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CAUDATE NUCLEUS NEURONAL RECORDINGS IN FREELY BEHAVING SD MALE RATS AGE DEFERENT VARIATION IN RESPONSE TO DOPAMINE AGONIST METHYLPHENIDATE (RITALIN): INTEGRATED BEHAVIORAL

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ABSTRACT
The study explores age-related differences in response to acute and chronic dopamine (DA) agonist methylphenidate (MPD) doses (0.6, 2.5, and 10.0 mg/kg) in freely behaving young and adult male rats. Simultaneous recordings of caudate nucleus (CN) neuronal activity and locomotor behavior aim to discern age-related variations in MPD response and potential correlations between behavioral expression and CN neuronal activity. The investigation reveals that chronic MPD administration at 0.6, 2.5, and 10.0mg/kg doses induces behavioral and electrophysiological tolerance in some animals and sensitization in others, compared to initial MPD dosages. Notably, significant differences exist in CN neuronal responses and behavioral expressions between the two age groups, highlighting age-dependent variations in responses to the DA agonist MPD exposure. Correlations were observed between the behavioral responses to varying MPD doses and the direction of CN neuronal response (excitation or attenuation) to the drug. These findings underscore the importance of evaluating drug effects on specific brain regions based on the animals' behavioral responses, particularly regarding chronic effects like sensitization and tolerance. Moreover, significant differences in both behavioral and electrophysiological responses were noted between young and adult rats in response to MPD. These distinctions underscore the necessity for more in-depth investigations to elucidate the impact of MPD on the developing brain and its potential long-term effects on neuronal function. The implication of this study is the critical need to assess neuronal responses within specific brain regions based on the behavioral responses exhibited by animals, particularly in the context of chronic MPD effects such as sensitization and tolerance. Understanding age-dependent variations is pivotal for comprehending the nuanced impact of MPD on neuronal function and behaviors.

KEYWORDS: Methylphenidate, behavior, neuronal recording, VTA, LC, DR, NAc, CN, & PFC, young, adult.

1. INTRODUCTION
Attention deficit hyperactivity disorder (ADHD) has witnessed a substantial surge in diagnosis and prevalence over the last decade (Cortese 2020; Jaeschke et al., 2021; Klein et al., 2019; Somkuwar et al., 2016). Methylphenidate (MPD), a psychostimulant, stands out as the primary pharmacological intervention for ADHD in both adolescents and adults (Accardo and Blondis, 2001; Ching et al., 2019; Rafal et al., 2021; Safer, 2016; Storebø et al., 2015; Wilens, 2008). However, the escalating diagnosis rates of ADHD have led to a parallel increase in both legal and illicit use of MPD (Bogle and Smith, 2009; Fond et al., 2016; Kim et al., 2020; Shin et al., 2023). MPD functioning as an indirect dopamine agonist, MPD binds to the dopamine transporter (DAT), impeding the reuptake of dopamine molecules from the synaptic cleft to the presynaptic terminals (Kuczenski & Segal 1997; Neugebauer et al., 2004; Wang et al., 2013). This mechanism contributes to the buildup of dopamine in the synaptic cleft, activating the brain's motivation and reward centers and potentially alleviating ADHD symptoms (Cooper et al., 2017; Neugebauer et al., 2004).

As the medical use of MPD has increased, so has its presence in the public The literature reveals MPD's growing role as a cognitive enhancement aid for children, college students, and professionals (Klein et al., 2019; Smith and Farah, 2011; Thanos et al., 2015; Volkow and Swanson 2008). The augmented usage of MPD in both young and adults has prompted a keen interest in investigating its effects, particularly considering the ongoing development of the central nervous system (CNS) in youngsters (Cohen et al., 2015; Marco et al., 2011; Wilcox et al., 2022). Previous

research indicates that chronic exposure to MPD at varying doses can result in either behavioral sensitization or tolerance in animals (Claussen and Dafny, 2015; Karim et al., 2017; 2018; Kharas et al., 2017; King et al., 2019; Venkataraman et al., 2019, 2020). This suggests potential genotypic variations influencing how the body adapts to psychostimulants (Zhang et al., 2023). Moreover, psychostimulants like MPD induce cross-sensitization with other stimulant, indicating an increased risk for polysubstance abuse (Bonate et al., 1997; Kharas et al., 2019; Thanos et al., 2015). This is of particular concern in young whose frontostriatal connections are still developing, and increased frontostriatal connectivity due to previous drug abuse may increase future drug abuse tendencies (Dafny and Young 2006; Koob and LeMoal, 2006). For these reasons, the effect of MPD on young and adult behavior and CN neural activities was made the focus of this study. While stimulants broadly impact the brain's reward circuit, this study zooms in on the caudate nucleus (CN). The CN, implicated in the pathophysiology of ADHD, exhibits a smaller volume in ADHD patients compared to controls (Schrimsher et al., 2002). Recent studies even suggest the possibility of predicting ADHD symptomatology in children based on CN asymmetry (Schrimsher et al., 2002). The CN is rich in dopaminergic neurons, thus the CN becomes a focal point for psychostimulants and addictive behaviors (Beckstead et al., 1988; Cooper et al., 2017). Its extensive connections, particularly with the prefrontal cortex, are crucial in understanding ADHD behaviors and drug addiction (Arnsten, 2009).

This focus is particularly pertinent in young, where developing frontostriatal connections coupled with previous drug abuse may elevate future drug abuse tendencies (Faraone et al., 2020; Klein et al., 2024; Koob and LeMoal, 2006). Hence, this study delves into the impact of MPD on both young and adult behavior and CN neural activities. This is of particular concern in young whose frontostriatal connections are still developing, and increased frontostriatal connectivity due to previous drug abuse may increase future drug abuse tendencies (Faraone et al., 2020, 2006; Klien et al., 2024; Koob and LeMoal, 2006). For these reasons, the effect of MPD on young and adult behavior and CN neural activities was made the focus of this study.

The study hypotheses are as follows: 1) Repetitive MPD administration will result in behavioral sensitization in some animals and behavioral tolerance in others; 2) The ratio of rats developing sensitization versus tolerance will significantly differ between young and adults; 3) Neuronal responses to chronic MPD will differ between animals expressing sensitization and tolerance; 4) Recorded neural responses of the CN will significantly differ between young and adult subjects. Notably, partial findings from young (Karim et al., 2018) and adult (Claussen and Dafny, 2015) observations have been previously reported.

2. METHODS

2.1 Animals: A total of 163 young and 174 adult Sprague-Dawley male rats were acquired at a post-natal age of 30 days (P30) for adolescents and 50 days (P-50) for adults. The rats were individually housed in enriched clear acrylic cages, serving both as home and testing environments. Utilizing the same cage for both purposes aimed to eliminate potential confounding variables that could influence the study's data. Following placement in their home cages, the rats were allowed 3-5 days to acclimate to a 12:12 h light/dark cycle (lights on at 06:00 h), with ad libitum access to food and water. Electrodes were bilaterally implanted into the Caudate nucleus (CN) under anesthesia, following a recovery period of 4-7 days. The experimental protocol was approved by the Animal Welfare Committee and adhered to the National Institute of Health Guide for Care and Use of Laboratory Animals.

2.2 Surgeries: Four Teflon insulated (except at the tips), 60-um diameter, Nickel-Chromium diamel wires were used as recording electrodes. Each of these electrodes was then secured to a 1 cm copper connector pin (A-M Systems, Inc.). Upon successful implantation, the pins were secured to an Amphenol plug, which was then secured to the skull with dental cement. Young rats were anesthetized with an intra-peritoneal (i.p) injection of 30mg/kg pentobarbital, while the adult rats were anesthetized with an i.p injection of 50 mg/kg pentobarbital. Their heads were then shaved and numbed with lidocaine hydrochloride topical gel. The rats were then placed on a stereotaxic instrument where an incision was made on the scalp and the skin, connective tissue, and muscle were removed to expose the skull. Bilateral holes were drilled above the CN at 0.5 mm anterior to bregma and 3.0 mm lateral from midline for adults and 0.5 mm anterior to bregma and 2.5 mm lateral from midline for young. Coordinates for the young rats were obtained from Sherwood and Timiras (1970) Adolescent Rat Brain Atlas. Coordinates for the adult rats were obtained from Paxinos and Watson’s (1986) Brain Atlas. Six anchor screws were inserted into the perimeter of the skull to help secure the Amphenol plug-in position and prevent accidental removal. One electrode (1.0mm in diameter) was implanted in front of the frontal sinus and served as a ground electrode. To observe the ongoing neuronal activity, the remaining four electrodes were inserted into the CN (two per hemisphere) at an initial depth of 3.2 mm for the young rats and 3.5 mm for the adult rats and connected to an oscilloscope and a Grass P511 series amplifier with its emitter Hi Z Probe close to the head of the rat. If the observed activity displayed a 3:1 signal-to-noise ratio, the electrode was secured to the skull using web glue cyanoacrylate surgical adhesive. However, if the ratio of the neuronal activity was less than 3:1, the electrode was inserted deeper in 5-10 um increments until the desired neuronal activity ratio was obtained. This procedure was repeated for the remaining electrodes. The copper pins securing the electrodes were then inserted into the Amphenol plugs, which were then

cemented to the skull using dental cement. Post-surgery, the rats were taken back to their home cage and given 4-7 days to recover under supervision. During the recovery days, the animals, along with their home cages, were placed into the experimental apparatus for 2-3 hours and connected to a wireless head-stage transmitter for acclimation to the experimental system.

2.3 Experimental apparatuses: Two different apparatus systems were used to simultaneously record neuronal and behavioral activity. The neuronal activity was monitored and recorded using a wireless head stage (Triangle BioSystems Int'l [TBSI], Durham, NC, USA) connected to the Amphenol plug on the head of each rat. The wireless TBSI head stage (4.5g) sent neuronal activity signals to the TBSI receiver, which was connected to a Cambridge Electronic Design (CED) analog-to-digital converter (Micro1401-3; Cambridge, England). This converter digitized the analog data to be collected and stored on a computer using Spike 2.7 CED software.

In addition to the TBSI recording neuronal activity signals, the study used an open-field computerized animal activity system (AccuScan system - Columbus, Ohio) located in the Faraday testing cage. It was used concomitantly to record the animal's behavioral locomotion (Claussen & Dafny 2015; Karim et al., 2028; Medina et al., 2022a & b) and its CN neuronal activity (Claussen & Dafny, 2015; Venkataraman et al., 2017, 2019, 2020). The animal's home cage fits into the behavioral recording apparatus, allowing for concomitant recording of the animal's behavior and CN neuronal activity. The open field system contained 16 x 16 infrared beams with sensors on the opposite side, five centimeters above the floor of the cage. Each time the rats crossed any of the infrared beams, it was counted by the AccuScan Analyzer, counting the breaking light beam movement, and the Oasis program then analyzed and calculated the beam disruption as a number of movements (NOM) and number of stereotypy (NOS) activity of the rats (Gaytan et al., 1997; 2000; Yang et al., 2003; 2006a, b, and c; 2011a and b). The NOM recorded the overall locomotor activity while the NOS activity counted the repetitive movements. Repetitive movements are described as movements with at least one second between each episode (Claussen & Dafny, 2015).

2.4 Experimental Protocol: On experimental day one (ED1), the rats were placed with their home cages in a Faraday testing cage, which served to reduce excess "noise" during the recording period. Prior to the recording sessions, the rats were given a twenty to thirty-minute period for acclimation during which the injected drug was prepared freshly, neuronal activity was monitored, and software parameters to capture the neuronal activity were set. Each age group (young and adult) was divided into the following four groups: saline (control) group, 0.6 mg/kg, 2.5 mg/kg, and 10.0 mg/kg MPD groups, respectively (Table 1). On ED1, all rats received an initial 0.8 mL saline injection. Upon injection, behavioral and electrical events were simultaneously recorded for sixty minutes to establish a baseline value (ED1 BL). This injection and recording were followed by either another saline injection to the control groups or an MPD injection of 0.6 mg/kg, 2.5 mg/kg, or 10.0 mg/kg doses to the respective MPD groups (Table 1). The behavioral and neuronal activity of the rats were recorded for an additional hour following the second injection. At the conclusion of the ED1 experiment, the rat was returned to the vivarium while remaining in its home cage. On experimental days two through six (ED2 – ED6), the animals received either a saline injection or an MPD injection of 0.6 mg/kg, 2.5 mg/kg, or 10.0 mg/kg doses (Table 1) while in their home cages without recording. On experimental days seven through nine (ED7 – ED9), the animals received no injections. This period is considered the "wash-out" period. On the final day of experimentation (ED10), the ED1 treatment and recording were repeated i.e., the rats received a 0.8 mL saline injection followed by either another saline injection or an MPD injection with neuronal and behavioral activity recordings an hour before and after the second injection (Table 1).

2.5 Drugs: Methylphenidate hydrochloride (MPD) was donated by Mallinckrodt (Hazelwood, MO, USA). MPD was administered at varying doses of 0.6, 2.5, and 10.0 mg/kg to both the young rats and the adult rats. These doses were selected based on previous dose-response studies (Gaytan et al., 1997; Kharas et al., 2019; Yang et al., 2006a, b, and c). Selected doses were prepared by dissolving the MPD salt in a 0.9% isotonic saline solution. The control injections consisted of 0.8 mL of 0.9% isotonic saline solution, the same solution used to prepare the MPD doses. All injections were equalized to 0.8 mL and were administered intraperitoneal (i.p) in the morning.

2.6 Histological Verification of Electrode Placement: Once the recording session of ED10 was completed, the animals were overdosed with sodium pentobarbital and then infused with 10% formaldehyde solution containing 3% potassium ferrocyanide. Next, a 20 mA DC current was passed through each electrode for 20 s to create a small lesion that would be used to identify the location of the implanted electrode tip. The brain was then removed from the rat's skull and set in 10% formaldehyde for a few days. Once removed from the solution, the brain was sliced into 40 um sections, stained with Cresol violet, and studied to verify that the tip of the electrode was properly implanted in the CN, as labeled by the lesion and the blue spot. Data evaluation from a specific electrode was considered valid if the electrode was correctly positioned in the CN and if the spike signals recorded on ED1 and ED10 from the same electrode displayed similar amplitudes and wave patterns.

2.7 Behavioral Analysis: The locomotor activity (NOM and NOS activity) was analyzed in both young and adult rats. Locomotor activity data were collected and

aggregated into 10-minute bins for a total of 60 minutes (6 bins/hr.) following saline injection or MPD administration using the OASIS software. Three comparisons were conducted for both young and adult rat groups: **1.** Acute MPD Effect – Locomotor activity following MPD administration on ED1 was compared to behavioral recordings following saline injection on ED1 (ED1 MPD/ED1 BL) to determine the initial effect of MPD. **2**. Baseline Changes – Locomotor activity following saline administration on ED10 BL, after six daily MPD injections and three washout days, was compared to behavioral activity following saline administration on ED1 (ED10 BL/ED1 BL) to determine if the drug elicited withdrawal behavior. **3**. Chronic MPD Effect – Locomotor activity following MPD rechallenge on ED10 was compared to behavioral activity recorded following MPD on ED1 (ED10 MPD/ED1 MPD) to determine if the six daily MPD injections elicited behavioral sensitization or tolerance. For each comparison, the critical ratio test C.R. = $\frac{E-C}{\sqrt{E+C}}$ = ± 1.96 = $p < 0.05$, where C is the control and E is the drug activity, was employed to identify behavioral sensitization or tolerance. Behavioral sensitization was defined statistically as a C.R. test value greater than 1.96, indicating significantly increased activity ($p < 0.05$). Behavioral tolerance was defined statistically as a C.R. value less than -1.96, indicating significantly decreased activity or no significant change in behavioral activity. After the C.R. test, animals were categorized into three subgroups: **a)** all animals, b) animals exhibiting behavioral sensitization, and **c)** animals exhibiting behavioral tolerance. Each subgroup was analyzed using the analysis of variance (ANOVA) test, followed by post-hoc Tukey analysis or Wilcoxon rank sum test, as appropriate, to compare subgroups (all, sensitized, or tolerant) and age groups (adults and young) for a given MPD dose (0.6, 2.5, and 10.0mg/kg). The significance for all comparisons was set at $P < 0.05$.

2.8 Electrophysiological Analysis

2.8.1 Spike Sorting: Spike 2 version 7 software (Cambridge Electronics Design – CED) was used for spike sorting, capturing data at sampling rates of up to 200 kHz. The program processed data with low and high pass filters (0.3 – 3 kHz) and used two window discriminator levels for positive and negative spikes (Fig. 1). Discriminated spikes were sorted based on amplitude and waveform, using 1000 waveform data points to define the desired spike pattern and amplitude. The algorithm ensured accurate spike sorting despite noise, false threshold crossing, and waveform overlap, yielding a sorting accuracy close to ninety-five percent (95%). Parameters used for spike sorting at ED1 were stored and applied at ED10 for consistency. Sorted neuronal activity was counted for each 60-minute segment and used for further analysis.

2.8.2 Electrophysiological Data Evaluation: A spreadsheet containing all electrophysiological data, including rat identity number, experimental day, MPD dosage, electrode numbers, and average firing rate based on 15-second interval bins for 60 minutes, was generated. The spreadsheet facilitated the creation of a histogram displaying sequential firing rates (Figure 2). Each 60-minute segment of neuronal activity was statistically compared. ED1 MPD counts were compared to the 60-minute segment following saline injection at ED1 (ED1 MPD/ED1 BL) to assess the MPD acute effects (Table 2&3). ED10 BL counts were compared to ED1 BL counts (ED10 BL/ED1 BL) to determine if consecutive six days of MPD exposure followed by three washout altered the ED10 BL. Additionally, ED10 MPD counts were compared to ED1 MPD counts (ED10 MPD/ED1 MPD) to calculate the chronic effect of MPD.

Previous studies have shown that daily saline injections for 42 days do not alter behavioral (Wilcox et al., 2022), locomotor, or electrophysiological activity. Thus, saline injections at ED1 were used as a control. To determine if MPD induced a significant response, the critical ratio test and Pearson's Chi-squared test ($p < 0.05$) were used to assess differences in neuronal activity recorded from young and adult rats activity (Claussen & Dafny, 2015; Jones & Dafny, 2014; Medina et al., 2022a & b; Venkataraman 2017, 2019, 2020).

3.0 RESULTS

3.1 Behavioral Results: A total of 337 animals were included, comprising 163 young and 174 adults. The young animals were divided into treatment groups of 15, 48, 51, and 59, and were treated with saline (control), 0.6 mg/kg MPD, 2.5 mg/kg MPD, and 10.0 mg/kg MPD, respectively. As for the adults, they were divided into groups of 15, 53, 57, and 49, and were treated with saline (control), 0.6 mg/kg MPD, 2.5 mg/kg MPD, and 10.0 mg/kg MPD, respectively (Fig. 3).

3.1.1 Effect of saline on behavioral activity (Figure 3A & 3B): Fifteen young animals and 15 adult animals were used as controls (saline). Minimal, nonsignificant changes in the number of movements (NOM) or the number of stereotypic (NOS) activities were observed following acute and repetitive saline exposure using post-hoc Tukey ($p < 0.05$). This pattern of minimal fluctuation was seen in both young and adults. It can thus be concluded that the NOM and NOS activities recorded on ED1 after saline administration can be used as baseline (BL) values when assessing the acute and chronic effects of MPD in both young and adult rats.

3.1.2 Effect of MPD (Fig. 3C-H): Both young and adult rats showed changes in NOM and NOS activity after acute and chronic exposure to 0.6, 2.5, or 10.0 mg/kg MPD. Animals were categorized into those exhibiting behavioral sensitization or tolerance. Sensitization involved a significant increase in activity, while tolerance indicated no significant change or a decrease (FIG. 3C-H) as compared to the initial MPD effects.

3.1.3 Effect of acute and chronic 0.6 mg/kg MPD on behavioral activity of all animals (Figures 3C & 3D. All): Fifty-three adults (Figure 3D) and 48 young rats (Figure 3C) were treated with 0.6 mg/kg MPD. When comparing adult and young animals, acute MPD exposure (ED1 MPD/ED1 BL) led to a significant ($p < 0.05$) increase in behavioral activity in both age groups, whereas chronic MPD exposure (ED10 MPD/ED1 MPD) after six daily MPD exposures and three washout days led to no significant difference compared to acute MPD in both age groups (Figs 3C & 3D All).

3.1.4 Effect of acute and chronic 0.6 mg/kg MPD on animals expressing either behavioral sensitization or tolerance (Figures 3C & 3D. Sensitized and Tolerant): Of the rats that were treated with 0.6 mg/kg MPD, similar numbers of adult (74%; 39/53) and young (81%; 39/48) animals developed behavioral sensitization (Figures 3C & 3D. Sensitized). In adult animals, similar locomotor activity was observed across acute (ED1 MPD/ED1 BL), baseline (ED10 BL/ED1 BL), and chronic (ED10 MPD/ED1 MPD) MPD exposure (Figure 3A. Sensitized). Young animals, however, had a significant ($p < 0.05$) increase in behavioral activity upon acute MPD exposure (ED1 MPD/ED1 BL) and a further significant ($p < 0.05$) increase in behavioral activity upon chronic MPD exposure (ED10 MPD/ED1 MPD) (Figures 3C. Sensitized). This further increase suggests that behavioral sensitization was developed.

Among the group of animals found to exhibit behavioral tolerance upon 0.6 mg/kg MPD exposure, acute MPD exposure (ED1 MPD/ED1 BL) elicited a significant ($p < 0.05$) increase in both age groups. Baseline (ED10 BL/ED1 BL) and chronic MPD exposure (ED10 MPD/ED1 MPD) were significant ($p<0.05$) different between adult and young animals (Figures 3C & 3D. Tolerant).

3.1.5 Effect of acute and chronic 2.5 mg/kg MPD on the behavioral activity of all animals (Figures 3E & 3F. All): Fifty-one young (Fig. 3E All and 57 adults (Figure 3F. All) rats were treated with 2.5 mg/kg MPD. The overall NOM in response to the 2.5 mg/kg MPD dose was compared between all adult and all young rats and exhibited significant ($F = 954$, $p < 0.001$) different. Compared to young rats, adult rats displayed a significantly ($p < 0.05$) greater increase in NOM and NOS activity in response to acute (ED1 MPD/ED1 BL) and chronic (ED10 MPD/ED1 MPD) administration of 2.5 mg/kg MPD (post-hoc Tukey test, $p < 0.05$).

3.1.6 Effect of acute and chronic 2.5 mg/kg MPD on animals expressing behavioral sensitization or tolerance (Figures 3E & 3F. Sensitized and Tolerant): Of the rats that were treated with 2.5 mg/kg MPD, 68% of adult rats (39/57) and 65% of young rats (33/51) developed behavioral sensitization (Figures 3E and 3F. Sensitized). Both adult (Figure 2F. Sensitized) and young (Figure 3F. Sensitized) animals started with similar baseline behavioral activity recorded on ED1 (ED1 BL). However, both acute (ED1 MPD/BL) and chronic (ED10 MPD/ED1 MPD) MPD exposure led to a more significant ($p < 0.05$) increase in behavioral activity in adult animals as compared to young animals. Additionally, behavioral activity recorded at ED10 after six daily MPD exposures and three washout days (ED10 BL) was significantly ($p < 0.05$) greater in young animals as compared to adult animals (Figures 3E & 3F. Sensitized). Thus, even though the behavioral activity recorded after MPD rechallenge on ED10 MPD was similar between adult and young animals, the magnitude of the change in behavioral activity after chronic MPD exposure (ED10 MPD/ED1 MPD) was significantly ($p < 0.05$) greater in adult animals.

Among behaviorally tolerant animals exposed to 2.5mg/kg MPD, baseline behavioral activity recorded at ED1 (ED1 BL) was similar between adults (Figure 3F. Tolerant) and young animals (Figure 3E. Tolerant). Acute (ED1 MPD/ED1 BL) and chronic (ED10 MPD/ED1 MPD) led to a more significant ($p < 0.05$) increase in behavioral activity in adult animals as compared to young animals. When comparing baseline (ED10 BL/ED1 BL) behavioral activity, young animals exhibited a significant ($p<0.05$) decrease in behavioral activity, whereas adult animals did not.

3.1.7 Effect of Acute and Chronic Administration of 10.0 mg/kg MPD on All Animals (Figures 3G & 3H. All): A total of 49 young (Figure 3G. All) and 49 adults (Figure 3H. All) were subjected to treatment with 10.0 mg/kg MPD. The overall NOM in response to the 10.0 mg/kg MPD dose was compared between all adult and young rats, revealing significant differences ($F = 4498$, $p < 0.001$). Adult rats, in comparison to their younger counterparts, exhibited a significantly greater increase in NOM and NOS activity following both acute and chronic administration of 10.0 mg/kg MPD (post-hoc Tukey test, $p < 0.05$).

3.1.8 Effect of Acute and Chronic Administration of 10.0 mg/kg MPD on Animals Expressing Behavioral Sensitization or Tolerance (Figures 3G & 3H): Out of the rats treated with 10.0 mg/kg MPD, an equal percentage (39%; 19/49) of adult and young animals developed behavioral sensitization. Young animals (Figure 3G Sensitized) exhibited a significantly greater increase in locomotor activity upon both acute (ED1 MPD/ED1 BL) and chronic (ED10 MPD/ED1 MPD) MPD exposure compared to adult rats (Figure 3G & H, Sensitized) ($p<0.05$). Young animals showed a significantly greater increase in behavioral activity at ED10 following six daily MPD exposures and three washout days (ED10 BL/ED1 BL) compared to adult animals (Figures 3G & 3H Sensitized) ($p<0.05$).

Similar patterns were observed among animals that developed behavioral tolerance. Adult animals (Figure 3G & H Tolerant) exhibited a significantly greater

increase in behavioral activity upon both acute (ED1 MPD/ED1 BL) and chronic (ED10 MPD/ED1 MPD) MPD exposure compared to young rats (Figure 3G. Tolerance) ($p<0.05$). Young animals displayed a significantly greater increase in baseline behavioral activity (ED10 BL/ED1 BL) compared to adult animals (Figures 3G & 3H. Tolerant) ($p<0.05$). In general, the effects of the three MPD doses on NOS were similar to the effects on NOM in both age groups.

3.2. Neurophysiological Results (Tables 2 & 3): A total of 1660 caudate nucleus (CN) neurons were recorded and evaluated, with 829 from adult animals and 831 from young animals, respectively. All neuronal recordings were histologically confirmed to be from the CN and exhibited similar spike waveform shapes and amplitudes on ED1 and ED10 (Tables 2 and 3). Ninety-eight, 219, 249, and 263 CN neurons were recorded from adult rats and evaluated following saline, 0.6, 2.5, and 10.0 mg/kg MPD exposure, respectively (Table 2). For CN neuronal activity recorded from young animals, 69, 257, 224, and 281 CN neurons were evaluated following saline, 0.6, 2.5, and 10.0 mg/kg MPD exposure, respectively.

3.2.1 Effects of Saline on CN Neurons: Ninety-eight and 69 CN neurons were recorded from adults and young rats following saline controls, respectively. Minimal changes in neuronal firing were observed following acute and repetitive saline exposure. This pattern of minimal fluctuation was observed in both young and adults, indicating that the injection of a solution and animal handling did not significantly affect CN neuronal activity. Therefore, it can be concluded that any significant changes in CN neuronal activity result from MPD administration.

3.2.2 Effect of 0.6 mg/kg MPD on CN Neurons Recorded from All Adult and Young Animals (Fig. 4A & 4B): A total of 219 CN neurons were recorded from adult rats, and 257 CN neurons were recorded from young rats exposed to 0.6 mg/kg MPD (Tables 2A and 3A). More adult CN neurons significantly ($p<0.05$) responded to 0.6 mg/kg MPD exposure compared to young CN neurons following acute ED1 MPD/ED1 BL and chronic ED10 MPD/ED1 MPD. There was no significant difference between adult and young animals in the percentage of neurons that responded to acute MPD (ED1 MPD/ED1 BL) (Fig. 4A) or chronic ED10 MPD/ED1 MPD (0.6 mg/kg MPD) in CN neuronal firing rate (Figures 4A & 4B).

3.2.3 Comparing the Effect of 0.6 mg/kg MPD on CN Neurons Recorded from Behaviorally Sensitized Animals (Tables 2B and 3B, and Figs 4C & 4D): A total of 202 CN neurons from young rats and 60 CN neurons from adults were recorded from behaviorally sensitized animals following 0.6 mg/kg MPD exposure (Tables 2B and 3B). Following acute 0.6 mg/kg MPD exposure, the majority of CN neurons recorded from adult animals (40/60; 67%) demonstrated an increase in firing rate, while the majority of CN neurons recorded from young animals (52/73; 71%) demonstrated a decrease in firing rate (Figure 4C). No significant differences were observed in CN neuronal responses between adult and young animals following chronic MPD (Figure 4D).

3.2.4 Comparing the Effect of 0.6 mg/kg MPD on CN Neurons Recorded from Behaviorally Tolerant Animals (Fig. 4E & 4F): A total of 159 CN neurons from adult rats and 55 CN neurons from young rats were recorded from behaviorally tolerant animals exposed to 0.6 mg/kg MPD (Table 2C & 3C). Upon 0.6 mg/kg, acute MPD exposure (ED1 MPD/ED1 BL), similar percentages of CN neurons recorded from adults (70%; 75/106) and young rats (74%; 23/31) exhibiting behavioral tolerance responded with an increase in neuronal firing rate (Figure 4E). Upon chronic 0.6 mg/kg MPD exposure (ED10 MPD/ED1 MPD), similar percentages of CN neurons were recorded from adults (73%; 115/157) and young (72%; 34/47) animals demonstrated a decrease in neuronal firing rate (Figure 3F).

3.2.5 Effect of 2.5 mg/kg MPD on CN Units Recorded from All Adult and Young Animals (Fig 4A & 4B): A total of 249 CN neurons were recorded from adults, and 254 CN neurons were recorded from young animals exposed to 2.5 mg/kg MPD (Tables 2A and 3A). Significantly ($p<0.05$), more CN neurons recorded from adults (Table 2A) were responsive to 2.5 mg/kg MPD exposure compared to CN neurons recorded from young rats (Table 3A). Following acute MPD (ED1 MPD/ED1 BL) Fig 4A, and chronic (ED10 MPD/ED1 MPD) MPD exposure (Fig 4B), there were no significant differences in the response direction (increase or decrease) between adult and young CN neurons (Fig 4A & Fig 4B).

3.2.6 Comparing the Effect of 2.5 mg/kg MPD on CN Neurons Recorded from Behaviorally Sensitized Animals (Fig 4C & 4D): Ninety CN neurons were recorded from adults, and 132 neurons were recorded from young behaviorally sensitized animals exposed to 2.5 mg/kg MPD (Tables 2B and 3B). Following acute 2.5 mg/kg MPD exposure, the majority of CN neurons (68%; 71/104) recorded from young exhibited an increase in firing rate, whereas the majority of CN neurons (57%; 42/74) recorded from adults exhibited a decrease in firing rate (Figure 4C). No significant difference was seen between adult and young CN neurons recorded following chronic (ED10 MPD/ED1 MPD) 2.5 mg/kg MPD exposure (Figure 4D).

3.2.7 Comparing the Effect of 2.5 mg/kg MPD on CN Neurons Recorded from Behaviorally Tolerant Animals (Fig 4E & 4F): A total of 144 CN neurons were recorded from adults and 92 neurons from young animals in behaviorally tolerant animals exposed to 2.5 mg/kg MPD (Table 2C & 3C). Upon both acute (ED1 MPD/ED1 BL) and chronic (ED10 MPD/ED1 MPD)

MPD exposure, significantly ($p<0.05$) more CN neurons recorded from adults responded with an increase in neuronal firing rate compared to CN neurons recorded from young (Figures 4E & 4F).

3.2.8 Effect of 10.0 mg/kg MPD on CN neurons recorded from all adult and young animal groups (Fig 4A & 4B): A total of 263 CN neurons were recorded from adults, and 281 from young animals, respectively (Tables 2A and 3A). Significantly ($p<0.05$), more CN neurons were responsive in adults across all three subgroups (acute, baseline, and chronic) compared to CN neurons recorded in young animals (Table 3A). No significant difference in the response direction was observed between CN neurons recorded in adults compared to young animals following acute 10.0mg/kg MPD exposure (Figure 4A). However, a significantly ($p<0.05$) greater percentage of CN neurons recorded from adult animals responded to chronic 10.0mg/kg MPD exposure (ED10 MPD/ED1 MPD) with an increase in neuronal firing rate compared to CN neurons recorded from young animals (Figure 4B).

3.2.9 Comparing the effect of 10.0 mg/kg MPD on CN neurons recorded from all adult and young animal groups (Fig 4A & 4B): 40 CN neurons were recorded from adults, and 124 from young animals (Tables 2B and 3B). Among CN neurons affected by acute 10.0mg/kg MPD exposure, a significantly ($p<0.05$) greater percentage of CN neurons recorded from adults responded with an increase in neuronal firing rate (Figure 4C). No significant differences in firing rates were observed between adult and young CN neurons following chronic 10.0mg/kg MPD exposure (Figure 4D).

3.2.10 Comparing the effect of 10.0 mg/kg MPD on CN units recorded from behaviorally tolerant animals (Fig 4E & 4F): In behaviorally tolerant animals exposed to 10.0 mg/kg MPD, 223 CN neurons were recorded from adults, and 157 from young animals. No significant difference in response direction (increase or decrease) was observed between adult and young CN neurons following acute (ED1 MPD/ED1 BL) 10.0mg/kg MPD (Figure 4E). However, following chronic 10.0mg/kg MPD exposure (ED10 MPD/ED1 MPD), significantly ($p<0.05$) more CN neurons recorded in adult animals responded with an increase in neuronal firing rate compared to CN neurons recorded in young animals (Figure 4F).

4.0 Comparing ED10 BL after six daily MPD exposures and three wash-out days in CN neurons recorded from adult and young animals (Table 2, 3 & Fig 5): Table 2 & 3 baseline (ED10 BL/ED1 BL) summarizes CN neuronal recordings at ED10 after six daily injections of MPD at 0.6, 2.5, and 10.0 mg/kg exposure, and three washout days of the three groups (All, Sensitized, and Tolerance) compared to ED1 BL. In general, significant ($p<0.05$) differences were observed between the two age groups in CN neurons recorded. CN neurons at ED10 BL recorded from young animals mainly exhibited increases in their firing rates, while those recorded from adult animals generally exhibited a decrease in their firing rate at ED10 BL compared to ED1 BL (ED10 BL/ED1 BL).

4.1 Effect of chronic MPD on CN units recorded from all adult and all young animals (Fig 5A): Following six daily 0.6 and 2.5 mg/kg MPD and three washout days, no differences were observed between CN neurons recorded from the two age groups regarding the ratio of CN neurons recorded at ED10 BL that were modulated in their firing rate direction (increase or decrease) compared to ED1 BL recording. However, following repeated 10.0 mg/kg MPD injection, significant ($p<0.05$) age differences were observed. In the adult group, the majority of CN neurons exhibited a decrease in their ED10 BL, while the opposite was noted in the recordings from the young groups (i.e., an increase in their ED10 BL/ED1 BL).

4.2 Effect of chronic MPD on CN neurons recorded from behaviorally sensitized adult and young animals (Fig 5B): The ED10 BL/ED1 BL in the CN neurons recorded from animals exhibiting behavioral sensitization to chronic MPD exhibited a significant ($p<0.05$) difference in the groups treated with 2.5 mg/kg MPD (Fig 5B). Following chronic 0.6 mg/kg MPD, most CN neurons in both age groups expressed attenuation in their ED10 BL/ED1 BL (Fig 5B 0.6 mg/kg MPD), while the opposite effects were observed following repeated 10.0 mg/kg doses (Fig 5B 10.0 mg/kg).

4.3 Effect of chronic MPD on CN neurons recorded from behaviorally tolerant adult and young animals (Fig 5C): The ED10 BL/ED1 BL in the CN neurons recorded from animals exhibiting behavioral tolerance to chronic MPD exposure exhibited a significant age difference in the number of CN neurons that expressed a significant ($p<0.05$) difference at ED10 BL/ED1 BL. Many CN neurons recorded from adult animals exhibited a significant decrease in their ED10 BL/ED1 BL, while most CN neurons recorded from behaviorally tolerant animals exhibited an increase in their ED10 BL/ED1 BL. Comparing the ED10 BL/ED1 BL pattern (increase/decrease) between the three groups (All, Sensitized, and Tolerance) exhibits a significant ($p<0.01$) difference between the age groups. This observation suggests that it is essential to evaluate neuronal responses to MPD based on their behavioral response to the chronic effects of the drug.

5. DISCUSSION

Methylphenidate (MPD) stands out as one of the most frequently prescribed psychostimulants for addressing attention-deficit hyperactivity disorder (ADHD) (Accardo and Blondis, 2001; Safer, 2016; Storebø et al., 2015; Wilens, 2008). Notably, recent years have witnessed a steady rise in the prescription of

psychostimulants, sparking concerns regarding the off-label usage of MPD (Bogle and Smith, 2009; Cohen et al., 2015; Kapur 2020; Kim et al., 2020; Som Kuwarr et al., 2016). The current landscape reveals a substantial number of young and adults employing misusing psychostimulant medications, including MPD, for purposes ranging from cognitive enhancement or recreational use (Challman and Lipsky 2000; Fond et al., 2016; Foschiera et al., 2022; Kim et al., 2020). Given the escalating abuse of psychostimulants, especially among individuals with developing brains and adults, it has become crucial to conduct studies assessing and comparing the long-term neuronal and behavioral effects of both acute and repetitive (chronic) doses of MPD exposure in both adult and young subjects.

The primary objectives of this study were threefold: 1) to investigate the impact of acute versus chronic MPD use on the behavior and CN neural activity of young and adult rats; 2) to discern individual differences in behavioral responses to the same acute or chronic MPD dose; and 3) to ascertain whether these individual differences in behavioral responses correlate with CN neuronal firing responses to MPD exposure.

Key findings from this study include 1) A dose-dependent increase in behavioral locomotor activity in both adult and young animals with escalating MPD doses, likely attributed to increased catecholamine (CA) levels in the synaptic cleft and subsequent heightened stimulation of postsynaptic CA receptors; 2) Varied age differences in behavioral responses to the same chronic MPD dose, including tolerance and sensitization, possibly linked to differences in receptor upregulation under the recording electrodes; and 3) Correlation between CN neuronal responses and animal behavior, with animals exhibiting behavioral tolerance showing a decrease in CN neuronal firing rate during repetitive (chronic) MPD exposure, while those exhibiting behavioral sensitization displayed an increase in CN neuronal firing rate compared to acute MPD exposure – indicating that changes in CN neuronal firing rate contribute to observable behavioral alterations.

Notable distinctions emerged in the responses to MPD between adult and young animals. Specifically, ED10 BL/ED1 BL behavioral activity after six daily MPD exposures and three washout days influenced young animals more than adults, aligning with previous research highlighting the developing susceptibility of the young CN to psychostimulants. Additionally, CN neuronal responses differed significantly between adults and young, with adults exhibiting higher intensity responses in the neuronal firing rate. This is consistent with previous study showing that the CN in young animals is still developing and potentially differ in susceptive to MPD as compared to adult (Medina et al., 2022a & b; Venkataraman et al., 2020).

The findings from this study underscore that the same dose of 0.6, 2.5, or 10 mg/kg MPD can induce behavioral and neuronal sensitization in some animals while causing behavioral and neuronal tolerance in others. This discovery is noteworthy, as sensitization and tolerance serve as experimental biomarkers indicating a drug's abuse potential, potentially leading to substance abuse disorder. Prior molecular studies have also demonstrated that stimulant abuse, such as MPD, may have long-term consequences due to underlying molecular modifications, including alterations in cellular and molecular plasticity, neuropil morphology, and gene expression. This puts individuals at risk not only for dependence on their current drug of abuse but also for possible cross-dependence with other drugs (Chao & Nestler 2004; Foschiera et al., 2022; Klein et al., 2019; Nestler, 2012; Quintero et al 2022).

MPD, akin to other psychostimulants, exerts its effects on the brain's reward circuit (Cooper et al., 2014; Russo & Nestler 2013), involving structures such as the nucleus accumbens, ventral tegmental area, prefrontal cortex, CN, and other brain areas (Dafny et al., 2022; Reyes-Vasquez et al., 2023). By preventing the reuptake of dopamine (DA), norepinephrine (NE), and serotonin (5HT), MPD enhances their availability in the synaptic cleft, intensifying the activity of the reward circuit (Campo et al., 2011; Dahl et al., 2022; Di Miceli et al., 2022). Studies have revealed that while acute exposure boosts behavioral and neural activity, chronic MPD exposure leads to sensitization in some animals and tolerance in others as compare to the initial MPD effects (Dafny et al., 2022; Reyes-Vasquez et al., 2023). This phenomenon is linked to synaptic plasticity and protein synthesis changes, particularly within the CN, where distinct pathways and receptors play pivotal roles. Stimulation of the CN direct pathway, rich in D1 DA receptors, upregulates genes and transcription factors associated with increased motivation and response to the drug, contributing to behavioral sensitization (Klein et al., 2019). On the other hand, the stimulation of the CN indirect pathway dominated by D2 DA receptors has resulted in the upregulation of the cAMP response binding element (CREB) (Chao and Nestler 2012; Nestler 2012). Consequently, this leads to significant attenuation of CN neuronal activity that regulates the ascending thalamic activity to the cortex, explaining the development of behavioral tolerance (Claussen & Dafny 2015; Reyes-Vasquez et al., 2023; Yan et al., 1999). These findings underscore the intricate interplay of MPD with various brain structures and neurotransmitters, necessitating further research to unravel the complexities of its impact on addictive behavior.

An additional possible explanation of the individual variations observed among animals in response to MPD in this study could stem from differences in the individual composition of the above-mentioned two pathways—the direct excitatory and the indirect inhibitory—that constitute the ascending striatal pathway

connecting the CN to other brain structures (Claussen & Dafny 2015; Venkataraman et al., 2020). Another potential explanation for the variation could be the uneven topographical location of D1 and D2 receptors. For instance, in one animal, the electrode might have been placed around D1 DA-dense receptors, while in another, it was situated around D2 DA-dense areas of the CN. This disparity could elucidate why repetitive (chronic) MPD exposure induces further excitation (sensitization) in some individuals while others develop less activity (tolerance). Alternatively, variations in the ratios of D1 or D2 DA receptors and differences in the direct-to-indirect ascending pathways may contribute to the observed variation. Individuals with more significant quantities of direct pathways would likely have more D1 receptors, leading to sensitization to chronic MPD exposure. Conversely, those with more indirect pathways would possess more D2 receptors, resulting in tolerance to chronic MPD exposure.

In summary, this study reveals that the intensity of MPD effects on the CN neuronal firing rates and behavioral expression differed significantly between age groups. Young animals demonstrated higher susceptibility to MPD, evidenced by changes in ED10 BL behavioral activity compared to ED1-BL behavioral activity and CN neuronal activity compared to adult animals. The ratio of animals expressing behavioral sensitization to tolerance did not differ significantly between age groups, contrary to the hypothesis. The most significant finding is that the neuronal recording from young animals responds differently to all MPD doses compared to adults. Furthermore, the same MPD doses of 0.6, 2.5, or 10 mg/kg led to either behavioral or neuronal sensitization or tolerance in some animals, emphasizing the importance of concurrently studying acute and chronic drug effects on animal behavior and evaluating neuronal responses in relation to behavioral responses.

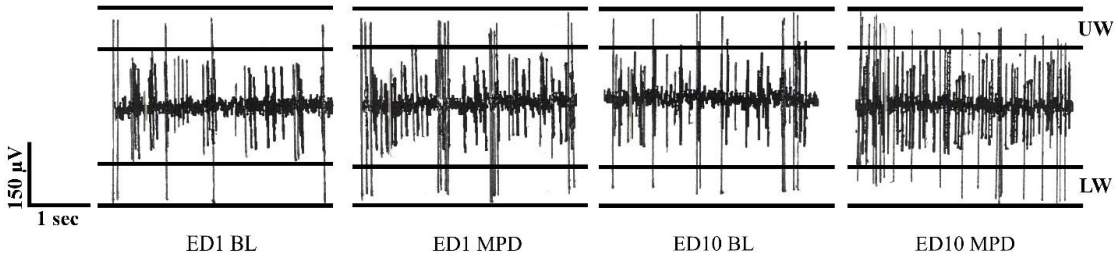

Figure 1: Displays four traces of analog recordings. A. Baseline recordings on experimental day 1 (ED1 BL). B. Recordings following 2.5mg/kg MPD on ED1 indicate that MPD elicits excitation compared to ED1 BL. C. Recordings following repeated 2.5mg/kg MPD on ED10 show MPD tolerance compared to ED1 MPD. The recording ED10 BL compared to ED1 BL shows withdrawal activity (ED10 BL/ED1 BL). The figure illustrates typical neuronal recordings and the upper(UW) and lower (LW) windows used in the first stage of spike discrimination. The number on the left of each trace summarizes the number of spikes in the trace.

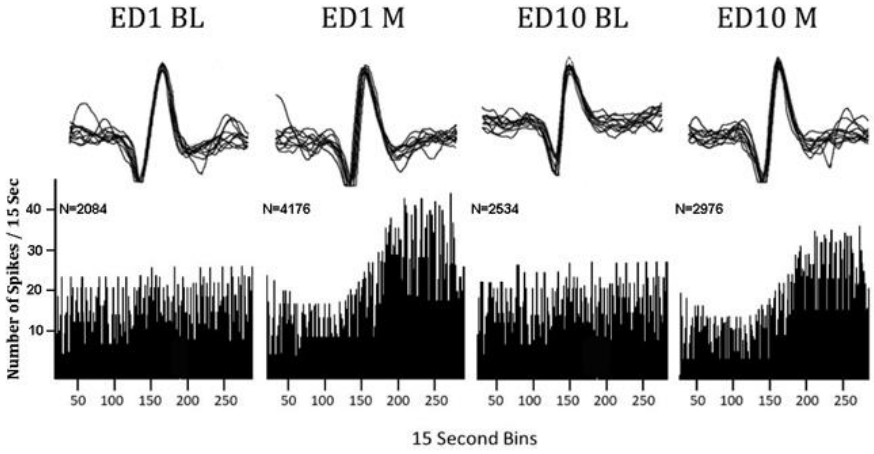

Figure 2: A histogram of CN neurons recorded from adult rats summarizes 60-minute sequential neuronal firing rates following acute and chronic 2.5mg/kg MPD exposure. N =represents the number of spikes over 60 minutes. The first panel, ED1 BL, shows the CN neuronal activity recorded at baseline on ED1. The second panel, ED M1 (ED1 MPD), shows the CN neuronal activity recorded after acute 2.5mg/kg MPD exposure, showing that MPD causes excitation. The third panel, ED10 BL, shows the CN neuronal activity recorded after previous exposure to six daily MPD administrations and three washout days, demonstrating a withdrawal response. The fourth panel, ED10 M (ED10 MPD), shows CN neuronal activity after chronic MPD administration, showing tolerance.

Above each histogram are 20 superimposed spikes sorted to produce the histograms, aiming to demonstrate that the same spike pattern was counted during each 60-minute recording session. The numbers above each histogram represent the total spikes per 60 min.

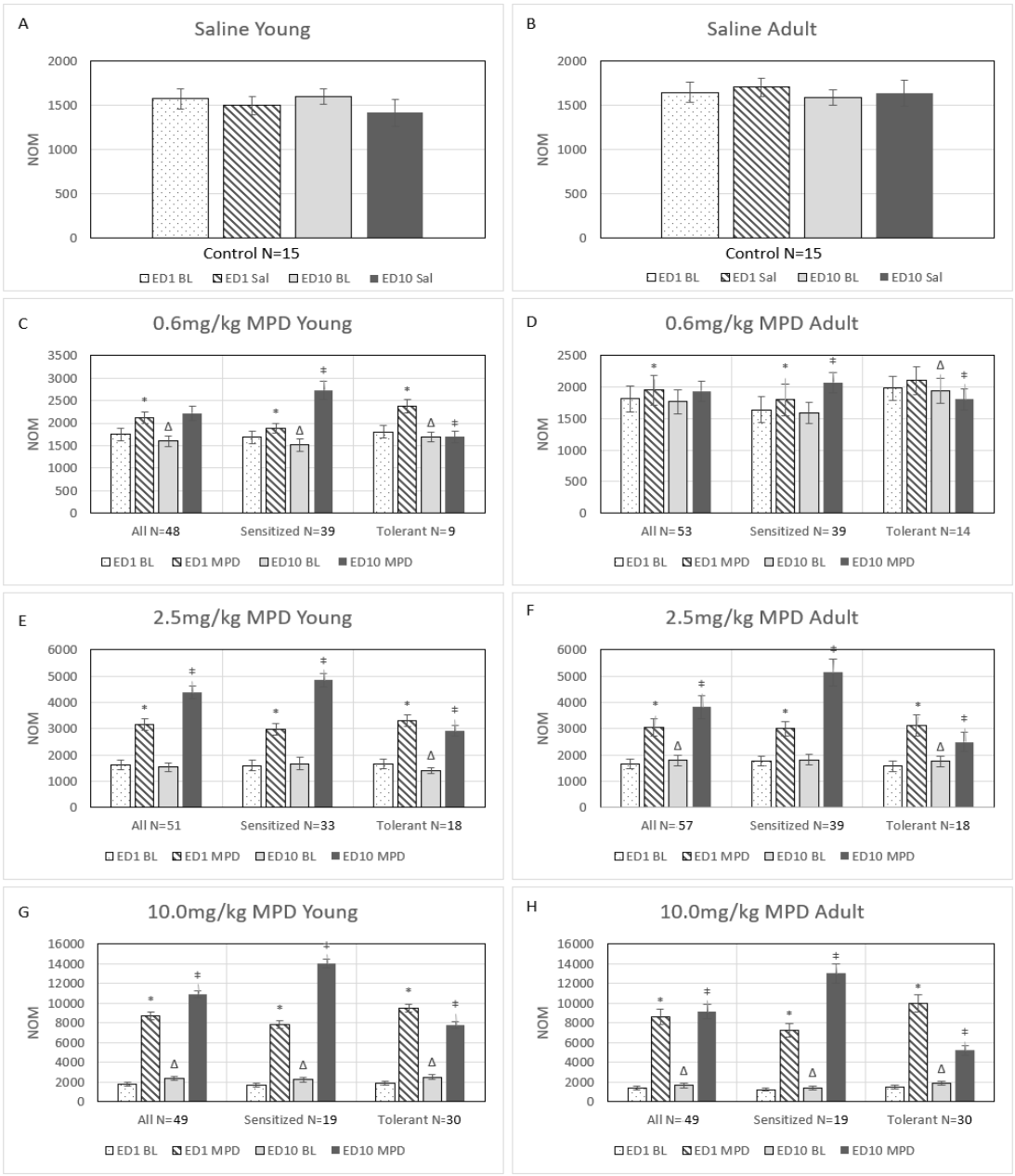

Figure 3: Summarizes all behavioral data (number of movements, NOM) for young and adult rat groups from the saline control in A and B, and each experimental MPD dose 0.6 mg/kg in C & D, 2.5mg/kg in E & F, and 10.0 mg/kg MPD in G & H. Each histogram is labeled from A-H for their respective age, with young on the left and adult on the right, and experimental groups (All, sensitized, and tolerance). N = represents the number of animals in each group. The rats in the experimental dose groups were divided into three subgroups: all animals, behaviorally sensitized animals, and behaviorally tolerant animals. The "All" group summarizes all animals for the respective MPD dose. The "Sensitized" group and the "Tolerant" group summarize 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 acute effect of MPD; ED10 BL/ED1 BL, comparing ED1 BL to ED10 BL to assess the impact of six daily MPD exposures and three washout days on ED10 BL; and ED10 MPD/ED1 MPD, comparing ED10 MPD to ED1 MPD to assess 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 adults, 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 is the standard deviation (SD). * = Indicates significant ($p < 0.05$) differences from ED1 BL (ED1 BL/ED1MPD, acute). Δ = Indicates significant ($p < 0.05$) differences between ED1 BL and ED10 BL (ED1 BL/ED10 BL, withdrawal). ‡ = Indicates significant ($p < 0.05$) differences from ED1 MPD (ED1 MPD/ED10 MPD, chronic).

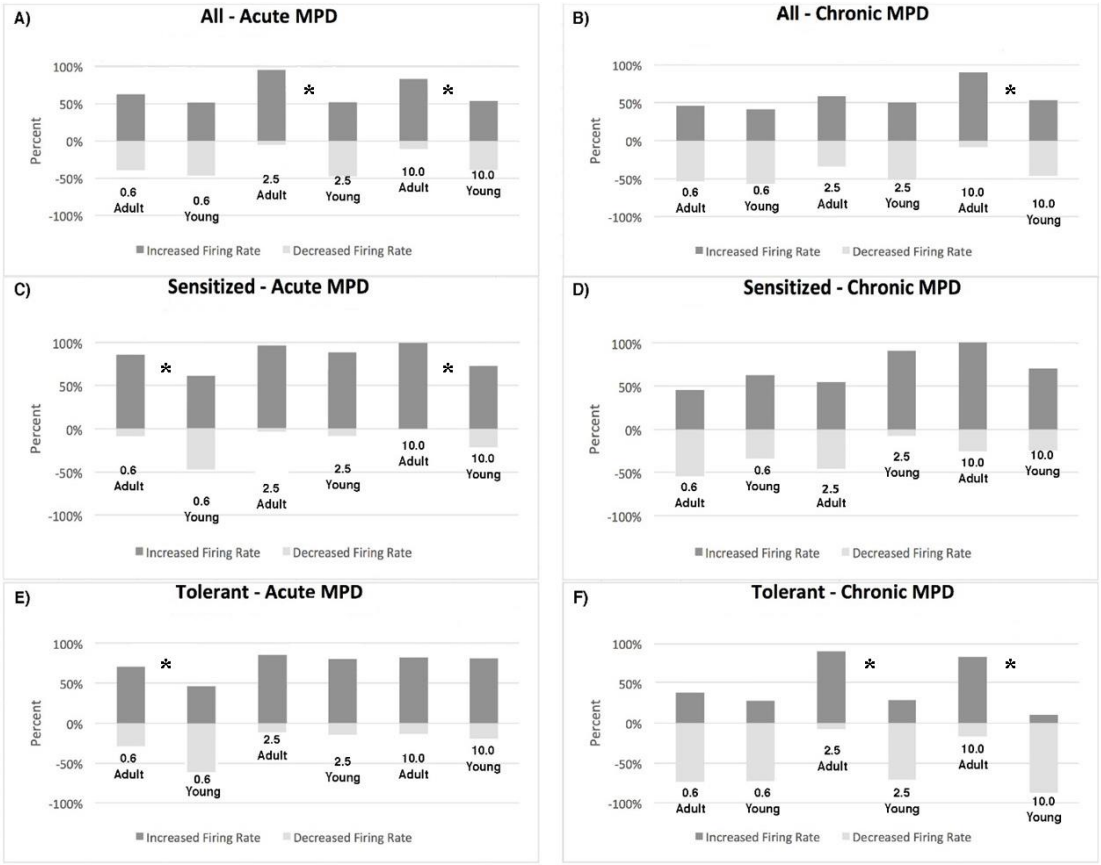

Figure 4: The figure summarizes the direction of responsiveness (increase or decrease in %) of how many CN neurons respond significantly to acute and chronic MPD doses. Each segment has three columns and three sections showing in percentage how many CN neurons respond significantly by either increasing 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). *= indicate significant ($p<0.05$) age differences in response direction.

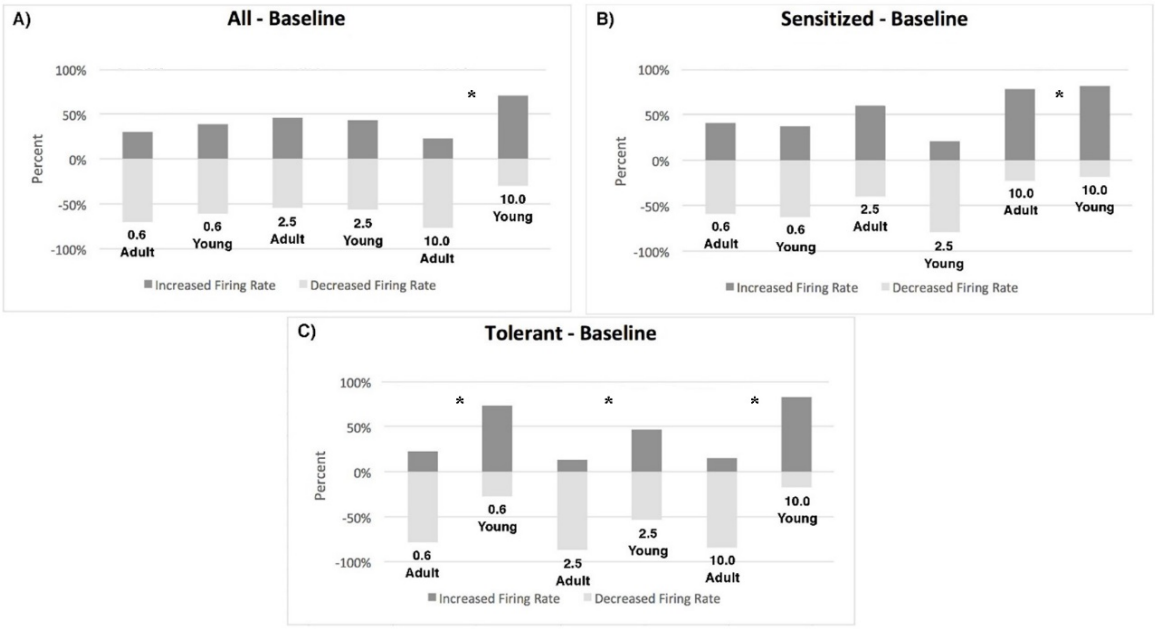

Figure 5: The figure summarizes in percentage how many CN neurons exhibit changes (increases or decreases) in firing rates on ED10 BL compared to ED1 BL. Figure 5A shows the CN neurons recorded from all animal groups. Figure 5B shows the CN neurons recorded only from behaviorally sensitized animals, and Figure 5C shows the CN neurons recorded from only the behaviorally tolerant adult and young animals, respectively. *= indicate significant ($p<0.05$) age differences in response direction.

Table 1: This table displays the animal groups and the MPD dose-response protocol followed for each group of young and adult animals. The four groups of animals used for each age are saline, 0.6, 2.5, and 10.0 mg/kg MPD. On experimental day 1 (ED1), animals were given an initial dose of saline. Recordings were taken for one hour to obtain baseline (BL), 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 were given an injection of the designated dose each morning. ED 7-9 were washout days where the animals received no injection. On ED10, the animals were given another dose of saline to obtain BL on ED10 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 those given on ED1. P = indicates the postnatal day age. * = indicates the behavioral and neuronal recording day.

Table 1 **Behavior + Electrophysiology**

Age Young	P - 40*	P - 41-45	P - 46-48	P - 49*
Age Adult	P - 60*	P - 61-65	P - 66-68	P - 69*
Experimental Day (ED)	ED1 Acute*	ED 2-6 Daily Administration	ED 7-9 Washout	ED10 MPD Rechallenge*
Saline	Saline / Saline	Saline	Washout	Saline / Saline
0.6 mg/kg	Saline / 0.6 mg/kg	0.6 mg/kg	Washout	Saline / 0.6 mg/kg
2.5 mg/kg	Saline / 2.5 mg/kg	2.5 mg/kg	Washout	Saline / 2.5 mg/kg
10.0 mg/kg	Saline / 10.0 mg/kg	10.0 mg/kg	Washout	Saline / 10.0 mg/kg

Table 2: Summarizes the CN neuronal responses following 0.6, 2.5, and 10.0 mg/kg MPD recorded from adult animals. In A, B, and C, the summary of the CN neuronal responses recorded from all the animals (2A), animals expressing behavioral sensitization in 2B, and animals expressing behavioral tolerance in 2C, respectively. Under Acute, Baseline, and Chronic are the numbers of CN neurons that responded significantly ($p < 0.05$) and their percentages (in brackets) that 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 percentage of CN neurons that did not respond to MPD following acute MPD (ED1 MPD/ED1 BL). The baseline (BL) activity of ED10 is compared to ED1 BL (ED10 BL/ED1 BL), as well as the chronic effect of each MPD dose (ED10 MPD/ED1 MPD).

Young CN Neurons Firing

A. Units recorded from ALL animals

		Acute (ED1 MPD/ ED1 BL)						Baseline (ED10 BL/ ED1 BL)						Chronic (ED10 MPD/ ED1 MPD)					
Dose	N	↑		↓		≠		↑		↓		≠		↑		↓		≠	
0.6	257	36	14%	68	26%	153	60%	33	13%	51	20%	173	67%	72	28%	48	19%	137	53%
2.5	224	95	42%	58	26%	71	32%	38	17%	49	22%	137	61%	109	49%	57	25%	58	26%
10	281	153	54%	67	24%	61	22%	161	57%	67	24%	53	19%	77	27%	158	56%	46	16%

B. Units recorded from behaviorally sensitized animals

		Acute (ED1 MPD/ ED1 BL)						Baseline (ED10 BL/ ED1 BL)						Chronic (ED10 MPD/ ED1 MPD)					
Dose	N	↑		↓		≠		↑		↓		≠		↑		↓		≠	
0.6	202	21	10%	52	26%	129	64%	24	12%	41	20%	137	68%	91	45%	46	23%	65	32%
2.5	132	71	54%	33	25%	28	21%	18	14%	69	52%	45	34%	89	67%	21	16%	22	17%
10	124	82	66%	23	19%	19	15%	93	75%	21	17%	10	8%	99	80%	19	15%	6	5%

C. Units recorded from behaviorally tolerant animals

		Acute (ED1 MPD/ ED1 BL)						Baseline (ED10 BL/ ED1 BL)						Chronic (ED10 MPD/ ED1 MPD)					
Dose	N	↑		↓		≠		↑		↓		≠		↑		↓		≠	
0.6	55	23	42%	8	15%	24	44%	19	35%	7	13%	29	53%	13	24%	34	62%	8	15%
2.5	92	18	20%	9	10%	65	71%	13	14%	15	16%	64	70%	15	16%	37	40%	40	43%
10	157	97	62%	23	15%	37	24%	102	65%	21	13%	34	22%	21	13%	102	65%	34	22%

Table 3: Summarizes the CN neuronal responses following 0.6, 2.5, and 10.0 mg/kg MPD recorded from young animals. In A, B, and C, the summary of the CN neuronal responses recorded from all the animals (2A), animals expressing behavioral sensitization in 2B, and animals expressing behavioral tolerance in 2C, respectively. Under Acute, Baseline, and Chronic are the numbers of CN neurons that responded significantly ($p < 0.05$) and their percentages (in brackets) that 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 percentage of CN neurons that did not respond to MPD following acute MPD (ED1 MPD/ED1 BL). The baseline (BL) activity of ED10 is compared to ED1 BL (ED10 BL/ED1 BL), as well as the chronic effect of each MPD dose (ED10 MPD/ED1 MPD.

Adult CN Neurons Firing

A. Units recorded from ALL animals

		Acute (ED1 MPD/ ED1 BL)						Baseline (ED10 BL/ ED1 BL)						Chronic (ED10 MPD/ ED1 MPD)					
Dose	N	↑		↓		≠		↑		↓		≠		↑		↓		≠	
0.6	219	92	42%	106	48%	21	10%	65	30%	153	70%	1	0%	103	47%	116	53%	0	0%
2.5	249	152	61%	77	31%	20	8%	113	45%	135	54%	1	0%	137	55%	112	45%	0	0%
10	263	187	71%	62	24%	14	5%	61	23%	202	77%	0	0%	133	51%	130	49%	0	0%

B. Units recorded from behaviorally sensitized animals

		Acute (ED1 MPD/ ED1 BL)						Baseline (ED10 BL/ ED1 BL)						Chronic (ED10 MPD/ ED1 MPD)					
Dose	N	↑		↓		≠		↑		↓		≠		↑		↓		≠	
0.6	60	40	67%	20	33%	0	0%	24	40%	35	58%	1	2%	43	72%	16	27%	1	2%
2.5	90	32	36%	42	47%	16	18%	54	60%	36	40%	0	0%	64	71%	20	22%	6	7%
10	40	38	95%	2	5%	0	0%	31	78%	9	23%	0	0%	29	73%	10	25%	1	3%

C. Units recorded from behaviorally tolerant animals

		Acute (ED1 MPD/ ED1 BL)						Baseline (ED10 BL/ ED1 BL)						Chronic (ED10 MPD/ ED1 MPD)					
Dose	N	↑		↓		≠		↑		↓		≠		↑		↓		≠	
0.6	159	75	47%	31	19%	53	33%	35	22%	124	78%	0	0%	42	26%	115	72%	2	1%
2.5	144	127	88%	5	3%	12	8%	19	13%	125	87%	0	0%	115	80%	28	19%	1	1%
10	223	138	62%	58	26%	27	12%	33	15%	184	83%	6	3%	184	83%	38	17%	1	0%

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