



Chronic Alterations in Behavior and Neuronal Morphology Associated With Methylphenidate Treatment During Development in Rats

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Stimulant drugs such as methylphenidate (MPD) are normally administered to children with neurodevelopmental disorders such as attention-deficit/hyperactivity disorder. Although there are studies examining the chronic postdrug behavioral effects of adults, the focus of such studies is on psychiatric syndromes such as anxiety, depression, or drug abuse. Little is known about the chronic effects on cognition, and executive function in particular. In the current study we examined the effect of MPD administration during the pre- and postpubertal developmental phases on behavior and neuronal morphology in adulthood. Injections of 0.5 mg/kg of Ritalin (2× daily) began immediately following weaning (postnatal day [P] 22) and continued until P60. Measures of executive functioning included open field testing (response inhibition), play behavior (flexibility and rule following), and a nonmatch to sample T-maze task (working memory). In order to control for the effects of maze training on neuronal morphology, a subset of rats did not participate in the T-maze training paradigm, but they were handled and transported to the testing area along with the animals that were included in behavioral testing. The results showed that prolonged, early MPD exposure enhances motor activity and negatively impacts on cognitive skills, producing a decline in performance on the T-maze task in adulthood. Play behavior was also altered during MPD treatment with saline-treated rats showing a distinct preference for the same-experience conspecifics when given a choice between saline or MPD-treated playmates. Furthermore, the anatomical results revealed that both training and MPD altered the dendritic fields of layer III prefrontal cortical regions, whereas the combination of MPD + training increased the dendritic field in layer V prefrontal neurons only. There were no drug or training effects on neurons of the parietal cortex, hippocampus, or nucleus accumbens. Thus, early exposure to MPD had chronic effects on executive function and the organization of prefrontal circuitry.

Public Significance Statement

Stimulant drugs such as methylphenidate (MPD) are normally administered to children with neurodevelopmental disorders such as attention-deficit/hyperactivity disorder, but little is known about the long-term effects of this drug exposure. Juvenile rats were given MPD for 37 days and their behavior and neuronal morphology studied later. In adulthood there were chronic effects of the drug treatment on both behavior and morphology of neurons in the prefrontal cortex.

Keywords: prefrontal cortex, methylphenidate, neuronal morphology, executive function

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Animals are continually faced with a changing environment that requires planning and the generation of novel actions—behaviors that are often referred to as executive functions (Leh, Petrides, & Strafella, 2010). Executive functions, which include attentional control, working memory, cognitive flexibility, and inhibition of inappropriate actions, are not the exclusive domain of the prefrontal cortex but they are heavily dependent on the integrity of the prefrontal cortex (Leh et al., 2010). The prefrontal cortex matures slowly (Petanjek et al., 2011), and as a result, executive functions mature slowly as well. There is an extensive literature on the negative effects of prefrontal cortex injury on executive functions (Kolb & Whishaw, 2015), but less is known about the effect of other factors such as the chronic effects of psychoactive drugs on executive functions. There is, however, evidence of chronic psychiatric effects of psychoactive drugs such as methylphenidate (MPD) on adults that received drug treatment as juveniles or adolescents (e.g., Bolaños, Barrot, Berton, Wallace-Black, & Nestler, 2003; Carlezon, Mague, & Andersen, 2003; Jordan & Andersen, 2017). It is not known, however, what chronic impact MPD-like drugs might have on the structure and function of the prefrontal cortex, *per se*. There is, however, considerable evidence that psychoactive drugs chronically alter the morphology of neurons in the prefrontal cortex of rodents (e.g., Robinson & Kolb, 2004). For example, psychomotor stimulants such as amphetamine, cocaine, and nicotine alter the morphology of prefrontal regions, although differently in the medial versus orbital prefrontal cortex of adult animals (Robinson & Kolb, 2004). These morphological changes have long-lasting functional effects and can reduce the later plasticity of both the prefrontal cortex and other cortical regions (Hamilton & Kolb, 2005; Kolb, Gorny, Li, Samaha, & Robinson, 2003). It is known that exposure to psychomotor stimulants in development has chronic effects on both many behaviors (Carlezon et al., 2003) and neuronal morphology (Diaz Heijtz, Kolb, & Forssberg, 2003), but no studies have been done looking at the chronic effect of juvenile drug exposure on executive functions (EF) and neuronal morphology.

The current study asked whether there was a persistent change in EF and/or neuronal mor-

phology after MPD was given to young animals. Importantly, the rats were given a clinically relevant dose and we used standard Long-Evans rats to allow comparison to the morphological results of our earlier drug experiments using juvenile and adult rats.

Our behavioral measures of executive function included juvenile play (a measure of flexibility and rule following; see Pellegrini, 2013; Pellis & Pellis, 1997) and delayed alternation (a measure of working memory). These behaviors also were chosen because prefrontal lesions disturb both of these behaviors (e.g., Euston, Gruber, & McNaughton, 2012; Uylings, Groenewegen, & Kolb, 2003), so it might be expected that MPD-induced alterations in prefrontal neural networks could influence these types of behaviors as well. We chose to use the same behavioral methods that we have used in previous studies, which have shown changes in dendritic morphology (e.g., Kolb, Cioe, & Comeau, 2008; Robinson & Kolb, 2004).

To aid in the determination of the potential influence of Ritalin on experience-induced plasticity, we also included “training” and “no-training” groups because we have shown previously that training alone alters dendritic morphology of the prefrontal cortex (PFC; Muhammad et al., 2013). In keeping with the notion that long-term use may produce alterations in the Nucleus Accumbens (NAc) as well as the PFC, we used Golgi-Cox analyses to determine structural changes that would in effect indicate altered circuitry in these regions.

Materials and Methods

Subjects

Twenty-one Long-Evans male rats chosen from four litters were used in the current study. On postnatal day (P) 21, the pups were randomly assigned using a cross litter design to receive one of two treatments: 1) MPD (0.5 mg/kg; $n = 10$) or 2) saline (0.9% NaCl dissolved in dH₂O solution; $n = 11$). Testing began at P21 and continued to approximately P100 (see Figure 1). Three animals were chosen randomly from three litters and two from the remaining litter for the saline group, and three from two litters and two from the other two for the MPD group. All of the animals were used in the drug-induced activity measure and play be-

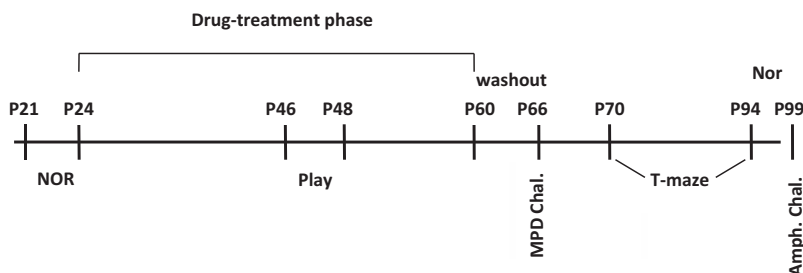


Figure 1. The experimental design and test timeline. P = postnatal day; NOR = novel object recognition task; washout = drug-free period; Chal. = acute drug administration; MPD = methylphenidate.

havior but only six MPD and saline animals were trained in the delayed alternation task. It is possible that using more than one rat per group per litter could reduce the power of the study due to possible litter effects, but none were observed.

Once weaned, all animals were housed in large, Plexiglas hanging tubs (39 × 57 × 21 cm) in mixed groups (MPD and saline) of five or six animals and maintained on a 12-hr light/dark cycle throughout the experiment. Animals were given ad libitum food and water except during the T-maze training period, at which time they were on a restricted food schedule. During the restricted food schedule, each animal received 20–25 g of rat chow at the end of daily testing. The following morning (at least two hours prior to testing), uneaten food pellets were removed. At the end of the experiment all rats were given an overdose of sodium pentobarbital and intracardially perfused with a 0.9% saline solution. The brains were harvested and postfixed in a Golgi-Cox solution for two weeks and then transferred to a 30% sucrose solution where they remained until being sectioned. Brains were sectioned at 200 μ m on a Vibratome and mounted for staining. Mounted sections were stained using the Golgi staining procedures set out by [Gibb and Kolb \(1998\)](#).

Drug Administration

Methylphenidate (MPD). The initial drug-treatment phase began when pups weighed about 60 g on average ($\sim$ P23). Rats received subcutaneous injections of MPD (0.5 mg/kg) or vehicle (0.9% saline in dH₂O vehicle solution) in the nape of the neck twice a day (a.m. and p.m.), spaced 6 hr apart for 37 consecutive days

($\sim$ P60). Prior to each injection, all rats were removed from their home cage and taken to the testing area, where they received the injections. Thus, all injections occurred in an “away” or novel environment. At the end of the drug-treatment period, daily injections were discontinued and subjects remained drug-free (washout period) five days ($\sim$ P65), at which time all animals received a single acute MPD challenge (0.5 mg/kg) to assess drug sensitivity.

Amphetamine challenge. On the final day of the experiment ($\sim$ P100), all rats were given a single intraperitoneal injection of d-amphetamine (1.0 mg/kg) and activity was monitored to test for any residual drug sensitivity. The rats were individually placed into the activity apparatus five minutes postinjection, and motor activity was recorded for 20 min. Rats were sacrificed immediately after testing and the brains harvested.

Behavioral Analyses

Activity apparatus. Animals were individually tested in the AccuScan Instruments Inc. Versamax animal activity monitoring system (open field) to measure motor response. The activity apparatus consisted of a clear Plexiglas cage measuring 42 cm × 42 cm × 30 cm with a removable Plexiglas lid. The cages were equipped with horizontal and vertical sensors (infrared beams) that monitored activity in selected intervals for a total of 20 min. Recorded measures of horizontal activity (number of beam breaks on the lower bar in each sample period), total distance (path traveled in centimeters), and the number of movements (number of separate horizontal movements identified as such by a one-second break in ambulatory ac-

tivity) were retrieved for analysis. Once the testing period was complete, the VersaDat function scanned the files for errors to verify the integrity of data collected. The files were then converted for import to an Excel spreadsheet.

Play behavior. Play behavior analysis was used to 1) detect obscure peculiarities in social behavior, and 2) observe possible changes in the typical pattern of play behavior in MPD-treated animals. To accomplish this, the animals were analyzed in groups of three during play. Group triads consisted of either two saline- and one MPD-treated animal or two MPD- and one saline-treated pup. The animals in the triads were not socially housed together. MPD-induced oddities in behavior were assessed by quantifying the willingness of saline-treated rats to initiate and participate in play with MPD-treated rats if given a choice. At the same time, initiation and play response were analyzed in MPD-treated animals. Subjects began habituation in the play arena at P42 (prior to Day 21 a.m. injections). Animals were introduced to the arena in groups of about 5–6 and left to acclimate to the new surroundings for 20–30 min each day for three consecutive days. Following the third habituation period, the rats were individually housed (isolated) for a 24-hr period. At the end of the 24-hr period, rats were taken to the play arena prior to the a.m. injection and placed inside the enclosure with playmates. The behavior was videotaped for a 10-min period with the experimenter out of the room. As subjects completed the task, they were removed from the enclosure and returned to their home environment, where they were once again group housed for the remainder of the experiment.

The video recording, analyzed frame by frame, was scored for attacks (initiated play with the snout on the nape of the playmate's neck), complete rotation defense, or evasion response (avoidance) generated in response to an attack (Pellis, Pellis, & Whishaw, 1992). The defense was scored as "complete rotation" when a rat had both fore- and hind limbs in the air while lying in a supine position. In the event that the rat turned away from the attacker instead of facing an attack, it was scored as evasion. The probability of complete rotation or evasion was calculated as the number of complete rotation or evasion divided by the total number of attacks carried out by the playmate (Pellis & Pellis, 2009).

T-maze task. Rats were habituated to the T-maze apparatus prior to the actual training phase. Once habituated, rats were trained to run to the end of the arms of the apparatus to obtain a food reward (piece of Froot Loops). Subjects were rewarded regardless of the arm they chose during the training phase. The testing phase began once all of the rats readily entered the arms to retrieve a reward. In the testing phase, rats were required to learn a nonmatch to sample paradigm of the T-maze task (similar to that used by Bartolini, Risaliti, & Pepeu, 1992). In short, each trial consisted of two components. In the first, one arm was blocked, forcing the subject into the open arm. In the second component, the block was removed and the rat was reintroduced to the maze (10-s delay). The fixed 10-s delay was chosen as we had previously found that this duration produced reliable changes in dendritic morphology (e.g., Kolb et al., 2008) and deficits in rats with PFC lesions (e.g., Nonneman & Kolb, 1979). To retrieve a reward, the rat had to choose the arm opposite of where the reward had just been obtained during component one. Subsequent trials began about 30 s after the end of the second component. Each subject was given 10 consecutive trials per day. A correct response on at least 8 out of 10 trials for four consecutive days was required to reach criterion. The rat was considered to have entered an arm when the head and both front feet were in the arm.

Anatomical Analysis

Neurons from select brain regions known to be affected by psychostimulant use were reconstructed using a camera lucida, and dendritic morphology was quantified for analyses. The cells included in the current study were pyramidal cells of the primary somatosensory cortex (Par 1, layer III), region three of the anterior cingulate cortex (Zilles Cg3, layer III and layer V), the dorsal agranular insular cortex (AID, layer III) described by Zilles (1985), CA1 hippocampal pyramidal neurons, and the core and shell of the nucleus accumbens.

To be included in the analysis, cells had to be 1) thoroughly impregnated by the Golgi-Cox solution so that the dendritic trees were clearly visible at 100 $\times$ and not obscured by blood vessels, neighboring processes, or debris, and 2) relatively intact with minimal breakage. The

cells were drawn using camera lucida (200 $\times$) and quantified by means of 1) dendritic branch order, a quantification of the number of bifurcations off each dendritic process that extends from the soma, and 2) Sholl analysis, a quantification of the number of processes that cross the rings (spaced 20 μm apart) of a concentric circle placed over the cell representation useful to quantify dendritic length. The means from five neurons in each hemisphere were calculated and used for later comparisons.

Spine density was also determined for fourth-order terminals of basilar dendritic trees in Cg3 LV and AID LIII. As with dendritic branch order and Sholl analysis, spine density for each hemisphere was determined by calculating the mean of five terminals (10 μm in length). Spines were drawn at 1000 $\times$ and included all spine protrusions that had a visible head. It was recognized that this method of evaluation is an underestimation of the total spine density as those spines on both the bottom and top of the dendrite would not be visible to the experimenter. Cells within each region were drawn by one of the experimenters blind to the groups until analysis.

Results

Behavioral Results

Motor activity. Horizontal activity and distance traveled (Figure 2A) as measures of motor activity illustrate the MPD-induced increase of motor activity that began on about Day 12 of treatment and increased over the course of the 37-day treatment period. The MPD challenge administered after a 5-day drug washout period demonstrates an increased behavioral sensitivity in pretreated MPD rats. Repeated-measures ANOVAs of the four 5-min intervals showed that 12 days of treatment was insufficient to produce increased motor activity in overall horizontal activity [$F(1, 19) = 1.51, p = .23$], or distance traveled [$F(1, 19) = 2.69, p = .12$]. On the last day of the treatment regimen (Day 37), repeated-measures ANOVAs of activity on Day 37 of treatment showed no overall effect of treatment on horizontal activity [$F(1, 19) = 0.08, p = .79$], nor distance traveled [$F(1, 19) = 0.20, p = .66$]. In view of the apparent effect of the drug on the fourth 5-min interval, a post hoc pairwise comparison was

performed and it revealed a significant increase in both by the fourth 5-min interval ($p < .05$).

MPD challenge. The challenge demonstrates an increased sensitivity as a result of previous drug exposure (see Figure 1). Repeated-measures ANOVAs of activity with treatment as the variable showed a significant effect of treatment on horizontal activity [$F(1, 19) = 5.41, p = .03$], and distance traveled [$F(1, 19) = 7.75, p = .012$]. This effect of the MPD challenge six days after the last MPD injection indicates a chronic effect of the MPD regime.

Adult amphetamine challenge. An amphetamine challenge was administered to all animals at the end of the experiment to assess the persistence of MPD-induced changes in motor activity. The animals used in the behavioral studies were separated from those animals that had been excluded from the T-maze training tests for this analysis in order to assess drug and T-maze training effects separately. Previous T-maze training enhanced the motor response to amphetamine in "drug naïve" rats. In contrast, whereas there were no differences between the untrained saline and MPD-treated rats, previous MPD use blocked the T-maze training-induced drug sensitivity exhibited by saline-treated animals (Figure 2B). An ANOVA of activity with treatment and T-maze training as variables showed a significant main effect of T-maze training on horizontal activity [$F(1, 17) = 4.34, p < .05$] and a marginal effect on distance traveled [$F(1, 17) = 4.14, p = .058$], but no significant main effect of treatment on either horizontal activity [$F(1, 17) = 0.006, p = .94$] or distance traveled [$F(1, 17) = 0.06, p = .82$], but a marginal interaction on horizontal activity [$F(1, 17) = 3.97, p = .063$], although not on distance traveled [$F(1, 17) = 3.09, p = .097$]. Thus, T-maze training significantly elevated the behavioral response to amphetamine in untreated rats for both horizontal ($p = .016$) and distance traveled ($p = .023$) but not in MPD pretreated rats ($ps > .81$).

T-maze task. Rats exposed to MPD as juveniles appeared to be at a disadvantage in the T-maze task, making almost twice as many errors as the untreated group ($M = 21, M = 12$, respectively) before reaching criterion as revealed by a one-tailed t test of errors with treatment as the variable, $t(11) = -1.76, p < .05$; Figure 3.

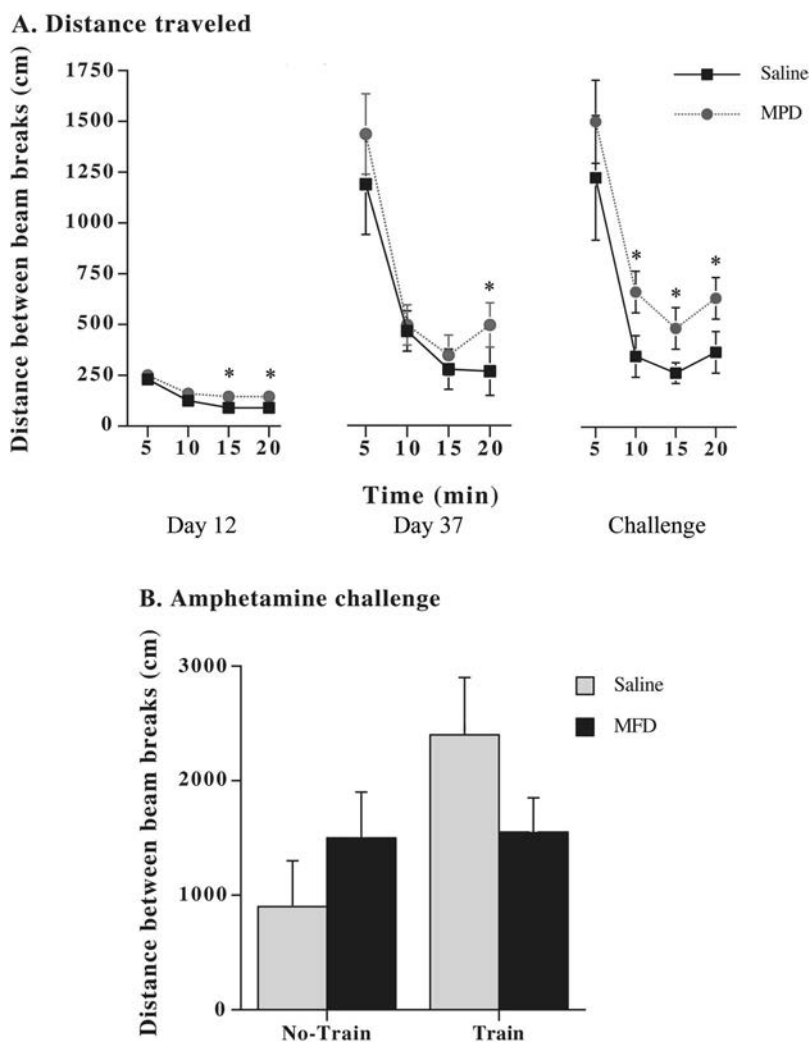


Figure 2. A. Activity during MPD treatment. B. Activity during amphetamine challenge. Data are $M \pm SEM$. MPD = methylphenidate. * $p < .05$.

Correlational analysis. Interestingly, performance on the T-maze task could be predicted using motor activity on the final day of the initial treatment regimen. A bivariate correlation test showed a strong positive correlation between T-maze errors and all three measure of motor activity: horizontal, $r = .995$, $p = .005$, distance traveled, $r = .996$, $p = .004$, and number of movements, $r = .997$, $p = .003$.

Play behavior. Analysis of play behavior was conducted using triads of saline- and MPD-treated rat, or two MPD- and one saline-treated rat. The results showed that saline-treated rats

were discriminative regarding their play partners (see Figure 4), choosing to initiate play significantly more often with saline-treated, rather than MPD-treated, animals [$F(1, 10) = 5.52$, $p = .04$], although they showed no discrepancy in avoidance behavior [$F(1, 10) = 0.79$, $p = .39$]. In contrast, MPD-treated rats did not show a playmate preference, initiating play equally between MPD- and saline-treated animals [$F(1, 10) = 0.87$, $p = .38$]. Interestingly, they did, however, use “avoidance” behavior more often when saline-treated animals were initiating the play [$F(1, 10) = 5.54$, $p = .04$].

T-Maze Errors

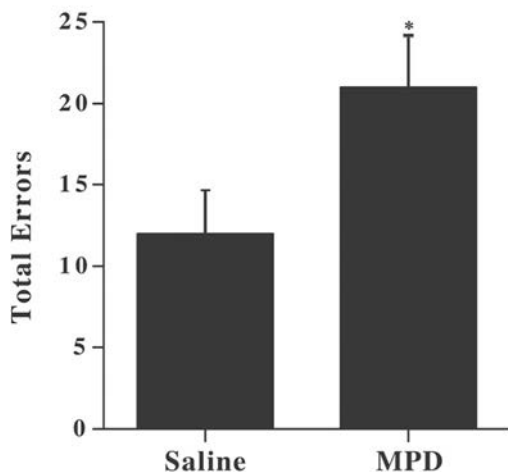


Figure 3. Mean errors in delayed-nonmatch-to-sample in T-maze. Data are $M \pm SEM$. MPD = methylphenidate. * $p < .05$.

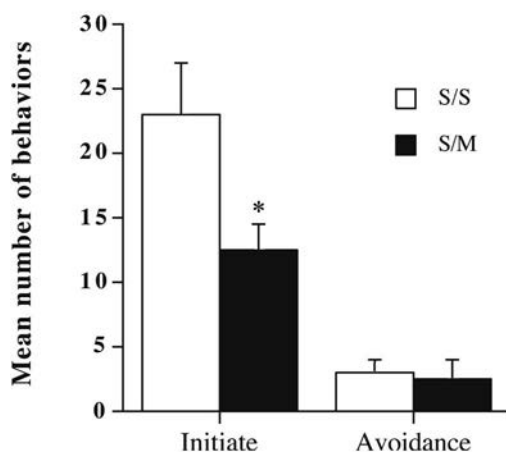
Anatomical Results

Analysis of dendritic morphology revealed that early MPD exposure as well as later T-maze training altered connectivity in the prefrontal cortex, albeit in different ways. The combination of early drug exposure and T-maze training also produced unique alterations in circuitry prefrontal cortical regions. In summary, MPD produced a decrease in spine density of the AID, but increased the apical and basilar dendritic field of Cg3 LIII, but not LV, neurons (see Figure 5). On the other hand, T-maze training produced the opposite effect in the AID, increasing basilar spine density. T-maze training, as with MPD, however, increased the dendritic field of LIII Cg3 neurons, but only in the basilar tree. Interestingly, the only significant alterations in Cg3 LV neuronal circuitry were found in the MPD + T-maze training group. The combination of experiences produced increases in basilar spine density as well as apical and basilar dendritic length. In contrast, the Cg3 LIII showed a decrease in the dendritic field as a result of the combined experience, an effect that was opposite of either MPD or T-maze training alone.

AID Layer III

Spine density. Prolonged MPD use in early life produced a reduction in basilar spine density of layer III AID pyramidal neurons of the prefrontal cortex. The effect was opposite of that of T-maze training, which increased the spine density of neurons in this region in both saline and MPD-pretreated rats (Figure 5A). The T-maze training-induced in-

A. Saline Play Behavior



B. MPD Play Behavior

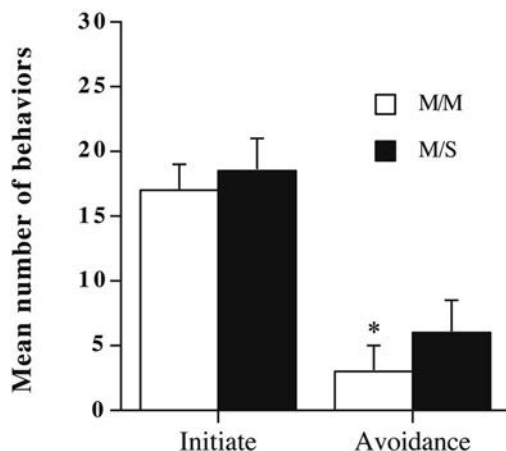


Figure 4. A. The number of times saline rats initiated play or used avoidance behavior in response to both saline (S/S) or MPD (M). B. A summary of the number of times MPD rats initiated play or used avoidance behavior in response to MPD (M/M) or Saline (M/S) rats. Data are $M \pm SEM$. MPD = methylphenidate. * $p < .05$.

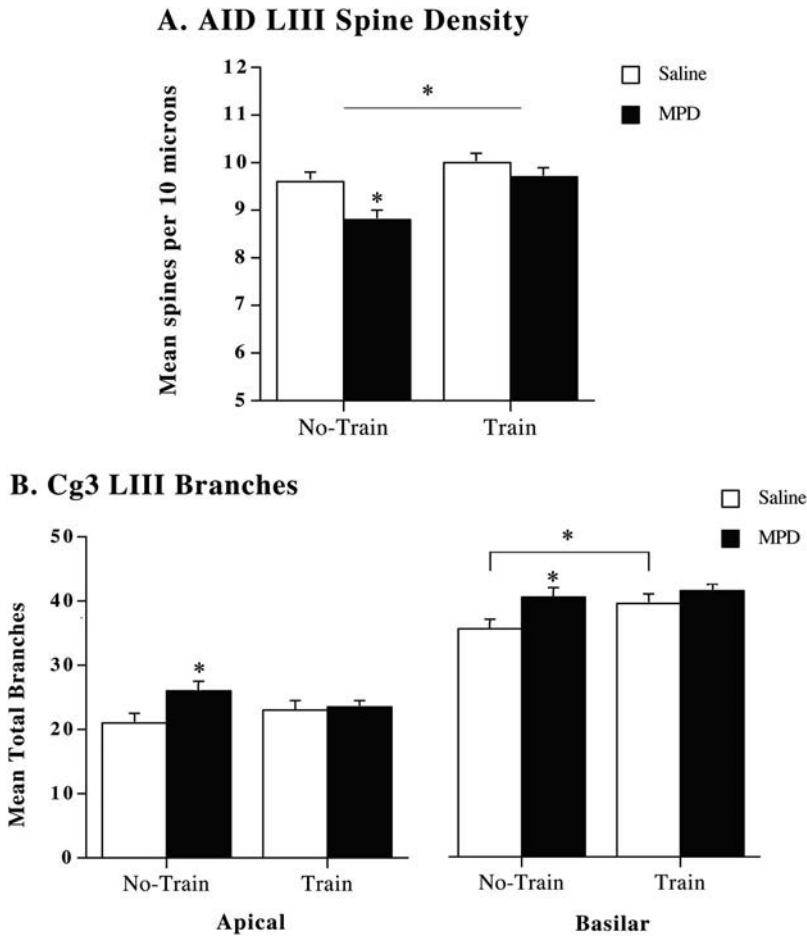


Figure 5. A. Mean spine density in basilar dendrites of layer III AID neurons. MPD alone produced a decrease in spine density, whereas training increased spine density. B. Summary of mean branch orders in layer III pyramidal neurons. The graph shows an MPD-induced increase in apical branch order that was blocked by subsequent training. In contrast, both MPD and training alone increased branch order in basilar dendrites but the combination had no further effect. Data are $M \pm SEM$. MPD = methylphenidate. * $p < .05$.

crease was robust and reversed the MPD-induced decrease found in animals that had not participated in the T-maze training. An ANOVA of spine density with treatment and T-maze training as variables showed a significant main effect of treatment [$F(1, 38) = 7.75, p = .008$] and T-maze training [$F(1, 38) = 18.48, p < .001$], but no interaction [$F(1, 38) = 0.28, p = .60$]. The pairwise comparison revealed that the treatment effect was significant in the No-Training group ($p = .036$), but not in the T-Maze Training group ($p = .09$).

Sholl and branch order. Measures of dendritic length (Sholl) and branch order revealed no other obvious changes (p values were all < 0.1) in the dendritic field of AID pyramidal cells (data not shown).

Cg3 LV Spine Density

Although neither MPD nor T-maze training alone affected spine density in layer V pyramidal cells, the combination of MPD + T-maze training increased spine density relative to either experience alone. An ANOVA of spine

density with treatment and T-maze training as variables showed no significant main effects of treatment [$F(1, 38) = 0.90, p = .35$] or T-maze training [$F(1, 38) = 1.08, p = .31$], but a trend for the interaction [$F(1, 38) = 3.34, p = .08$]. The pairwise comparison revealed a significant increase in the spine density of rats that had received MPD and T-maze training relative to MPD treatment ($p = .03$) or T-maze training ($p = .04$) alone (data not shown).

Cg3 LIII

Sholl and branch order. Both MPD and training produced changes in layer III of the Cg3. In summary, although MPD increased branch order of apical dendrites, subsequent T-maze training reversed the effect (Figure 5B). Basilar branch order was also increased by both MPD and T-maze training alone, but the combination of the two had no additional impact. The Sholl analyses also highlighted the effect of both T-maze training and MPD. Whereas T-maze training created a decrease in apical dendritic length in rats pretreated with MPD, the basilar dendrites showed an MPD-induced increase in length that was unaffected by subsequent T-maze training.

An ANOVA of apical branch order showed a significant main effect of treatment, [$F(1, 38) = 8.13, p = .007$], but not T-maze training, [$F(1, 38) = 0.34, p = .57$], nor interaction [$F(1, 38) = 3.19, p = .08$]. The MPD effect was only significant in the untrained animals, however ($p < .05$). In the basilar dendritic field, an ANOVA of dendritic branch order showed a significant main effect of treatment [$F(1, 38) = 14.08, p = .001$] and T-maze training [$F(1, 38) = 7.51, p = .009$], but no interaction [$F(1, 38) = 1.04, p = .32$].

In contrast, an ANOVA of apical dendritic length (Sholl) showed no significant main effect of treatment [$F(1, 38) = 0.29, p = .59$], nor training [$F(1, 38) = 0.55, p = .46$], but a significant interaction [$F(1, 38) = 3.96, p = .05$]. Whereas an ANOVA of basilar dendritic length showed a significant main effect of treatment [$F(1, 38) = 6.22, p = .017$], but not T-maze training [$F(1, 38) = 1.62, p = .21$] or interaction [$F(1, 38) = 0.22, p = .64$].

Spine density. The spine density for layer III could not be drawn as the staining had become too dark by the time that we decided to

include layer III in addition to layer V. The dendritic arbor had already been drawn and thus was not affected.

NAc core and shell. Long-term use of MPD in juvenile rats did not result in any significant changes in dendritic morphology of medium spiny neurons in either the shell or core of the nucleus accumbens. ANOVAs with treatment and T-maze training as variables showed no significant effects of treatment [$F(1, 36) = 0.62, p = .44$], training [$F(1, 36) = 0.01, p = .91$], or interaction [$F(1, 36) = 0.17, p = .90$] in the shell. Similarly, the core also showed no significant effect of treatment [$F(1, 36) = 2.19, p = .15$], T-maze training [$F(1, 36) = 0.26, p = .61$], or interaction [$F(1, 36) = 0.48, p = .49$].

Par 1. We found no effect of either MPD or training in the dendritic field of layer III Par1 neurons. An ANOVA of apical dendritic branching showed no significant main effect of treatment [$F(1, 38) = 0.007, p = .93$] or training [$F(1, 38) = 0.91, p = .35$], nor interaction [$F(1, 38) = 0.25, p = .62$]. An ANOVA of apical dendritic length also showed no significant main effect of treatment [$F(1, 38) = 0.41, p = .53$] training [$F(1, 38) = 0.49, p = .49$] or interaction [$F(1, 38) = 0.10, p = .75$]. Similar results were found for basilar branch order and length (ps all $> .40$).

CA1. Analysis of basilar branch order and length showed no influence of either condition. An ANOVA of basilar dendritic branch order showed no significant main effect of treatment [$F(1, 38) = 1.08, p = .31$], T-maze training [$F(1, 38) = 0.14, p = .71$], or interaction [$F(1, 38) = 1.34, p = .25$]. An ANOVA of basilar dendritic length also showed no significant main effect of treatment [$F(1, 38) = 0.09, p = .76$], T-maze training [$F(1, 38) = 0.32, p = .57$], or interaction [$F(1, 38) = 0.64, p = .43$].

Discussion

The results of the current experiment highlight a number of novel phenomena that support previous findings demonstrating the potential for permanent behavioral and anatomical alterations following early MPD exposure. First, early MPD exposure adversely affected behavior as measured by altered social behavior in young animals and produced a deficit in working memory. Second, adult rats pretreated with MPD during the preadolescent and adolescent

period showed unique patterns of PFC circuitry, but there was no apparent effect of MPD on neurons in nucleus accumbens, hippocampus, or parietal cortex. In addition, just the training of animals in the T-maze produced significant changes in PFC circuitry that were independent of those of MPD.

Altered Behavioral Functions as a Consequence of Long-Term MPD Use

Our behavioral measures included juvenile play (a measure of flexibility and rule following; see [Pellegrini, 2013](#); [Pellis & Pellis, 1997](#)) and delayed alternation (a measure of working memory). Performance on both measures was altered by MPD.

We predicted that early MPD could lead to long-term changes in motor activity in response to the drug. Although this did not show throughout the time on the drug regime, there was an increase in MPD-induced activity in the challenge test after six days of drug washout, showing a chronic effect of the drug exposure. The potential for early MPD use to produce chronic sensitivity to stimulants, thereby increasing the risk for addiction in later life, was not supported by the current results, however. We found no evidence of a persistent stimulant sensitivity following early drug exposure, as demonstrated by the results of the amphetamine challenge in adulthood.

Altered social interaction as a consequence of adolescent MPD use has been reported elsewhere ([Pellis & McKenna, 1995](#); [Vanderschuren, Stein, Wiegant, & Van Ree, 1995](#)). To our knowledge, however, this is the first time that the effects of prolonged, chronic MPD use on play behavior has been examined using triads. The rationale for using triads in the current study was to determine if there were peculiarities in social behavior that may go undetected by the observer but that could be inferred by the response/acceptance of saline and MPD-treated play partners. Indeed, it appears that there are. The play behavior results of the current study highlight two phenomena. First, although experimenters detected no obvious abnormalities in the behavior of MPD-treated rats, the reluctance of saline-treated rats to initiate play with MPD rats indicates that the treatment altered behavior in such a way that was detectable to “normal” rats. In contrast, MPD-treated rats ini-

tiated play equally among their conspecific partners. Similar results have been found in play interactions in juvenile rats following neonatal orbitofrontal cortex (OFC) lesions, in which case controls initiated fewer play attacks on OFC lesion-partners relative to intact play-partners ([Pellis et al., 2006](#)). Second, when MPD-treated rats were placed in triads that included another MPD-treated rat, they used avoidance behavior more often when play was initiated by the saline-treated partner. One explanation for the latter finding may be that MPD rats produce fewer ultrasonic vocalizations in initiating play. Juvenile rats emit many 50-kHz ultrasonic vocalizations during play behavior, and these appear to function to promote play, so fewer vocalizations by the MPD rats could have influenced the behavior of the MPD rats toward one another ([Kisko, Himmler, Himmler, Euston, & Pellis, 2015](#)). We note, however, that deaf rats show no decrease in play behavior ([Siviy & Panksepp, 1987](#)).

It is noteworthy that in the current study play behavior was assessed when the rats were still undergoing MPD treatment. The rationale was that in doing so we could take advantage of the peak play phase that occurs between P30 and P40 in rats ([Pellis & Pellis, 2009](#)). Due to extended time between last injection and play behavior (about 18 hr) and the fast metabolism rate of low doses of stimulants such as MPD (see [Field & Pellis, 1994](#)), however, it is unlikely that there was an acute stimulant-induced effect on observed play behavior. Furthermore, in a parallel study ([Comeau, 2007](#)), MPD treatment was only from P22 to P34. Play behavior measured on P42 showed similar results to those shown here, demonstrating that there was a chronic effect of MPD on play behavior.

Working memory is one of the core components of executive function ([Diamond, 2013](#)). There was a clear effect of MPD on performance in the T-maze in the current study: MPD-treated rats made almost twice as many errors as saline-treated in the T-maze task. We interpret this deficit as one of working memory, but it is possible that it could be related to a difference in rule learning or some other factor such as increased activity or perseveration. Indeed, the high correlation between open field activity and T-maze errors suggests that hyperactivity could be a factor. In addition, rats with PFC lesions do show increased perseverative responding in

many learning tasks, including delayed alternation (Nonneman & Kolb, 1979). Increased activity or perseveration could reflect a deficit in response inhibition, which would reflect a deficit in EF.

It may be surprising that given the low dose of MPD used in the current study, we would find behavioral deficits in adulthood. The findings suggest that the effects of early drug experience are persistent and may indeed influence subsequent behavior and learning.

Altered Connectivity in the Adult Brain as a Consequence of Early MPD Use and T Maze Training

The potential for psychostimulants to produce enduring alterations in cortical and subcortical connectivity has been reported previously by this lab and in collaboration with others following adult stimulant use (Adriani & Laviola, 2004; Andersen & Navalta, 2004). Importantly, these studies have linked the aberrant connectivity to behavioral indices of psychostimulant addiction and alterations in subsequent experience-induced plasticity. One of the questions asked in the current study was whether similar effects would be produced by low-dose psychostimulant use in young animals. It was predicted that, due to the late development of the PFC and related circuitry, and the fact that the immature brain is both highly plastic and vulnerable to experiential influences into adolescence (see Diaz Heijtz et al., 2003), MPD use during this period would influence developing circuitry. Furthermore, as T-maze training may also influence circuitry, it was predicted that MPD use would disrupt these changes, providing a possible explanation for some of the behavioral effects reported previously. Indeed, the area that was most affected by stimulant use in the current study was the prefrontal cortex, with no effect of either MPD or T-maze training in the NAc, hippocampus, or parietal cortex.

The anatomical findings in the current study illustrate that both prolonged MPD use and T-maze training result in pronounced alterations in PFC circuitry. Especially interesting was the finding that T-maze training and MPD produced very distinct patterns of connectivity, as did the combination of the two experiences. Thus, MPD use produced a decrease in the dendritic

field and spine density of basilar AID pyramidal cells, increased both the basilar and apical dendritic fields of layer III Cg3 neurons, and increased spine density in layer V Cg 3 neurons. In contrast, the effects of T-maze training were restricted to basilar fields of layer III neurons, producing increases in both the AID and Cg3. Furthermore, unlike MPD, training altered connectivity of layer V Cg3 neurons, producing a decrease in the apical dendritic field that was blocked by MPD exposure.

The finding that behavioral training alters PFC organization is not novel and has been seen in a variety of studies both in our lab and others (e.g., Comeau, McDonald, & Kolb, 2010; Kolb et al., 2008; Muhammad et al., 2013). Curiously, in the current study we found no training-related changes in NAc, parietal cortex, or hippocampus, suggesting that these structures were not strongly recruited for the delayed alternation task. In contrast, other types of training are known to change other brain regions. Thus, for example, rats learning visual tasks show changes in the visual cortex (Greenough & Chang, 1988; Greenough, Juraska, & Volkmar, 1979), whereas rats learning motor tasks show changes in the motor cortex contralateral to the trained paw (Greenough, Larson, & Withers, 1985; Kolb et al., 2008; Withers & Greenough, 1989).

Interestingly, although persistent changes in the dendritic morphology of prefrontal and NAc neurons in both young and adult rats have been reported following psychostimulant use (e.g., Robinson & Kolb, 1999), we found no chronic changes in circuitry of NAc neurons from animals exposed to MPD.

These findings indicate that the pattern of experience-induced plasticity is regulated by the type of experience. Furthermore, as MPD blocked training-induced modifications in Cg3 layer III and V neurons, it also suggests that experience-induced alterations in circuitry have the capacity to modulate subsequent changes, perhaps adversely. Combined with the behavioral influence of early MPD use, we may now begin to understand the functional significance of at least some of these anatomical alterations on behavior.

It should be noted that the behavioral effects reported here were produced with what is considered a very low dose of MPD, even by clinical standards. The absence of behavioral sen-

sitization and later sensitivity to stimulant use may very well be related to the dose used. Direct comparisons with other studies that have focused on the addictive capabilities of early MPD exposure are therefore not practical. Nonetheless, based on the current findings we may expect that increasing the dose over a prolonged treatment period during development could result in one of two scenarios. It may be that a higher dose would produce tolerance, resulting in a decline in the motor activating effects. Alternatively, the behavioral effects produced by a higher dose may more closely mimic addictive behavior, including an increased sensitivity to drugs of abuse in later life.

Finally, attention-deficit/hyperactivity disorder (ADHD) is a common neurodevelopmental disorder that is often characterized by deficits in executive functions (Brown, 2013). ADHD is often treated with stimulant drugs such as amphetamine or MPD, and these treatments have been shown to improve executive functions as measured on a range of neuropsychological tests (e.g., Adler, Clemow, Williams, & Durell, 2014). But the effectiveness of the drug is dependent on having the drug on board. The question of whether there are long-term effects of MPD in humans has not yet been answered. An extensive literature review on this issue concluded that “despite countless publications and extensive administration, especially to children, we have insufficient data to judge the long-term effects and risks of methylphenidate taking” (Konrad-Bindl, Gresser, & Richartz, 2016, p. 2635). And, in those studies finding long-term behavioral effects of MPD, the behavioral changes studied were psychiatric (e.g., anxiety, depression) and not measures of EF.

Finally, we would be remiss not to identify a possible limitation of this study. In particular, MPD injections were subcutaneous twice daily for 37 days. This procedure was chosen so we could compare our data to a similar study by Diaz Heijtz et al. (2003) in which a clinically relevant dose of amphetamine was given during the juvenile period. Our current results were similar to the earlier study but it is entirely possible that both studies were confounded by the stress of repeated injections. Future studies would probably be advised to use oral administration of the drug to reduce this possibility.

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