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Research Article

The Serotonergic Signaling and the Dorsal Raphe (DR) Neurons in Adolescent Rats are the Most Engaging in Response to Acute and Chronic Methylphenidate as Compared to Other Neuronal Activities Recorded from Other Five Brain Areas

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Abstract

Methylphenidate (MPD) is a CNS stimulant known for its treating behavioral disorders. The increase in its use among "normal" individuals for cognitive enhancement and recreation has become a serious public health problem. This study reveals that acute MPD exposure elicits mainly increases in neuronal and behavioral activity in dose response characteristics. Chronic exposure of MPD compared to acute (0.6, 2.5 or 10.0 mg/kg) MPD, elicits in some animals electrophysiological and behavioral sensitization and in other animals tolerance. When neuronal recording evaluations were performed based on the animals' behavioral responses to chronic MPD the majority of the neurons recorded from animals expressing behavioral sensitization, responded to chronic MPD by a further increase in firing as compared to the initial MPD exposure. And, the majority of the neurons recorded from animals expressing behavioral tolerance, responded to chronic MPD by a decrease in their firing rate as compared to the initial MPD exposure. Each of the brain area studies (VTA, LC, DR, NAc, PFC & CN) responds with significant differences to MPD suggesting that each one of the above brain areas exhibits different roles in the response to MPD. The DR neurons were the most responsive to MPD. The study demonstrates that it is essential to evaluate neuronal activities responses to psychostimulants based on the animal's behavioral responses from several brain areas simultaneously to obtain accurate information of the role of each of them in response to a drug. MPD elicits symptoms that are characteristic of substance abuse disorders.

Keywords: Methylphenidate (MPD); Behavior; Neuronal recording; VTA; LC; DR; NAc; PFC; CN

Introduction

Methylphenidate (MPD) is a psychostimulant used to treat behavioral disorders such as Attention Deficit Hyperactivity Disorder (ADHD) [1-3]. MPD use and abuse has increased dramatically in recent years for cognitive enhancement and recreational purposes by ordinary children and adults [4-8]. MPD modulates monoamine signaling in brain areas associated with addiction and reward by competing with DA, NE and 5HT transporters for re-uptake from the synaptic cleft into the presynaptic terminals [1,9,10]. This is of particular concern as MPD abuse is extremely dangerous with intravenous or intranasal consumption having higher mortality than amphetamines and cocaine [8,11-14]. Moreover, the use of MPD can lead to undesirable behavioral consequences that lead to severe depression, dependence, overdose and even death [15].

Exposure of psychostimulants such as MPD results in the inhibition and alteration of biochemical, molecular, morphological, neuronal activities and behavioral expression that leads to plasticity in the central nervous system [16-22]. Psychostimulants including MPD modulate the release and concentration of the monoamine system that includes Dopamine (DA), Norepinephrine (NE) and serotonin (5HT) in the CNS mainly by binding to DA, NE and 5HT transporters [9,23,24]. The main source of monoamines is from the Ventral Tegmental Area (VTA), Locus Coeruleus (LC) and Raphe Nucleus (DR). These nuclei ascend and descend to and from the Nucleus Accumbens (NAc), Prefrontal Cortex (PFC), Caudate Nucleus (CN) and more brain sites known as the motive/reward neuronal circuit [25-28].

The major source of DA for the motive neuronal circuit is the A-10 neurons of the VTA which serve as the origin of DA to the mesocorticolimbic system that underlies the brain reward circuit [16]. The major VTA ascending neurons are to the NAc and the PFC, which are critical for the initiation of reward function *via* the mesolimbic

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system [29,30]. Dopaminergic and glutaminergic signaling within the VTA have been shown to be critical for an animal's response to MPD. Since the VTA is the main source of DA to this neuronal circuit, and MPD modulates the dopaminergic system, it is essential to study the effect of MPD on the VTA neurons [31].

MPD injection has also been shown to increase extracellular NE levels [9,32,33]. Increases in NE concentration are known to affect attention and maintain arousal and alertness of the Central Nervous System (CNS) [32,34]. MPD enhances noradrenergic function by binding to NE Transporters (NET) this blocking NE reuptake from the synaptic cleft to the presynaptic terminals [33]. NE is synthesized within the LC and it is known that LC neurons discharges correlates with NE concentration [35,36]. LC noradrenergic neurons project broadly throughout the CNS [37,38]. Experiments suggest that the LC-NE circuit modulates both attention and arousal related processes [39] and MPD binds to NET as mentioned above, thus facilitating the NE effect on the post synaptic terminal. Since the LC area is the principal source of NE, and MPD modulates the noradrenergic system it is essential to study the effect of MPD on LC neurons.

Most of the studies that have investigated MPD properties have concentrated mainly on its effect on the DA system and some on the NE system. There is some experimental evidence that suggests that MPD modulates the serotonergic system as well [24,40,41]. The Dorsal Raphe Nucleus (DR) is known to be one of the major sources of serotonin (5HT) in the mammalian brain. The DR neuron firing rates correlate with diverse behavioral events, such as rewards, condition cues and impulsive behaviors [42]. MPD exposure modulates the serotonin transporters and the serotonin (5HT) levels [23,43]. This modulation alters the balance of the monoamine system and is in part responsible for the therapeutic properties of MPD. The 5HT system that originates from DR ascends to the PFC and has been implicated in the etiology of treatment of several behavioral disorders such as ADHD, schizophrenia, obsessive-compulsive disorder and cognitive sequelae of certain drugs of abuse [44]. Therefore, the DR was selected to be an additional target for this study.

The Nucleus Accumbens (NAc) belongs to the neuronal reward circuit that is critical for mesocorticolimbic functions, such as motivation and emotion [29,45-48]. The NAc plays a key role in the function of the neuronal circuit underlying psychostimulants and the constructs of the neuronal function [49]. Changes in the accumbal DA system have been shown to be critical for neuronal reward circuit responses. MPD has been shown to cause an increase in dopaminergic transmission from the VTA to the NAc, and up regulation of DA has been shown to lead to increased locomotion [50]. The NAc mediates reward behavior *via* dopaminergic projections from the VTA [51]. In addition, the NAc acts as a functional interface between the mesolimbic and motor systems [52], and mediates the rewarding effects of psychostimulants [53]. Chronic application of psychostimulants into the NAc causes behavioral sensitization, while NAc destruction modulates the behavioral response to the drug such as behavioral sensitization [48,54-56]. Sensitization is a sustained increase in behavioral activity, beyond the acute effects following repetitive psychostimulant exposure, and serves as one of the experimental biomarkers that a drug has the potential in causing dependence and addiction [22,45,48]. Therefore, the NAc was selected as a target for this study.

The Prefrontal Cortex (PFC) is a cortical area that participates in decision making and complex behavioral expression [57,58]. The PFC is also involved in the higher cortical processing and cognitive functions that separate animals from humans, such as language, writing and more [58]. The PFC is one of the CNS areas compromising the neuronal motive circuit that participates in the executive function and the brain neuronal system that underlies the effects of psychostimulants on the brain [49,59,60]. An important function of the PFC is its participation in initiating and carrying out goal-oriented actions, i.e. executive functions. It has been shown that drug abuse outcomes are due to the loss of control over executive function [60], i.e., drug seeking becomes less a conscious choice and more an impulse. It was reported that the PFC is composed largely of DA receptors and has reciprocal connections with the VTA and the other brain sites belonging to the brain reward circuit [49]. Understanding the effect of MPD on the PFC is of importance since MPD is abused by many ordinary children and adults for cognitive enhancement and recreational purposes [6]. Thus, the PFC was selected as an area of interest and the target in our study.

The Caudate Nucleus (CN) is a unique, large structure that belongs to both the brain motive/reward circuit as well as to the extrapyramidal motor system [61,62]. The CN participates in many behavioral functions and makes reciprocal connections with many cortical and subcortical sites which include the VTA, NAc and PFC that participate in mediating behavioral responses to chronic psychostimulant administration such as sensitization and tolerance [22,54,63-65]. The CN has been shown to be critical for learning and memory. Animal studies have shown that memory is enhanced with increasing CN DA and impaired by CN dopaminergic ablation [66], consistent with the observation that DA mediates memory consolidation. CN destruction modulates the behavioral effect of acute and chronic responses to psychostimulants [67,68]. Specific glutaminergic CN ablation modulates the acute and chronic effect of MPD [69], indicating the role of the CN in response to psychostimulants. The CN dopaminergic and glutaminergic signal pathways have been shown to be critical for the effect of MPD [67,69,70]. Specific destruction of the CN dopaminergic system extinguishes the response to MPD [67,69]. Therefore, the CN was selected as a target in this study.

The above six brain structures belong to the brain motive/reward circuit and are known to play a significant role in the development of reward seeking behaviors involved in substance abuse disorders [27,41,60]. Most of the electrophysiological studies of psychostimulants investigate only one brain area. Previously we studied each of the above six brain areas following acute and chronic MPD exposure [71-75]. The objective of this investigation is to repeat our study on the neuronal properties following acute and chronic MPD on the above six brain areas recorded simultaneously, i.e., on the neuronal circuit participating in MPD function, simultaneously with the behavioral response to acute and chronic MPD exposure.

The aim of the present study was to test the following 1) the acute dose of 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg will elicit mainly increases in firing rates in all the six brain areas (VTA, LC, DR, NAc, PCF, and CN); 2) the repetitive (chronic) doses of either 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD will elicit similar and/or different behavioral expression such as behavioral sensitization or behavioral tolerance, as compared to the effects of the initial MPD dose; 3) the neuronal activity recorded from behaviorally sensitized animals will be different or the same as compared to the neuronal activity recorded from behavioral tolerance animals as compared to the effect of the initial (acute) MPD effects; 4) Each of the repetitive (chronic) MPD

doses (0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD) will elicit the same and/or different effects in each of the six brain areas studied, i.e., the percentage of neuronal units' response and the direction of the responses such as excitation or attenuation as well the intensity of the firing rates will be the same and/or different; and, 5) the percentage numbers of how many neuronal units will exhibit increases in firing following 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD compared to the percentage of neuronal units that exhibit decreases in their firing rates to acute or chronic MPD exposure will be the same and/or different in each of the six different brain area studies.

Material and Methods

Animals

Male Sprague Dawley (SD) rats (Harlan Indianapolis, IN, USA) obtained at postnatal day 30-32 were placed individually in the enriched home cage that also served as the test cage in a controlled room with a 12-hour light/ dark schedule (light on at 06:00 hours) and given water and food as needed for 5 to 6 days of acclimation prior to bilateral electrode implantation in the Ventral Tegmental Area (VTA), Locus Coeruleus (LC), Dorsal Raphe (DR), Nucleus Accumbens (NAc), Prefrontal Cortex (PFC) and Caudate Nucleus (CN). The electrophysiological concomitant with the behavioral recording started at age P-40 for ten consecutive days, and all recordings and injections were done in the home cages (i.e., the home cage was used also as the test cage to eliminate the novelty of the test cage as a potential confounding factor in treatment).

Electrode implantation

Animals were anesthetized with 30 mg/kg pentobarbital, their heads were shaved and covered with lidocaine hydrochloride topical gel and placed in a stereotactic holder. An incision was made on the head to expose the skull by removing the skin, the muscle, and the connective tissue. Bilateral holes were drilled into the skull above the following six brain areas: VTA- posterior (P) from Bregma 6.0 mm and lateral (L) from the midline 0.2 mm, LC- P 9.3 mm and L 1.0 mm, DR- P 7.8 mm and L 0.2 mm, NAc- anterior (A) from Bregma 1.2 mm, L- 6.0 mm, PFC- A 3.0 mm, L- 0.6 mm and CN A- 0.5 mm, L- 3.0 mm [76]. Additional holes were drilled in the frontal skull for the reference electrodes. At vacant spots laterally six to eight screws were inserted to secure the implanted electrodes and the head plug holding the electrodes. Nickel-chromium, Teflon-coated electrodes (Except at the tips) 60 µm in diameter were used as the recording electrodes. Electrodes were inserted individually into the six brain areas bilaterally at initial depths of 8.0 mm, 6.6 mm, 6.4 mm, 6.4 mm, 2.6 mm and 3.6 mm in the VTA, LC, DR, NAc, PFC and CN, respectively. During electrode placement, the neuronal activity was monitored using a Grass P-511 amplifier with its cathode follower connected to an audio monitor and oscilloscope. When spike activity exhibited at least a 3:1 signal to noise ratio the electrodes were fixed to the skull; otherwise, the electrode was lowered in steps of 5 µm to 10 µm increments until a 3:1 spike to noise ratio was obtained and then the electrodes were fixed to the skull with dental cement. Each electrode was connected to a copper pin and all the pins were inserted into an Amphenol plug, which was fixed onto the skull with dental acrylic cement. Rats were allowed to recover from the surgical procedure for 5 to 7 days. During this recovery period, rats were daily placed in their home cage in the experimental set up and connected to the wireless head stage (Triangle BioSystem Inc. TBSI, Durham, NC USA) for about two hours to adapt and acclimate to the recording system. The first recording day started at P-40 age. All the experimental procedures were approved by the University of Texas Health Science Center Welfare Animal Committee in Houston, TX, in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

Drug

Methylphenidate (MPD) hydrochloride was obtained from Mallinckrodt (Hazelwood, MD, USA). MPD was dissolved into a 0.9% isotonic saline solution and the 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD doses were calculated as a free base. All MPD injections were equalized to a volume of 0.8 ml with 0.9% saline to keep all injection volumes the same for all animals. Injections consisted of 0.8 ml isotonic saline solution (0.9% NaCl) MPD and were given intraperitoneally.

In previous studies using MPD dose responses experimental from 0.1 mg/kg to 40.0 mg/kg MPD treatment on rats neuronal and behavioral activities, it was observed that neuronal and behavioral effects of MPD were start to obtained from MPD doses of 0.6 mg/kg and above [45,62,65,77-79]. Therefore, these three MPD doses were selected to be used in this study as low, medium and high doses.

Experimental protocol

The behavioral locomotor activity and the neuronal activity were recorded concomitantly using the TBSI and a computerized animal activity monitoring system (Opto-M3, Columbus, OH, USA). The TBSI system consists of a wireless head stage (weight less than 4.5 gm) and a remote receiver. The TBSI head stage was connected to the rat's head cap containing the electrode pins and the electrical signals (sampling notes up to 200 Hz) sent through a transmitter to a receiver that was connected to an analog-to-digital converter (Micro 1402-3: Cambridge Electronic Design (CED)). The neuronal activity from each electrode was collected and stored on a PC using the CED spike 2.7 software.

Four groups of animals were used: control (saline) group, and 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD treatment groups. On experimental day 1 (ED1), rats were placed with their home cage in a Faraday testing cage to reduce interfering noise. The TBSI wireless head stage was connected to the electrode pins of the skull cap and animals were allowed to acclimate for an additional 30 minutes prior to the recording session. After saline injection, behavioral locomotor activity and the neuronal activity from all the electrodes were recorded simultaneously for one hour post injection. The 60 min recording following saline injection served as the Baseline (BL) activity. Following this 60 min recording additional injections were given: either saline to the control group or 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD to the MPD experimental groups (Table 1), and recordings (electrical and behavioral) were resumed for an additional 60 minutes. On ED2-ED6, rats received daily injections of either saline (the control group) or single doses of 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD in their home cage to initiate the MPD chronic effect [78]. On ED 7, 8 and 9 the rats underwent washout days, in these days no injections were given. On ED10, injections and neuronal and behavioral recordings were resumed identically to ED1.

Histological verification of electrode location

At the end of recording on ED10, an overdose of sodium pentobarbital was administered followed by intracardial perfusion of 10% formalin solution containing 3% potassium ferrocyanide. A 20 µA DC current was passed through each electrode for 20 seconds to

Table 1: Experimental Protocol: Table 1 displays the 4 animal groups for the adolescent animals, and the drug protocol that was followed for each group. The four groups of animals were: saline, 0.6 mg/kg, 2.5 mg/kg 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 (ED1 BL), followed by one of the four designated injections of saline, 0.6 mg/kg, 2.5 mg/kg 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 in their home/test cage. ED 7-9 is 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 (ED10BL) followed by the designated MPD dose and recordings were taken, identical to that given on ED1 (ED10MPD).*Indicates the recording day.

	Experimental Days (ED)			
Treatment Groups	**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

produce a small lesion in the recording site. The brain was excised and stored in the 10% formalin for subsequent histological procession. The position of each of the implanted electrodes was confirmed by the location of the lesion and the Prussian blue spot using the Rat Brain Atlas [76]. Only recordings obtained from electrodes confirmed histologically to be within the aim in targets (VTA, LC, DR, NAc, PFC and CN) and exhibited similar spike amplitude and pattern at ED1 and ED10 were evaluated.

Data Acquisition

Behavioral data

Locomotor activity was recorded in each session for 60 minutes. Six bins of data each of 10 minutes for a 60 minutes total were used to analyze each animal's locomotor behavior following acute-experimental day 1 (ED1) and chronic- Experimental Day 10 (ED10) MPD exposure. The paired t-test was used to determine the effect of the acute MPD by comparing the ED1 MPD/ ED1 BL, and the chronic effect of MPD was determined by comparing ED10MPD/ ED1MPD [22,46,71,74,75,80-82]. Lee et al. [63], Yang et al. [62] calculated that six consecutive daily injections are considered chronic as it equals drug exposure for 0.86% of the rat's lifespan. The human equivalent of 0.86% lifespan would be 33 weeks of treatment, based on an average lifespan of 78 years, and thus is considered chronic [62,63,80]. Animals that had significant ($P<0.05$) increase in locomotor activity at ED10 MPD compared to ED1 MPD (ED10 MPD/ED1 MPD) were classified as exhibiting behavioral sensitization following six daily MPD exposures and three washout days as compared to the initial (acute) MPD effect on ED1, and animals that exhibited significant decreases ($P<0.05$) on ED10 MPD/ ED1 MPD were classified as exhibiting behavioral tolerance. The mean values for these two subgroups, sensitized and tolerant, were exported from oasis software and each group was further analyzed using Analysis of Variance (ANOVA) with reported measures. In addition, a post hoc Tukey test was run to compare significant ($P<0.05$) behavioral changes observed between experimental days ED1 and ED10 within each group using SPSS software version 21.

Neuronal data

The recorded neuronal activity from each of the electrodes was replayed offline for neuronal spike sorting and statistical analysis using the CED spike 2.7 software. The neuronal spikes were captured by the program and processed using low-pass and high-pass filters (0.3 kHz-3.0 kHz). There were two window discrimination levels, one for spikes in the positive direction and another for spikes exhibiting negative direction. The selected spikes that enter the window were used to create a template, which selected 1000 waveform data points from the chosen spikes to be evaluated. The algorithm used to capture the selected neuronal spike pattern allows the extraction of templates that provide high dimensional reference points that can be used to discriminate consistent and accurate spike sorting regardless of the influence of noise, false threshold crossing, and waveform overlap (Figures 1 and 2). All temporally displaced templates are compared with the incoming selected spike events to find the best fitting template that yields the minimum residue variance. A template matching procedure is then used, so that if the distance between the template and waveform exceeds some threshold (80%), the waveforms are rejected to allow the spike sorting accuracy in the reconstructed data of about 95%. Spikes with peak duration and pattern outside of these parameters were rejected. The templates that were used to analyze the ED1 file were then loaded onto the ED10 file of the same electrodes from the same animal the evaluate the ED10 neuronal activity. This ensured that the spike amplitude and pattern sorted from ED10 was the same as the one recorded on ED1 (Figure 2). Once spike sorting was completed, the data was exported into a spreadsheet. Statistical comparisons were made for each neuronal unit as follows: (1) unit firing rate after the initial MPD exposure (ED1 MPD) was compared to unit firing rates following saline on ED1 (ED1 BL), i.e. ED1 MPD/ ED1 BL; (2) unit firing rates after saline exposure on ED10 was compared with unit firing rates obtained after saline on ED1 (ED10 BL/ ED1 BL) to find out whether six daily MPD (0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD) exposure and three washout days modulates the ED10 BL. Significant differences in baseline would indicate withdrawal; and, (3) unit firing rates after MPD rechallenge at ED10 was compared to unit firing rates after MPD exposure on ED1 (ED10 MPD/ ED1 MPD) to determine the MPD chronic effect. Significant changes and direction of the change (increase or decrease) for each neuronal unit was determined by the Critical Ratio (CR) test [16,22,70,73-75,78,80,82,83]. In addition to the above evaluation of all the recorded units, the data analysis obtained from each of the six brain areas (VTA, LC, DR, NAc, PFC and CN) were divided into two subgroups based on how the rats responded behaviorally to chronic 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MDP exposures as compared to the initial response to MPD: (1) neuronal activity data recorded from animals expressing behavioral sensitization and ED10 compared to ED1 (ED10 MPD/ED1 MPD) and (2) the neuronal recordings obtained from animals that express behavioral tolerance, respectively.

Statistical analysis

All the statistical tests and analysis are performed in R 4.0.5. To examine whether there are significant differences in the percentages of neuronal units responding to MPD among six brain regions, the χ^2 test is performed. If the χ^2 test result indicates that the percentage of responding neuronal units is significantly different amount among the six brain regions with P-value less than the 005, the logistic regression model is then used to fit the data and post hoc comparisons are performed to identify the regions(s) with significantly different response rates compared to other regions. The post hoc comparisons

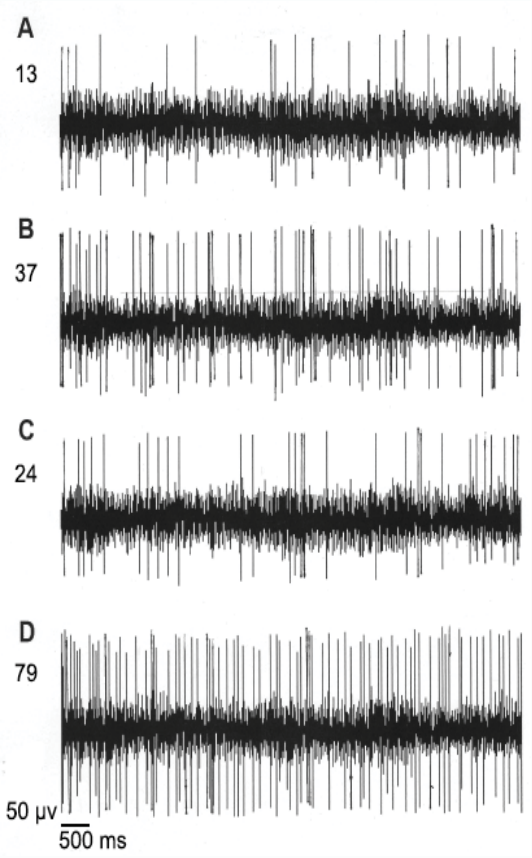

Figure 1: Representative Analog of Neuronal Activity from CN: Figure 1 is a 5 second representative analog neuronal spikes activity recorded from the CN. (A). Represents the neuronal spike activity at experimental day 1 baseline (ED1 BL). (B). Represents the neuronal spike activity after acute 2.5 mg/kg MPD exposure on ED1 (ED1 MPD). (C). Represents the BL spike activity at ED10 (ED10 BL). (D). Represents the neuronal spike activity after rechallenging 2.5 mg/kg MPD on ED10 (ED10 MPD). The numbers to the left of each figure are the total number of spikes for each 5 second tracing. Comparing 37 spikes in Figure 1B to ED1 BL in 1A trace-13 spikes show that MPD cause excitation compared to ED1 BL. Comparing ED10 BL in 1C- 24 spikes to ED1 BL 13 spikes show electrophysiological withdrawal. Comparing 79 spikes in Figure 1D to Figure 1B-acute MPD effects-37 spikes, shows neuronal sensitization in this recording.

are done with the function contrast in the R package "emmeans". The Bonferroni correction method is used to adjust p-values for multiple comparisons. We also analyze the ratio of neuronal units responding to MPD with increased *vs.* decreased firing rates among six different brain regions using the χ^2 test. If there are significant differences among the different brain regions, post hoc comparisons are performed to identify the regions(s) with significantly different ratios of neuronal units responding to MPD with increased *vs.* decreased firing rates when compared to other regions.

Results

Locomotor behavioral expression

A total of 157 adolescent male SD rats were evaluated, 8, 8, 49, 44, 48 were used following time (control), saline, 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD, respectively.

Time and saline (Sal) control (Figure 3): Animals in the time control group during all eleven recording days exhibit similar levels of locomotor activity (Figure 3). Comparing the locomotor activity of the time control of experimental day 1 (ED 1) to the other recording

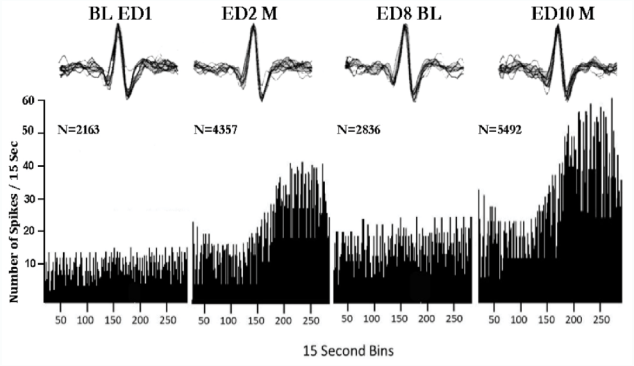

Figure 2: Histogram of NAc units: Figure 2 represents a histogram of NAc units recorded from animals summarizing 60 min sequential neuronal firing rates/15-seconds following acute 2.5 mg/kg MPD exposure.The neuronal spikes under ED1 BL represents the recordings on ED 1 after saline injection (ED1 BL). The recordings under ED1 MPD show increased neuronal spikes activities after MPD administration on ED1. ED10 BL compares to ED1 BL shows increased spikes on experimental ED10 after six daily MPD exposures and three washout days, as compared to ED1 BL (ED10 BL/ED1 BL). The recordings under ED10 MPD/ED1 MPD show further increases in neuronal spikes activities following MPD rechallenge on ED10, i.e. neuronal sensitization is expressed. Similar observation were perform to each of the 3036 neuronal activities recorded.

days show no significant differences (One way RM ANOVA F=0.0427, P<0.874). Comparing the locomotor activities of ED 1 following Sal injection to its Baseline (BL) recording at ED1 and to the other recording days following Sal injections, show no significant differences between the recording days (One way RM ANOVA F=0.0398, P<0.908) demonstrating that the handling, injection procedure, and environment had no effect on the animals' locomotor behavior during all the experimental days (Figure 3).

Effect of Acute and Chronic 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD on Horizontal Activities (HA) (Figure 4): Figure 4 summarizes the effect of 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD on HA of all three groups (all, sensitized, tolerant groups respectively).

Acute effect of MPD on All groups (ED1 MPD/ED1 BL, Figure 4 left side): Acute 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD elicits significant (F=27,751, P<0.01; F=29.045, P<0.001; F=41.867, P<0.001) difference compared to ED1 BL by increase it's HA (Figure 4 All ED1 MPD/ED1 BL) respectively using the RM ANOVA.

All Groups ED10 BL/ ED1 BL all group: The ED10 BL activities after six days of repetitive (chronic) 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD respectively and three washout days exhibit F=24.014, P<0.05; F=23.422, P<0.05; F=26.59, P<0.001 (one way RM ANOVA) increase in ED10 BL/ED1 BL HA respectively (Figure 4 All). The further increases in HA at ED10 express withdrawal behavioral activities.

ED10 MPD/ED1 MPD All Groups: The chronic effects of 0.6, 2.5 and 10.0 mg/kg MPD compared to initial effect of MPD at ED1 MPD (ED10 MPD/ED1 MPD) resulted in further significant increases in HA (F=24.862; P<0.001, F=24.147; P<0.001; F=25.632; P<0.001) as compared to the initial MPD effects. The further significant increase indicates that behavioral sensitizations were expressed.

Effect of Acute 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD on HA of the Behavioral sensitized groups (Figure 4 middle histograms ED1 MPD/ED1 BL): The HA recorded from the behavioral sensitized

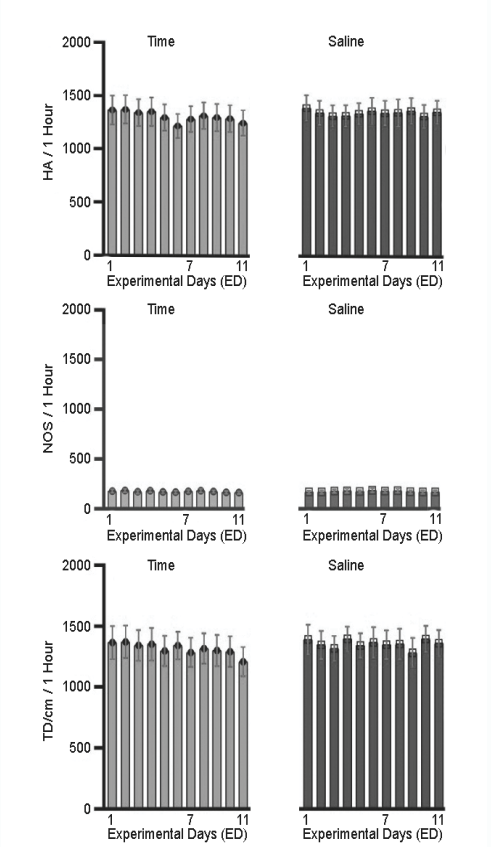

Figure 3: Time and saline control groups (each N=8) of three different locomotor activities of behavioral expression recorded for one hrs/day for sequential 11 experimental days. Horizontal Activity (HA) summarizes the total number of movements activities. Number of Stereotypic (NOS) movements summarizes repetitive movements recorded from the same sensor with at least one sec. interval between the movement. Total Distance (TD) traveling summarizes in cm the TD traveling in cm/1 hr. During the eleven recording days both animals 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.

group following acute 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD elicits significant increases (F=23.842, P<0.01; F=30.425, P<, P,0.001; F=26.322, P<0.001) compared to ED1 BL HA (Figure 4 sensitized ED1 MPD/ED1 BL) respectively using the RM ANOVA as compared to ED1 BL activity (Figure 4 Sensitizes).

ED10 BL/ED1 BL HA in Behavioral sensitized groups (Figure 4 middle histograms): The ED10 BL activities after six days of repetitive (chronic) 0.6, 2.5 and 10.0 mg/kg MPD exhibit significant increase F=39.742, P<0.001; F=37.891, P<0.01; F=47.372, P>0.001 (one way RM ANOVA) in ED10 BL/ED1 BL (Figure 4 sensitized). These significant increases in ED10 BL/ED1 BL indicate behavioral withdrawal in all the three MPD groups.

ED10 MPD/ED1 MPD Sensitized groups: The chronic effects of 0.6, 2.5 and 10.0 mg/kg compared to initial effect of MPD at ED1 MPD (ED10 MPD/ED1 MPD) resulted in significant increased F=35.892, P<0.001; F=44.245, P<0.001; F=58.913, P<0.001 as compared to the acute effect of 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD on ED1. These additional significant increases indicate that behavioral sensitizations were expressed.

Acute effect of MPD Behaviorally Tolerant Groups (ED1 MPD/ ED1 BL, Figure 4 right side histogram): The behaviorally tolerant animals groups responded to acute 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD with a significant increase in HA (F=10.052, P<0.01; F=38.672, P<0.001; F=54.556, P<0.001) to acute 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD respectively.

ED10 BL/ED1 BL on HA of Behavioral tolerance groups (Figure 4 right histograms): The ED10 BL activities after six days of repetitive (chronic) 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD and three washout days exhibited significant increases in their ED10 BL/ ED1 BL following 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD (F=23.452, P<0.001; F=27.582, P<0.001; and F=28.894, P<0.001) respectively (one way RM ANOVA; Figure 4 tolerance).

ED10 MPD/ED1 MPD tolerance group (Figure 4 right histograms): The rechallenge (chronic) effects of 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg after six daily MPD exposure and three washout days compared to initial effect of MPD at ED1 MPD (ED10 MPD/ED1 MPD) resulted in significant reduction in locomotor activity behaviors compared to the initial effects (ED1 MPD) of 0.6. mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD respectively. This significant decrease (F=36.104, P<0.001; F=32.534, P<0.001; F=42.354, P<0.001) as compared to the acute effect of the drug on ED1 indicates that behavioral tolerance was expressed. Similar observations with minor insignificant fluctuation were observed following NOS and TD traveling.

The ratio of how many animals expressed behavioral sensitization *vs.* behavioral tolerance to chronic 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD: The ratio of how many animals expressed behavioral sensitization *vs.* behavioral tolerance to chronic 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD was significantly (P<0.05) different using the Chi Square test (27/22; 30/14 and 39/9) following 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD respectively (Figure 4, the numbers below the histograms).

Neurophysiological result

A total of 3086 neuronal units were recorded (Table 2), 405, 409,479, 503, 459, and 831 neuronal units from the VTA, LC, DR, NAc, PFC and CN respectively. All the above neuronal units were identified histologically to be recorded from the above brain target and exhibited similar neuronal amplitude and spike wave patterns at experimental day 1 (ED1) as well on ED10.

Control groups: effects of acute and repetitive saline on neuronal unit activities: A total of 304 neuronal units were recorded following acute and repetitive saline injections (Table 1), 36, 56, 57, 41, 45 and 69 from the VTA, LC, DR, NAC, PFC and CN respectively (Table 2). Saline injection resulted in only few (2%, 2%, 3%, 1%, 3% and 4% of VTA, LC, DR, NAC, PFC and CN respectively) neuronal unit changes in their firing rate. That is, no significant neurons change their neuronal activities following single or repetitive saline injection indicating that the injection procedure and handling of the animals has no effects on the recorded neuronal activities from the above six brain area. Therefore, any significant changes from baseline (ED1 BL) neuronal activities on ED1 MPD are the result of the acute MPD effects.

Neuronal response to 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD (Figures 5-7): It was observed that in each of the chronic MPD dose groups some of the animals expressed behavioral sensitization and some animals expressed behavioral tolerance. Therefore, the

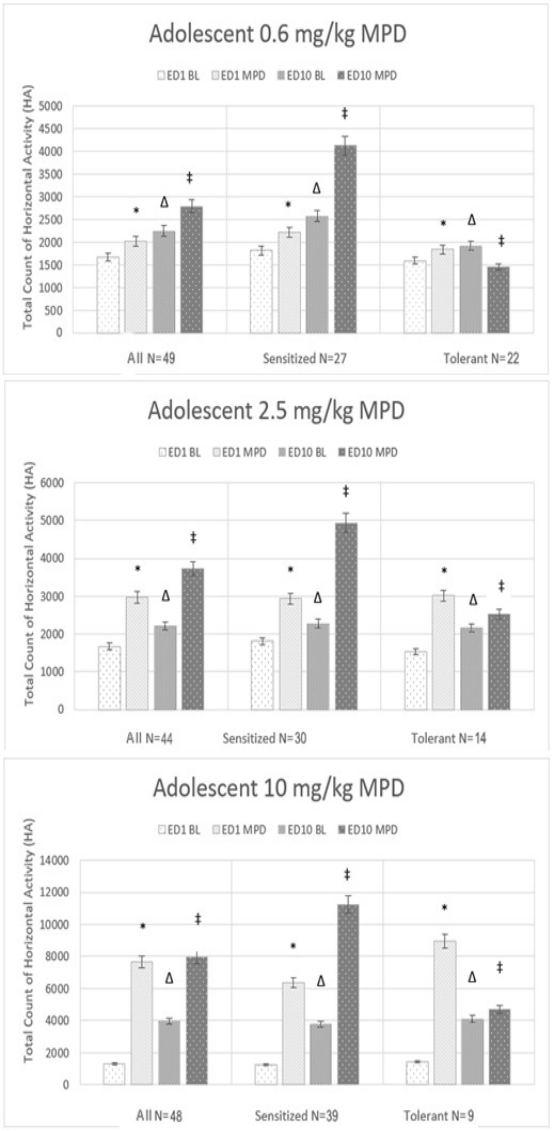

Figure 4: Behavioral Responses to MPD: Figure 4 summarizes the behavioral data of each experimental dose (0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD). All animal groups for each experimental dose were also divided into 2 subgroups; sensitized animals and tolerant animals group. All group- summarizes all the animals for a particular MPD dose. Sensitized group-summarizes only animals that expressed behavioral sensitization following chronic MPD. Tolerance group- summarizes only animals that expressed behavioral tolerance to chronic MPD as compared to the initial MPD response respectively. The left column of each group shows the total Horizontal Activity (HA) following saline injection, i.e. baseline activity on experimental day 1(ED1 BL). The second to the following column shows the activity after acute MPD (ED1 MPD). The third column shows the baseline activity on experimental day 10 (ED10 BL), and the furthest right column of each group shows the activity following chronic MPD on experimental day 10 (ED10 MPD).

neuronal responses recorded from animals' expression of behavioral sensitization were evaluated separately from the neuronal recording recorded from animals expressing behavioral tolerance (Figures 5-7).

Acute response to MPD 0.6 mg/kg in animals' expressing behavioral sensitization (Figure 5A): We compared the proportion of neuronal units in six brain regions that responded to MPD acutely recorded from animals exhibiting behavioral sensitization to chronic 0.6 mg/kg MPD. It was found that the proportions of responding neuronal units are significantly different among six brain regions with χ^2 (5,461)=66.7 (p<0.0001). We then fit the data to a logistic regression model and performed the post hoc comparison. The proportion of neuronal units responding to MPD acutely are significantly lower in regions CN (36%) and LC (30%) when compared to the average response rates of all other regions, with adjusted p-values 0.001 and 0.004, respectively. However, the neuronal response in DR region (89%) was significantly higher than the average of all other neuronal responses from the other regions (p<0.0001).

We also compared the ratio of neuronal units responding to MPD with increased *vs.* decreased firing rates among six different brain regions. We found that the ratios are significantly different among these regions with χ^2 (5,220)=24.37 (p=0.0002). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased in the region CN is significantly lower than the average of all other regions (adjusted p-value <0.0001).

Acute responses to MPD 0.6 mg/kg in animals expressing behavioral tolerance (Figure 5B): For animals expressing behavioral tolerance to repeated 0.6 mg/kg MPD as compared to its initial (acute) effect, we found that the proportion of responding neuronal units are significantly different among six brain regions with χ^2 (5,446) = 38.86 (p<0.0001). The proportion of neuronal units responding to MPD acutely are significantly lower in regions NAc (29%) and PFC (29%) when compared to the average response rates of all other regions, with adjusted p-values 0.005 and 0.02, respectively, while the proportion of responding neuronal units in region LC (71%) is significantly higher than the average of all other regions (p<0.0001).

In addition, we found that the ratio of neuronal units responding to MPD with increased *vs.* decreased firing rates significantly differed among these regions, with χ^2(5,188)=25.56 (p=0.0001). Further post hoc comparison indicated that the ratio of neuronal units with increases *vs.* decreases in the region CN is significantly higher than the average of all other regions (adjusted p-value=0.04), while the ratio in LC is significantly lower than that in other regions (adjusted p-.value=0.008).

Baseline activity to MPD in animals expressing behavioral sensitization (Figure 5C): The neuronal baseline activities (ED10BL/ ED1BL) after six daily 0.6 mg/kg MPD exposures and three washout

Table 2: The table summarizes the numbers of neuronal units (N=) Recorded from adolescent animals from the Ventral Tegmental Area (VTA), Locus Coeruleus (LC), Dorsal Raphe (DR), Nuclease Accumbens (NAc), prefrontal cortex PFC) and from the Caudate Nucleus (CN) following single (acute) saline 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD respectively.

	Saline	0.6 mg/kg MPD	2.5 mg/ mg/kg MPD	10.0 mg/kg MPD	Total
VTA N=	36	115	137	117	405
LC N=	56	109	132	112	409
DR N=	57	127	142	153	479
NAc. N=	41	152	143	167	503
PFC. N=	45	147	165	102	459
CN N=	69	257	224	281	831
Total N =	304	907	943	932	N=3086

days are significantly different among the six brain regions with χ^2 (5,461)=103.99 (p<0.0001). The proportion of neuronal units with modified ED10BL are significantly lower in regions CN and VTA when compared to the average firing rates of all other regions, with adjusted p-values 0.0001 and 0.004, respectively. However, the ED10BL changes in region DR (95%) is significantly higher than the average of all other regions (p<0.0001).

We also compared the ratio of neuronal units with increasing ED10BL compared to those with decreasing ED10BL among six different brain regions. We found that the ratios are significantly different among these regions with χ^2 (5, 238)=31.30 (p<0.0001). Further post hoc comparison indicated that the ratio of neuronal units with increases *vs.* decreases in firing rates is significantly lower than the average of all other regions in the NAc (adjusted p-value=0.013), while the ratio in the PFC is significantly higher than that in other regions (adjusted p-value=0.025).

Baseline activity to MPD 0.6 mg/kg in animals expressing behavioral tolerance (Figure 5D): The neuronal baseline activities (ED10BL/ ED1BL) after six daily 0.6 mg/kg MPD exposure and three washout days are significantly different among the six brain regions with χ^2 (5,446)=26.59 (p<0.0001). The proportion of neuronal units with modified acute BL were significantly lower in the PFC region, but significantly higher in the VTA region, when compared to the average firing rates of all other regions (adjusted p-values 0.0001 and 0.02, respectively).

In addition, we found that the ratio of neuronal units with increasing *vs.* decreasing ED10BL was significantly different between the six brain regions, with χ^2 (5,196)=11.50 (p=0.04). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased firing rates in the CN region is significantly higher than the average of all other regions (adjusted p-value=0.01). This indicates a push-pull relationship between the PFC and the CN.

Response to MPD 0.6 mg/kg rechallenge in animals expressing behavioral sensitization (Figure 5E): A separate evaluation was performed on the MPD chronic effect. The first one was comparing the effect of chronic MPD at ED10 to the ED1BL (ED10 MPD/ ED1BL). The second evaluation was performed comparing the effect of chronic MPD to the acute effect of MPD for each dose, respectively (ED10 MPD/ED1MPD).

The proportion of neuronal units responding to MPD rechallenge are significantly lower in the VTA region when compared to the average response rates of all other regions, with adjusted p-values 0.0002. However, the response rate in the DR region (90%) was significantly higher than the average of all other regions (p=0.009).

We also compared the ratio of neuronal units responding to MPD with increased firing rates *vs.* decreased firing among the six brain regions. We found that the ratios are not significantly different among these regions with χ^2 (5, 329)=9.04 (p=0.11). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased firing rates in the NAc is significantly lower than the average of all other regions (adjusted p-value=0.013), while the ratio in the PFC is significantly higher than those in other regions (adjusted p-value 0.025).

Response to MPD 0.6 mg/kg rechallenge in animals expressing behavioral tolerance (Figure 5F): The proportion of responding neuronal units to MPD at rechalenge is significantly different among the six brain regions, with χ^2 (5,446)=47.45 (P, 0.0001). The proportion of neuronal units responding to MPD acutely is significantly lower in the PFC (P<0.0001), but significantly higher in the CN (p<0.001), when compared to the average rates of all other regions.

In addition, we found that the ratios of neuronal units responding to MPD with increased *vs.* decreased firing rates are not significantly different among these regions, with χ^2 (5,232)=9.13 (p< 0.10).

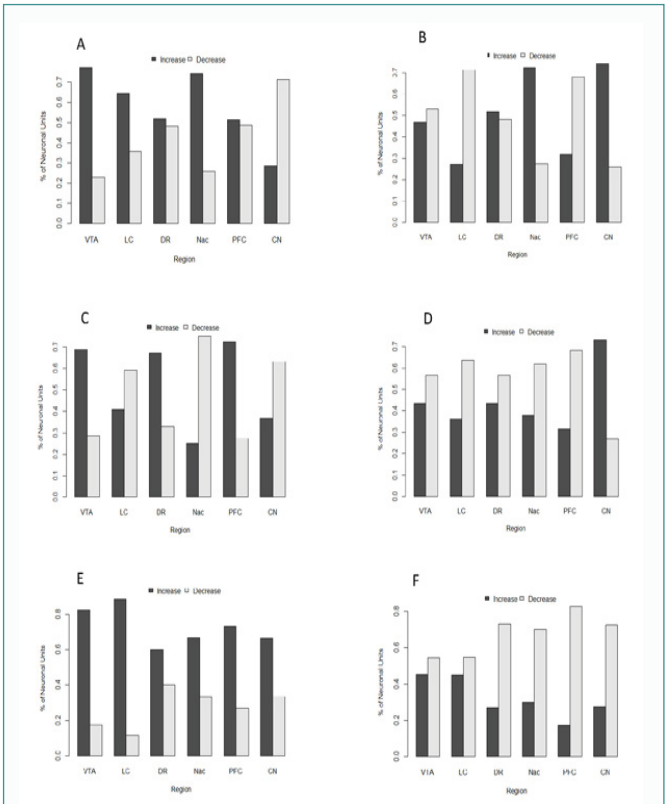

Figure 5: Neuronal Response to 0.6 mg/kg MPD: Figure 5 summarizes the percentage of neuronal units in six regions responding to 0.6 mg/kg MPD with increased or decreased significant firing rates. The figures on the left-hand side (5A, 5C, 5E) represent responses from those animals expressing behavioral sensitization. The figures of the right-hand side (5B, 5D, 5F) represent responses from those animals expressing behavioral tolerance. The figures in the top row (5A, 5B) represent the responses following acute MPD (ED1 MPD/ED1 BL), those in the middle row (5C, 5D) represent the changes to the baseline responses (ES10 BL/ED1 BL), and those in the bottom row (5E, 5F) represents responses following chronic or rechallenge MPD (ED10 MPD/ED1 MPD).

Acute Response to MPD 2.5 mg/kg in animals expressing behavioral sensitization (Figure 6A)

We compared the proportion of neuronal units responding to acute treatment of MPD In six brain regions from animals expressing behavioral sensitization to chronic MPD. We found that the proportion of responding neuronal units were significantly different among six brain regions with χ^2 (5,514)=16.27 (p<0.006). We then fitted the data to a logistic regression model and performed the post hoc comparison. The proportion of neuronal units responding to MPD acutely in the DR (89%) was significantly higher compared to the average response rates of all other regions (adjusted p-value=0.05).

There were no significant differences found among the ratios of neuronal units responding with increased *vs.* decreased firing rates in different regions. In all the six regions, we can see that there were

more neuronal units responding with increased firing rates than those with decreased firing rates, in response to 2.5 mg/kg MPD.

Acute response to MPD 2.5 mg/kg in animals expressing behavioral tolerance (Figure 6B): For animals expressing behavioral tolerance to chronic MPD, we found that the proportion of responding neuronal units was significantly different among the six brain regions with χ^2 (5,427)=21.42 (p< 0.0006). The proportion of neuronal units responding to MPD acutely in the CN (29%) was significantly lower compared to the average response rates of all other regions, with adjusted p-values 0.004, while the proportion of responding neuronal units from the PFC (63%) was significantly higher than the average of all other regions (p=0.02).

There were no significant differences found among the ratios of neuronal units responding with increased *vs.* decreased firing rates in different regions, although we found that the ratio of neuronal units with increased *vs.* decreased activity was lower in the VTA than all other regions.

Baseline activity to 2.5 mg/kg MPD in animals expressing behavioral sensitization (Figure 6C): The proportion of neuronal units that changed their baseline activity after six daily doses of 2.5 mg/kg MPD exposure and three washout days was significantly different among the six brain regions with χ^2 (5,514)=19.91 (p=0.001). The proportion of neuronal units in the CN was significantly lower (66%) (Adjusted p-value=0.001), but significantly higher in the DR (94%) (Adjusted p-value=0.03), compared to the average of all other areas. The changes in ED10BL compared to ED1BL represent withdrawal activity.

We also compared the ratio of neuronal units with either increasing or decreasing changes in ED10BL/ED1BL activity among six different brain regions. We found that the ratios are significantly different among these regions with χ^2 (5,395)=96.65 (p<0.0001). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased firing rates in the CN was significantly lower than the average of all other regions (adjusted p-value <0.0001).

Baseline activity to 2.5 mg/kg MPD in animals expressing behavioral tolerance (Figure 6D): The proportion of neuronal units that changed their baseline activity after six daily doses of 2.5 mg/kg MPD exposure and three washout days was significantly different among six brain regions, with χ^2 (5,427)=40.30 (p<0.0001). The proportion of neuronal units with baseline changes with significantly lower in the CN (30%), but significantly higher in the LC (72%) when compared to the average of all other regions (p-values 0.0001 and 0.002, respectively).

In addition, we found that the ratios of neuronal unit activities showing increased *vs.* decreasing firing rates was significantly different among these brain regions, with χ^2 (5,319)=29.53 (p<0.0001). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased activity was significantly lower in the DR (p=0.006), but significantly higher in the PFC (p=0.0001), when compared to the average of all other regions.

Response to 2.5 mg/kg MPD rechallenge in animals expressing behavioral sensitization (Figure 6E): The proportion of responding neuronal units to MPD at ED10 compared to ED1 (ED10 MPD/ED1 MPD) was significantly different among six brain regions with χ^2 (5,514)=12.15 (p=0.03). The proportion of neuronal units responding to MPD rechallenge in the VTA (78%) is only marginally lower compared to the average of all other regions (p-value=0.06).

There were no significant differences found among the ratios of neuronal units responding with increased *vs.* decreased firing rates in the different regions. In all the six regions, we found that there were more neuronal units responding with increased firing rates than those responding with decreased firing rates.

Response to 2.5 mg/kg MPD rechallenge in animals expressing behavioral tolerance (Figure 6F): The proportion of responding neuronal units to MPD at ED10 as compared to ED1 (ED10 MPD/ED1 MPD) are marginally different among six brain regions with χ^2 (5,427)=10.73 (p=0.06). The proportion of neuronal units responding to chronic 2.5 mg/kg MPD recorded from behaviorally tolerant animals were the most responsive in PFC (73%), and the least responsive in VTA (44%). The ratios on neuronal units responding to MPD with increased *vs.* decreased firing rates were significantly different among the six regions, with χ^2 (5,248)=19.76 (p=0.001). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased firing rates in the PFC was significantly lower than the average of all other regions (adjusted p-value=0.015).

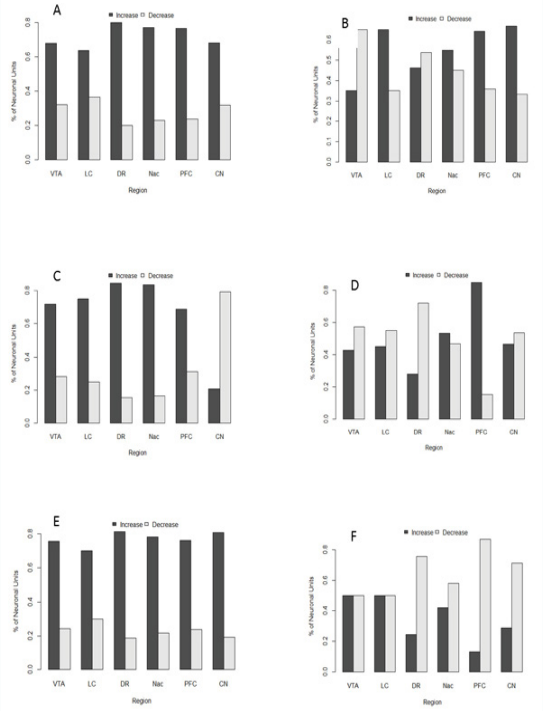

Figure 6: Neuronal Response to 2.5 mg/kg MPD: Figure 6 summarizes the percentage of neuronal units in six regions responding to 2.5 mg/kg MPD with increased or decreased significant firing rates. The figures on the left-hand side (6A, 6C, 6E) represent responses from those animals expressing behavioral sensitization. The figures of the right-hand side (6B, 6D, 6F) represent responses from those animals expressing behavioral tolerance. The figures in the top row (6A, 6B) represent the responses following acute MPD (ED1 MPD/ED1 BL), those in the middle row (6C, 6D) represent the changes to the baseline responses (ES10 BL/ED1 BL), and those in the bottom row (6E, 6F) represents responses following chronic or rechallenge MPD (ED10 MPD/ED1 MPD).

Acute response to 10.0 mg/kg MPD in animals expressing behavioral sensitization (Figure 7A)

For animals expressing behavioral sensitization to chronic MPD, there were no significant differences among the proportions of neuronal units responding to acute treatment of MPD in six brain

regions. In all six regions, more than 80% of the neuronal units had significant changes in response to MPD acute treatment.

The ratios of neuronal units responding to MPD with increased *vs.* decreased firing rates are significantly different among six brain regions, with χ^2 (5,491)=33.55 ($p<0.0001$). The ratio of neuronal units with increased *vs.* decreased firing rates is significantly lower in the DR (adjusted p-value <0.0001), but significantly higher in the VTA (p=0.01), compared to the average of all other regions.

Acute response to 10.0 mg/kg MPD in animals expressing behavioral tolerance (Figure 7B): The neuronal recordings from animals expressing behavioral tolerance to chronic MPD, showed the proportion of responding neuronal units to acute MPD treatment are significantly different among six brain regions with χ^2 (5,357)=19.99 (p=0.001). The proportion of neuronal units responding to MPD acutely in the DR (67%) was significantly lower compared to the average of all other regions (adjusted p-value=0.02).

The ratios of neuronal units responding to MPD with increased *vs.* decreased firing rates was significantly different among six brain regions, with χ^2 (5,284)=26.60 ($p<0.0001$). The ratio of neuronal units with increased *vs.* decreased firing rates is lower in the DR adjusted p-value=0.002), compared to the average of all other regions.

Baseline activity to 10.0 mg/kg MPD in animals expressing behavioral sensitization (Figure 7C): The neuronal recordings from animals expressing behavioral sensitization to chronic MPD, exhibited no significant differences among the proportions of neuronal units responding to MPD at baseline in six brain regions. In all six regions, more than 85% of neuronal units have a significant change responding to MPD acute treatment.

We also compared the ratio of neuronal units responding to MPD with increased *vs.* decreased firing rates among six different brain regions. We found that the ratios are significantly different among these regions with χ^2 (5,525)=17.16 (p=0.004). The ratio of neuronal units with increased *vs.* decreased firing rates is significantly lower in the DR (adjusted p-value=0.01).

Baseline activity to 10.0 mg/kg MPD in animals expressing behavioral tolerance (Figure 7D): The proportion of neuronal units that changed their baseline activity after six repetitive MPD exposures and three washout days were marginally different among the six brain regions with χ^2 (5,357)=11.60 (p=0.04). The proportion of neuronal units that changed their BL was significantly lower in the NAc (62%) when compared to the average response rates of all other regions (adjusted p-value 0.01).

In addition, we found that the ratios of neuronal units exhibiting increased *vs.* decreased firing rates was significantly different among these regions, with χ^2 (5,273)=64.48 ($p<0.0001$). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased firing rates was significantly lower in the DR ($p<0.0001$), but significantly higher in the CN (p=0.0001), when compared to the average of all other regions.

Response to 10.0 mg/kg MPD rechallenge in behaviorally sensitized animals (Figure 7E): For animals expressing behavioral sensitization to chronic MPD, there were no significant differences among the proportions of neuronal units responding to MPD rechallenge in six brain regions. In all six regions, more than 80% of neuronal units had a significant change responding to chronic MPD treatment.

The ratios of neuronal units responding to MPD rechallenge with increased *vs.* decreased firing rates are significantly different among six different brain regions, with χ^2 (5,568)=65.69 ($p<0.0001$). The ratio of neuronal units with increased *vs.* decreased firing rates is significantly lower in the DR (adjusted p-value<0.0001), but significantly higher in the NAc (p=0.01).

Response to 10.0 mg/kg MPD rechallenge in behaviorally tolerant animals (Figure 7F): The proportion of responding neuronal units to MPD at baseline was marginally different among six brain regions with χ^2 (5, 357)=32.13 ($p<0.0001$). The proportion of neuronal units that responded to MPD was significantly lower in the DR (55%) (Adjusted p-value <0.0001), when compared to the average of all other regions.

The ratios of neuronal units responding to MPD with increased *vs.* decreased firing rates was significantly different among the six brain regions, with χ^2 (5, 280)=19.58 (p=0.002). Further post hoc comparison indicated that the ratio of neuronal units with increased *vs.* decreased firing rates in the region CN is significantly lower than the average of all other regions (Adjusted p-value=0.014).

Discussion

The mesocorticolimbic catecholaminergic system is the neuronal circuit recognized as the primary circuit involved in reinforcement learning and is influenced by many drugs of abuse including alcohol,

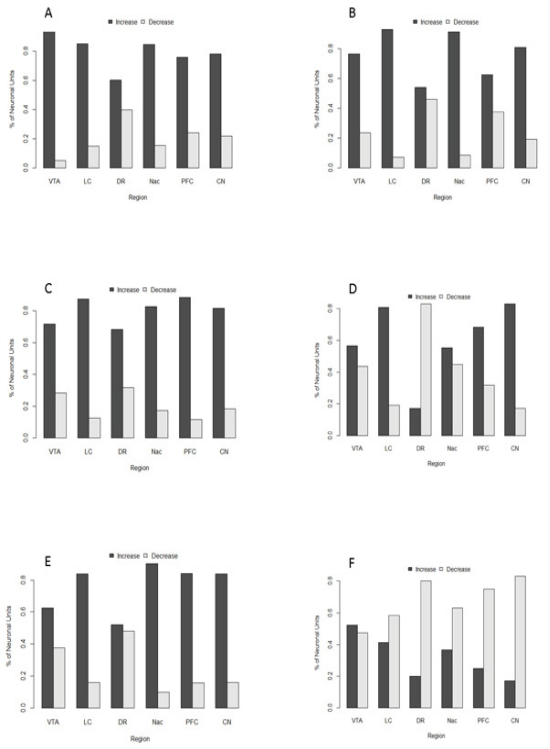

Figure 7: Neuronal Response to 10.0 mg/kg MPD: Figure 7 summarizes the percentage of neuronal units in six regions responding to 10.0 mg/kg MPD with increased or decreased significant firing rates. The figures on the left-hand side (7A, 7C, 7E) represent responses from those animals expressing behavioral sensitization. The figures of the right-hand side (7B, 7D, 7F) represent responses from those animals expressing behavioral tolerance. The figures in the top row (7A, 7B) represent the responses following acute MPD (ED1 MPD/ED1 BL), those in the middle row 7C, 7D) represent the changes to the baseline responses (ES10 BL/ED1 BL), and those in the bottom row (7E, 7F) represents responses following chronic or rechallenge MPD (ED10 MPD/ED1 MPD).

cocaine, and methamphetamines [27,44,60,84]. This system includes the VTA, LC and DR where DA, NE and 5HT neurons project to the NAc, CN and PFC, and participate in motivation, memory, cognition, and learning. The NAc is believed to play a strong role in mediating addictive behaviors, especially the Medium Spiny Neurons (MSNs) which express either D1 or D2 receptors and result in neuronal excitation or inhibition, respectively [22,46,85,86]. The PFC has also been shown to provide inhibitory feedback and modulation on the activity of the neurons in both the VTA and the NAc. And, while the catecholaminergic pathways are the most well-known to contribute to drug addiction and behavior, there are also studies that suggest the serotonergic systems, such as those in the DR, mitigate addiction and cravings as well [33,40,41].

Methylphenidate, one of the most used drugs for the treatment of ADHD [8], it affects the reward pathway by inhibiting the reuptake of DA, NE and 5HT from the synaptic cleft, thereby prolonging and increasing the amounts of these neurotransmitters available in the post-synaptic cleft [8,16,74,80,81]. The LC, VTA, DR, NAc, PFC and CN have all demonstrated participation in response to MPD with direct correlations between the behavioral activity of the animals and the neuronal firing rates in the given area. That is, when the majority of their neurons respond to the drug rechallenge as compared to the initial MPD effects by excitation, behavioral sensitization was observed and *vice versa* [16,46,70,74,80,81,87]. When each of the six brain areas was analyzed separately in previous studies, it appears that each of the six different brain areas had similar responses to MPD and similar expressions between behavioral and neuronal activities. The goal of this study was to record simultaneously the neuronal activities from all six of the above brain areas and statistically analyze the response in each area based on their behavioral responses to chronic MPD and compare them to each other to determine if their responses to acute and chronic MPD are similar or if each area plays a different role in response to the drug.

The main findings of the study are that in the above six brain areas 1) MPD exposure elicits increases in locomotor behaviors and neuronal activity in dose response characteristics; 2) the same chronic exposure of MPD as compared to acute 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD, elicits in some animals behavioral and neuronal sensitization and in others behavioral and neuronal tolerance; 3) when the neuronal recordings are evaluated based on the animals' behavior, most of the neurons that were recorded from animals expressing behavioral sensitization respond with further increases in firing rates, and most of the neurons that were recorded from animals expressing behavioral tolerance respond with decreases in their firing rates, as compared to the initial response to MPD exposure. 4) each of the six brain areas studied (VTA, LC, DR, NAc, PFC, and CN) respond significantly differently to MPD, suggesting that each of the above brain areas have a different role in the response to MPD and the DR neuronal units were the most affected by MPD exposure; 5) this study demonstrates that it is essential to evaluate neuronal activities based on the animals' behavioral responses from several brain areas simultaneously to obtain accurate information about the response to a drug; and, 6) MPD elicits symptoms that are characteristic of substance abuse disorders (sensitization and tolerance), and therefore MPD has the potential to elicit dependency.

Overall, the acute dose response effect of MPD was significantly different in the six brain areas we investigated. Moreover, when comparing ED10 BL to ED1 BL after six daily 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD doses and three washout days, the neuronal activity recorded from behaviorally sensitized animals exhibited mainly increases in their ED10 BL neuronal activity compared to ED1 BL activity. On the other hand, the neuronal activity recorded from behaviorally tolerant animals exhibited mainly decreases at ED10 BL activity compared to ED1 BL. These are similar to the findings in the previous studies that study each one of the above six brain area separately that there are direct correlations between the neuronal response direction (increase or decrease) and the behavioral response, i.e., behaviorally sensitized animals' express mainly increases in neuronal firing rates and behaviorally tolerant animals' express mainly decreases in neuronal firing rates on the above six brain areas respectively. There were, however, significant differences between the six brain areas in the intensity of the neuronal firing rates and the number of neurons in percentage, that modulate their firing rates as result of the six daily MPD and three wash out days on ED10 BL/ED1 BL.

Despite confirming the correlations between the behavioral and neuronal activities for the six areas, there are new findings in this study that demonstrate significant differences to acute as well to chronic MPD between the responses in each brain area when they are recorded together as compared to when each of the brain areas are studied alone. In general, the percentage of significant responsiveness to the three MPD doses, as well as the direction of the response. That is, increasing or decreasing their firing rates demonstrated statistically significant differences for all the six brain areas when comparing the recordings obtained from behaviorally sensitized to behaviorally tolerant animals. In the 0.6 mg/kg groups, the neuronal recordings from behaviorally sensitized animals had the most responsive units in number (in the percentage) of neuronal units affected significantly by MPD in the recording from the DR and least responsive units in the CN, LC, and VTA, respectively. For the neuronal recordings from behaviorally tolerant animals to the 0.6 mg/kg MPD dose, the most responsive units were in the LC and CN while the least responsive units were in the NAc and PFC. In conclusion, based on their neuronal responses to 0.6 mg/kg MPD, each of the six brain areas plays a different role in the acute response to 0.6 mg/kg MPD. Following acute and chronic MPD, the DR neuronal recordings obtained from behaviorally sensitized animals were the most neurons that were modulated significantly by all the three MPD doses used, while the recording for the behaviorally tolerant animals the acute response to MPD elicits mostly modulation of the LC neurons and the chronic response to MPD was mostly modulate the neuronal recording from the CN in response to the 0.6 mg/kg MPD dose group.

For the recordings following 2.5 mg/kg MPD groups, those obtained from behaviorally sensitized animals show that the DR neurons were more active than the neurons in the other five brains areas, while the neuronal recordings from the behaviorally tolerant animals showed that the neurons recorded from the PFC, LC and NAc exhibited the highest responsiveness to 2.5 mg/kg MPD.

For the 10.0 mg/kg MPD groups, the neuronal recordings from behaviorally sensitized animals were highly responsive to the drug in all six brain areas without significant differences between them. However, the neuronal recordings from behaviorally tolerant animals indicate that most of the responsive units were in the VTA, LC, and PFC, while significantly less responsive units were in the DR. Overall, in the 10.0 mg/kg MPD groups, all the animals exhibited high levels of responsiveness in every brain area except that behaviorally tolerant

animals showed significantly lower responsiveness in DR. These findings suggest that the 0.6 and 2.5 mg/kg MPD are physiological doses, and that these doses elicit in each of the six brains areas different responses which suggests that each site has a different role in the response to MPD, and the behavioral expression is the result of activation of all of these brain areas, with the DR having the highest response for the behaviorally sensitized animals. The 10.0 mg/kg MPD elicits in all the six brain areas similar effects, indicating that this dose is a mega dose, and the effects are not specific between the structures or the behaviorally sensitized and tolerant animals.

In summary, the observations indicate that the responses to MPD in each brain structure are different from each other, suggesting that each of the six different brain areas respond differently to MPD. The effect of the lower (0.6 mg/kg MPD) and the middle (2.5 mg/kg MPD) doses exert different response patterns in each of the VTA, LC, DR, and NAC. PFC and CN structures while the MPD high dose (10.0 mg/kg MPD) exerts similar effects on all six of the brain structures. Therefore, we suggest that each of the above six brain areas has a different role in the response to MPD and the behavioral expression following acute and chronic MPD is the summation of push-pull interactions between these brain areas. This observation is with agreement that the calming effect of MPD on ADHD patients is at least in part due to the effect of MPD on the DR and the 5HT system [10], i.e., the serotonergic system exert significant role in the effects of MPD.

Conclusion

The DR neuronal units were the most affected by acute and chronic 0.6 mg/kg and 2.5 mg/kg MPD as compared to the neuronal units recorded from VTA, LC, NAc, PFC and CN neurons. While following 10.0 mg/kg MPD in general all the six brain area units were similarly affected. However, the number of neuronal units that responded by increased or decreased firing rates was significantly different among the six brain areas.

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