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Research Article

Methylphenidate Elicits Long Term Sex Difference Effects

*Victor T Wilcox, Sarah D George, Pamela B Yang, Cruz Reyes-Vazquez and Nachum Dafny**

Department of Neurobiology and Anatomy, University of Texas McGovern Medical School, USA

Abstract

Methylphenidate (MPD) is a psychostimulant widely used to treat ADHD. MPD consumption has increased among ordinary people as a cognitive enhancer and recreational drug across multiple age groups. Due to the close pharmacological profile it shares with cocaine and amphetamines there is concern that MPD has the potential to elicit dependence and abuse. The object of this study was to determine whether the effect of using MPD at a young age remains in adulthood, and for how long, in female and male ordinary subjects. Behavioral activity was recorded using a computerized monitoring system before and after acute and chronic MPD exposure. Five different locomotors of behavioral expression were recorded. In all groups the Baseline (BL) activity on experimental day 1 (ED1 BL) was obtained followed by 6 daily Saline or MPD injections followed by 3 to 37 washout days and MPD re-challenge at ED 11, or at ED 27, or at ED 36, or at ED 42. The rechallenge effect of each of the MPD dose groups at ED 11, ED 27, ED 36 or ED 42 exhibited behavioral sensitization in some animals and behavioral tolerance in other animals. Sex differences were observed in all five different behavioral expressions. The observations suggest that each of the five behavioral expressions studied are regulated by different neuronal circuitry, and that when MPD is used for six days, its effects last for prolonged periods of time.

Keywords: Methylphenidate; Dose response; Sex; Behaviors; Sensitization; Tolerance

Introduction

Methylphenidate (MPD), more widely known as Ritalin is a, drug currently used to treat ADHD [1-3]. MPD is a stimulant like cocaine and methamphetamines, and is known to have similar effects [4]. Due to its chemical classification, much controversy has arisen as to whether MPD is an addictive substance, or has the potential to become one. MPD remains one of the most prescribed drugs for children with behavioral disorders. Its use is up 15% in school-aged children in some areas of the United States, and from 3% to 6% in Europe, especially in urban areas. MPD use often continues into adulthood [2,5,6]. Furthermore, its popularity as a cognition enhancer has increased both its legal and illegal use often in college age and professional adults [7]. This is particularly concerning as previous meta-analyses have found that while MPD initiation during childhood seems to mitigate or even lower the risks of Substance Abuse Disorder (SUD), chronic behavioral changes and SUD are increased with later initiation at or beyond the adolescent stage [8-10]. Estimates that ADHD and SUD range from 9.5% up to 58% of MPD users and lifetime prevalence of cocaine-use is doubled from around 10% to over 20% for those with ADHD.

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***Corresponding author:** Nachum Dafny, Department of Neurobiology and Anatomy, University of Texas McGovern Medical School, Houston, Texas, 77025, USA, E-mail: Nachum.dafny@uth.tmc.edu

Recently, MPD has increased in popularity as a cognitive enhancement and is abused for recreation [1]. Much of MPD research has been done on animal models, namely rats, due to their chemical and behavioral similarities shared with humans. Various limitations not addressed in previous studies include differences in sex, and how long MPD continues to impact tolerance or sensitization following repetitive (chronic) use. Recently, the effects of MPD used for a short time have been shown to cause abuse of the drug [3]. Animal studies have shown a significant effect of MPD on the dopaminergic neuronal system, suggesting evidence for its potential addiction [11]. Another animal study has shown that the time of introduction to MPD treatment, specifically in adolescence, results in different chronic effects of neurotransmission [12,13]. Although much has been reported about MPD use, the high prescription and abuse rates among youth and adults of both sexes, calls for a more detailed and definitive observation of its effects. The present study addresses how long its effects will last once a subject has used MPD for short times. Expression of behavioral tolerance and/or sensitization was used to assess the lasting effects of MPD. These two components, behavioral tolerance and sensitization, are commonly used as a biomarker to determine the drug duration effects and if it has the potential and the properties to elicit dependence [4, 14-16]. The study provides insight for how long MPD will last in the system and its potential as an addictive substance of abuse.

Methods

One hundred and twenty-eight post-natal (P) days 33 to 34 female and 128 male Wistar-Kyoto (WKY) rats were purchased from Harlem Laboratory (Indianapolis, IN. USA). They were divided to the following groups: Saline (control), 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD female groups and four similar male groups. Each group consisted of 4 subgroups (Table 1) each N=8 × 16, groups=128 rats for each sex, totaling 256 rats. The rats were acclimated for 5 to

7 days to a 12h light, 12h dark cycle (05:30-17:30h light cycle). The room temperature was kept at 21°C ± 2°C, with 37%-42% humidity throughout the experiment. Food and water were provided as needed. After 6 to 7 days adaptation at P-40 behavioral recording was started. Rats were kept in the same enriched cages for the duration of the experiment i.e., the home cage was also used as the test cage to eliminate environmental effects on the animal's behavioral responses to the drug. All the animals were injected with saline at experimental day 1 (ED1) followed by 120 min locomotor behavioral recordings to obtain the baseline (ED1 BL). Over the following six days (ED2 to ED7) saline, or 0.6 mg/kg, 2.5 mg/kg, or 10.0 mg/kg MPD was given followed by three washout (no treatment) days (ED8 to ED10). Then the 4 female and the 4 male sub-groups were re-challenged at ED 11 with the same treatment as at ED2 to ED7. These 8 sub-groups animals of group 1 were sacrificed at the end of the recording at ED11 (Table1). In the second 8 sub-groups were given similar treatment at ED1 to ED7 (saline, or 0.6 mg/kg, 2.5 mg/kg, or 10.0 mg/kg MPD) followed by 26 washout days and were re-challenged with their designated MPD at ED 27 (group 2). In the third 8 sub-groups designated MPD was given for six consecutive days followed by 35 washout days and at ED 36 MPD rechallenge was given followed by additional 120 min behavioral recording. In the fourth 8 sub-groups after the six daily MPD exposures, at ED 42 MPD re-challenge was given, and recording were resumed for 120 min post injection respectively (Table 1). The saline group was used as the control group for each sex grouping. All injections were in 0.8 cc volume i.p given at about 08:00.

Apparatus

A Computerized Animal Activity Monitoring (CAAM) system was used to record the locomotor activity during the experiment. The home testing chamber consisted of a clear acrylic box. The home/test cage were 40.5 cm × 40.5 cm × 31.5 cm each fitted with sets of 16 infrared beams and on the opposite side 16 motion sensors located 6 cm and 12 cm above the floor (Accuscan Instruments Inc, Columbus, OH). Once a rat was moving, the infrared beams were interrupted (albino rats have red colorblindness). The system recorded beam interruptions at the frequency of 100 Hz. The counts and times of the interrupted beams from each sensor were transmitted to the Accuscan Analyzer. Cumulative counts were compiled and downloaded every 10 minutes for a total time of 2 hours post-injection into OASIS data collection software and organized into various locomotor indices as follows: Total Distance (TD) travelling forward in centimeters during the experiment; Number of Movements (NM) and Horizontal Activity (HA) counts the total number of interrupted beams to the lower level sensors during the experiment; Number of Stereotypic Movements (SM), counts the number of repetitive episodes with at least a one second interval before the initiation of another episode of the same beam, which represents stereotypic behaviors such as spooning or sniffing; and, Vertical Activity (VA) [17-20].

Drugs

Methylphenidate hydrochloride (MPD) was donated by Mallinckrodt (Hazelwood, MO). MPD was dissolved in 0.9% NaCl (saline) solution, with doses calculated as free base. MPD doses were given intraperitoneally and total volume was maintained at 0.8 cc with 0.9% saline solution. Three doses of 0.6 mg/kg, 2.5 mg/kg, and 10 mg/kg MPD were used. All injections were administered intraperitoneally at around 08:00. The time of injection and the drug dosages used were selected based on our previous MPD dose response studies that showed that starting from 0.6 mg/kg MPD, the drug caused changes in the locomotor behavioral activities [14,16, 21-25]. Dosing schedule over the time period of the experiment is presented in Table 1.

Experimental protocol (Table 1)

All animals received an injection of saline on experimental day 1 (ED1) at age P-40, followed by 6 consecutive daily injections on ED2-7 of either saline (Sal control), or 0.6 mg/kg MPD, or 2.5 mg/kg MPD or 10.0 mg/kg MPD. This was followed by a 3-day washout period (ED8-10) where no drugs were administered in group1 and its 4 subgroups (Sal, 0.6, 2.5 and 10.0 mg/kg MPD) female and similar 4 male subgroups i.e., total 8 subgroups. On ED 11 these 8 subgroups rats were re challenged with the same MPD dose as at ED 2 to 7, and recording of the animals' behavior was resumed on ED 11, i.e., in these 8 subgroups recordings were done on ED 1, 2, 11. In another similar 8 subgroup MPD rechallenges were given on ED 27, and recording of the animal's behavior was done on ED 1, 2, 27. In another 8 subgroup MPD rechallenge was given on ED 36, and recording of the animals' behavior was done on ED 1, 2, 36. In another 8 subgroup MPD rechallenge was given on ED 42, and recording of the animals' behavior was done on ED 1, 2 and 42 for 120 min post each injection respectively (Table 1).

Data analysis

Locomotor activity of five different behavior expressions (HA, TD, NM, VA & SM) were recorded before and after MPD exposure as described above (Table 1). The recordings following saline injection on ED-1 represent the baseline control [14-16,21,22,26-28]. The recordings on ED-2 MPD compared to ED-1 BL represent the acute effect of the drug. Comparison of the recording obtained on ED-11, 27, 36 and 42 MPD respectively to ED 2-MPD represents the chronic effect of the drug and tests whether the animals expressed behavioral sensitization or tolerance [14-16,19,21,22,28]. Repeated measure analysis of variance (ANOVA) with post-hoc analysis using Fischer's LSD test was used to determine statistically significant differences among the various days and drugs. Bonferroni adjusted pairwise comparisons were made. Post-hoc Tukey test was used to compare between different groups for all ANOVA that produced significant results. All tests were considered significant at $p<0.05$ for all comparisons.

Table 1: Show the experimental protocol of four WKY female groups' rats. Similar four groups were used for WKY male rats. The different between the groups were when after six daily repetitive Methylphenidate (MPD) and Wash-Out (W.O) days the MPD rechallenge was given. Each groups had four subgroups as follow: Saline (BL), 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD (Figures 2 and 3).

Group 1	ED 1 BL	ED 2 to ED 7 MPD	ED 8 to ED 10 W.O.	ED 11 MPD
Group 2	ED 1 BL	ED 2 to ED 7 MPD	ED 8 to ED 26 W.O.	ED 27 MPD
Group 3	ED 1 BL	ED 2 to ED 7 MPD	ED8 to ED 35 W.O.	ED 36 MPD
Group 4	ED 1 BL	ED 2 to ED 7 MPD	ED 8 to ED 42 W.O.	ED 42 MPD

ED: Experimental Day; BL: Base-Line

Results

Control (Figure 1)

Eight females and 8 male rats were used for time control groups and additional 8 female and 8 males as a saline control group. Their locomotor activities were recorded nonstop for 42 days (no injection). Additional 8 groups were used for the saline control animals-after ED 1 receding with no injection, from ED 2 to ED 42 animals received 0.8 ml saline daily. In the time and the saline control groups there were minor non-significant difference fluctuations between the

42 recording days in all the five locomotor behavioral expressions recorded as compared to ED 1 (Figure 1). Therefore, we used the saline injection on ED 1 in the drug groups as control for MPD acute effects.

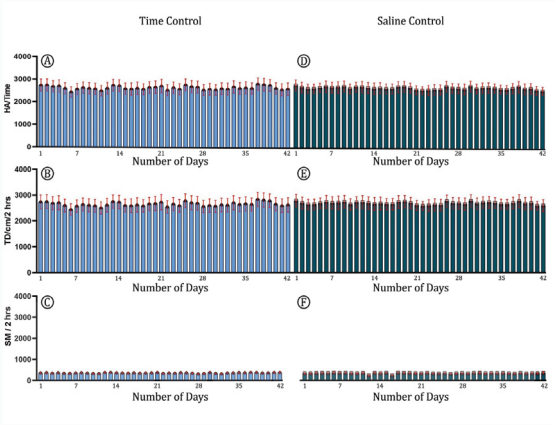


Figure 1: The figure summarizes the Horizontal Activity (HA), Total Distance (TD), traveling and Number of Stereotypic (NOS) activity of 42 sequential days (time control) and the effect repetitive 42 daily saline injections (saline control). Recordings were obtained for two hours from 08:00 to 10:00 AM each day demonstrating that the locomotor activity of WKY female rats with minor?? Non-significant remained similar for 42 days and following daily saline injections.

Experimental groups (Figures 2 and 3)

Comparing the activity after the first MPD injection (ED 2 MPD) to ED 1 saline (ED 1BL) provide the MPD acute effects. Comparing ED 11, or 27, or 36 and 42 after MPD to ED-2 after MPD provide to determine the chronic effects of the drug. Figure 2 summarizes the Horizontal Activity (HA), Total Distance (TD) traveled, and Number of Movements (NM), Vertical Activity (VA) and Stereotypic Movements (SM) measured in female WKY rats for a total of 2 hr. Post injection of Sal at ED 1, and 0.6, 2.5 and 10.0 mg/kg MPD at ED 2, 11, 27, 36 and 42. Figure 3 summarizes similar experimental results from males.

Acute 0.6 mg/kg, 2.5 mg/kg, and 10.0 mg/kg MPD on female (Figure 2)

The acute dose of 13.0 mg/kg MPD elicited a significant increase in the Horizontal Activity (HA), Number of Movements (NM), and Stereotypic Movements (SM) (Figure 2A, C, and E), while the same MPD dose (0.6 mg/kg) elicits significant (P<0.05) decrease to the Total Distance (TD) traveling and Vertical Activity (VA) Figure 2B and D, i.e. this 0.6 mg/kg MPD dose elicits different effects on different locomotor behaviors. Acute 2.5 and 10.0 mg/kg MPD elicits a significant (P<0.05) increase in all five locomotor behaviors with dose response characteristics, meaning, with each increased dose, locomotor activity was further increased (Figure 2).

Chronic effect of 0.6 mg/kg MPD on females (Figure 2)

Rechallenge of 0.6 mg/kg MPD at ED 11,or at ED 27, or at ED 36, or at ED 42 as compared to the initial MPD effect at ED 2 for HA resulted in significant (P<0.05) decrease, increase, no change and significant increases compared to ED 2 (Figure 2A) respectively. For TD, the repeated 0.6 MPD at ED 11, 27, 36, and 42 as compared to the initial effect at ED2 elicited no significant effect at ED 11, 27, and 36, while at ED 42 the MPD injection elicited significant (P<0.05) increase as compared to the initial effect of MPD at ED2 respectively (Figure 2B). The same repeated 0.6 mg/kg MPD effect on NM exhibits different outcomes. At ED 11, 27, 36, and 42 MPD elicits significant (P<0.05) decrease, decrease, no change, and significant (P<0.05) increase respectively (Figure 2C). The same repeated 0.6 mg/kg MPD elicited on VA behavior at ED 11, 27, 36, and 42 compared to the acute MPD at ED2, no effect, no effect, significant (P<0.05) increase, and significant (P<0.05) increase respectively (Figure 2D). While the same repeated (chronic) dose on SM elicits different effects i.e., at ED 11, 27, 36, and 42 as compared to the effect of acute 0.6 mg/kg MPD at ED2, further significant (P<0.05) increase effects were observed at ED 11, ED 27, no different at ED 36 while significant (P<0.05) increase at ED 42 respectively. In conclusion, each locomotor behavior (HA, VA, TD, NM, and SM) was affected differently by repeated (chronic) 0.mg/kg MPD exposure.

Chronic effect of 2.5 mg/kg MPD on female (Figure 2)

The rechallenge (Chronic) 2.5 mg/kg MPD at ED 11, or at ED 27, or at ED 36, or at ED 42 was significantly (P<0.05) further increased in locomotion as compared to the initial 2.5 mg/kg MPD effects given at ED2 in all the 4 different locomotor activity behaviors (HA, TD, NM, and SM) accept VA respectively (Figure 2A-E 2.5 mg/kg MPD).

Chronic effect of 10.0 mg/kg MPD on females (Figure 2)

The same repeated (chronic) 10.0 mg/kg MPD at ED 11, or at 27, or at ED 36, or at ED 42 as compared to the initial (acute) 10.0 mg/kg MPD in each of the 5 locomotor behaviors exerted different effects (Figure 2A-E 10.0 mg/kg MPD). The HA at ED 11 responded to the drug in the same way as on ED 2 i.e., no significant difference compared to the MPD initial effects at ED 2, while at ED 27, 36 and 42 further significant (P<0.05) increases were observed as compared to the initial (acute) MPD at ED2. This further increase is interpreted as expression of behavioral sensitization (Figure 2A). However, the same MPD dose (10.0 mg/kg) differently affects the TD traveling behavior, i.e. at ED 11 significant (P<0.05) decreases were observed as compared to the initial effect of MPD at ED 2, no difference at ED 27 compared to ED 2, and further significant (P<0.05) decrease on ED 36 and ED 42. This further significant decrease compared to the initial effect is interpreted as expression of tolerance (Figure 2B). Similar observations were noted for the NM and VA (Figure 2C and D) but the intensity of decreases at ED 11, 27, 36, and 42 were of varying degrees (Figure 2 C and D). However, the same MPD dose of 10.0 mg/kg on SM exhibited a significantly (P<0.05) different pattern. At ED 11 compared to ED 2 MPD tolerance was observed, at ED 27 and ED 36 MPD had similar effects as at ED 2, and while at ED 42 compared to ED 2 behavioral sensitization were observed (Figure 2E).

Acute effect of 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD on males (Figure 3)

The acute dose of 0.6 mg/kg MPD elicited a significant increase in the Horizontal Activity (HA), Number of Movements (NM), and Stereotypic Movements (SM) (Figure 3A,C, and E), while the same MPD dose (0.6 mg/kg) elicited a significant (P<0.05) decrease to the Total Distance (TD) traveling and Vertical Activity (VA) as shown in Figure 3B and D; i.e. this 0.6 mg/kg MPD dose elicits different effects on different locomotor behaviors. Acute 2.5 mg/kg and 10.0 mg/kg MPD elicits a significant (P<0.05) increase in all five locomotor behaviors with dose response characteristics, meaning that with each increased dose, locomotor activity was further increased (Figure 3).

Chronic effect of 0.6 mg/kg MPD on males (Figure 3)

Repeated 0.6 mg/kg MPD at ED 11, or at ED 27, or at ED 36, or

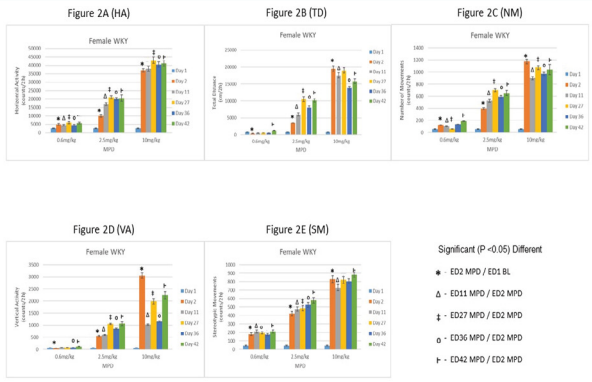


Figure 2: The figure summarizes the effect of acute and chronic dose response of 0.6 mg/kg, 2.5 mg/kg and 10.0 mg/kg MPD on four female WKY rat groups and their Horizontal Activity (HA), Total Distance (TD), traveling in cm, Number of Movements (NM), Vertical Activity (VA), and Stereotypic Movements (SM). All four groups were recorded following saline at experimental day 1 (ED 1)- left blue column is all histograms, following six daily injections of 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD at ED 2 to ED 7, followed by several washout days. MPD rechallenge of either 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg MPD was given at ED 11 to group 1; at ED 27 to group 2; at ED 36 to group 3 and at ED 42 to group 4 of the same dose given at ED 2 to ED 7. Significant (P<0.05) differences noted within and between each dose group of each locomotor expression and between the five different locomotor activities behavior expression.

at ED 42 as compared to the initial (acute) MPD effect at ED2 for HA resulted in significant (P<0.05) decrease at ED11, no difference at ED 27 significant decrease at ED 36, and significant (P<0.05) further increase on ED 42 as compared to the effect of the same MPD dose of 0.6 mg/kg at ED2 (Figure 3A 0.6mg/kg). The 0.6 mg/kg MPD effects on TD traveling was different from the effects of MPD on HA (Figure 3B). The NM behavior expression response to MPD rechallenge at ED 11 with no difference compared to ED 2 MPD (acute MPD). The MPD rechallenge at ED 27 resulted in a significant (p<0.05) decrease in behavioral activity as compared to the initial dose of MPD at ED2, while at ED 36 and ED 42 the reverse effect was observed, i.e., further significant (P<0.05) increase in NM was observed (Figure 3C). The VA expressed different response patterns to the same 0.6 mg/kg MPD dose i.e. significant (P<0.05) decrease at ED 11, significant (P<0.05) increase on ED 27, no difference at ED 36, and significant (P<0.05) increase on ED 42 as compared to the effect of the same MPD dose of 0.6 mg/kg on ED 2 (Figure 3D). SM exhibited completely different response patterns i.e., all the rechallenge MPD treatments at ED 11, 27, 36, and 42 resulted in no difference from the initial (acute) effect at ED 2 (Figure 3E).

Chronic effect of 2.5 mg/kg MPD on males (Figure 3)

The rechallenge 2.5 mg/kg MPD at ED 11 compared to the initial MPD exposure at ED 2 on HA and TD showed no difference compared to ED 2 MPD, while the rechallenge 2.5 mg/kg at ED 27, or at ED 36, or at ED 42 compared to ED 2 MPD, the initial administration, exhibited further significant (P<0.05) increases (Figure 3 A and B). However, NM, VA and the SM each responded differently to rechallenge MPD (Figure 3C-E). The NM exhibited significant (p<0.05) attenuation at ED11 following MPD compared to the initial MPD effect of the same dose at ED2, i.e. expressing tolerance, while the responses to rechallenge MPD at ED 27, 36, and 42 were similar to the response obtained following the initial MPD exposure at ED 2 (Figure 3C). The VA in ED 11, 27, 36, and 42 responded to 2.5 mg/kg MPD in significant (P<0.05) further increase as compared to the same MPD dose given at ED 2, i.e. expressing behavioral sensitization, however the intensity between the days were significantly (p<0.01) different (Figure 3D). The SM activity following MPD rechallenge exhibited a completely different response pattern to MPD exposure. Rechallenge MPD exposure at ED 11 and 27 as compared to the initial MPD exposure at ED 2 were about the same, while ED 36 and ED 42 exhibited further significant (P<0.05) increases as compared to the initial effect of MPD at ED 2, i.e., behavioral sensitization was expressed at ED 36 and 42 (Figure 3E).

Chronic effect of 10.0 mg/kg MPD on males (Figure 3)

The rechallenge 10.0 mg/kg MPD at ED 11, 27, 36 and 42 as compared to the initial MPD exposure at ED2 responded by significant (P<0.01) decreases, i.e., expressing behavioral tolerance in all five locomotor behaviors studied. However, the degree of attenuation (tolerance) in percentage was significantly (P<0.01) different among the five indices (HA, TD, NM, VA, and SM). TD traveling exhibited the greatest reduction in percentage at ED 11, 27, 36, and 42 and the SM behavior exhibited less than the other four behavioral expressions (Figure 3 10 mg/kg).

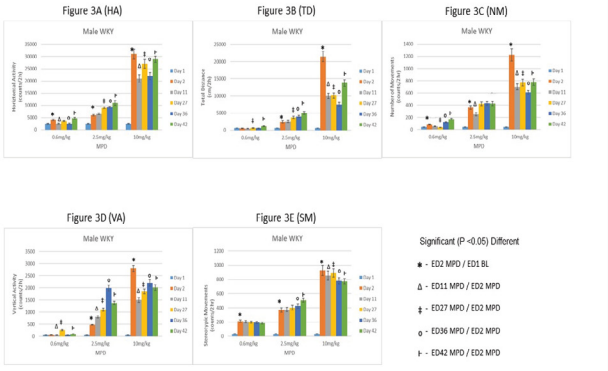
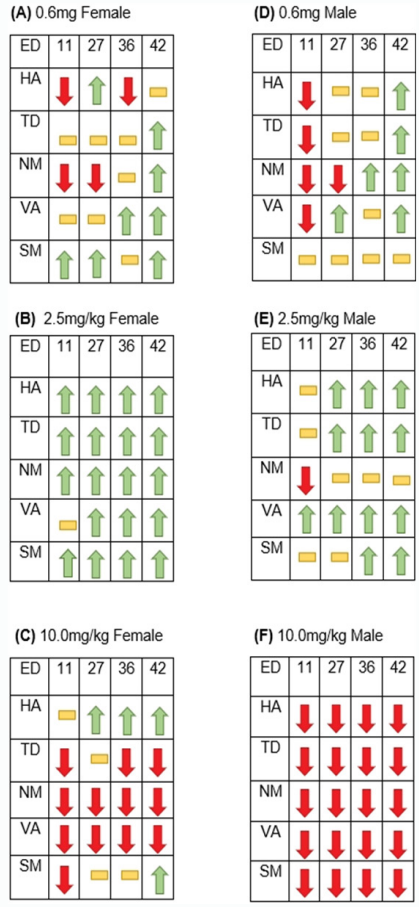


Figure 3: Life expectancy of WKY rats is 2 years=100%. Life expectancy of female in USA at 2019 was 78 years. 78 × 360 day/year=28,080 days=100%. (1) Percentage of WKY rat compare to human life. (2) Equal to 477 days of human life. (3) Equal to 15.9 month of human life. (4) Equal to 1.3 year of human life.

Comparison between females and males to repetitive MPD exposure (Table 2)

Table 2 summarizes the response direction following MPD exposure of either significant increase (arrow up) or significant decrease (arrow down) or no significant change of the five recorded locomotor expression activities recorded in females (Table 2A-C) and males (Table 2D-F). The table shows that each of the five behavioral expressions in female rats response to repetitive 0.6 mg/kg MPD exposure are significantly (p<0.01) different from the male groups (Table 2A compared to Table 2D) using the chi square test. Similar observations were observed following rechallenge 2.5 mg/kg MPD, i.e. each locomotor behavior of the female group responded significantly differently from the male group (Table 2B compared to Table 2E). Following rechallenge 10.0 mg/kg MPD exposure, differences between the sexes were expressed by the HA and SM, while the TD, NM, and VA behaviors expression responded similarly to rechallenge MPD (Table 2C compare to Table 2F).

Table 2: The table summarizes how each of the female and male subgroups responded to MPD rechallenge at experimental day 11 (ED 11) or at ED 27, or at ED 36, or at ED 42 to each of the five behavioral expression – HA: Horizontal Activity; TD: Total Distance Traveling; NM: Number of Movements; VA: Vertical Activity; and SM: Stereotypic Movements. ↑ indicate significant (P<0.05) increase, ↓ indicate significant (P<0.05) decrease and 6% indicate non-significant effects as compared to the initial effects of MPD at ED 2. The table demonstrated significant (P< 0.01) differences in the direction of the responses to the MPD rechallenge exposure between the ED 11, 27, 36, and 42 and between the five different behavioral expression (HA, TD, NM, VA, and SM as well between the sexes.



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	(1)	(2)	(3)	(4)
6 Days	1.70%	477 Days	15.9 months	1.3 years of human life
11 Days	3.10%	8.71 Days	290 months	2.4 years of human life
27 Days	7.50%	2.106 Days	70.2 Months	5.9 years of human life
36 Days	10.00%	2.808 Days	93.6 Months	7.8 years of human life
42 Days	11.70%	3.285 Days	109.5 Months	9.1 years of human life

Discussion

The study investigated for how long the consecutive six days of MPD consumption still indicates behavioral effects. The main findings of this study are: 1) Five different locomotor behavior expressions were measured, each respond similarly to acute MPD but differently to chronic MPD exposure, suggesting that each of the five locomotor behavioral expressions are regulated by different neuronal circuits. 2) The same dose (0.6 mg/kg, 2.5. mg/kg or 10.0 mg/kg) of chronic MPD caused behavioral sensitization in some animals and behavioral tolerance in other animals. Behavioral sensitization or tolerances are one of the experimental biomarkers indicating dependent and addiction. 3) The effect of MPD rechallenge at ED 11 after six daily MPD exposures of 0.6 mg/kg, 2.5 mg/kg or 10.0 mg/kg reveal that behavioral sensitization or behavioral tolerance developed during the six daily MPD exposures. While MPD rechallenge at ED 27, 36 and 42 reveals that behavioral sensitization and tolerance remain for prolonged periods of time with intensity fluctuations. 4) Once MPD was used, its effects on locomotor behavior remain for long periods of time in female and male rats.

The study expands on previous investigations into the acute and chronic effects of MPD commonly known under the brand name Ritalin in female and male WKY strain rats. The study uses field arrays to measure locomotor behavioral expression (indices) of Wistar-Kyoto (WKY) rats in groups of males and females in response to three different doses of MPD: 0.6 mg/kg, 2.5 mg/kg, and 10.0 mg/kg to gauge the differences in acute and repetitive (chronic) behavioral effects of MPD administration. Rats have shown behavioral response to stimulants in similar fashion to humans in multiple studies [30,31]. As opposed to studies that rely on higher technology such as brain implants, the open field array is a time and cost-effective method to track the locomotor behavioral activity of five different neuronal circuits. The open field array system we used has 3 levels of 16 sensors each and provides 20 points of data related to rat activity and movement in three dimensions, though our experience supports using five of those 20 that measure: Horizontal Activity (HA), Total Distance (TD) travelled, Numbers of Movements (NM), Vertical Activity (VA), and frequency of Stereotypical Movement (SM) activity expression. These measurements have proven effective in identifying behavior modifications from acute and chronic MPD use. The newer, higher technology options can differentiate amongst five neuronal circuits involved in the behavior though with more invasive trauma to the subjects that can confound behavior results and at greater cost. Processing results can be more challenging as separating the circuits can mask tolerance and sensitization in ANOVA analysis. Combining open field assays with some newer technologies may help identify relationships between individual movement behaviors and individual neuronal circuits and cellular changes.

Electrophysiological and behavioral recordings following MPD treated rats were found to have higher no glutamate levels activating metabotropic glutamate receptors suggesting phasic signals as a potential therapeutic mechanism and also suggesting the potential of such recordings from distinct neuronal circuits to identify differences in MPD treatment effects in particular neuronal circuits [32-35]. Tissue analyses have identified interesting patterns of astrocyte atrophy and loss of blood-brain barrier immunological privilege and inflammation with higher doses of MPD. Interestingly low-dose MPD can increase cellular junction integrity supporting MPD being of potential benefit in cognition and treatment of ADHD at lower dosage, while higher dosages lead to inflammatory and degenerative effects undermining beneficial effects of the drug [36,37]. Combining these techniques with open field assay has the potential to identify specific behavioral changes in HA, TD, NM, VA and SM patterns that correlate to underlying physiological and histological changes. To relate behavioral changes in acute and chronic MPD use to humans, we have previously used an extrapolation based on the life expectancy of the rats *vs.* human life expectancy [27,38]. We calculated that 6, 11, 27, 36, and 42 days of a rat's life are equivalent to 1.3, 2.4, 5.9, 7.8, and 9.1 years in human life (Table 3), i.e., the six daily MPD exposure in rats can be considered long term consumption of the drug, and

once used its effects remain for long periods of time at least 9.1 years (Table 3). Moreover, comparing the effects of MPD dose response experiments after six daily MPD exposures and three washout days (i.e., ED 11) to those with longer washout days at ED 27, 36 and 42 for the 0.6 and 2.5 mg/kg MPD reveal significantly different ($p<0.01$) within each sex and between the sexes, while following 10.0 mg/kg MPD it was not significantly different in both sexes. This observation suggests that the 0.6 mg/kg and the 2.5 mg/kg MPD are physiological doses while the 10.0 mg/kg MPD mega dose has nonspecific effects.

Previous studies have focused on differences in behavioral response of WKY *vs.* Spontaneous Hypertensive/Hyperactive Rats (SHR) which serves as a good model for ADHD including greater tendency for cocaine self-administration consistent with increased SUD in humans with ADHD [9,10,39-41]. This current study uses only WKY rats, originally bred as normotensive controls for SHR rats, but in sex sorted groups to study the role gender plays in behavioral response such as sensitization and tolerance with acute and chronic MPD administration. The dose of 10.0 mg/kg MPD is relatively high compared to human dosages, but rats more rapidly metabolize MPD and those dosage ranges have worked in previous studies to show statistically significant differences in dose response and sensitization *vs.* tolerance [18,39].

The present study addresses the role of sex in acute and chronic effects of MPD use, and in agreement with past studies that show statistical significance of sex differences in response to cocaine administration with a variance in the liver enzyme response as well as in temperature changes suggesting CNS differences [42]. Female rats have also been observed to more rapidly become sensitized to cocaine but only in adulthood secondary to the effects of Estradiol and Progesterone which were seen to increase dopamine (DA) release, reuptake, and turnover as well as making DA receptor alterations and up regulating DA site density [43]. The present study found no statistical difference in locomotor activity between the control groups whether they underwent saline injection, or not, evidence that animal handling and the needle injection of MPD is not a confounding factor in the study. All rats showed the highest chronic behavioral response to the 0.6 mg/kg dose while the 2.5 mg/kg elicited a delayed response after ED11 and 40% of the rats showed increased response after washout without statistically significant differences between male and female rats [44-46].

At the 10.0 mg/kg (highest) MPD dose, half the rats had decreasing behavior with chronic exposure as compared to the initial MPD dose, while the other half demonstrated further significant increases in response after washout as compared to the initial exposure which elicited the greatest response in 80% of the subjects. The primary differences between responses for the two sexes were in the percentage that had chronic effects from MPD dosing 86% for males *vs.* 66% for females and a lack of increase in vertical activity for the females at the lowest dose of MPD. Hormonal effects may play a factor in activity differences between the sexes as well as the later sexual maturity of the female specimens. Considering the controversial nature of treating millions of children with MPD, the appreciated differences between male and females suggest further study of sex differences with use are warranted as they may impact clinical decisions for human patients. The variations in locomotor activity expression between sexes and between the five different locomotor behavior expressions suggest that the each of these regulate by different neuronal circuits and that MPD affects each neuronal circuit differently and argue that distinct neuronal circuits are involved and warrant identification and individual study to determine the effects of MPD on each circuit. The present study focused on WKY rats to study sex effects in a non-ADHD rat model. But further study is warranted to identify sex differences in MPD in ADHD models such as SHR rats and possibly other genetic strains, both with early and late initiation of MPD, particularly to determine sex impacts on chronic behavioral changes and subsequent tendency towards SUD. Furthermore, studies to provide correlation between the open field assay results, electrophysiological measurements, and histological analyses may also improve the usefulness of open field assay allowing for cost effective studies at greater scale.

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