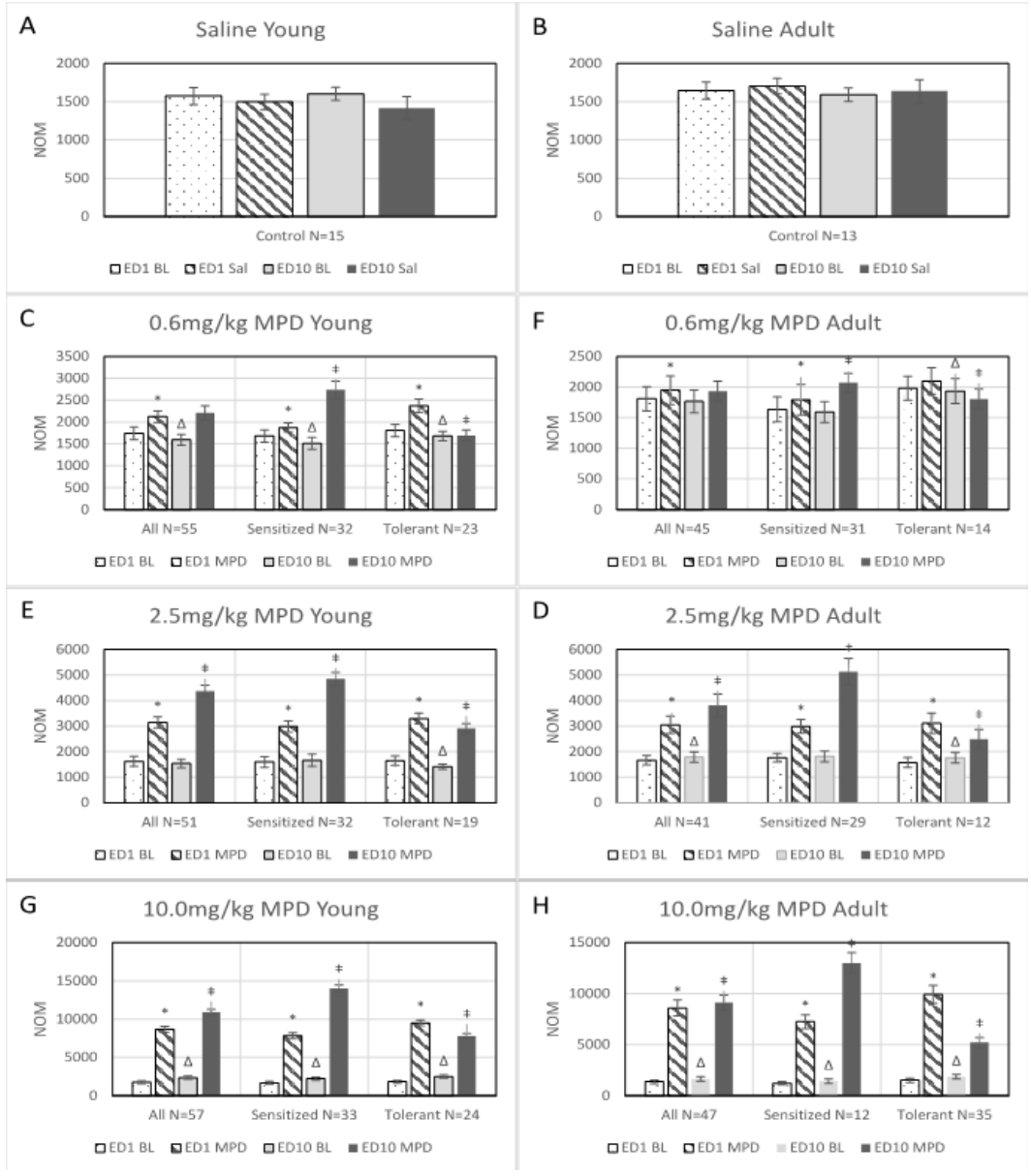

Figure 4. Summarizes all the behavioral data (Number of movements, NOM) for young and adult rat groups from the saline control in **A** and **B** and each experimental MPD dose (0.6 mg/kg (**C & F**), 2.5mg/kg, 10.0 mg/kg MPD (**G & H**)). Each histogram is labeled from A-H for their respective age with young on the left and adult on the right and experimental group (All, sensitized, and tolerance). N represents the number of animals in each group. The rats in the experimental dose groups were divided into 3 subgroups: all animals, behaviorally sensitized animals, and behaviorally tolerant animals. "All" group summarizes all the animals for the respective MPD dose. "Sensitized" group and "Tolerant" group summarizes only animals that expressed either behavioral sensitization or tolerance to chronic MPD at ED10 after six daily MPD exposures (0.6, 2.5, 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: ED1 BL, ED1 MPD, ED10 BL, and ED10 MPD organized into three comparisons per subgroup: ED1 MPD/ED1 BL, to obtain the MPD acute effect ED10 BL/ED1 BL, ED1 BL is compared to ED10 BL to obtain whether the six daily MPD exposures and three washout days affect ED10 BL. ED10 MPD/ED1 MPD. ED10 MPD is compared to ED1 MPD to obtain the chronic MPD effect. The NOM of young ED1 MPD is compared to the NOM of adult ED1 MPD to examine the behavioral difference between the acute response of MPD for young and adult; and the NOM of young ED10 MPD is compared to the NOM of adult ED10 MPD to examine the difference in behavior in response to chronic MPD in young and adult. Above each column are the standard deviation (SD). *Indicates significant ($p < 0.05$) differences from ED1 BL (ED1 BL/ED1MPD, acute). ΔIndicates significant ($p < 0.05$) differences from ED1 BL from ED10 BL (ED1 BL/ED10 BL, withdrawal). ‡Indicates significant ($p < 0.05$) differences from ED1 MPD (ED1 MPD/ED10 MPD, chronic).

to the young animals in their locomotive behavioral response following acute and rechallenge of MPD on ED10 when compared to acute administration of MPD on ED1 (ED10 MPD/ED1 MPD). Adult rats, however, did demonstrate a significant increase ($p<0.05$) in locomotor activity on ED10 after rechallenging of MPD when compared to locomotor activity following acute MPD administration (ED10 MPD/ED1 MPD). This increase in activity is considered expression of behavioral sensitization.

Behavioral sensitized group 0.6 mg/kg MPD (Figure 4C and 4D, Sensitized): When animals were sorted into subgroups based on individual behavioral response to MPD on ED10 as compared to ED1 MPD (ED10 MPD/ED1 MPD) using the CR test and the student t-test, 31 individual adult rats and 32 individual young rats demonstrated a significant increase ($p<0.05$) in locomotor activity following MPD rechallenge on ED10 as compared to activity on ED1 (ED10 MPD/ED1 MPD, chronic). These animals were classified as expressing significant behavioral sensitization. Both young and adult rats demonstrated significant ($p<0.05$) increase in locomotor activity on ED1 following acute administration of MPD as well (ED1 MPD/ED1 BL). Only the young rats of the sensitization group demonstrated a significant ($p<0.05$) change in ED10 BL compared to ED1 BL (ED10 BL/ED1 BL, withdrawal).

The ratio of adults showing behavioral sensitization to 0.6 mg/kg MPD is 31 out of 45, or 68.9%. The ratio of young showing behavioral sensitization to 0.6 mg/kg MPD is 32 out of 55, or 58.2%.

Behavioral tolerance group 0.6 mg/kg MPD (Figure 4C and 4D, Tolerant): Fourteen adults and twenty-three young rats exhibited behavioral tolerance to repetitive 0.6 mg/kg MPD compared to the initial MPD effects (ED10 MPD/ED1 MPD). Both age groups demonstrated a significant decrease ($p<0.05$) in locomotor activity following rechallenge of MPD on ED10 when compared to activity post MPD injection on ED1 (ED10 MPD/ED1 MPD). These animals were classified as expressing behavioral tolerance. The adult and the young rats demonstrated a significant ($p<0.05$) increase in locomotor behavior following acute 0.6 mg/kg MPD administration of MPD on ED1. Both age groups demonstrated significant ($p<0.05$) changes in ED10 BL/ED1 BL activity.

The ratio of adult rats showing behavioral tolerance to 0.6 mg/kg MPD is 14 out of 45, or 31.1%. The ratio of young rats showing behavioral tolerance to 0.6 mg/kg MPD is 23 out of 55, or 41.8%. Using the Chi square test results in significant ($p<0.05$) difference between the two age groups to 0.6 mg/kg MPD.

Behavioral response to acute and chronic 2.5 mg/kg MPD All group (Figure 4E and 4F, All): Forty-one adult and fifty-one adolescent rats received acute and chronic injections of 2.5 mg/kg MPD. When analyzed as whole populations (all group), both adults and adolescents demonstrated a significant increase in behavioral response ($p<0.05$) following acute administration of MPD on ED1 (ED1 MPD/ED1 BL). The ED10 BL activity after six daily MPD exposure and three wash out days (ED10 BL/ED1 BL) exhibit significantly ($p<0.05$) increase in adult rats and was not significantly changed in young rats. Following MPD rechallenge at ED10 both age group rats demonstrated a significantly ($p<0.05$) increased behavioral response compared to initial response of MPD on ED1 (ED10 MPD/ED1 MPD).

Behavioral sensitized group 2.5 mg/kg MPD (Figure 4E and 4F, Sensitized): Twenty-nine adults and thirty-two young rats exhibited behavioral sensitization to chronic MPD exposure. Both groups displayed a significant ($p<0.05$) increase in locomotor activity following acute MPD administration (ED1 MPD/ED1 BL) and no difference between ED10 BL/ED1 BL after six daily 2.5 mg/kg MPD and three wash out days. Following MPD rechallenge at ED10, both adult and young rats demonstrated a significantly ($p<0.05$) further increased behavioral response to MPD compared to response to MPD on ED1 (ED10 MPD/ED1 MPD).

The ratio of adult rats showing behavioral sensitization to 2.5 mg/kg MPD is 29 out of 41, or 70.7%. The ratio of adolescents showing behavioral sensitization to 2.5 mg/kg MPD is 32 out of 51, or 62.7%.

Behavioral tolerance group 2.5 mg/kg MPD (Figure 4E and 4F, Tolerant)

Twelve adults and nineteen young rats demonstrated behavioral tolerance to chronic MPD exposure (**Figure 4E and 4F**)**.** Both age groups displayed a significant ($p<0.05$) increase in locomotor activity following acute MPD administration on ED 1 (ED1 MPD/ED1 BL). Young and adult rats had a significant ($p< 0.05$) change in locomotor activity of ED10 BL compared to ED1BL; with young exhibiting a decrease in activity and adults exhibiting an increase in activity (ED10 BL/ED1 BL). Both groups demonstrated a significant ($p <0.05$) decrease in locomotor activity following rechallenge of MPD on ED10 compared to activity with MPD on ED1 (ED10 MPD/ED1 MPD). Both groups demonstrated a significant ($p<0.05$) decrease in locomotor activity following rechallenge of MPD on ED10 compared to activity with MPD on ED1 (ED10 MPD/ED1 MPD).

The ratio of adult rats showing behavioral tolerance to 2.5 mg/kg MPD is 12 out of 41, or 29.3%. The ratio of young rats showing behavioral tolerance to 2.5 mg/kg MPD is 19 out of 51, or 37.3%.

Behavioral response to acute and chronic 10.0 mg/kg MPD All groups (Figure 4G and 4H, All): Forty-seven adults and fifty-seven young rats received acute and chronic injections of 10.0 mg/kg MPD. When analyzed as whole populations, both adult and young rats demonstrated a significant increase in behavioral activities ($p<0.05$) following acute administration of 10 mg/kg MPD on ED1 (ED1 MPD/ED1 BL). Both age groups exhibited significant ($p<0.05$) increase in locomotor activity on ED 10 BL/ED1 BL. However, the young animals exhibited significantly ($p<0.05$) greater increase in ED10 BL activity compared to adult ED10 BL. Following chronic 10.0 mg/kg MPD, both age group of rats demonstrated a significantly increased behavioral response ($p<0.05$) following rechallenge of MPD on ED10 MPD/ED1 MPD (**Figure 4G and 4H**, All).

Behavioral sensitization group 10.0 mg/kg MPD (Figure 4G and 4H, Sensitized): Twelve adult and thirty-three young rats exhibited behavioral sensitization to chronic 10 mg/kg MPD exposure. The behavioral sensitized subgroups displayed a significant ($p<0.05$) increase in locomotor activity following acute 10 mg/kg MPD administration (ED1 MPD/ED1 BL), and a significant ($p< 0.05$) further increase in locomotor activity following MPD rechallenge on ED10 MPD/ED1 MPD. Behaviorally sensitized adult and young rats demonstrated a significant ($p<0.05$) increase

in ED10 BL/ED1 BL, while the adult group exhibited significantly ($p<0.05$) more locomotor activity compared to young.

The ratio of adults showing behavioral sensitization to 10 mg/kg MPD is 12 out of 47, or 25.5%. The ratio of young showing behavioral sensitization to 10 mg/kg MPD is 33 out of 57, or 57.9%.

Behavioral tolerance group 10.0 mg/kg MPD (Figure 4G and 4H, Tolerant): Thirty-five adult and twenty-four young rats demonstrated behavioral tolerance to chronic 10 mg/kg MPD exposure. The behavioral tolerance groups displayed a significant ($p<0.05$) increase in locomotor activity following acute MPD administration (ED1 MPD/ED1 BL) while the adult group exhibited more locomotor activity than the young. A significant ($p<0.05$) decrease in locomotor activity following rechallenge of MPD on ED10 (ED10 MPD/ED1 MPD) was observed in the adult and the young groups respectively. Both groups had a significant ($p<0.05$) increase of ED10 BL activity compared to ED1 BL (ED10 BL/ED1 BL) i.e., expressing withdrawal.

The ratio of adult rats showing behavioral tolerance to 10.0 mg/kg MPD is 35 out of 47, or 74.5%. The 10.0 mg/kg MPD dose is the only dose that the majority of the adult rats became behaviorally tolerant to the effects of MPD. The ratio of young rats showing behavioral tolerance 10.0 mg/kg MPD is 24 out of 57, or 42.1%.

Comparing the number of movements (NOM) among the three groups: all, sensitized, and tolerance: Comparing the effect of acute 0.6, 2.5. and 10 mg/kg MPD (ED1 MPD/ED1 BL), the change in ED10 BL/ED1 BL after six daily MPD and three wash out days, and the effect of chronic MPD (ED10 MPD/ED1 MPD) between the two age groups reveal significant ($P<0.001$) difference in response to MPD between adult and young animals using the Chi square test.

Dorsal raphe (DR) neuronal activity

A total of 860 DR neurons were recorded: 356 DR neurons were recorded from adult rats and 504 DR neurons were recorded from young rats. All the above DR neuronal evaluated recordings were histologically confirmed to be recorded from the DR nucleus. All the neuronal recordings in the study exhibited similar waveform spikes and amplitudes on ED10 as compared to ED1.

Control recording: 27 and 57 DR neurons were recorded from adult and young rats respectively as controls following acute and repetitive saline injections (**Table 2A**).

Of the 27 DR neurons recorded from adults, none showed any significant changes in neuronal activity following the initial injection of saline on ED1 (ED1 Sal/ED1 BL). 1 DR neuron records showed an increase in baseline activity, 2 neurons showed a decrease in baseline activity, and 24 neurons had no change in baseline activity on ED10 BL/ED1 BL. Following saline rechallenge on ED10 Sal/ED1 Sal, 1 DR neuron showed an increase in neuronal activity, while another 1 neuron showed a decrease in neuronal activity and 25 neurons had no change in activity (**Table 2A**, saline).

Of the 57 DR neurons recorded from young rats, 1 neuron showed an increase in neuronal activity following acute injection of saline on ED1, and 1 neuron showed a decrease in neuronal activity, and 55 neurons had no change in neuronal activity (ED1 Sal/ED1 BL). 3 neurons showed an increase in baseline neuronal activity, 2 neurons showed a decrease in baseline neuronal activity, and 52 neurons did not have a change in baseline neuronal activity on ED10 (ED10 BL/ED1 BL). Upon rechallenge with saline on ED10 (ED10 Sal/ED1 Sal), 2 neurons showed an increase in neuronal activity, while 1 neuron showed a decrease in neuronal activity, and 54 had no change in neuronal activity upon saline rechallenge (**Table 3A**, saline). These observations indicate that the few DR neurons that exhibit alterations in BL in DR activity following saline injection were random, and that the saline injections, animal handling, and laboratory conditions did not have a significant effect on neuronal activity in DR neurons in both age groups. Therefore, any significant changes in neuronal recording from ED1 saline (i.e., ED1 BL) are presumably due to the effects of MPD exposure.

Effect of 0.6 mg/kg MPD on DR neurons **recorded from all animals:** 122 DR neuronal activity following acute and chronic administration of 0.6 mg/kg MPD were recorded from adult animals. Of these DR neurons, 48% (58/122) showed a significant ($p<0.05$) change in their neuronal firing rate following acute administration of MPD on ED1 MPD/ED1 BL, with a majority of them showing a significant ($p<0.05$) increase in firing rate (**Table 2A**, All). Of these 122 DR neurons at ED10 BL/ED1 BL, 89% (108/122) showed a significant ($p<0.05$) change, with an even split of 47% (51/108) showing an increase in firing rate and 53% (57/108) showing a decrease in activity after six daily of 0.6 mg/kg MPD (ED1 to ED6) and three wash out days (ED7 to ED9). Following MPD rechallenge on ED10 (ED10 MPD/ED1 MPD), 87% (106/122) responded with a significant ($p<0.05$) change in neuronal activity. Of these DR responding neurons, 45% (48/106) responded with a significant ($p<0.05$) increase in activity while 55% (58/106) responded with a significant decrease in activity (**Table 2A**, 0.6 mg/kg MPD All).

One hundred and thirty-seven DR neurons were recorded from all young rats group **(Table 3A)**. Of these neurons at ED1 MPD/ED1 BL, 47% (67/137) responded with a significant ($p<0.05$) change in neuronal firing activity following acute administration of MPD on ED1, with a majority 52% (35/67) responding by an increase in firing rate. When comparing ED10 BL/ED1 BL neuronal activity, 48% (65/137) showed a significant ($p<0.05$) change in their neuronal firing rate at ED10 BL/ED1 BL. Of these neurons, the majority 60% (39/65) showed a significant ($p<0.05$) increase in firing rate, while 40% (26/65) demonstrated a significant ($p<0.05$) decrease in firing rate. Upon rechallenge of MPD on ED10, 48% (66/137) responded by a significant ($p<0.05$) change in neuronal firing rate. Of these responding neurons, 39% (26/66) responded by a significant ($p<0.05$) increase in firing rate, while 61% (40/66) responded by a significant decrease in firing rate (**Table 3A**, 0.6 mg/kg MPD, All).

DR neuronal activity recorded from animals expressing behavioral sensitization to chronic 0.6 mg/kg MPD: Twenty-seven DR neurons were recorded from adult animals that demonstrated behavioral sensitization to chronic exposure of 0.6 mg/kg MPD (**Table 2B**). Of these neurons at ED1 MPD/ED1 BL, 41% (11/27) showed a significant ($p<0.05$) change in firing rate, with 45% (5/11) demonstrating an increase in firing rate. Of these DR neurons at ED10 BL/ED1 BL 74% (20/27) of them exhibited a significant ($p<0.05$) difference after six daily 0.6 mg/kg MPD exposure and three washout days on ED10 BL. Of these neurons, 55% (11/20) exhibited an increase in firing rate at ED10 MPD/ED1 MPD following MPD rechallenge (ED10 MPD/ED1 MPD). Fifty-six percent (15/27) of the DR neurons responded with a significant

($p<0.05$) change in neuronal firing rate. A majority 67% (10/15) responded by a significant ($p<0.05$) increase in firing rate (**Table 2B**, 0.6 mg/kg MPD, Sensitized).

Seventy DR neurons were recorded from young animals that demonstrated behavioral sensitization to chronic exposure of 0.6 mg/kg MPD (**Table 3B**). Of these responding neurons at ED1 MPD/ED1 BL, 90% (63/70) responded with a significant ($p<0.05$) change in firing rate, with 61% (43/70) demonstrating an increase in firing rate. When comparing ED10 BL/ED1 BL 86% (60/70) of DR neurons responded with a significant ($p<0.05$) change in firing rate. Of these neurons, a majority 55% (33/60) responded with a significant increase in firing rate. Upon rechallenge of MPD on ED10 (ED10 MPD/ED1 MPD), 91% (64/70) of DR neurons responded with a significant ($p<0.05$) change in their firing rate. Of these neurons, a majority 65% (42/64) responded to 0.6 mg/kg MPD with an increase in firing rate (**Table 3B**, 0.6 mg/kg MPD Sensitized).

DR neuronal activity recorded from animals expressing behavioral tolerance to chronic 0.6 mg/kg MPD: Ninety-five DR neurons were recorded from adult animals demonstrating behavioral tolerance to chronic 0.6 mg/kg MPD (**Table 2C**). Of these DR neurons, 48% (46/95) responded with a significant ($p<0.05$) change in neuronal firing rate following acute injection of MPD, with a majority 72% (33/46) responded with an increase in firing rate. When comparing ED10 BL/ED1 BL, 92% (87/95) displayed a significantly ($p<0.05$) different in their BL activities at ED10. Of these DR units, 44% (38/87) displayed an increased baseline neuronal firing rate and 56% (49/87) expressed decrease in their ED10 BL/ED1 BL. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 98% (93/95) of DR neurons responded with a significant ($p<0.05$) change in firing rate. Of these neurons, the majority 58% (54/93) responded with a decrease in firing rate (**Table 2C**, 0.6 mg/kg MPD, Tolerance).

Sixty-seven DR neurons were recorded from young animals demonstrating behavioral tolerance to chronic 0.6 mg/kg MPD (**Table 3C**). Of these neurons, 46% (31/67) responded with a significant ($p<0.05$) change in firing rate following acute injection of MPD, with 45% (14/31) responding with an increase in firing rate and 55% (17/31) responded with decrease in their firing rate. When comparing ED10 BL/ED1 BL, 42% (28/67) showed a significant ($p<0.05$) change in their firing rate after six daily MPD exposure and three wash out days. Of these neurons, 54% (15/28) showed an increase in firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 57% (38/67) responded with a significant ($p<0.05$) change in firing rate. Of these DR neurons, the majority 74% (28/38) responded by decreasing their firing rate and 26% (10/38) responded with a significant increase in firing rate (**Table 3C**, 0.6 mg/kg MPD, Tolerance).

Effect of 2.5 mg/kg MPD from DR units recorded from all the animals: Ninety-four DR neuronal activity following administration of 2.5 mg/kg MPD were recorded from adult animals. Of these DR neurons, 57% (54/94) responded with a significant ($p<0.05$) change in their neuronal firing rate at ED1 MPD/ED1 BL, with a majority 91% (49/54) exhibiting a significant ($p<0.05$) increase in firing rate. Of these 94 DR neurons, 71% (67/94) showed a significant ($p<0.05$) change in ED10 BL/ED1 BL, with 52% (35/67) showing an increase in firing rate. Following MPD rechallenge on ED10 MPD/ED1 MPD, 79% (74/94) responded with a significant ($p<0.05$) change in their neuronal activity. Of these DR responding neurons, a majority 61% (45/74) responded with a significant ($p<0.05$) increase in neuronal activity (**Table 2A**, 2.5 mg/kg MPD All).

One hundred and forty-two DR neurons were recorded **from young animals**. Of these neurons, 69% (98/142) responded with a significant ($p<0.05$) change in their neuronal firing activity following acute administration of MPD with 54% (53/98) responding by an increase in firing rate. When comparing ED10 BL/ED1 BL, 60% (85/142) showed a significant ($p<0.05$) change in neuronal BL after six daily MPD exposure and three wash out days. Of these neurons, 58% (49/85) showing a significant ($p<0.05$) increase in firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 68% (96/142) DR neurons responded with a significant ($p<0.05$) change in their neuronal firing rate. Of these responding neurons, an even split of 50% (48/96) showed a significant ($p<0.05$) increase or decrease in firing rate (**Table 3A**, 2.5 mg/kg MPD All).

DR neuronal activity recorded in animals expressing behavioral sensitization to chronic 2.5 mg/kg MPD: Forty-two DR neurons were recorded from adult animals that demonstrated behavioral sensitization to chronic exposure of 2.5 mg/kg MPD (**Table 2B**). Of these DR neurons at ED1 MPD/ED1 BL, 43% (18/42) responded with a significant ($p<0.05$) change in firing rate, with 94% (17/18) responded with an increase in firing rate. Eighty-three percent (35/42) of DR units demonstrated a significantly ($p<0.05$) different on ED10 BL/ED1 BL. Of these neurons, 63% (22/35) DR units exhibited a decrease in their firing rate and 37% (13/35) exhibited an increase in firing rate. Eighty-eight percent (37/42) of DR neurons responded with a significant ($p<0.05$) change in their neuronal firing rate upon rechallenge of MPD on ED10 (ED10 MPD/ED1 MPD). Of these neurons, 54% (20/37) showed a significant ($p<0.05$) increase in firing rate (**Table 2B**, 2.5 mg/kg MPD, Sensitized).

Sixty-two DR neurons were recorded from young animals that demonstrated behavioral sensitization to chronic exposure of 2.5 mg/kg MPD (**Table 3B**). Of these DR neurons at ED1 MPD/ED1 BL, 89% (55/62) responded with a significant ($p<0.05$) change in firing rate, with 89% (49/55) responded with an increase in their firing rate. When comparing ED10 BL/ED1 BL, 89% (55/62) of DR neurons showed a significant ($p<0.05$) change in firing rate. Of these neurons, a majority 80% (49/55) showed a significant increase in firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 95% (59/62) of DR neurons responded with a significant ($p<0.05$) change in firing rate. Of these neurons, a majority 81% (48/59) responded by an increase in firing rate (**Table 3B**, 2.5 mg/kg MPD, Sensitized).

DR neuronal activity recorded in animals expressing behavioral tolerance to chronic 2.5 mg/kg MPD: Fifty-two DR neurons were recorded from adult animals demonstrating behavioral tolerance to chronic 2.5 mg/kg MPD (**Table 2C**). Of these DR neurons at ED1 MPD/ED1 BL, 71% (37/52) responded by a significant ($p<0.05$) change in neuronal firing rate, with a majority 89% (33/37) responded by an increase in firing rate. When comparing ED10 BL/ED1 BL, 62% (32/52) displayed a significantly ($p<0.05$) difference in ED10 BL. Of these neurons, 66% (21/32) displayed an increased BL neuronal firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 71% (37/52) of DR neurons responded with a significant ($p<0.05$) change in firing rate. Of these DR neurons, 62% (23/37) responded by an increase

in firing rate (**Table 2C**, 2.5 mg/kg, Tolerance).

Eighty DR neurons were recorded from young animals demonstrating behavioral tolerance to chronic 2.5 mg/kg MPD (**Table 3C**). Of these neurons at ED1 MPD/ED1 BL, 63% (50/80) responded with a significant ($p<0.05$) change in firing rate following acute injection of MPD, with 72% (36/50) responded by an increase in firing rate. When comparing ED10 BL/ED1 BL, 51% (41/80) showed a significant ($p<0.05$) change in firing rate. Of these DR neurons, 46% (19/41) exhibited an increase in their ED10 BL while 54% (22/41) showed a decrease in firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 61% (49/80) responded by a significant ($p<0.05$) change in firing rate. Of these neurons, 76% (37/49) responded with a significant decrease in firing rate (**Table 3C**, 2.5 mg/kg MPD, Tolerance).

Effect of 10 mg/kg MPD on DR neurons recorded from all animals group: One hundred and thirteen DR neuronal activity were recorded following 10.0 mg/kg MPD administration from adult all animals **(Table 2A)**. Of these DR neurons at ED1 MPD/ED1 BL, 73% (83/113) responded by a significant ($p<0.05$) change in neuronal firing rate, with a majority 87% (72/83) exhibiting a significant ($p<0.05$) increase in firing rate. Of the 113 DR neurons recorded at ED 10 BL/ED1 BL after six daily MPD exposure and three wash out days, 87% (98/113) DR neurons showed a significant ($p<0.05$) change in their ED10 BL/ED1 BL, with majority 62% (61/98) exhibited decrease in their ED10 BL while 38% (37/98) showing an increase in firing rate. Following MPD rechallenge on ED10 MPD/ED1 MPD, 89% (100/113) of DR neurons responded with a significant ($p<0.05$) change in their neuronal activity. Of these DR responding neurons, the majority 61% (61/100) 49% (39/80) responded with a significant ($p<0.05$) decrease in their neuronal activity (**Table 2A**, 10.0 mg/kg MPD, All).

One hundred and sixty-eight DR neurons were recorded from young rats. Of these neurons at ED1 MPD/ED1 BL, 86% (145/168) responded with a significant ($p<0.05$) change in neuronal firing rate activity, with 55% (80/145) demonstrating an increase in firing rate. When comparing ED10 BL/ED1 BL activity, 80% (135/168) of DR neurons showed a significant ($p<0.05$) change in their neuronal baseline on ED10 BL. Of these neurons, 53% (72/135) showing a significant ($p<0.05$) increase in firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 83% (139/168) neurons responded with a significant ($p<0.05$) change in neuronal firing rate. Of these responding neurons, 53% (73/139) responded by a significant ($p<0.05$) increase in firing rate (**Table 3A**, 10.0 mg/kg MPD, All).

DR neuronal activity recorded from animals expressing behavioral sensitization to chronic 10.0 mg/kg MPD: Twenty DR neurons were recorded from adult animals that demonstrated behavioral sensitization to chronic exposure of 10.0 mg/kg MPD (**Table 2B**). Of these DR neurons at ED1 MPD/ED1 BL, 75% (15/20) responded by a significant ($p<0.05$) change in their firing rate, all the 15 neurons demonstrating an increase in firing rate. At ED10 BL/ED1 BL, 95% (19/20) of DR neurons demonstrated a significantly ($p<0.05$) change in their neuronal firing rates. Of these 19 DR neurons 79% (15/19) exhibited decrease in their ED10 BL activity while 21% (4/19) exhibited increase in their firing rates. Comparing ED10 MPD/ED1 MPD 95% (19/20) of DR neurons responded with significant ($p< 0.05$) change in their neuronal firing rates. Of these neurons, 16% (3/19) responded with a significant ($p<0.05$) increase in their firing rate and 84% (16/19) responded to MPD rechallenge by significant ($p<0.05$) decrease in their neuronal activity (**Table 2B**, 10.0 mg/kg MPD, Sensitized).

One hundred and nine DR neurons were recorded from young animals that demonstrated behavioral sensitization to chronic exposure of 10.0 mg/kg MPD (**Table 3B**). Of these DR neurons, at ED1 MPD/ED1 BL, 95% (103/109) responded with a significant ($p<0.05$) change in firing rate, with 70% (73/109) demonstrating an increase in firing rate. When comparing ED10 BL/ED1 BL, 83% (90/109) of neurons showed a significant ($p<0.05$) change in firing rate. Of these DR neurons, the majority 59% (53/90) showed a significant increase in firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 94% (102/109) of DR neurons responded with a significant ($p<0.05$) change in firing rate. Of these neurons, 66% (67/102) responded with an increase in firing rate (**Table 3B**, 10.0 mg/kg MPD, Sensitized).

DR neuronal activity recorded from animals expressing behavioral tolerance to chronic 10.0 mg/kg MPD: Ninety-three DR neurons were recorded from adult animals demonstrating behavioral tolerance to chronic 10.0 mg/kg MPD (**Table 2C**). Of these neurons at ED1 MPD/ED1 BL, 74% (69/93) responded with a significant ($p<0.05$) change in their neuronal firing rate, with a majority 86% (59/69) responding with an increase in firing rate. When comparing ED10 BL/ED1 BL, 84% (78/93) of DR units displayed a significantly ($p<0.05$) different baseline. Of these DR neurons, 41% (32/78) displayed an increased baseline neuronal firing rate and 54% (46/78) exhibited decrease in their ED10 BL. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 87% (81/93) of DR neurons responded with a significant ($p<0.05$) change in firing rate when compared to responses from acute injection of MPD. Of these neurons the majority 56% (45/81) responded by a significant ($p<0.05$) decrease in their firing rate, and 44% (36/81) responded with an increase in firing rate (**Table 2C**, 10.0 mg/kg, Tolerance).

Fifty-nine DR neurons were recorded from adolescent animals demonstrating behavioral tolerance to chronic 10.0 mg/kg MPD **Table 3C**). Of these neurons at ED1 MPD/ED1 BL, 65% (45/59) responded with a significant ($p<0.05$) change in firing rate, with 24% (11/45) responded with an increase in firing rate and the majority 76% (34/45) by a decrease in their firing rate. When comparing ED10 BL/ED1 BL 68% (40/59) showed a significant ($p<0.05$) change in firing rate. Of these neurons, 55% (22/40) showed an increase in firing rate. Upon rechallenge of MPD on ED10 MPD/ED1 MPD, 58% (34/59) responded with a significant ($p<0.05$) change in their firing rate. Of these DR neurons, 18% (6/34) responded with a significant increase in firing rate and the other 82% (23/34) responded with a significant ($p<0.05$) decrease in firing rates as compared to the initial MPD effects respectively (**Table 3C**, 10.0 mg/kg MPD, Tolerance).

DR neurons response direction (increase or decrease) to MPD (Figure 5)

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

In **Figures 5A** and **5D** are the percentages of neurons that responded significantly ($p<0.05$) to acute 0.6, 2.5 and or 10.0 mg/kg MPD (Fig 5A and D left column). In the middle 3 columns are

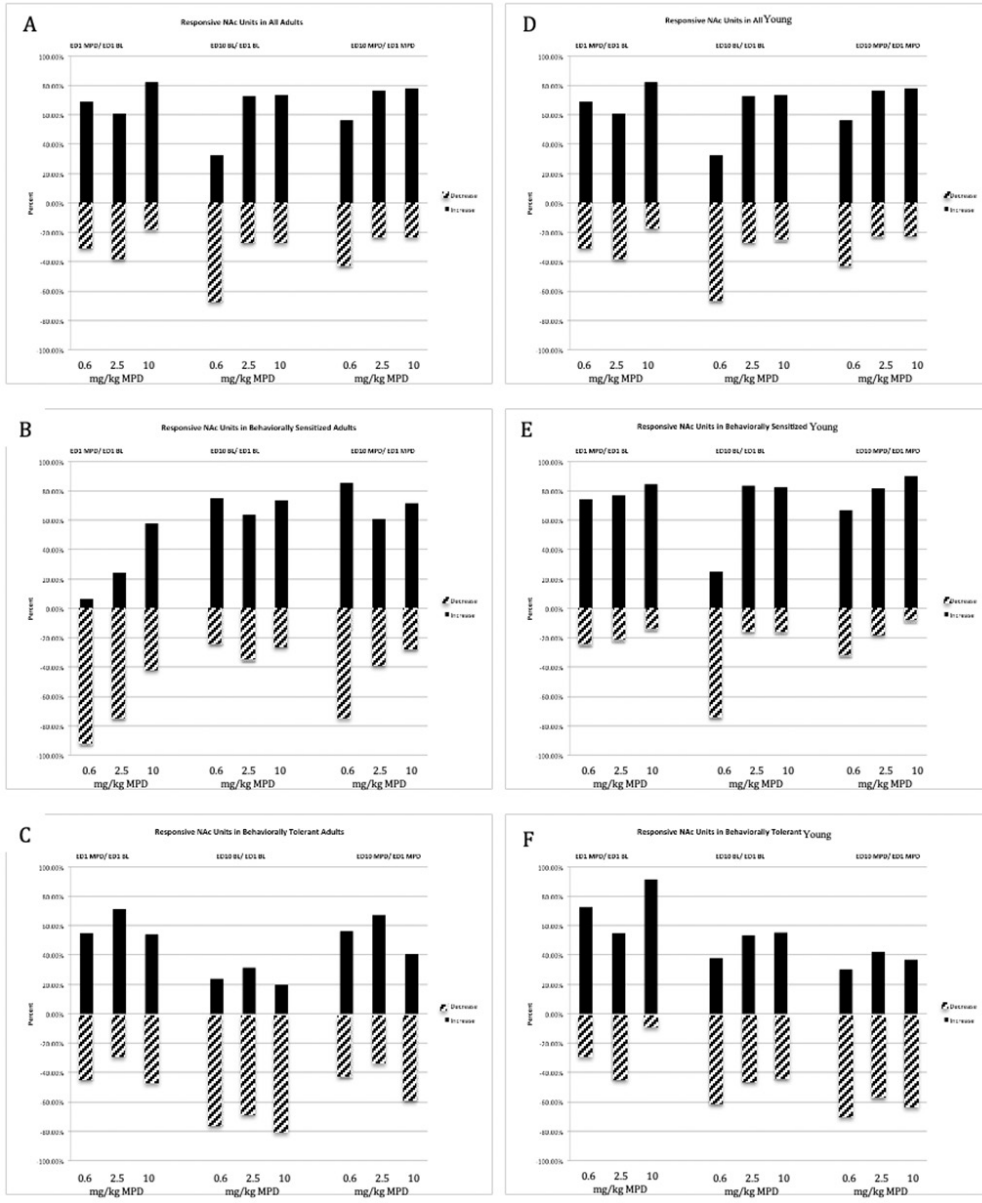

Figure 5. The figure summarizes the responsiveness direction (increase of decrease in % of how many DR neurons respond significantly to acute and chronic MPD doses. Each segment has three columns and three sections showing in percentage how many DR neurons respond significantly by either increasing or 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 **Figures 5A** and **5D** are the DR units recorded from all the animals. In **Figures 5B** and **5E** are the DR neurons recorded only from behaviorally sensitized animals, and in **Figures 5C** and **5F** are the DR neuronal units recorded from only the behaviorally tolerant adult and adolescent animals, respectively.

the changes in ED10 BL compared to ED1 BL (ED10 BL/ED1 BL), and in the right column of each of **Figures 5A-5F** are the comparisons between ED10 MPD compared to ED1 MPD (ED10 MPD/ED1 MPD).

Comparing within each age, the **Figures 5A-5C** show significant ($p<0.05$) 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 DR neurons 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 ($p<0.05$) differently from those recorded from behaviorally tolerant animals. Similar observations are seen in the young animal groups (**Figures 5D-5F**).

Comparing adult to young all groups (**Figures 5A and 5D**) no significant difference in response direction is seen between the ages. However, when comparing the recording obtained from animals expressing behavioral sensitization to MPD (**Figures 5B and 5E**) to

those expressing behavioral tolerance (**Figures 5C and 5F**) significant ($p<0.05$) 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, TD traveling activity responded similarly to NOM.

Discussion

Most of the studies investigating the properties of MPD have focused mainly on MPDs effects on the DA system, and some of the NE system. There are limited studies that suggest that serotonin signaling participates in addiction, vulnerability, and cravings, and that MPD mitigates the serotonergic system as well [20,30,31,51,61,62]. During development i.e., in adolescence, overproduction of synaptic connections and receptors occurs, followed by pruning or competitive elimination occurs [18,43,63]. In addition, acute and chronic administration of MPD in adult rats has been shown to elicit significant changes in DR neuronal activity [53,54]. At therapeutic dosages, MPD has been shown to alter the expression of dopaminergic regulator genes in the sensorimotor striatum, a region also involved in learning and habit formation [64]. 5-HT reuptake inhibitors have been shown to potentiate MPD-induced gene regulation in the adolescent striatum [65]. In addition, 5-HT and serotonergic adaption has been suggested to implicated in the formation of addiction [51,52]. The objective of this study was to investigate whether the acute and chronic dose response effects of MPD on young rats is the same or different than the effects of MPD on adult male rats using concomitant behavioral and neuronal recording from the dorsal raphe (DR) area. Each animal in this study was also evaluated independently based on their behavioral response to repetitive (chronic) MPD exposure as compared to the initial (acute) response to MPD to determine whether they express behavioral sensitization or tolerance.

The main findings of the study are **1)** The same chronic dose of 0.6, 2.5 and 10.0 mg/kg MPD elicits in some animals' behavioral sensitization and in others behavioral tolerance, **2)** Significant age differences in response to MPD were observed in the ratio of how many adolescents (young) compared to adult rats expressed behavioral sensitization or behavioral tolerance to chronic MPD as compared to the initial (acute) MPD effects. **3)** In general, the ratio of DR neuronal responses to acute and chronic 0.6, 2.5 and 10.0 mg/kg MPD had similar response directing from **all** the young and **all** adult rat groups, in terms of total responsiveness: how many DR neurons responded to MPD with either excitation or attenuation in their firing rate. However, when the DR neuronal responses were evaluated based on the animal's behavioral responses to chronic MPD and separately divided to neuronal recording obtained from the animals that exhibit behavioral tolerance from the recording from animals that expressed behavioral sensitization into their own groups, the DR neuronal recordings from these two age subgroups had significantly different ratios of how many DR neuron units responded by excitation or attenuation of the firing rates when comparing to the acute and chronic MPD administration from both age groups. **4)** In general DR neurons recorded from animals exhibiting behavioral tolerance to chronic MPD treatment are significantly more likely to respond to MPD with decreases in their firing rates as compared to the initial (acute) MPD effect, while DR neuronal activity recorded from animals expressing behavioral sensitization tended to respond to chronic MPD by further increasing their firing rates as compared to the acute MPD effect. However, significant differences between the two age groups were observed in the percentage of how many DR neurons responded to each acute and chronic MPD doses as well on the behavioral responses to MPD. This observation indicates that the effect of MPD on animals' behaviors and on DR in young animals is significantly different from the effect of MPD on DR neurons recorded in adult animals. In addition, this observation supports our hypothesis that the same chronic dose of 0.6, 2.5 and 10.0 mg/kg MPD will elicit in some animals' behavioral sensitization and in others behavioral tolerance, and that there is a correlation between the DR neuronal response to chronic MPD and the behavioral expression to chronic MPD. Moreover, the observation indicates that it is essential to evaluate drug effects on the brain based on the chronic effects of the drug on the subject's behavior.

The results from evaluating all the DR neuronal recording from either young rat groups or adult rat groups were not significantly different. However, when the data evaluation from all the animals were divided into data obtained from a behavioral sensitization separated from data obtained from behavioral tolerance group based on behavioral recordings to chronic MPD as compared to initial MPD effects, it was found that the DR neurons recorded from behavioral sensitized animals exhibited significant ($p<0.001$) differences in response to MPD rechallenge on ED10 as compared to the DR neurons recorded from behavioral tolerance animals. Furthermore, the ratio of how many young rats as compared to adult rats express behavioral sensitization or tolerance to the same MPD dose was found to have a significant ($p<0.001$) difference. These correlations suggest a different effect of MPD on young brain development compared to adult. The difference in response to MPD by age needs further study. An understanding of these effects may pave the way for preventing or treating abuse of psychostimulants.

The present observations are consistent with previous studies using the same MPD doses recorded from other brain areas [18,36,53-55,66]. We proposed the following mechanism of MPD action: **1)** Following acute MPD exposure, MPD binds to DA, NE and 5-HT transporters, preventing the reuptake of DA, NE and 5-HT from the synaptic cleft into the presynaptic terminals [53,54]. This increases the DA, NE, and the 5-HT levels in the synaptic cleft leading to increases in locomotion and in DR neuronal activities [18,53,54]. MPD is metabolized and the DA, NE, and the 5-HT levels return to pre-MPD levels, and the animals' behavioral expression and the DR neuronal firing rates return to baseline levels [67,68]. **2)** Repeated MPD exposure has been associated with plasticity in the mesocorticolimbic system [67,69]. Molecular and cellular plasticity in the brain requires changes in gene expression. Gene expression is controlled by a series of DNA-binding proteins known as transcription factors. Several transcription factors have been implicated in this regulation such as CREB and DFos B.

Following acute MPD exposure, young and adult animals respond in general in similar patterns with minor non-significant differences. The main differences in the acute responses to MPD are in the intensity of the responses that expressed in the firing rate of DR neurons and in the effect of ED10 BL/ED1 BL. However, significant differences were observed between the age groups following chronic MPD exposure to 0.6 and 2.5 mg/kg MPD. These

different effects between young and adult rats may be explained by age differences in the upregulations of transcription factors that lead to different levels of protein synthesis [69]. The age differences can also be explained by varying plasticity processes that are ongoing in young animals as compared to adult animals. To explain why some animals respond to chronic MPD with behavioral sensitization and others with behavioral tolerance, we can assume that MPD elicits sensitization is the result of extracellular signal related to Kinase phosphorylation (ERK) that leads to increased levels of DFosB, AMPA receptor subunits, ionotropic transmembrane glutamate receptors that mediate synaptic transmission in the central nervous system, and subsequent increases in the neuronal and behavioral activities [49,50,68,70]. While in behaviorally tolerant animals, the same chronic MPD exposure results in the upregulation of cAMP response element binding protein (CREB), which leads to decreases in AMPA receptor subunits and decreases in neuronal and behavioral activities [70]. The reason why MPD in some animals elicits upregulation in CREB and in DFos B needs further studies.

In addition to serotonergic neurons, the DR also contains GABAergic inhibitory neurons as well as glutamatergic, dopaminergic and substance P-containing neurons [71]. It was reported that the DR serotonergic neurons exhibit moderate regular firing patterns and exhibit a long duration action potential as compared to non-serotonergic DR neurons [71-73]. Based on the moderate firing rates and the neural spikes duration that was used to sort and analyze the recorded spikes, it is likely that most of the DR units recorded in this study were serotonergic.

Immunohistochemistry studies have demonstrated that withdrawal from chronic MPD treatment results in upregulation of serotonin transporter (SERT) and consequently SERT density, thus suggesting that 5-HT may play a part in participating in the long-term potentiation following repetitive MPD exposure [26]. This observation indicates that DR neurons recorded from young were more sensitive to MPD than the DR neurons recorded from adult animals, and that expressed mainly by the ED10 BL/ED1 BL neuronal activity that was significantly higher in the recording obtained from young animals as compared to those DR neurons recorded from adult animals. Since modulation of ED10 BL/ED1 BL indicates withdrawals, it raises concerns that MPD use in young increases potential for abuse as compared to adult since behavioral withdrawal is one of the experiment biomarkers indicating that a drug has the characteristics and the potential to elicit dependence [18,60].

Modulation of 5-HT neurotransmission by MPD and drugs of abuse was reported, as well its role in drug addiction and vulnerability to drug relapse [51]. While the dopaminergic and glutaminergic signals have definitive roles in the neuroplasticity underlying addiction, there are limited studies of the serotonergic signals that participate in the expression of drug dependence [51]. Additional support of the 5-HT role in expression of drug abuse is provided by Sora et al. [74]. In these studies, mice with genetically deleted DA and NE transporters show that these mice continued responding to reward properties of drugs of abuse, such as cocaine, as evidenced by self-administration and condition placed-preference experimental model [74,75], indicating that non-CA mechanisms also contribute to psychostimulant effects.

Salman et al [76] reported that 2.5 mg/kg MPD exposure increases the metabolism of 5-HT and improves performance in water maze tests which suggest, the significant therapeutic effects of MPD are also by increasing 5-HT in the synaptic cleft and reduces GABA-a receptor mRNA expression to release excitatory glutamate from the inhibitory influence of GABA. We agree with Kirby et al., [51], and Ruocco et al., [77], that serotonergic signals play a role in psychostimulant action and as evidenced from our observations that chronic MPD exposure elicits withdrawal, sensitization, and tolerance.

Can six daily MPD exposures be considered chronic effects of the drug? In our opinion - **Yes** from the following: Life expectancy of the average male human in Europe and USA before the 2020 COVID pandemic was about 78 years. 78 X 360 days/years = 28,080 days = 100%. Rat life expectancy is 2 years=100%. Six days of rat's life is 1.7%. 1.7% of human life is 477 days or 15.9 month or 1.3 years of human life. 1.3 years of daily treatment to our opinion can be considered as chronic treatment [59,78].

Conclusion

The study provides additional evidence that the DR and the serotonergic signaling participates in the behavioral expression of MPD and the role of the serotonergic signaling in the response to a psychostimulant, i.e., serotonin (5-HT) signaling and the DR play an important role in the mechanism of action of MPD therapy in behavioral disorders, and that the DR neurons express neurophysiological withdrawal, sensitization, and tolerance to chronic MPD. This study supports Kirby et al. [51] and Ruocco et al. [77] observations that serotoninergic signaling plays a role in the expression of chronic psychostimulant and that there are significant age differences in response to MPD exposure. In summary**,** this study provides additional evidence of the role of the DR and the serotonergic signaling in response to psychostimulants.

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