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Characterization of anxiety-related responses in male rats following prolonged exposure to therapeutic doses of oral methylphenidate

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ABSTRACT

Increases in the rates of attention-deficit/hyperactivity disorder (ADHD) diagnosis and the prescribed use of methylphenidate (MPH) in recent years have raised concerns over the potential effects of early MPH exposure on brain structure and function in adulthood. Animal studies have shown that long-term MPH exposure can modify anxiety-related behaviors and related neural circuitry in adulthood. The present study employed a battery of behavioral tests and repeated testing to assess the long-term effects of MPH exposure on anxious responding. Male Wistar rats beginning on post-natal day 27 were exposed to 4 or 7 weeks of twice daily MPH administration at doses of 2, 3, or 5 mg/kg. MPH was administered orally and on weekdays only in order to approximate drug treatment in clinical populations. Behavioral testing began 18 days following the last drug administration. Our results indicate that prolonged oral MPH treatment at therapeutic doses has little or no enduring effects on anxious behaviors. However, a comparison of MPH groups that received treatment for 4 or 7 weeks suggests that the two treatment periods influenced anxious behaviors in observably different manners in adulthood; namely, a more prolonged period of exposure produced less anxiety relative to the shorter period of MPH exposure as indicated by behaviors in the light–dark transition, elevated plus-maze, and fear conditioning tests. These findings were interpreted as evidence of the importance of considering length of drug exposure in pre-clinical studies aimed at investigating the effects of MPH exposure in ADHD populations.

1. Introduction

Attention-deficit hyperactivity disorder (ADHD) is among the most commonly diagnosed psychiatric disorders in children and adolescents (Swanson et al., 1998). The disorder is characterized by persistent deficits in attention, inhibition and executive function (Barkley, 1997) and has been associated with a wide range of negative social, economic, and psychological outcomes (Swanson et al., 1998). Methylphenidate (MPH), a nonamphetamine psychostimulant, is the principle treatment for ADHD (Solanto, 1998). It has been found to be safe and effective at therapeutic doses (Biederman and Faraone, 2005), but the precise long-term effects of prolonged MPH treatment remain unclear (Volkow and Insel, 2003). Because rates of ADHD diagnosis and the prescribed use of MPH have risen significantly in recent years (Pastor and Reuben, 2008), there are concerns over the potential effects of early MPH exposure on brain structure and function in adulthood (Faraone et al., 2000; Spencer et al., 1996; Wilens et al., 2002).

An abundance of evidence indicates that early childhood and adolescence are periods characterized by substantial neural developmental changes, particularly in brain systems underlying cognitive, motivational and emotional functions (Giedd et al., 2004; Spear, 2000). Thus, it is not surprising that the introduction of stimulant medications in this dynamic context can interfere with normally developing neurotransmitter systems and produce long-term structural and behavioral changes (Spear, 2000). For example, MPH has been shown to modify dopaminergic systems responsible for appropriate responding to affective stimuli (Brandon et al., 2003; Gray et al., 2007; Moll et al., 2001), and chronic MPH administration in young rats alters anxiety-related behaviors, increases plasma levels of corticosterone, and produces depressive-like effects in adulthood (Bolaños et al., 2003; Britton et al., 2007; Carlezon et al., 2003). Considering the role that emotion plays in normal cognitive processes (Gray, 2004) and in psychopathology (Pezze and Feldon, 2004), further studies are required to elucidate the effects of MPH on models of anxious behaviors.

Most of the studies that have examined MPH-induced changes in emotional responding to date have employed an intraperitoneal (i.p.) route of drug administration. Critics of animal studies of MPH question the clinical validity of employing an i.p. route of drug administration

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because MPH is administered orally and often with food to ADHD children, which results in a much slower and more gradual rate of absorption (Gerasimov et al., 2000; Kuczenski and Segal, 2005). Moreover, for the most part, prior studies have examined behavior in a single session using two or more anxiety tests, when research has shown that repeated testing can be useful for determining whether treatment effects persist upon re-exposure to the testing apparatus (Henderson et al., 2004). The present study addressed these concerns by employing an oral route of drug administration at doses that achieve plasma and brain levels of MPH in rats that correspond to those administered clinically (Wheeler et al., 2007) and measuring behavioral responses to a battery of emotional stimuli in adulthood.

2. Method

2.1. Animals

Subjects were 86 male rats of Wistar descent (Harlan, Mexico) raised in the INDICASAT AIP colony. Rats were weaned on post-natal day (PN) 21, and housed in groups of three in polyurethane tubs. The colony was maintained at 22 ± 2 °C and kept on a reversed 14:10 h light/dark cycle (lights on at 15:00 h and off at 05:00 h). Animals were provided free access to water throughout the experiment. Procedures were conducted in accordance with the National Institutes of Health regulations relating to the care and use of laboratory animals (Publication No. 85-23) and INDICASAT AIP policies.

2.2. Drugs and treatment procedure

Feeding and oral drug administration procedures used in the current study were developed by Chuhan and Taukulis (2006) and LeBlanc-Duchin and Taukulis (2004, 2007) for use with rodents. For four consecutive days (PN 23–26), animals were weighed, handled by the experimenters (20–30 s per rat per day) and habituated to the feeding procedure. During habituation, animals were placed individually in holding tubs where they were fed 4–6 g of wet chow made up of one part rodent food (Harlan 2018S) and two parts distilled water. No drug was administered during habituation. On PN 26 animals were randomly assigned to one of the drug treatment groups (control, 2, 3, or 5 mg/kg MPH). These doses are considered low to moderate and within the range of clinical relevance when delivered orally (Gerasimov et al., 2000; Kuczenski and Segal, 2005; Swanson and Volkow, 2003). MPH administration was initiated on PN 27 (experimental day 1) and was maintained twice daily on weekdays only (weekends were 'drug holidays') over a four-week (PN 27–53) or seven-week (PN 27–71) period, each corresponding roughly to peri-adolescence through young adulthood periods in humans (Anderson, 2005). Experiments took place in four replications, and 4- and 7-week drug exposure studies were not conducted simultaneously. Random assignment resulted in the following groups: 4-week treatment [control ($n = 11$); 2 mg/kg MPH ($n = 11$); 3 mg/kg MPH ($n = 11$); 5 mg/kg MPH ($n = 11$)]; 7-week treatment [control ($n = 10$); 2 mg/kg MPH ($n = 11$); 3 mg/kg MPH ($n = 10$); 5 mg/kg MPH ($n = 11$)].

The drug solution was prepared by dissolving pulverized MPH (Ritalin, 10 mg, Novartis) tablets in distilled water at a concentration of 10 mg/2 ml. Animals were weighed, and the drug solution was added to the wet rodent chow at the corresponding dose using a micropipette. Control and MPH-treated animals were fed the wet chow twice a day during the dark portion of their light/dark cycle (07:30 h and 13:30 h). Consistent with previous work utilizing the same methods (Bethancourt et al., 2009; Chuhan & Taukulis, 2006; LeBlanc-Duchin and Taukulis, 2004, 2007), animals adapted readily to the procedures and consumed all the wet chow during feeding. Additional food pellets (approximately 5–7) were provided each weekday evening. Animals were provided free access to food pellets on weekends.

2.3. Behavioral tests

The schedule of behavioral testing is presented in Table 1. The order of tests was selected such that animals would be exposed to the least invasive tests before being evaluated on the more invasive tests, and several days of recovery time were allowed between tests to reduce the effects of prior test experiences on later tests (McIlwain et al., 2001). Behavioral testing began 18 days following the last MPH treatment. We continued to monitor animal weights approximately three times per week after the last drug administration, throughout behavioral testing and during the week that followed. All tests were conducted during the dark portion of the light/dark cycle in a room separate from the animal colony between 07:30 and 13:30 h, and the order in which each animal was tested was randomized for each session. Animals were transported from the colony to the testing room in opaque plastic tubs. The testing arena, chambers and related materials were cleaned with 30% isopropyl alcohol between tests. Test sessions were recorded with a digital camera for off-line analysis. All behavioral measures were scored by experimenters blind to the animals' treatment group. The inter-rater reliability analyses were over .90 in each case.

2.3.1. Open field activity

Locomotor activity was evaluated in an open field arena (100 × 100 cm) made of white plastic. The walls were 36 cm high, and the floor was divided into 16 squares of 25 × 25 cm (four center squares and 12 peripheral squares). An overhead light bulb provided dim illumination of the field (70 lx). The objective of employing dim lighting was to decrease the aversiveness of the test (Deacon, 2006) in order to assess treatment effects on locomotion and habituation to a novel environment prior to exposing the animals to the anxiety test battery. Masking noise was provided by a dehumidifier that was kept on during all test sessions. Animals were exposed to the open field for 5 min on each of three consecutive days. Each session began when an animal was placed in the open field facing a corner. The number of squares crossed was used as a measure of locomotor activity and was recorded when a rat removed all four paws from one square and entered another.

2.3.2. Light–dark transition

The apparatus was made of plastic and consisted of two compartments. One compartment (34 × 47 × 27 cm) was painted white and was strongly illuminated by white light (800 lx), while the other (25 × 47 × 27 cm) was painted black, not illuminated and covered by a black roof. The two compartments, separated by a wall, were connected by an opening of 10 × 10 cm. Each rat was placed in the center of the light compartment, facing the opening, and behavior was recorded for 5 min on each of three consecutive days. The following behaviors were recorded: the latency to enter the dark compartment, time spent in the light compartment, number of whole-body transitions between compartments, and number of head pokes made from the dark to the light compartment without entering the light compartment. A head poke was employed as a measure of risk assessment and was recorded

Table 1
Experimental schedule.

Procedure		Length of MPH exposure	
		4 weeks	7 weeks
A	Habituation to feeding	PN 23–26	PN 23–26
B	Oral drug administration (b.i.d.)	PN 27–53	PN 27–71
C	Open field testing	PN 71–73	PN 89–91
D	Light–dark transition	PN 85–87	PN 103–105
E	Elevated plus-maze	PN 92–94	PN 110–112
F1	Contextual fear conditioning	PN 99	PN 117
F2	Contextual fear testing	PN 100–101	PN 118–119

PN = age of animal (post-natal day) at the time of each procedure.

when an animal crossed the threshold between compartments with only part of its body.

2.3.3. Elevated plus-maze

The apparatus was made of white plastic and consisted of four elevated arms (50 cm from the floor) 50 cm long and 11 cm wide. The arms were arranged in a cross-like configuration, with two opposite arms being open and two being enclosed (30 cm high walls). A central platform was located at the intersection of the four arms under an illumination of 800 lx. Each rat was placed on the central platform and its behavior was recorded for 5 min on each of three consecutive days. The following behaviors were recorded: the latency to enter an open arm, number of entries and time spent in the open arms, and number of head pokes made from the central platform into an open arm without entering the arm (risk assessment), all of which are indices of plus-maze anxiety (Lister, 1987). Locomotor activity was assessed by recording the number of entries into the closed arms and the total number of arm entries.

2.3.4. Fear conditioning

Fear conditioning was conducted in an operant box (27×28×30 cm) contained within a sound- and light-attenuating chamber. A 5 W house lamp was located on the wall of the chamber. The floor consisted of 16 stainless steel bars connected to a shock generator (H13-16; Coulbourn Instruments). A computer software program (Graphic State, Coulbourn Instruments) controlled shock presentations. Shock intensity and duration (1.0 mA; 2 s) were selected on the basis of previous studies that showed reliable conditioning using these parameters (Bethancourt et al., 2009; Britton and Astheimer, 2004; Phillips and LeDoux, 1992). A low-light level camera placed inside the chamber recorded all sessions. Freezing, defined as the absence of movement except that required for breathing (Bouton and Bolles, 1980; Fanselow, 1980), was used as the index of behavioral fear and was assessed using a standard time-sampling procedure every 4 s and converted to percent freezing. Training began by placing the animal in the conditioning chamber and turning off the house lamp. A 160 s period preceded the shock, followed by 30 s during which no further stimuli were delivered. The rat was placed back into the test chamber with the house light off for 4 min at 24 and 48 h after training in order to assess retention of contextual fear. At the conclusion of each session, animals were returned to their home cage.

2.4. Data analysis

Results obtained from four-week and seven-week drug exposure periods were analyzed separately. Body weight was analyzed with two-factor (group×day) repeated measure analysis of variance (ANOVA). Treatment effects in each behavioral test were analyzed using two-factor ANOVA with repeated measures (group×test session). In addition, minute-by-minute analyses of locomotor activity in the open field, percent time spent on the open arms of the elevated plus-maze and freezing during retention tests were conducted using repeated measures ANOVA for each test day (group×minute) to reveal within-session patterns of activity. Significant effects were followed by post hoc Tukey test when appropriate. Values of $p<.05$ were considered statistically significant.

3. Results

3.1. Body weight

Animals treated with MPH for 4 and 7 weeks gained weight at the same rate as controls. Weights averaged 75 g ($SD=10.4$) at PN 27 (first day of MPH treatment), 226 g ($SD=13.3$) at PN 53 (last day of 4-week treatment), and 310 g ($SD=15.4$) at PN 71 (last day of 7-week treatment). A significant main effect of day confirmed

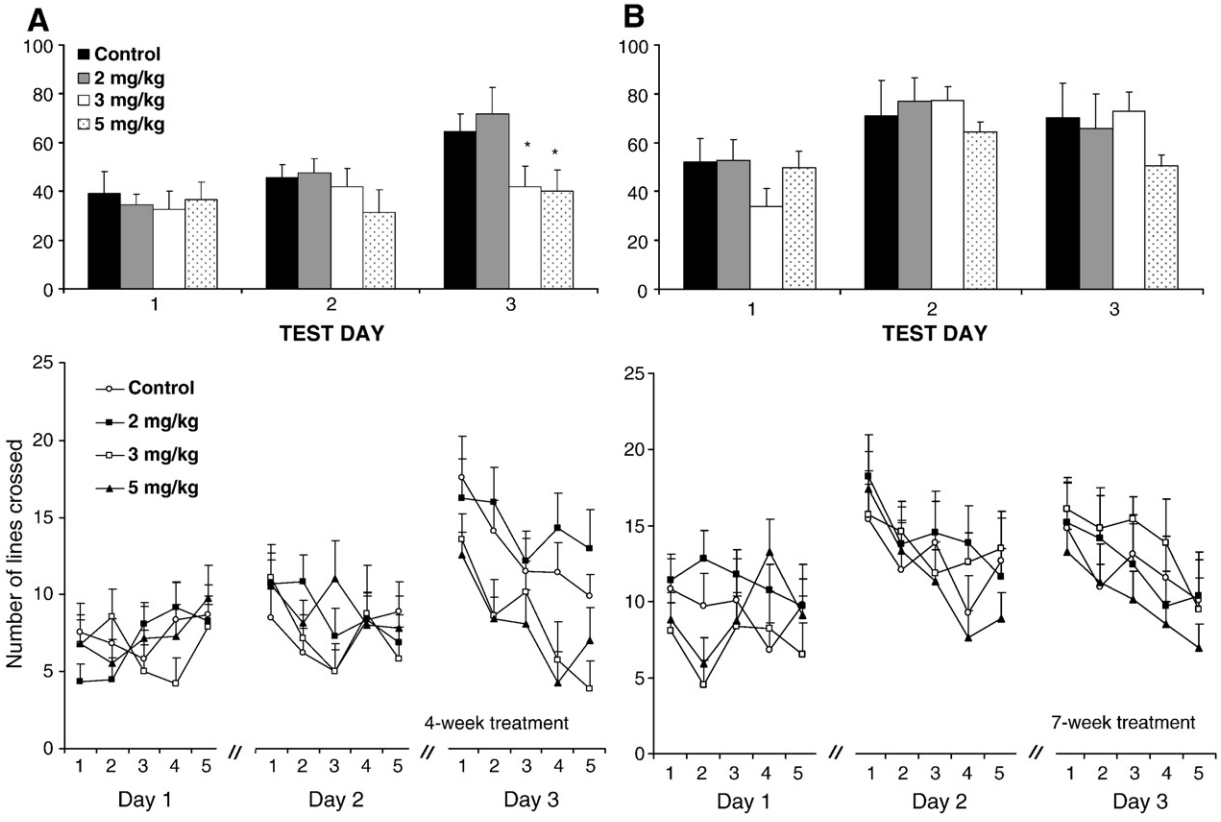

Fig. 1. Locomotor activity in three open field tests in animals treated with MPH for 4 weeks (A) and 7 weeks (B). Data are average number of lines crossed + S.E.M. on each test day (top) and across five 1-min intervals (bottom).*$p<.05$ relative to control group.

that animal weights increased significantly over the course of drug treatment [4 week exposure: $F(19,760) = 620.08$, $p < .001$; 7 week exposure: $F(34,1292) = 1365.84$, $p < .001$], and after drug treatment [4 week exposure: $F(8,320) = 223.28$, $p < .001$; 7 week exposure: $F(8,304) = 172.48$, $p < .001$].

3.2. Open field activity

Locomotor activity in the open field test was measured 18 days following the last MPH treatment. For animals exposed to MPH for 4 weeks, analysis of the sum of activity over each 5-min test revealed a main effect of test day [Fig. 1A top; $F(2,80) = 8.85$, $p < .01$]. Post hoc tests confirmed that the average number of lines crossed was significantly greater on the third test relative to the first test ($p < .05$). However, although group averages clearly demonstrate increases in activity across sessions for the control and 2 mg/kg MPH groups, no change in locomotor activity is apparent for the 3 and 5 mg/kg MPH groups. Within-session analysis of activity revealed normal habituation to the testing arena on the second and third tests (Fig. 1A bottom), that is, decreases in activity for all groups across the 5-min period. Nevertheless, although patterns of activity were similar across groups, ANOVA revealed a significant effect of MPH treatment on the amount of activity on the third test day [$F(3,40) = 4.83$, $p < .01$]. Post hoc tests revealed that the 3 and 5 mg/kg MPH groups were less active relative to controls ($ps < .05$) and 2 mg/kg MPH group ($ps < .01$).

Seven weeks of MPH treatment produced no effects for any measure in the open field (Fig. 1B). Only the test day had a significant effect on locomotion [$F(2,76) = 13.56$, $p < .001$]. Post hoc comparisons indicated that animals regardless of treatment condition crossed more squares on the sand third day relative to the first day (Fig. 1B top; $ps < .05$). Lastly, within-session analyses showed that re-exposure to the testing arena on the second and third sessions produced similar rates of habituation across treatment groups (Fig. 1B bottom).

Table 2
Light–dark transition test measures grouped by dose and length of exposure.

Treatment group and measures	Test Day		
	1	2	3
4 weeks MPH exposure			
Control ($n = 11$)			
Latency to enter dark (s)	14.5 ± 3.2	11.1 ± 5.2	4.2 ± 0.5
Light–dark transitions	6.0 ± 0.7	8.2 ± 1.7	9.8 ± 1.4
Dark-to-light head pokes	16.8 ± 1.4	16.8 ± 1.3	12.3 ± 1.4
2 mg/kg MPH ($n = 11$)			
Latency to enter dark (s)	13.4 ± 2.2	6.1 ± 1.1	4.0 ± 0.5
Light–dark transitions	4.2 ± 0.6	4.5 ± 0.8	7.6 ± 1.3
Dark-to-light head pokes	16.2 ± 1.7	16.0 ± 2.4	14.6 ± 1.6
3 mg/kg MPH ($n = 11$)			
Latency to enter dark (s)	18.9 ± 5.2	5.5 ± 1.0	5.1 ± 0.6
Light–dark transitions	5.5 ± 1.5	8.2 ± 1.5	10.6 ± 1.4
Dark-to-light head pokes	15.2 ± 1.4	15.2 ± 1.3	12.8 ± 1.8
5 mg/kg MPH ($n = 11$)			
Latency to enter dark (s)	29.6 ± 4.6*	9.3 ± 2.0	4.9 ± 0.4
Light–dark transitions	4.1 ± 1.4	5.2 ± 1.1	7.1 ± 1.3
Dark-to-light head pokes	14.8 ± 1.2	17.3 ± 2.3	11.9 ± 1.6
7 weeks MPH exposure			
Control ($n = 10$)			
Latency to enter dark (s)	25 ± 11.4	7.3 ± 1.0	3.0 ± 0.3
Light–dark transitions	6.7 ± 1.6	11.8 ± 2.1	13.1 ± 2.2
Dark-to-light head pokes	15.3 ± 1.9	17.6 ± 1.7	10.3 ± 1.6
2 mg/kg MPH ($n = 11$)			
Latency to enter dark (s)	26.9 ± 6.7	15.5 ± 5.8	4.1 ± 1.0
Light–dark transitions	7.9 ± 1.4	12.7 ± 1.8	13.1 ± 2.0
Dark-to-light head pokes	17.6 ± 1.8	14.7 ± 1.9	11.1 ± 1.6
3 mg/kg MPH ($n = 10$)			
Latency to enter dark (s)	25.9 ± 3.7	6.5 ± 0.8	5.4 ± 1.0
Light–dark transitions	5.0 ± 1.2	8.1 ± 1.7	12.1 ± 1.4
Dark-to-light head pokes	17.8 ± 1.6	19.3 ± 2.5	11.7 ± 1.5
5 mg/kg MPH ($n = 11$)			
Latency to enter dark (s)	26.7 ± 9.1	19.5 ± 8.7	11.5 ± 6.6
Light–dark transitions	4.5 ± 0.9	7.9 ± 1.2	9.0 ± 1.0
Dark-to-light head pokes	18.9 ± 1.6	18.6 ± 2.4	10.9 ± 1.0

Values are means ± S.E.M. *$p < .05$ vs. controls on the same day.

3.3. Light–dark transition

Behaviors in the light–dark transition test are summarized in Table 2 and Fig. 2. In animals treated with MPH for 4 weeks, the latency to enter the dark compartment on each test day depended on the drug dose [group × test day interaction, $F(6,78) = 3.05$, $p < .05$]. Post hoc analyses revealed that animals treated with 5 mg/kg exhibited a longer average latency on the first day of testing than controls and the 2 mg/kg MPH group ($ps < .05$), and a marginally longer latency than the 3 mg/kg group ($p = .06$) to enter the dark compartment (Table 2). MPH treatment also affected the average time spent in the light compartment [$F(3,39) = 2.83$, $p = .05$], with the low MPH dose (2 mg/kg) group spending significantly less time in the light compartment relative to controls and higher dose MPH groups (Fig. 2A; $ps < .05$). There was also a main effect of test day [$F(2,78) = 7.69$, $p < .01$] indicating that across treatment groups the time spent in the light on the third day was greater relative to the first and second days ($ps < .05$). Significant main effects of test day indicate that animals across groups performed fewer pokes [$F(2,78) =$ 8.87, $p < .001$] and more transitions between compartments [$F(2,78) =$

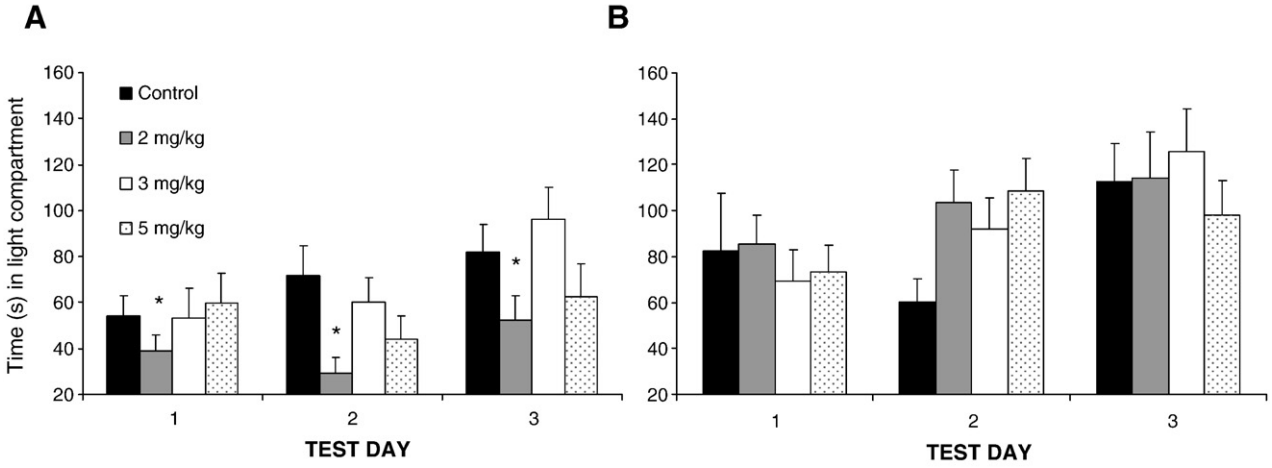

Fig. 2. Exploratory activity in three light–dark transition tests in animals treated with MPH for 4 weeks (A) and 7 weeks (B). Data are average times (s) spent in the light compartment + S.E.M. on each test day.*$p < .05$ relative to control group.

15.36, $p<.001$] on the third test day relative to the first and second day. A marginal main effect of MPH treatment in number of transitions indicated fewer transitions for 2 and 5 mg/kg MPH groups relative to controls ($ps<.07$, see Table 2).

MPH exposure for 7 weeks did not affect any behavior in the light–dark transition test. However, there was a significant main effect of test day for the latency to enter the dark compartment [$F(2,76)=18.06$, $p<.001$; Table 2], time spent in the light compartment [$F(2,76)=5.35$, $p<.05$; Fig. 2B], frequency of poking [$F(2,76)=24.04$, $p<.001$; Table 2], and number of light–dark transitions [$F(2,76)=29.69$, $p<.001$; Table 2]. Significant changes in each of these behaviors occurred between the first and last test days: decreased latency to enter the dark compartment, increased time in the light, fewer pokes, and increased transitions ($ps<.05$).

3.4. Elevated plus-maze

Anxiety-related behaviors in the plus-maze are displayed in Fig. 3. In animals treated with MPH for 4 weeks, the latency to enter an open arm (Fig. 3A) decreased on the last day of testing [main effect of test day: $F(2,80)=5.19$, $p<.01$]. One-way ANOVA performed on the last day of testing [$F(3,40)=2.87$, $p<.05$] indicated that the 2 and 3 mg/kg MPH groups showed significantly longer latencies to enter an open arm than controls ($ps<.05$); the 5 mg/kg group exhibited borderline longer latencies ($p=.07$). A main effect of test day [$F(2,80)=8.97$, $p<.001$] indicated that animals spent more time on the open arms on the last day of testing relative to the first and second day ($ps<.01$; Fig. 3B). Within-session analyses of time spent on the open arms (data not shown) revealed a significant effect of MPH treatment [$F(3,40)=3.17$, $p<.05$]

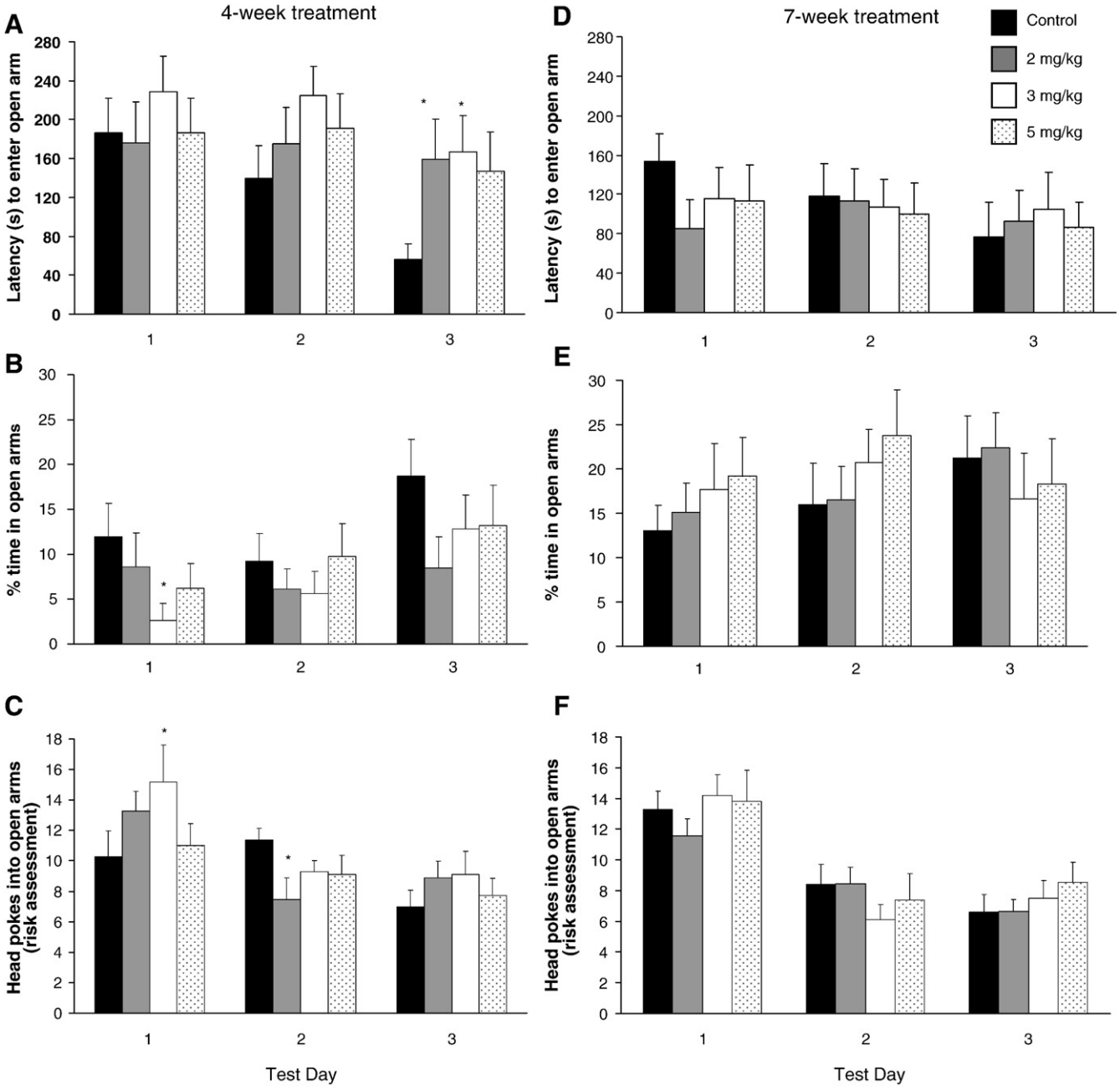

Fig. 3. Anxiety-related behaviors in three elevated plus-maze tests in animals treated with MPH for 4 weeks (A–C) and 7 weeks (D–F). Data are averages + S.E.M. of latency to enter an open arm (A,D), percent open-arm entries (B,E), and frequency of head pokes into open arms (C,F). *$p<.05$ vs. control group.

Table 3
Elevated plus-maze measures of locomotion grouped by dose and length of exposure.

Treatment group and measures	Test day		
	1	2	3
4 weeks MPH exposure			
Control ($n=11$)			
Total arm entries	14.8 ± 2.2	18.5 ± 2.0	18.3 ± 2.4
# closed arm entries	5.8 ± 0.7	7.2 ± 0.5	7.9 ± 0.9
2 mg/kg MPH ($n=11$)			
Total arm entries	13.4 ± 1.2	15.8 ± 2.2	17.8 ± 2.6
# closed arm entries	6.1 ± 0.7	6.5 ± 1.0	6.7 ± 0.9
3 mg/kg MPH ($n=11$)			
Total arm entries	12.1 ± 2.1	17.6 ± 2.0	22.9 ± 2.7
# closed arm entries	6.0 ± 1.0	8.0 ± 0.9	8.6 ± 0.7
5 mg/kg MPH ($n=11$)			
Total arm entries	12.8 ± 1.9	16.0 ± 3.7	19.5 ± 3.7
# closed arm entries	5.8 ± 0.7	6.5 ± 1.1	7.2 ± 0.9
7 weeks MPH exposure			
Control ($n=10$)			
Total arm entries	10.6 ± 1.7	12.7 ± 2.2	16.6 ± 1.7
# closed arm entries	5.9 ± 1.1	7.9 ± 0.9	8.5 ± 0.8
2 mg/kg MPH ($n=11$)			
Total arm entries	8.9 ± 1.3	10.6 ± 1.7	13.2 ± 1.8
# closed arm entries	6.2 ± 0.9	7.7 ± 1.5	8.9 ± 1.1
3 mg/kg MPH ($n=10$)			
Total arm entries	8.2 ± 0.8	10.4 ± 1.1	10.7 ± 1.4
# closed arm entries	5.8 ± 0.5	6.7 ± 0.7	8.0 ± 0.8
5 mg/kg MPH ($n=11$)			
Total arm entries	9.6 ± 1.1	12.1 ± 1.1	11.0 ± 0.8
# closed arm entries	6.7 ± 1.0	8.1 ± 0.9	8.2 ± 0.8

Values are means ± S.E.M.

and minute [$F(4,160)=3.11$, $p<.05$] only on the first day of testing. Post hoc tests showed that animals treated with 3 mg/kg MPH spent less time in the open arms than controls ($p<.05$; Fig. 3B), and overall, the average time animals spent on the open arms decreased over the course of the session. However, no effects were found for the number of entries into open arms (data not shown). A significant group × day interaction for the number of pokes into the open arms [Fig. 3C; $F(6,80)=2.35$, $p<.01$] suggests that risk assessment on each test day differed by group. Post hoc analyses showed that the group treated with 3 mg/kg MPH performed significantly more pokes than controls on the first day of testing (Fig. 3C), whereas animals treated with 2 mg/kg exhibited significantly fewer pokes than controls on the second day of testing ($ps<.01$); no differences were evident by the last day of testing. Four-week MPH treatment did not affect locomotor behavior in the plus-maze, but a main effect of test day for entries into closed arms [$F(2,80)=6.20$, $p<.01$] and total number of arm entries [$F(2,80)=16.89$, $p<.001$] revealed that locomotor activity increased over the course of test days across groups (see Table 3).

There were no effects of 7-week MPH treatment for any measure in the plus-maze (Fig. 3D–F). Significant main effects of test day revealed that the average percent of entries into the open arms increased on days 2 and 3 relative to the first day [$F(2,76)=18.95$, $p<.001$; data not shown], while the average number of pokes into open arms decreased over the course of days [$F(2,76)=40.00$, $p<.001$; Fig. 3F]. No effects were found in the latency to enter or time spent on open arms (Fig. 3D and E). Locomotor activity increased over the course of test days across groups; there was a main effect of test day for entries into the closed arms [$F(2,76)=11.90$, $p<.001$] and the total number of arm entries [$F(2,76)=17.31$, $p<.001$; Table 3].

3.5. Fear conditioning

All animals responded to the shock stimulus during the conditioning session in an observable manner (e.g., running, jumping) and gradually ceased activity consistent with studies employing similar procedures (Fanselow, 1982, 1990). Accordingly, there was no effect of MPH dose or length of exposure on the number of freezing responses

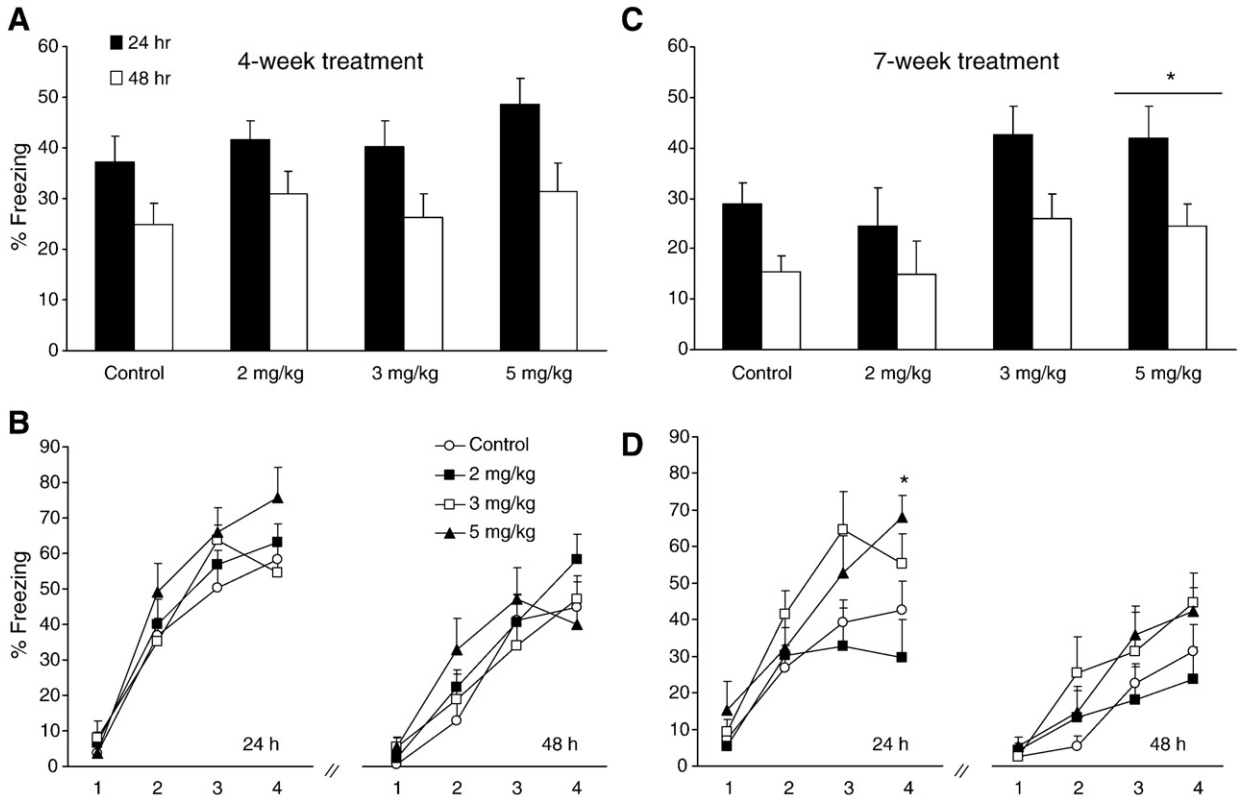

Fig. 4. Fear retention in animals treated with MPH for 4 weeks (A,B) and 7 weeks (C,D). Percent freezing responses recorded at 24 and 48 h following conditioning (A,C). Within-session % freezing recorded during each test at four 1-min intervals (B,D). Data are averages + S.E.M. *$p<.05$ vs. control group.

performed before or after shock presentation during conditioning ($ps > .40$).

Fear retention tests of animals treated with MPH for 4 weeks revealed no effects of MPH treatment. All animals regardless of treatment condition exhibited more freezing at 24 h relative to the 48 h testing time [Fig. 4A; $F(1,40) = 46.56$, $p < .001$], and within-session analysis of each retention test showed similar patterns of freezing across treatment conditions (Fig. 4B). In contrast, 7 weeks of MPH treatment (Fig. 4C) produced main effects of group [$F(3,38) = 2.89$, $p < .05$] and test session [$F(1,38) = 20.68$, $p < .001$]. Post hoc analyses showed that average percent freezing across both test sessions was greater for the 5 mg/kg group relative to controls and the 2 mg/kg group, while average freezing was greater for the 3 mg/kg group relative to the 2 mg/kg group ($ps < .05$). Minute-by-minute analysis of freezing during the 24 h retention test (Fig. 4D) revealed a significant main effect of minute [$F(3,114) = 42.36$, $p < .001$] and a group × minute interaction [$F(9,114) = 2.37$, $p < .05$]. The highest MPH dose (5 mg/kg) produced more freezing than controls during the last minute of testing ($p < .05$), while the lowest dose (2 mg/kg) produced significantly less freezing than the 3 and 5 mg/kg doses during the last 2 min of testing ($ps < .05$). Fear responses during the 48 h test (Fig. 4D) revealed only a main effect of minute, [$F(3,114) = 26.20$, $p < .001$]; animals across treatment groups displayed significant increases in freezing between the first and last minutes of the test session [one-way ANOVA comparing minute 1 and 4, $F(1,38) = 70.32$, $p < .001$].

4. Discussion

We examined the effects of prolonged oral MPH administration at therapeutic doses on a number of anxiety-related behaviors. Our aim was to mimic as closely as possible the drug doses, route of drug administration, and repeated exposure to stressful events experienced by clinical populations. Moreover, we examined behaviors long after the last MPH administration to determine whether drug-related effects persist after the cessation of treatment when the drug has been excreted from the system. After four or seven weeks of twice daily treatment, MPH effects endured after repeated testing in only two instances: (1) 4 weeks of MPH treatment at doses of 3 and 5 mg/kg elicited a significant decrement in locomotor behavior in the open field relative to untreated animals, and (2) 4 weeks of MPH treatment at a dose of 2 mg/kg increased anxiety in the light–dark transition test as evidenced by less time spent in the light compartment relative to controls. The remaining results of the present experiments indicate that prolonged MPH exposure using orally administered therapeutic doses has little or no enduring effects on anxious behaviors. Considering the evidence that long-term intraperitoneally administered MPH increases anxiety- and stress-related responding in adulthood in a variety of tasks including the elevated-plus maze and fear conditioning (Bolaños et al., 2003; Britton et al., 2007; Carlezon et al., 2003), the present findings question the potential of early MPH exposure to modify emotional responses when oral administration techniques and therapeutic doses are employed.

Studies have shown that oral MPH administered acutely (Kuczenski and Segal, 2002) and chronically (LeBlanc-Duchin and Taukulis, 2007) at similar doses to those employed in the current study does not produce increases in locomotor activity. Accordingly, no treatment effects on open field locomotion were observed after 7 weeks of MPH administration, consistent with recent studies conducted in our laboratory (Bethancourt et al., 2009). Four weeks of MPH exposure at the two highest doses (3 and 5 mg/kg) produced decreases in locomotor behavior in the open field on the last day of testing. Because MPH treatment did not produce effects on locomotion in the plus-maze or fear conditioning tests regardless of length of exposure, our results provide further evidence that chronic treatment with low doses of oral MPH does not produce hyperactivity or interfere with habituation as is the case following chronic MPH administered by intraperitoneal injections (Carlezon et al., 2003; Yang et al., 2007). Moreover, our findings are consistent with the clinical observation that prolonged MPH use at therapeutic doses does not cause hyperactivity at adulthood (Faraone et al., 2000; Volkow and Insel, 2003).

Traditional animal models of anxiety-related behaviors often employ repeated measures designs in order to assess treatment effects in test-experienced subjects (File, 1995; Henderson et al., 2004). Our aim in the present study was to use repeated testing in order to address potential enduring treatment effects on anxious behaviors upon re-exposure to stressful contexts. To our knowledge, no previous study has examined the effects of MPH on behaviors in the light–dark transition test. However, a recent study that characterized the behavior of mice after repeated testing in the light–dark box provides evidence that the anxiogenic properties of the light compartment decrease by the time of retest (Henderson et al., 2004). In our study, the anxiogenic properties of the light compartment persisted in animals treated with 2 mg/kg MPH for 4 weeks, whereas 7 weeks of treatment at the same dose produced no effects on anxious behavior by the last day of testing (see Fig. 2). In sum, it appears as though the lowest MPH dose applied over 4 weeks produced increases in anxiety as indicated by this task. However, the results of the remaining tests in the battery indicate that other measures of anxiety were not altered in an enduring manner by treatment at the lowest dose of MPH.

In plus-maze testing, all MPH groups in the 4-week treatment condition exhibited increases in the latency to enter an open arm on the last test session, suggesting increased anxiety even after repeated testing. However, the lack of an effect of MPH in the percent time spent on the open arms and the number of entries into open arms provides evidence that animals behaved similarly to controls in other open-arm measures of anxiety. Previous studies assessing MPH-induced effects on plus-maze behaviors have yielded mixed results; increases (Bolaños et al., 2003), decreases (Gray et al., 2007), and no change (LeBlanc-Duchin and Taukulis, 2004) in anxious behaviors were observed following chronic MPH treatment. Further, the procedures employed in the studies, namely intraperitoneal injections (Bolaños et al., 2003; Gray et al., 2007) or the choice of an oral dose much higher than those used in clinical settings (10 mg/kg; LeBlanc-Duchin and Taukulis, 2004), limit the relevance to the clinical uses of MPH. In the present study, oral MPH treatment did not suppress activity in the open arms, even over the course of repeated testing, suggesting an absence of MPH effects on anxiety (Pellow et al., 1985).

The results of fear conditioning indicate that 7 weeks of oral MPH treatment at the highest dose (5 mg/kg) produced more robust freezing at 24 h but not at 48 h after training, whereas 4 weeks of MPH treatment did not affect fear retention at any dose examined. Fear conditioning is the only task in the test battery that involved an explicit associative learning component, so it is likely that the enhanced freezing produced by the 5 mg/kg dose is related to an effect of MPH on associative learning processes, rather than on anxiety, particularly in light of no group differences in responding to the shock stimulus during training. This interpretation is also supported by the lack of an effect of the 5 mg/kg dose on other anxiety measures in the test battery. MPH has been shown to improve learning and memory in ADHD children (see Pietrzak et al., 2006 for review) and to enhance hippocampal long-term potentiation, a putative molecular mechanism of learning and memory (Dommett et al., 2008), providing support for its ability to modify learned associations. Moreover, chronic MPH treatment modifies hippocampal-dependent tasks such as object recognition memory (Bethancourt et al., 2009; Chuhan and Taukulis, 2006; LeBlanc-Duchin and Taukulis, 2007), Morris water maze learning (Zeise et al., 2007) and learned contextual fear (Bethancourt et al., 2009; Britton et al., 2007), suggesting that the underlying hippocampal circuitry is at least one brain system affected by early MPH exposure. Oral MPH at low doses similar to those employed in the current study has also been shown to increase norepinephrine levels in the hippocampus in the short-term without causing hyperactivity, which may underlie its efficacy when administered at clinically relevant doses (Kuczenski and Segal, 2002).

Accordingly, it is conceivable that long-term treatment with low doses of MPH may promote neurochemical effects that enhance hippocampal-dependent emotional memory consolidation possibly through its interactions with the noradrenergic system (LeBlanc-Duchin and Taukulis, 2004). In a recent study conducted in our laboratory, chronic MPH treatment (2 mg/kg) administered intraperitoneally resulted in enhanced freezing in the context in which shock was experienced and not in a different context (Britton et al., 2007), supporting the view that MPH treatment may be capable of strengthening the association formed during fear conditioning. In the present study, our objective was to examine MPH effects on long-term retention of learned fear; however, we recognize that the inclusion of a shorter-term test (i.e., 2–3 h after conditioning) combined with a longer test period may have revealed a more detailed pattern of behavior that was not evident under our conditions. Although the enhancement of freezing at the 24 h retention test is indicative of drug-induced modifications in behavior, no group differences emerged at 48 h, suggesting that MPH treatment does not produce enduring changes in the expression of fear memory. Similarly, in a preliminary study using the same oral dosing methods over the course of 7 weeks, our laboratory demonstrated a transient increase in freezing 24 h following conditioning that returned to control levels at 48 h (Bethancourt et al., 2009).

Because behavioral testing in the 4- and 7-week exposure conditions began two weeks after the last drug administration (see Table 1), the effects of MPH on anxious behaviors in each exposure condition were analyzed independently. We reasoned that although animals had reached early adulthood by the first test in both exposure conditions, we would not be able to rule out the contribution of age effects on observed differences. Nevertheless, it is worth noting that a visual comparison of MPH groups between exposure conditions revealed a pattern with regard to the effects of length of drug exposure on anxiety-related behaviors across tests. In light–dark testing, 7-week treatment groups on average spent a greater amount of time in the light compartment on the second and third test days relative to 4-week MPH groups (see Fig. 2). Likewise, a comparison of MPH groups in plus-maze behaviors reveals that 7-week treatment produced shorter latencies to enter an open arm and greater time spent on the open arms relative to 4 weeks of MPH treatment (see Fig. 3). In each case, 7-week treatment appears to produce less anxious behavior relative to 4-week treatment. In fear retention, comparisons between groups across exposure conditions shows that the higher MPH doses (3 and 5 mg/kg) produced equivalently robust freezing regardless of the length of treatment, whereas the lowest dose of MPH (2 mg/kg) produced less freezing after 7 weeks of treatment relative to 4 weeks (see Fig. 4). Although control groups also appear to differ between exposure conditions, statistical analyses revealed that only the 2 mg/kg groups differed across 4- and 7-week exposure conditions ($p = .04$); all other comparisons revealed non-significant differences ($ps > .10$). Taken together, it appears that the 4- and 7-week treatment periods influenced anxious behaviors in observably different manners in adulthood; namely, when treatment is begun on the same post-natal day, a more prolonged MPH exposure produced less anxiety relative to a shorter period of drug exposure. Our results are consistent with those of developmental studies that indicate that the duration of drug exposure is a critical factor on the impact of psychostimulants on behavior (Anderson, 2005; Bolaños et al., 1998), and underscore the need for pre-clinical studies to assess the effects of length of drug exposure on cognitive and behavioral processes in adulthood.

To summarize, this study provides behavioral evidence that chronically administered MPH beginning at adolescence produces little or no enduring effects on anxious behaviors. This study also suggests a number of factors that should be taken into account in future research. First, drug effects were examined using normal animals and not animal models of ADHD, and thus the results primarily address the question of how MPH affects the normal brain. Nevertheless, increases in MPH use and difficulties in ADHD diagnosis raise the possibility that healthy children are undergoing psychostimulant treatment inadvertently and support the need for studies aimed at determining the impact of psychostimulant use under normal conditions (Biederman and Faraone, 2005). Second, because psychostimulant effects on behavior and brain function are sensitive not only to the length of treatment (Thanos et al., 2007), but also to the age at which treatment is applied (Anderson, 2005), future animal studies should include subjects at earlier developmental periods than those represented in the present study, especially in light of increased MPH use in children as young as two years of age (Zito et al., 2000). Lastly, although we did not detect significant enduring effects of MPH exposure on emotional behaviors, more complex tests of higher cognitive functions may reveal effects of MPH on brain function that were not detectable using the procedures we employed. Evidence of enduring MPH-produced changes in learning and behavior (Bolaños et al., 2003; Britton et al., 2007; Carlezon et al., 2003; LeBlanc-Duchin and Taukulis, 2007) and brain structure and function (Brandon et al., 2003; Gray et al., 2007; Moll et al., 2001) provide a rationale for further inquiry into the risks involved in extensive drug treatment during early development.

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