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Long-Term Exposure to Oral Methylphenidate or *dl*-Amphetamine Mixture in Peri-Adolescent Rhesus Monkeys: Effects on Physiology, Behavior, and Dopamine System Development

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The stimulants methylphenidate and amphetamine are used to treat children with attention deficit/hyperactivity disorder over important developmental periods, prompting concerns regarding possible long-term health impact. This study assessed the effects of such a regimen in male, peri-adolescent rhesus monkeys on a variety of cognitive/behavioral, physiological, and *in vivo* neurochemical imaging parameters. Twice daily (0900 and 1200 hours), for a total of 18 months, juvenile male monkeys (8 per group) consumed either an unadulterated orange-flavored solution, a methylphenidate solution, or a *dl*-amphetamine mixture. Doses were titrated to reach blood/plasma levels comparable to therapeutic levels in children. [^{11}C]MPH and [^{11}C]raclopride dynamic PET scans were performed to image dopamine transporter and D_2-like receptors, respectively. Binding potential (BP_{ND}), an index of tracer-specific binding, and amphetamine-induced changes in BP_{ND} of [^{11}C]raclopride were estimated by kinetic modeling. There were no consistent differences among groups on the vast majority of measures, including cognitive (psychomotor speed, timing, inhibitory control, cognitive flexibility), general activity, physiological (body weight, head circumference, crown-to-rump length), and neurochemical (ie, developmental changes in dopamine transporter, dopamine D_2 receptor density, and amphetamine-stimulated dopamine release were as expected). Cytogenetic studies indicated that neither drug was a clastogen in rhesus monkeys. Thus, methylphenidate and amphetamine at therapeutic blood/plasma levels during peri-adolescence in non-human primates have little effect on physiological or behavioral/cognitive development.
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INTRODUCTION

The psychomotor stimulants methylphenidate (MPH) and amphetamine (AMPH) have been successful treatments for attention deficit/hyperactivity disorder (ADHD) in children (MTA Cooperative Group, 1999, 2004a, b). Understanding the long-term effects of extended exposure to such medications, especially when treatment occurs across important developmental periods, has been a source of concern for physicians and parents alike. Effects on physiological development, in particular, have been noted in children undergoing treatment for ADHD. For example, growth rates of children medicated for ADHD were reduced compared with population norms or un-medicated children (Mattes and Gittelman, 1983; MTA Cooperative Group, 2004b), although the mechanism and duration of the effect are unclear (Poulton and Cowell, 2003; Rapport and Moffitt, 2002).

Certain side effects of daily exposure also have been of concern. Sleep disturbances in those with ADHD are well documented, although their exact nature and severity remain disputed (Cohen-Zion and Ancoli-Israel, 2004; Spruyt and Gozal, 2011). Although stimulant administration itself can affect sleep, depending upon dose and time of administration (Efron *et al*, 1997; Williamson *et al*, 1997), two studies reported no difference in nocturnal activity in medicated and un-medicated children with ADHD,

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suggesting that when stimulant therapy is not administered late in the day, sleep is neither improved nor impaired (Cohen-Zion and Ancoli-Israel, 2004).

Both MPH and AMPH increase synaptic dopamine (DA) either by blocking DA transporter (DAT) uptake of DA (MPH and AMPH) or promoting DA release (AMPH), suggesting that the dopaminergic system plays a critical role in the neuropharmacology of ADHD. Despite the benefit of stimulant medications for ADHD treatment, stimulants can produce long-term changes in DA function and in behavioral responses to stimulants (Robinson and Becker, 1986; Segal *et al*, 1981; Spear and Alderton, 2003). For example, decreases in neurotransmitter receptors or uptake proteins were reported when low to moderate stimulant doses were administered chronically in monkeys and children (Ginovart *et al*, 1999; Vles *et al*, 2003). Chronic stimulant administration also can modify the amount of DA released into the synapse. Castner *et al* (2000) showed that chronic low-dose AMPH exposure resulted in reduced DA released by an acute AMPH challenge in monkeys. Similar reductions in MPH-stimulated DA release were reported in detoxified cocaine users (Volkow *et al*, 1997), suggesting a general effect of long-term stimulant administration. Such data on long-term effects of chronic stimulant administration on the DA system raise concerns regarding developmental effects of such a regimen on the DA system.

Certain types of cognitive deficits, particularly in executive function, which encompasses planning and execution of complex behavioral sequences, are characteristic of ADHD. Performance on tasks that measure aspects of executive function, such as time perception/estimation, response inhibition, working memory, and attentional set formation, are impaired in children diagnosed with ADHD, and performance improves with stimulant medication (Baldwin *et al*, 2004; Kempton *et al*, 1999; Mehta *et al*, 2004; Tannock *et al*, 1989).

Finally, there have been concerns raised regarding possible genetic toxicity of MPH in children (El-Zein *et al*, 2005). In that report, there were increases in chromosomal aberrations (CAs) in peripheral lymphocytes of children treated with MPH for 3 months. In addition, in 11 of those children, there were increases in sister chromatid exchanges (SCEs) and micronuclei (MN). Later clinical and pre-clinical studies have failed to support the conclusions of that report (Morris *et al*, 2009; for a review see, Morris *et al* (2012), Ponsa *et al* (2009), Tucker *et al* (2009), and Witt *et al* (2008)), but concerns for parents and physicians remain.

This study evaluated the effects of chronic oral MPH or AMPH administration in pre-adolescent male macaques on a variety of physiological, neurological, and behavioral measures to address a range of concerns regarding the impact of chronic stimulant administration to juveniles. Macaques are useful as developmental models because non-human primate cortical structures are similar to humans in the frontal cortical and striatal regions important in ADHD (Mostofsky *et al*, 2002; Sowell *et al*, 2003) and are shown to mediate psychomotor and executive function (Haber, 2003; Middleton and Strick, 2002). Onset of adolescence in macaques is at 3–4 years of age and thus allows study of the effects of a lengthy period of drug exposure before puberty. The effects of twice-daily oral doses of either AMPH or MPH, 7 days per week, were evaluated, compared with a vehicle control condition, across a total of 18 months of drug administration and again 6 months after dosing ended to determine whether there were any lasting effects of the extended exposure to stimulant medication.

MATERIALS AND METHODS

Subjects

Male rhesus monkeys (*Macaca mulatta*) ($N = 24$) of Chinese origin, purchased as yearlings, served as subjects. Reverse osmosis (RO) water was continuously available. Access to food (Harlan Teklad) was relatively constant until behavioral training sessions began around 24 months of age. Biscuits consumed were counted daily, and the total delivered was adjusted such that at least one remained uneaten daily. Once-daily dosing sessions began, food was delivered around 1600 hours along with a piece of fresh fruit. Any remaining food was removed at 0900 h, before sessions in which a drug solution was orally available and/or behavioral testing was to be conducted. The procedures in this study were approved by the Johns Hopkins University Animal Care and Use Committee.

Equipment

Each cage was equipped with a custom-made, stainless-steel 'intelligence' panel, which was equipped with all components needed for the behavioral procedures except for the intra-dimensional (ID)/extra-dimensional (ED) set-shifting task, which used custom-made portable devices with touch screens for implementing procedures from the Cambridge Neuropsychological Testing Automated Battery (Weed *et al*, 1999, 2008a) and the object retrieval and detour task, which used a custom-made device for positioning a clear Plexiglas box with one open side in front of the monkey's cage (Gray *et al*, 2006).

Study Design

An overview of the study timeline is provided in Table 1. During baseline, all monkeys were trained on the behavioral tasks and were introduced to the oral self-dosing procedure with the drug vehicle. Once trained, monkeys were assigned to one of three experimental groups ($n = 8$ per group) matched on body weight and baseline drinking behavior. During the subsequent 6-month treatment period, the control, AMPH, and MPH groups engaged in twice-daily oral self-dosing sessions with, respectively, vehicle, AMPH, and MPH 7 days per week. This initial treatment period was followed by an approximately 2-month break, during which oral dosing was temporarily suspended. Following the break, an uninterrupted 12-month treatment period ensued, after which oral self-dosing was discontinued permanently ('post-treatment'). Table 1 also shows the periods in the study in which the various assessments, described below, were conducted.

Oral Self-Dosing Procedure

Oral self-dosing training involved a phase of habituation to drinking a bitter solution as has been reported previously

Table 1 Timeline of Chronic Drug or Vehicle Treatment and the Months in which Physiological Development, Cytogenetic, Cognitive-Behavioral (SSRT, DE/MP, ID/ED, and/or ORD tasks), and PET Assessments were Conducted

Assessment	Baseline	6-Month treatment						Break		12-Month treatment												Post-treatment					
Month in study	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Cumulative months of treatment		1	2	3	4	5	6			7	8	9	10	11	12	13	14	15	16	17	18						
Physiol. develop.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Cytogenetic	X			X			X								X						X						X
Activity	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
SSRT	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
DE/MP	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
ID/ED	X							X							X						X						
ORD									X						X						X						
PET	X							X														X					X

General activity level was collected continuously throughout all conditions. Each block in the body of the table represents 1 month.

(Weed and Hienz, 2006; Weed *et al*, 2008b). It involved providing signaled 15-min access through a drinkometer on the intelligence panel to 100 ml of orange drink at 0900 and 1200 hours daily. Across days, quinine was added gradually until a concentration of 0.32 mg/ml was being consumed reliably. Once the experimental groups were formed, quinine was removed and MPH or AMPH was added at a low concentration for the drug groups. The control monkeys continued drinking unadulterated Tang solution. MPH and AMPH concentrations were increased gradually over, respectively, 4–6 and 12–14 weeks of the 6-mnth treatment period. Blood was sampled periodically at 1000 or 1300 hours to determine the mg/kg per session intake that produced blood levels in the therapeutic range (ie, MPH: 15–25 ng/ml (Patrick and Markowitz, 1997; Swanson and Volkow, 2003); AMPH: 50–60 ng/ml of *d*-AMPH and 15–25 ng/ml *l*-AMPH (Brown *et al*, 1978; Greenhill *et al*, 2003; Tulloch *et al*, 2002)). For blood sampling, monkeys were sedated with ketamine hydrochloride + midazolam hydrochloride, intramuscularly. Samples were analyzed for drug levels by National Medical Services (NMS) Labs (Willow Grove, PA) using gas chromatography/mass spectrometry. Lower level of quantification for *d*- and *l*-AMPH was 20 ng/ml, and lower level of quantification for MPH was 2 ng/ml. Blood sampling occurred 5–6 times per group over the course of the drug exposure periods. Dose targets, based on blood/plasma results, were 12–16 mg/kg per session for MPH and 0.7–0.8 mg/kg per session for AMPH.

Physiological Development. Monkeys were sedated, as described above, once a month to enable measurement of body weight, rump–crown lengths, and head circumference. Blood samples drawn after 12 months of exposure were analyzed for testosterone levels as described previously (Mattison *et al*, 2011).

Cytogenetic Changes. Blood was sampled at baseline; after 3, 6, 12, and 18 months of treatment (2 h after the 0900-hour dose); and 6 months after treatment ended to determine if exposure to MPH or AMPH induced increased frequency of CAs and MN (%MN is reported from 1000 cells counted per subject), or increased the number of SCEs in peripheral blood lymphocytes. These studies were conducted under contract with SRI International (Menlo Park, CA), using standard assays with methods similar to those reported previously (El-Zein *et al*, 2005; Ponsa *et al*, 2009; Tucker *et al*, 2009; Witt *et al*, 2008). Frequency of cells with CA and MN were analyzed using Fisher's exact test (significance level of $\alpha = 0.05$, one-tailed) because of the dichotic nature of these data. SCE data were analyzed with two-way ANOVA described below.

Monitoring General Activity. Actical accelerometer monitors (Mini Mitter, Bend, OR) for continuously recording activity were mounted to collars (Primate Products, Woodside, CA) fitted to each monkey before collection of the behavioral baseline data. Data were downloaded each month while the monkeys were sedated for growth measurements.

Behavioral Models of Executive Function.

Stop signal reaction time: The stop signal reaction time (SSRT) procedure is considered to measure the ability to inhibit an action. Each trial began with a blinking light over the lever. Inserting one hand into the pellet receptacle, while depressing the lever with the other, changed the blinking to continuous illumination (the 'hold' signal). After 1 to 7 s, the yellow 'hold' light changed to white (the 'release' signal); and simultaneously, one of the two response keys was illuminated white. Releasing the lever and pressing the response key within 1.5 s resulted in food pellet delivery and a 10-s inter-trial interval. Withdrawing the hand from the pellet receptacle terminated the trial and initiated a 10-s timeout, during which no lights were illuminated and responses had no programmed consequences. To promote stimulus control of the monkey's behavior, some trials (20%) were 'catch' trials in which the release stimulus was never presented and the trial ended after the monkey held the lever for 7 s (Weed *et al*, 2003). Of the non-catch trials, 25% were 'stop' trials in which the usual response key changed from white to red and the second response key was

trans-illuminated white. Touching the white key resulted in pellet delivery, while touching the red key ended the trial with no reward. The interval before the key turned from white to red was manipulated to determine the time at which a monkey successfully avoided pressing the red key and pressed the white key on 50% of those trials. To achieve this 50% success rate, the interval between the release of the lever and the onset of the red light started at 250 ms. If the monkey successfully inhibited pressing the red key at this delay, the interval lengthened by 50 ms in the next trial, making it harder for the monkey to stop in time. If the monkey then failed to inhibit pressing the key, the interval was shortened by 50 ms in the next trial to make it easier to inhibit the response. The SSRT was conducted from 1000 to 1100 hours, 3 days per week.

Delay estimation/motor preparation: This task, considered to measure time/delay estimation, was similar to the SSRT. Briefly, the blinking light became continuous after 1–3 s, and a pellet was delivered when the lever was released within 1.5 s of that change. There were no catch or stop trials. Reaction time was evaluated as a function of the length of the interval before the stimulus change. The delay estimation/motor preparation (DE/MP) task compares reaction time with no temporal cues (1-s release stimulus) with reaction time when the timing of the release stimulus is 100% predictable 3-s release stimulus; Decamp and Schneider, 2004). The DE/MP task was conducted from 1300 to 1400 hours, 3 days per week.

ID/ED set-shifting task: This task assesses attentional set shifting (Dias *et al*, 1996; Weed *et al*, 2008a; Weed *et al*, 1999). A portable device with a touch screen was positioned in front of the monkey's cage. Training and testing was as described previously (Weed *et al*, 2008a). The IE/ED task was conducted at the same time of day for each animal (1100 h or 1400 hours) and took 4.7 days, on average, to complete the entire sequence of stimuli. All other behavioral testing was suspended during ID/ED testing. The dependent measure was the number of errors until criterion performance at each stage (12 correct responses in 15 trials).

Object retrieval detour: This task is used to measure inhibitory control. It involved positioning a clear Plexiglas box with a single open side in front of the cage, providing the opportunity for the monkey to retrieve a miniature marshmallow positioned within the box. On different trials, the box was positioned with the open side facing the cage, facing left, or facing right and the marshmallow was placed either at the front of the box near the opening or back of the box (for more details, see Gray *et al* (2006)). A trial was considered 'hard' when the box opening was not directly facing the monkey and the marshmallow was placed deep inside the box (Gray *et al*, 2006; Jentsch *et al*, 2000; Rutten *et al*, 2008). Performance of the task was tested at baseline and after 6, 12, and 18 total months of treatment. Two habituation sessions were conducted during the week before five acquisition sessions in the following week. The task was conducted at the same time of day for each animal (1100 or 1400 hours). Dependent measures were the percentage of trials in which the food item was retrieved on the first reach, errors due to touching closed sides of box (barrier reaches), and latency to initiate a reach, which were analyzed for all trials and for the subset of trials defined as hard.

PET Procedures

Each monkey received two dynamic PET scans 1 week before baseline ended, after 6 and 18 total months of treatment, and 6 months after treatment ended. PET scans occurred no earlier than 1 week after discontinuation of treatment to allow drug to be cleared from the body so that self-dosed drug did not compete with PET radiotracers. One scan used [^{11}C]MPH to determine DAT binding, and the other used [^{11}C]raclopride to determine DA D_2 receptor binding. Magnetic resonance imaging (MRI) sessions were conducted for each monkey approximately 1 month before each round of PET scans so that regions of interest (ROIs) for quantification of radioligand binding could be manually determined from those MRIs. Anesthesia during the PET scan was maintained using propofol (titrated to 15–30 mg/kg/h) (Wilcox *et al*, 2008). The dynamic PET scans were performed on a high-resolution research tomography PET scanner, a state-of-the-art three-dimensional-only dedicated brain tomography with <2 mm spatial resolution and a bore length sufficient to view the entire monkey brain (de Jong *et al*, 2007; Horti *et al*, 2006; Sossi *et al*, 2005) as described previously (Rahmim *et al*, 2005; Wilcox *et al*, 2008). The [^{11}C]MPH scans were conducted first and [^{11}C]raclopride scans were conducted approximately a week later. For DAT imaging studies, a bolus of [^{11}C]MPH (average injected dose 6.8 mCi ± 0.14 (SEM); average specific activity 6775 mCi/μmol ± 304 (SEM)) was injected intravenously and dynamic images of 30 frames were reconstructed for the 90 min of data acquisition in each study. The binding potential (BP_{ND}) of [^{11}C]MPH was estimated by fitting a simplified reference tissue model (SRTM) (Lammertsma and Hume, 1996). ROI of the cerebellum was used as reference tissue input in fitting ROI time–activity curves (TACs). For D_2-like receptor imaging studies, a bolus injection of high-activity [^{11}C]raclopride was followed by continuous infusion (average injected dose 18.8 mCi ± 0.27 (SEM); average specific activity 9816 mCi/μmol ± 677 (SEM)) as described previously (Guilarte *et al*, 2006; Zhou *et al*, 2006). An intravenous bolus of *d*-AMPH (2 mg/kg in 1 ml/kg, administered over 2 min) was given 45 min after initiation of the scan. The BP_{ND}s of [^{11}C]raclopride pre- and post-*d*-AMPH bolus were estimated by fitting an extended SRTM (Zhou *et al*, 2006) to ROI TACs. The *d*-AMPH-induced DA release (DAR%) was estimated as the percentage change in BP_{ND} by taking the percentage difference in BP_{ND} obtained before [BP_{ND}(baseline)] and after [BP_{ND}(AMPH)] *d*-AMPH administration: $DAR\% = 100[BP_{ND}(\text{baseline}) - BP_{ND}(\text{AMPH})]/BP_{ND}(\text{baseline})$. PET measures of BP_{ND} values and DAR% were compared across baseline, 6, and 18 months of treatment, and 6 months after treatment ended.

Drugs

dl-MPH hydrochloride was obtained from Mallinckrodt Pharmaceuticals (St Louis, MO); *d*-AMPH sulfate and *l*-AMPH sulfate were obtained from Sigma-Aldrich (St Louis, MO); and quinine sulfate was obtained from Sigma-Aldrich. *d*-AMPH used in PET studies was dissolved in 0.9% saline and filter-sterilized before intravenous administration. Otherwise, all compounds were taken via the oral route. The two forms of AMPH were mixed to produce a 75% *d*-AMPH and 25% *l*-AMPH mixture, comparable to that contained in prescription Adderall. All were dissolved in RO water for stock solutions. Quinine and the MPH and AMPH solutions were added to an orange drink prepared with Tang powder (23 g/l) in RO water. Drug doses and concentrations refer to the salts.

Statistical Analysis

Unless otherwise noted, dependent measures from behavioral, PET, and cytogenetic assessments were analyzed using repeated-measures ANOVAs with the within-subjects factor of time point (eg, baseline, following 6, 12, and 18 months of treatment, and 6 months after treatment was permanently discontinued) and the between-groups factor of treatment (control, AMPH, MPH). The Holm–Sidak method was used for α-adjusted (family-wise $\alpha = 0.05$) *post-hoc* comparisons (Sigmaplot 11; Systat Software). *Post-hoc* comparisons were conducted comparing control animals to the drug-treated animals overall (ie, across time points) and within each time point, and comparing baseline to later time points overall (ie, across treatment groups) and within treatment groups.

RESULTS

Oral Self-Dosing

Consistent volumes of Tang or drug + Tang mixtures were consumed. Monkeys in the AMPH group reached or exceeded 90% of their final target doses on an average of 83% of days (range: 64–94%) and on average consumed 0.99 mg/kg per session of *dl*-AMPH (range 0.81–1.15 mg/kg per session). Monkeys in the MPH group reached or exceeded 90% of their final target doses on 86% of days (range: 77–94%); on average, they consumed 10.7 mg/kg per session when the target was 12 mg/kg per session and 16.5 mg/kg per session (range 8.89–13.1 mg/kg per session) when the target was 15 mg/kg per session (range 15.5–18.7 mg/kg per session). Mean MPH plasma levels were 18 and 22–32 ng/ml following the 0900 and 1200 hours drinking sessions, respectively. Mean AMPH plasma levels were 64–97 and 93–124 ng/ml following the 0900 and 1200 hours drinking sessions, respectively.

Physiological Development

Measures of head circumference, crown-to-rump length, and weight were consistent with normative growth rates for rhesus monkeys reported by Rawlins *et al*. (1984), and observed in monkeys trained on similar behavioral tasks in which responding was motivated by a food reinforcer (MR Weed, unpublished observation). There was no statistically significant difference in the number of biscuits consumed among the groups over the duration of the study (AMPH: min = 12.8 ± 0.51, max = 13.7 ± 0.36; MPH: min = 13.2 ± 0.30, max = 15.3 ± 0.41; control: min = 11.7 ± 0.41, max = 14.5 ± 0.30). There was no effect of chronic drug ingestion for either AMPH or MPH on any growth measure across the 18 total months of exposure, or 6 months after drug was no longer available (Figure 1).

There was a significant group difference in testosterone levels at 12 months of treatment ($F_{2,20} = 3.72$, $p = 0.042$; 263.1 ± 61.14, 496.4 ± 80.20, 624.5 ± 131.1 for control, AMPH, and MPH monkeys, respectively). Testosterone levels for MPH monkeys were higher than those for control monkeys according to *post-hoc* comparisons. The differences in age of testes descent were not statistically significant (38.6 ± 0.84, 37.5 ± 0.48, 36.4 ± 0.30 for control, AMPH, and MPH groups, respectively).

Cytogenetic Changes

Transient effects on SCE were noted at 3 and 6 months, but not at any time afterward (Figure 2, top), as confirmed by repeated-measures ANOVA on the factor of time ($F_{5,105} = 177$, $p < 0.0001$) and treatment ($F_{2,105} = 33.8$, $p < 0.0001$), and their interaction ($F_{10,105} = 15.6$, $p < 0.0001$). *Post-hoc* tests indicated differences from control monkeys for only the

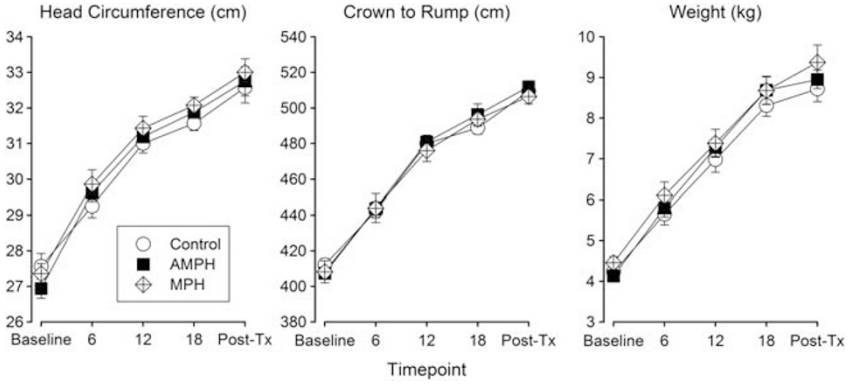

Figure 1 Physiological development for control, amphetamine (AMPH), and methylphenidate (MPH) groups over the duration of the study. Growth measures were taken for head circumference (left panel), crown-to-rump length (center panel), and weight (right panel). Data are the mean ± SEM, and $n = 8$ for each treatment group. 'Post-Tx' = post-treatment period.

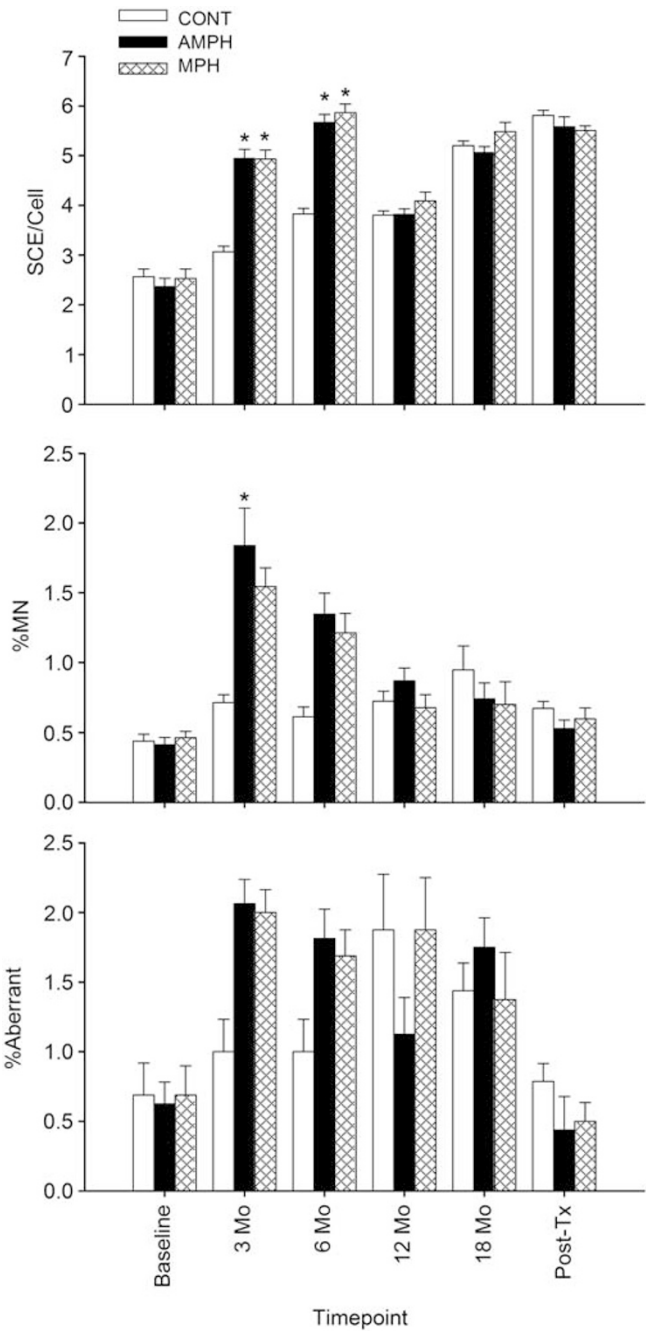

Figure 2 Frequency of sister chromatid exchange (SCE) per cell (top graph), %micronuclei (%MN) (middle graph), and %aberrant (percentage of cells with chromosomal aberrations; bottom graph) over the duration of the study for control, amphetamine (AMPH), and methylphenidate (MPH) monkeys. 'Post-Tx' = post-treatment period.

3- and 6-month time points ($p<0.001$ for both), but not other time points ($p>0.05$ for others). Similarly, transient changes in the percentage of cells with MN in the AMPH group with the control group were detected at 3 months (Fisher's exact $p=0.028$), with no differences at any time afterward (Figure 2, middle). There was no significant effect of AMPH or MPH on the percentage of cells with CA compared with control monkeys for each time point (Fisher's exact $p>0.05$; Figure 2, bottom).

General Activity

General activity was analyzed in three ways. First, for each monkey, hourly (0–2300) averages were calculated across the 7 days of each week at the time periods of interest (ie, the last week of baseline and the week at the end of 6, 12, and 18 months of treatment; see Figure 3). Two-factor (group by hour) repeated-measures ANOVAs were conducted (note that post-treatment data were not analyzed due to loss of data from half of the control monkeys). There was a significant main effect of hour at each time period ($F_{23,46}=106$, $p<0.001$; $F_{23,46}=96$, $p<0.001$; $F_{23,46}=22$, $p<0.001$; $F_{23,46}=33$, $p<0.001$ for baseline, and after 6, 12, and 18 months of treatment, respectively). There was no significant effect of group at any time period. There was a significant interaction of group and hour during the last week of the 6-month treatment period ($F_{46,437}=1.6$, $p<0.006$). According to *post-hoc* comparisons, activity levels were higher in control monkeys compared to AMPH monkeys at 1200, 1400, and 1500 hours and higher in control monkeys compared to AMPH and MPH monkeys at 2100 hours. Other than the last week of the 6-month treatment period, there were no significant main effects of group or hroup × hour interactions.

In the second analysis of general activity, activity counts were grouped in 3-h time bins starting at midnight, resulting in eight bins (0000–0300 hours, 0300–0600 hours, etc). Thereafter, 7-day averages were calculated for each monkey for each of the 8 time bins for each of the 4 weeks comprising a period of interest (ie, baseline, 6 cumulative months of treatment, the first 4 weeks of the break period, and 12 and 18 cumulative months of treatment). Similar to the first activity analysis, this allowed a two-factor (group by time bin) repeated-measures ANOVA to be conducted for each week. The results of the statistical analyses are summarized in Table 2. There was a significant main effect of group in the 2nd and 3rd weeks of baseline and in the 2nd week of the break, and a significant group by time bin interaction in the 3rd week of the last month of treatment. Where significant differences were obtained, activity levels of control monkeys were higher than those of the AMPH or MPH groups or both.

In the third type of analysis, which focused only on daylight hours when AMPH and MPH likely would have had the largest acute effects, activity was totaled from 0900 to 1500 hours for each monkey for each day and averaged for all 4 weeks of baseline, the last 4 weeks of the 6-month treatment period, the first 4 weeks of the break period, the 4 weeks comprising the 12th month of treatment, the 4 weeks comprising the 18th month of treatment, and the first 4 weeks of the post-treatment period. Separate one-way (week) repeated-measures ANOVAS, one for each group (AMPH, MPH, and control) were conducted with *post-hoc* comparisons to the last week of baseline. For the AMPH group, there was a main effect of week ($F_{27,177}=2.6$, $p<0.001$). According to *post-hoc* comparisons, activity in the 2nd week of the break and each of the first 4 weeks of the post-treatment period was lower than activity during the 4th week of baseline. For the MPH monkeys, there was a main effect of week ($F_{27,176}=2$, $p=0.004$); and according to *post-hoc* analysis, activity during the 2nd week of the post-treatment period was lower than in the last week

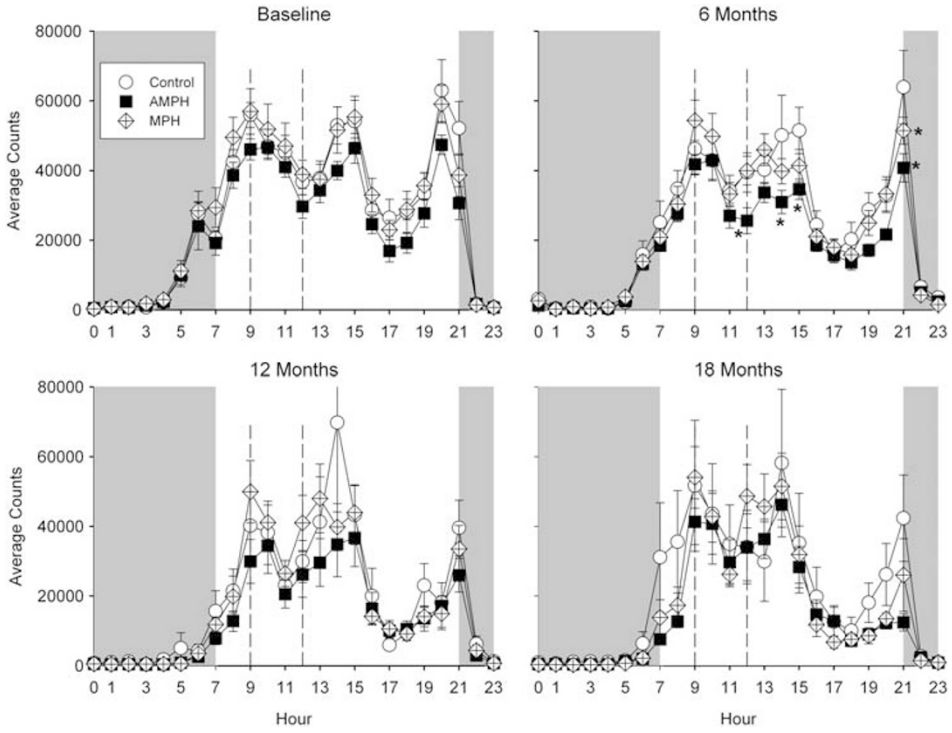

Figure 3 Hourly activity counts over 24 h for control, amphetamine (AMPH), and methylphenidate (MPH) groups at baseline, and after 6, 12, and 18 months of treatment. The *x* axis represents hour in the day and the *y* axis represents averaged total activity counts. Shaded areas on each graph represent the time when the room lights were off, and vertical dashed lines indicate the two daily drink sessions (0900 and 2400 hours). Data are the mean ± SEM, and $n = 8$ for each group.

of baseline. For control monkeys, there was no main effect of week, but recall that post-treatment period data were excluded from analysis due to the loss of activity data in that group.

Behavioral Models of Executive Function

SSRT. All monkeys were successfully trained to perform the SSRT task. Three dependent measures were analyzed for 1 week of sessions (ie, three sessions) at each of the time points (baseline, and 6, 12, and 18 total months of treatment). The measures were: median reaction time (lever release latency once release light was illuminated), median touch latency (time from lever release till key press), and median stop latency (time from lever release till white, not red, key was pressed on stop trials). These measures represent, respectively, simple reaction time, gross motor speed, and ability to inhibit responding. There was a significant main effect of group for stop latency ($F_{2,21} = 5.1$, $p = 0.01$). *Post-hoc* analysis revealed that stop latency for the MPH group was significantly lower than for the control group under baseline conditions (ie, before the beginning of drug dosing; Figure 4, top). There were no statistically significant group differences in lever release latency (simple reaction time) or touch latency (gross motor speed) at any time point (data not shown).

DE/MP. All animals in the control group acquired the DE/MP task by the end of baseline; that is, they demonstrated timing behavior by releasing more quickly after 3-s hold times than after 1-s hold times. In the MPH group, all but one animal acquired the task, and in the AMPH group, five of the eight monkeys acquired the task. Only those animals that acquired the task were used for analysis. The dependent measure was the difference in lever release latency when the hold light was on for 3 s relative to 1 s. Data were compared for 1 week of sessions (ie, 3 sessions) at baseline and after 6, 12, and 18 months of treatment. Figure 4 (bottom) shows the difference between latencies 1 and 3 s after the release stimulus was presented. Two-way (group and time period) repeated-measures ANOVA indicated no significant main effect of group or a group-by-time period interaction. There was a main effect of time period ($F_{3,6} = 3.3$, $p = 0.03$), and *post-hoc* comparisons revealed that DE/MP performance at 6-month treatment differed significantly from baseline for the AMPH group only.

ID/ED. All animals successfully performed the ID/ED task. Each administration of the ID/ED used a different stimulus set and, as previously reported (Weed *et al*, 1999), there were differences in the gross number of errors as a function of the stimulus sets (Figure 5a and b). Repeated-measures ANOVA indicated a significant main effect of ID/ED stage at all time points: baseline ($F_{7,14} = 14.93$, $p < 0.001$); 6 ($F_{7,14} = 30.56$, $p < 0.001$), 12 ($F_{7,14} = 8.91$, $p < 0.001$), and 18 total months of treatment ($F_{7,14} = 16.00$, $p = 0.001$). Overall, the pattern of errors was as expected, the numbers of errors differed from stage to stage, and there were differences between overall performance at each time point (most likely due to intrinsic differences in difficulty of the stimulus sets and/or experience with the testing, rather than differences in the animals). Within each stage, there were no statistically significant differences in the number of errors

Table 2 Summary of Statistical Analysis of General Activity in 3-h Bins, Averaged Across 7 Days of Each of Weeks 1–4 Comprising Each Time Point of Interest

Source	Week 1	Week 2	Week 3	Week 4
Baseline				
Group main effect	NS	$F_{2,19} = 3.7$, $p = 0.044$[a]	$F_{2,21} = 3.86$, $p = 0.037$	NS
Group × time bin	NS	NS	NS	NS
0000–0300 h	NA	NS	NS	NA
0300–0600 h		NS	NS	
0600–0900 h		NS	NS	
0900–1200 h		NS	Cont > AMPH	
1200–1500 h		NS	Cont > AMPH	
1500–1800 h		NS	NS	
1800–2100 h		NS	NS	
2100–0000 h		NS	NS	
6th Month of cumulative treatment				
Group main effect	NS	NS	NS	NS
Group × time bin	NS	NS	NS	NS
First 4 weeks of the break				
Group main effect	NS	$F_{2,19} = 4.19$, $p = 0.031$	NS	NS
Group × time bin	NS	$F_{14,133} = 2.98$, $p < 0.001$	NS	NS
0000–0300 h	NA	NS	NA	NA
0300–0600 h		NS		
0600–0900 h		NS		
0900–1200 h		Cont > Amph		
1200–1500 h		Cont > AMPH; Cont > MPH		
1500–1800 h		Cont > AMPH; Cont > MPH		
1800–2100 h		Cont > Amph		
2100–0000 h		NS		
12th Month of cumulative treatment				
Group main effect	NS	NS	NS	NS
Group × time bin	NS	NS	NS	NS
18th Month of Cumulative Treatment				
Group main effect	NS	NS	NS	NS
Group × time bin	NS	NS	$F_{14,105} = 1.97$, $p = 0.027$	NS
0000–0300 h	NS	NS	NS	NS
0300–0600 h	NS	NS	NS	NS
0600–0900 h	NS	NS	Cont > AMPH; Cont > MPH	NS
0900–1200 h	NS	NS	NS	NS
1200–1500 h	NS	NS	NS	NS
1500–1800 h	NS	NS	Cont > AMPH; Cont > MPH	NS
1800–2100 h	NS	NS	NS	NS
2100–0000 h	NS	NS	NS	NS

Abbreviatons: NA, not applicable (*post-hoc* comparisons not conducted if no main effect and no interaction); NS, not significant.
[a]*Post-hoc* comparisons of MPH and control and AMPH and control monkeys were not significant.

made between the groups at any time point. Attentional set shifting is measured in the ID/ED task by the increase in errors on the ED shift (the ED stage) relative to the ID stages. Figure 5b presents raw errors at these stages for each time point. Errors were generally higher on the ED than the ID stage; however, there were no significant differences among the groups in the measure of attentional set shifting at any time point. Neither was there any statistically

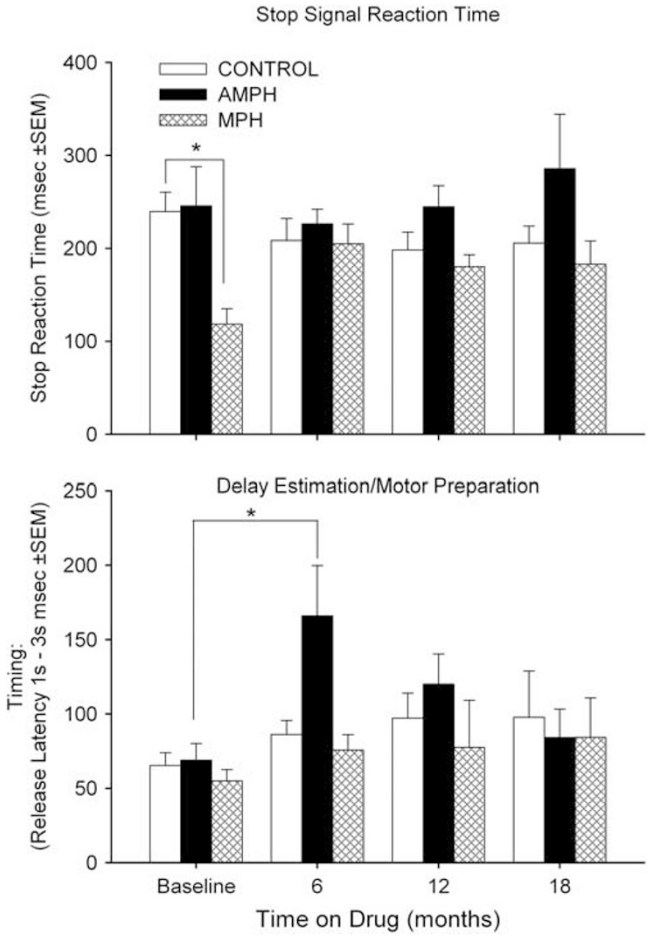

Figure 4 Stopping time data from the stop signal reaction time (SSRT) procedure for control, amphetamine (AMPH), and methylphenidate (MPH) groups at baseline, and after 6, 12, and 18 months of treatment (top). The *x* axis represents time on drug, and the *y* axis represents stopping time in milliseconds, calculated as touch latency minus stop latency. Data are the mean ± SEM, $n = 8$ for each group. Difference between release latencies (RLs) at 1 and 3 s in the delay estimation/motor preparation (DE/MP) task for control, AMPH, and MPH groups at baseline, and after 6, 12, and 18 months of treatment (bottom). The *x* axis represents time on drug, and the *y* axis represents RL1–RL3 in ms. Data are the mean ± SEM, and $n = 8$ for each group.

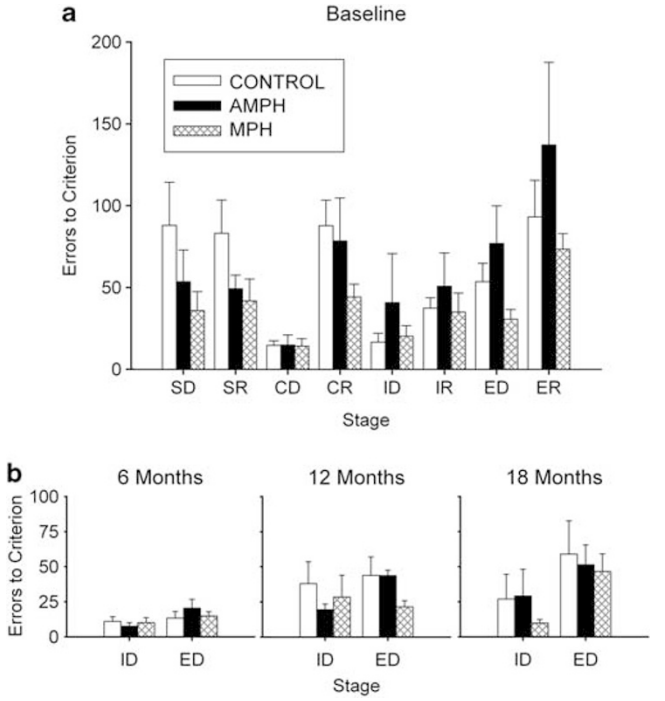

Figure 5 Errors to criterion for the different stages of the intra-dimensional/extra-dimensional (ID/ED) task for control, amphetamine (AMPH), and methylphenidate (MPH) groups at baseline, and after 6, 12, and 18 months of treatment. The top panel (a) shows performance under baseline conditions for all of the stages of the ID/ED task. The bottom panel (b) shows performance on the ID and ED stages of the ID/ED task at all time points studied. Data are the mean ± SEM, and $n = 8$ for each group.

significant effect of group in the size of the difference between errors in the ED and ID stages.

Object retrieval detour. Figure 6 presents the data from the object retrieval detour (ORD) task at each time point for each group. Data were collected for three parameters: the percentage of trials in which food was retrieved on the first reach, barrier reaches (ie, errors), and latency to initiate a reach into the box. Data are presented for all trials (left graphs) and 'hard' trials (as previously defined; right graphs). There was a significant main effect of time point on the percentage of hard trials where the food reinforcer was retrieved on the first reach ($F_{2,4} = 3.73$, $p = 0.03$), but no significant effect of group or a group by time point interaction. *Post-hoc* comparisons revealed that there were fewer trials on which food was retrieved on the first reach after 6 months compared to 12 months of treatment and 18 total months of treatment.

PET Measures of DAT, D_2-like Receptors, and %DAR

Figure 7 presents the data from the PET studies evaluating DAT ([^{11}C]MPH BP_{ND}; top graph), DA D_2 receptor ([^{11}C]raclopride BP_{ND}; middle graph), and AMPH-stimulated %DAR (bottom graph) in the striatum at baseline, after 6 months of treatment and after 18 total months of treatment, and after 6 months of post-treatment. There was a significant main effect of time point ($F_{3,6} = 9.31$, $p < 0.001$) for DAT BP_{ND} in the striatum, but no main effect of group or a group by time point interaction. DAT BP_{ND} values for control monkeys were higher during baseline than 6 months after then end of treatment according to *post-hoc* comparisons. DAT BP_{ND} values were higher for MPH monkeys after 18 total months of treatment compared with baseline values, according to *post-hoc* comparisons. *Post-hoc* comparisons of group DAT BP_{ND} were not significant at any time point.

Similar to DAT binding, there was a significant main effect of time point for binding of [^{11}C]raclopride to D_2 receptors in the striatum ($F_{3,6} = 5.89$, $p = 0.001$). Overall there was a decrease in D_2 receptor BP_{ND} values, compared to baseline, across groups after 18 months of dosing and 6 months after treatment ended, (Figure 7, middle graph). There were no statistically significant differences between treatment Groups at any time point.

There was also a main effect of time point ($F_{3,6} = 5.56$, $p = 0.002$) for AMPH-stimulated DA release (%DAR;

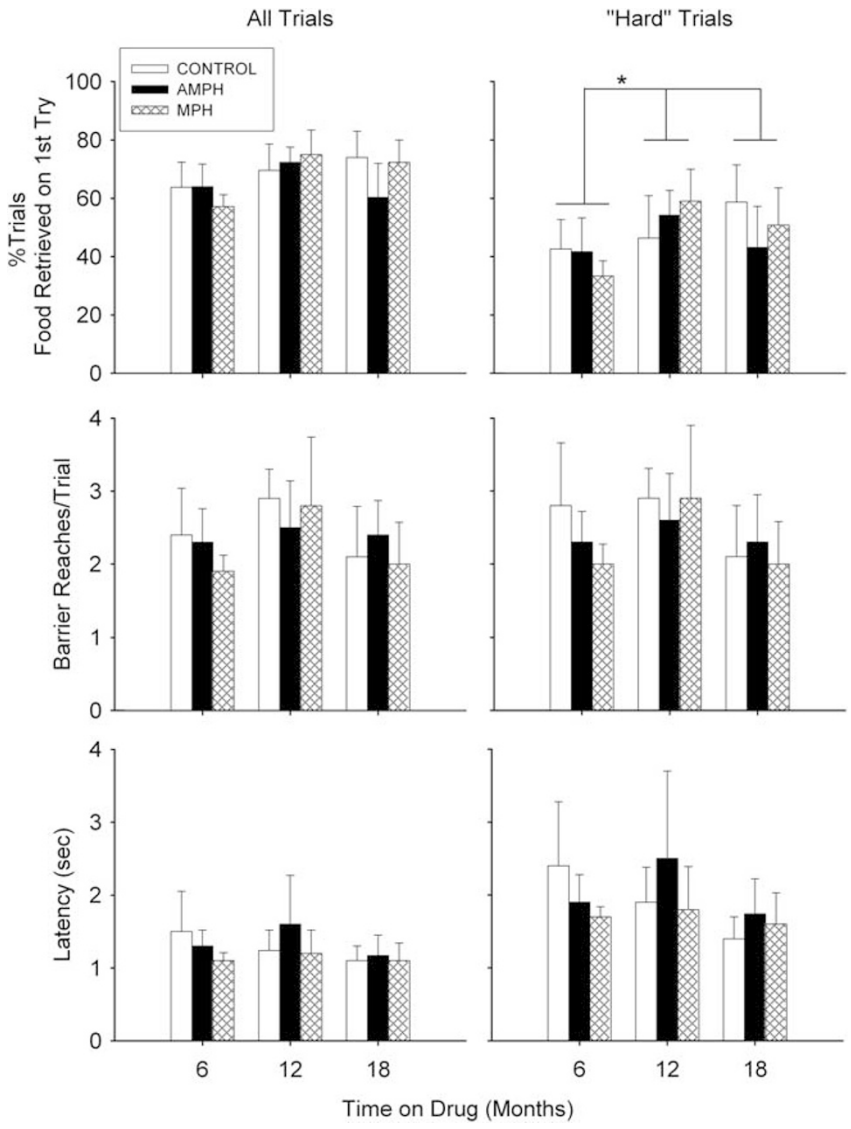

Figure 6 Performance on the object retrieval detour (ORD) task for control, amphetamine (AMPH), and methylphenidate (MPH) groups after 6, 12, and 18 months of treatment. Data are presented for all trials (left panels) and trials defined as 'hard' (eg, when the box opening was not directly facing the monkey and the food reinforcer was placed far inside the box; right panels). Data from three measures are presented: percent of trials where the food reinforcer was retrieved on the first reach (top panels), average errors/trial or 'barrier reaches' (middle panels), and latency to initiate a reach (bottom panels). Data are the mean ± SEM, and $n = 8$ for each group.

Figure 7, bottom graph). Overall, there was an increase in the percentage change in D_2 receptor BP_{ND} following injection of *d*-AMPH after 18 months of treatment and 6 months after treatment ended, compared to baseline. The main effect of group and the interaction between group and time point were not statistically significant.

DISCUSSION

The results of this study clearly show that prolonged exposure to therapeutically relevant doses of AMPH or MPH in peri-adolescent rhesus monkeys had little effect on development in terms of growth, activity, executive/cognitive function, DAT and D_2 BP_{ND} values, or *d*-AMPH-induced %DAR. These results are similar to a previous study with juvenile macaques and chronic MPH in an operant test battery that showed no change in many behavioral/cognitive measures with a low dose of MPH (Rodriguez *et al*, 2010).

Given the lack of effects found in these studies, it is important to reiterate that the blood levels produced by the oral self-dosing procedures were in the clinically relevant range. Drug exposure levels were on the higher side of the clinically relevant range with both compounds. The plasma levels of *dl*-AMPH and MPH achieved in this study were above peak concentrations reported in children treated with those compounds (Greenhill *et al*, 2003; Patrick and Markowitz, 1997). One important caveat is that the oral self-dosing procedure in this study resulted in lower bioavailability of MPH than did the oral dosing procedure used by Rodriguez *et al*. (2010) (40.84–134.55 ng/ml in their study and 18–32 ng/ml in this study). The cause of this difference in exposure is not clear, but likely related to the large difference in dosing volume used in the two

procedures (approximately 100 ml in this study *vs* 0.5 ml/kg in the study by Rodriguez *et al*. (2010)), with the lower volume likely encouraging retention in the monkey's cheek pouch resulting in more efficient buccal absorption. Most importantly, our previous PET study indicated that oral doses of MPH similar to these resulted in roughly 50% occupancy of the DAT by MPH in rhesus macaques (Wilcox *et al*, 2008), an occupancy level shown to result from therapeutic doses of oral MPH in humans (Volkow *et al*, 1998).

The major advantage of the oral self-dosing procedure was that it enabled an automated, reliable, non-stressful administration of both MPH and AMPH, with all animals in the study receiving their treatments at exactly the same time. Models such as these that use voluntary consumption will naturally have some variability in dosing. Overall the 'compliance' in drinking typically exceeded estimates of compliance in ADHD patients (eg, 74.11% at 1 week and 72.75% at 3 months; Ibrahim *et al*, 2002). Therefore, the level of consistent exposure of the monkeys to AMPH or MPH throughout the study can be considered therapeutically relevant.

Reports of the physiological effects (eg, growth rates and sleep patterns) of ADHD treatment medications show variable results. For example, some studies reported that growth rates were reduced (Mattes and Gittelman, 1983; MTA Cooperative Group, 2004b) and that the effect may be transient or long lasting (Poulton and Cowell, 2003; Rapport and Moffitt, 2002). In this study, there were no differences between the control group and either the AMPH or the MPH groups on any growth rate measure (weight, head circumference, and crown-to-rump length). These results are consistent with another chronic MPH study using juvenile macaques where growth rates were not affected (Morris *et al*, 2009).

Interestingly, the increase in testosterone found in the MPH monkeys after 12 months of drug exposure compared with the control monkeys is opposite to that found in a recent study of chronic MPH exposure in rhesus monkeys, in which MPH-exposed monkeys exhibited lower testosterone levels (Mattison *et al*, 2011). The lack of baseline measurements and measurements across months of dosing is a limitation of the testosterone data set in this study; the study by Mattison and co-workers included many more time points. However, the reason for the discrepancy between the studies is unclear. Further research may be necessary to disentangle the effects of MPH on testosterone levels in male peri-adolescent rhesus monkeys.

There was no persistent induction of the markers of genetic toxicity (CA, MN, and SCE) with either drug. There were statistically significant but very small and transient increases in MN and SCE at the early time points (when blood was taken after 3 and 6 months of drug delivery). Following those changes, the measures were similar to the control Group for more than a year. In the case of the SCE changes, the transient increases were small enough as to be within the ranges seen between baseline and 18 months in the control Group. In the case of the MN changes, the magnitude was less than that expected from a diagnostic X-ray or angiography in humans (Norman *et al*, 2001). Overall, the absence of persistent effects indicates that these drugs are not clastogens in rhesus monkeys. The lack of clastogenic effects in this study is similar to one previous report in rhesus monkeys (Morris *et al*, 2009) and several reports in ADHD patients (Ponsa *et al*, 2009; Tucker *et al*, 2009; Witt *et al*, 2008).

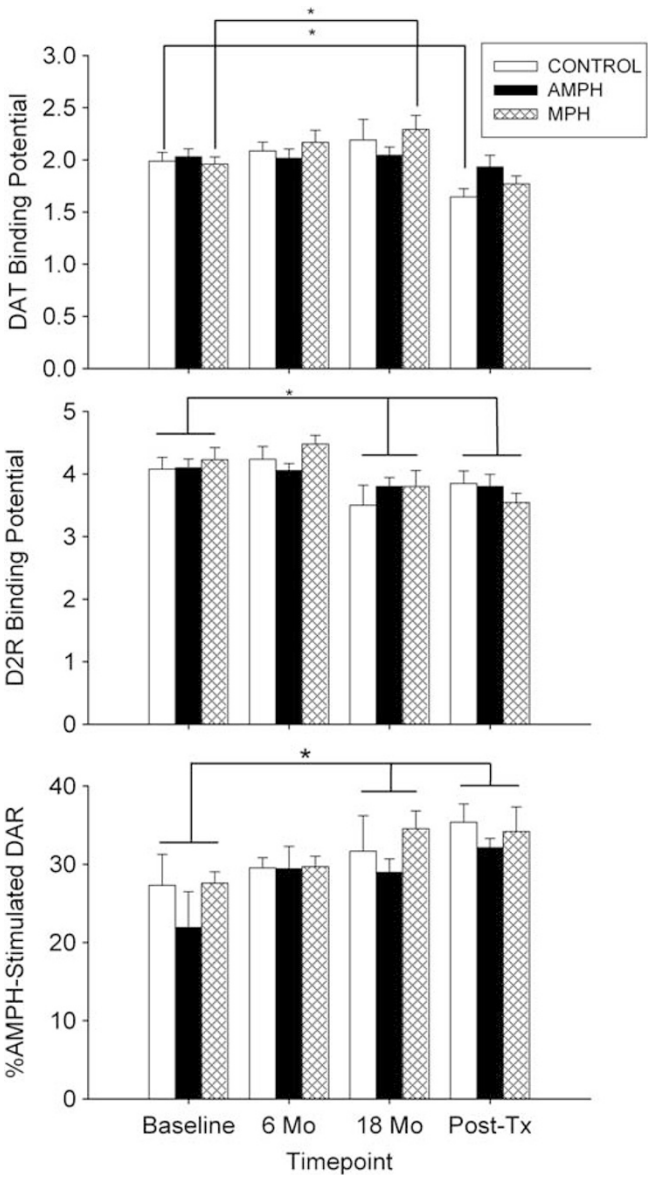

Figure 7 Dopamine transporter (DAT) binding potential (top graph), dopamine D_2 receptor binding potential (middle graph), and %change in D_2 receptor binding potential following intravenous (i.v.) bolus infusion of *d*-amphetamine (bottom graph) in the striatum for control, amphetamine (AMPH), and methylphenidate (MPH) groups at baseline, after 6 and 18 months of treatment, and 6 months after treatment ended. Data are the mean ± SEM, and $n = 8$ for each group. 'Post-Tx' = post-treatment period.

When the data on general activity were analyzed in hourly time bins, only at the 6-month treatment period were there any statistically significant differences between groups, and the activity of control monkeys was higher than that of the AMPH and MPH monkeys. Similarly, when the data were analyzed in 3-h time bins, statistically significant effects of group or group by time bin interactions were obtained during the 3rd week of baseline, the 2nd week of the break, and the 3rd of the last 4 weeks of the 12-month treatment

period. The time of day in which these between-group differences in activity level occurred varied; but in all cases, activity levels of control monkeys were higher than those of AMPH, MPH, or both groups of monkeys. The fact that such differences were obtained before any drug exposure, during drug exposure, and during time off drug, and were always in the same direction, suggest that the differences were not due to drug exposure or termination of that exposure. With respect to activity data, once drug delivery was stopped, within-group analyses of general activity for the AMPH monkeys suggested a decline in activity during the 2nd week of the break and also during all of the first 4 weeks of the post-treatment period relative to the last week of baseline. Within-group analysis for the MPH monkeys showed a decline in activity during the 2nd week of the post-treatment period relative to the last week of baseline. Although not conclusive, it is possible that the decreases in activity were the result of abrupt drug termination. Overall the lack of robust drug effects on activity is consistent with two previous studies that reported no difference in nocturnal activity between medicated and un-medicated children with ADHD, suggesting that stimulant therapy neither improved nor impaired sleep (Cohen-Zion and Ancoli-Israel, 2004).

The behavioral battery used in this study assessed a number of aspects of executive function, including inhibitory control (ORD and SSRT tasks), time estimation (DE/MP task), and cognitive flexibility (ID/ED task). Performance on all tasks was similar in the AMPH, MPH, and control groups under baseline conditions, and consistent with previous reports (Dias *et al*, 1996; Weed *et al*, 2003, 1999). All in all, there were no important or systematic effects of long-term exposure to these doses of AMPH or MPH on any cognitive measure. Previous reports have suggested that acute administration of psychomotor stimulants can affect performance on the cognitive tests used in these experiments; however, few studies have used a gradually escalating, chronically administered low dose. The very gradual increase in dose was employed in this study to eliminate rejection of the drinking solution, and it is possible that the slow increase altered the behavioral effects of AMPH and MPH relative to an acute oral administration in naïve animals.

It is important to note several caveats regarding these studies. The magnitude and length of these studies precluded the use of more than one dose group for these drugs. A dose–response function that included doses higher than the therapeutic range would have allowed determination of a safety margin for these treatments; however, this would have required doubling or tripling the current efforts. Similarly, studies of acute and chronic treatments would have improved the ability to interpret these data, but again would have required doubling the number of animals used. Fortunately, a growing body of literature for both compounds has investigated the acute and chronic effects of higher doses of these compounds—doses more typical of abused doses have significant effects (Castner *et al*, 2000; Morris *et al*, 2009). These studies are an important addition to this literature.

Another important caveat of these studies is that the animals used in this study were healthy normal monkeys and not intended to model the ADHD population. The overall goal of this study was to investigate the developmental effects of AMPH and MPH treatments *per se*. It remains a possibility that the effects of stimulant drugs such as AMPH and MPH may differ in humans and non-humans with biological characteristics indicative of ADHD. Nevertheless, these results demonstrate that chronic, 18 months exposure to therapeutically relevant, low doses of AMPH or MPH does not result in significant effects on growth rates, sleep patterns, cognitive/executive function, or on dopaminergic system development, including changes in DAT and DA D_2 receptor densities and *d*-AMPH-evoked DA release. Consistent with other reports, cytogenetic studies suggested that neither AMPH nor MPH is a clastogen. Overall, these results are consistent with a growing body of evidence in laboratory animals and humans that suggest that at therapeutic doses, AMPH and MPH have little, if any, long-term detrimental effects.

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DISCLOSURE

The authors declare no conflict of interest.
In the past 3 years, the authors have received compensation for professional services as follows: Dr Ator's work has been funded by the NIH and she has received funding from Helsinn Healthcare to conduct an abuse liability evaluation of an unrelated compound. She has received compensation for consulting on abuse liability evaluation from Bristol Myers Squibb and F Hoffmann LaRoche. Dr Riddle's, Dr Wilcox's, and Dr Zhou's work has been funded by the NIH. Dr Soto's work has been funded by the NIH; he has received compensation, unrelated to his scientific work, for database/software consulting from Shands Hospital at the University of Florida. Dr Weed has been an employee of Bristol Myers Squibb since the end of the PET scans that occurred following the 18 months of treatment in this study. Bristol Myers-Squibb provided no financial support for these studies and had no scientific involvement. Dr Wong's work has been funded by the NIH, Avid, Biotie, GE, Intracellular, Johnson & Johnson, Eli Lilly, H Lundbeck, Merck, Orexigen, Otsuka, F Hoffmann LaRoche, and Sanofi-Aventis. He has received compensation for consulting from Amgen.

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