Differences in Methylphenidate Dose Response between Periadolescent and Adult Rats in the Familiar Arena-Novel Alcove Task^[S]

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ABSTRACT

Methylphenidate is a psychostimulant widely used in the treatment of attention deficit hyperactivity disorder. In this study, the effects of two nonstereotypy-inducing doses of methylphenidate (2.5 and 5.0 mg/kg s.c.) were examined in periadolescent [postnatal days (P) 35 and 42] and young adult (P70), male Long-Evans rats using a three-period locomotor activity paradigm that affords inferences about exploration, habituation, and attention to a novel stimulus (an "alcove") in a familiar environment in a single test session. In the first test period, P35 and P42 rats were more active than P70 rats, and methylphenidate increased locomotion in a dose-related manner. The introduction of a novel spatial stimulus in the third test period revealed a significant interaction of dose and age such that P70 rats exhibited dose-related increases in distance traveled, but

P35 rats did not. Furthermore, methylphenidate dose-relatedly disrupted the rats' tendency to spend increasing amounts of time in the alcove across the test period at P70 but not at P35. Brain and serum methylphenidate concentrations were significantly lower at P35 than at P70, with intermediate levels at P42. Developmental differences in dopaminergic neurochemistry were also observed, including increased dopamine content in the caudate-putamen, nucleus accumbens, and frontal cortex and decreased densities of D₁-like receptors in the frontal cortex in P70 than in P42 rats. These results raise the possibility that children and adults may respond differently when treated with this drug, particularly in situations involving response to novelty and that these effects involve developmental differences in pharmacokinetics and dopaminergic neurochemistry.

Introduction

Methylphenidate is the most widely used drug in the treatment of attention deficit hyperactivity disorder (ADHD), a neurodevelopmental disorder involving alterations in atten-

tion, response to novelty, habituation, and other processes. The disorder affects roughly 5% of school-aged children (Polanczyk et al., 2007), but it is increasingly recognized as affecting both children and adults (Simon et al., 2009). Methylphenidate increases dopaminergic signaling by blocking reuptake (Wilens, 2008). This effect on dopamine and perhaps also effects on norepinephrine reuptake appear to ameliorate some of the frontolimbic dysfunction believed to underlie ADHD and thus improve attention and consequently decrease general activity in individuals with the disorder (Tripp and Wickens, 2009). However, the therapeutic mechanism of the drug is not fully understood. Although the drug has demonstrated efficacy in treating the core symptoms of ADHD in both children and adults (Wilens, 2008), methodological variations in clinical studies make it difficult to ascertain whether efficacy of the drug differs between children and adults (Faraone and Glatt, 2009). However, some studies suggested that younger children exhibit lower therapeutic efficacy and more side effects (Wilens et al., 2002).

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ABBREVIATIONS: ADHD, attention deficit hyperactivity disorder; FA-NA, familiar arena-novel alcove; P, postnatal day; SCH 23390, 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol; WIN 35,428, (–)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate monohydrate; ANOVA, analysis of variance.

Although there are several well established tests of attentional function in rodents, such as the sustained attention task (Skjoldager and Fowler, 1991; Brockel and Fowler, 1995), these tests require considerable training of the animals. Consequently, the training and execution phases of these tests in rapidly maturing rodents can span several critical developmental periods (for example, preadolescence, periadolescence, and adolescence), potentially confounding the interpretation of the results. To address this issue, we designed an exploration/habituation/spatial change paradigm, the familiar arena-novel alcove (FA-NA) task, based on the observation that the restlessness, fidgeting, and generally unnecessary gross body movements associated with ADHD tend to be less pronounced in novel environments than in familiar environments (Sleator and Ullmann, 1981; Sagvolden and Sergeant, 1998). The FA-NA task affords inferences about habituation and attention to a novel stimulus in a familiar environment in a single test session without prior training of the animals and is thus able to capture data at discrete points in time (that is, a single day).

In previous studies using the FA-NA task, the introduction of the novel spatial stimulus was highly effective in evoking dose-related effects of methylphenidate in young adult rats (Fowler et al., 2010). The FA-NA task also revealed developmental differences, with the youngest rats [postnatal day (P) 28] exhibiting patterns of behavior that differed from those of young adult (P70) rats with respect to level of activity, degree of habituation, and novelty-induced stimulation of activity. Adult patterns of behavior emerged by P49, with P35 rats exhibiting immature patterns of behavior and P42 rats exhibiting intermediate patterns (Levant et al., 2010). The periadolescent period, which can be considered to span from P33 to P42, is also characterized by more general activity, higher incidence of play, and poorer learning and retention than observed in younger and older rats (Spear and Brake, 1983).

Psychostimulants such as methylphenidate are widely used in the treatment of ADHD in both children and adults; however, it is not clear whether mature and immature individuals respond similarly or differently, and treatment strategies for each age group remain to be optimized (Wilens et al., 2002; Wigal, 2009). Accordingly, in this study, the FA-NA task was used to compare the dose-response effects of methylphenidate in periadolescent and young adult rats. We will show that periadolescent and adult rats exhibit different methylphenidate-related responses in the FA-NA task that are associated with developmental differences in pharmacokinetics and dopaminergic neurochemistry.

Materials and Methods

All experiments were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996) and were approved by the University of Kansas Medical Center Animal Care and Use Committee.

Animals and Husbandry. Male Long-Evans rats (P35, P42, or P70; $n = 11$ –16 for the behavioral studies and 10 for the neurochemical assessments; each from a different litter) were bred in-house from breeding stock obtained from Harlan (Indianapolis, IN) as part of a larger diet study. Rats were group-housed (three per cage) in a temperature- and humidity-controlled animal facility with a 12-h light/dark cycle (lights on at 06:00 AM). Except during the behavioral

assessment sessions, rats had ad libitum access to water and chow (AIN-93G; Teklad, Indianapolis, IN). Rats were weighed regularly and were accustomed to handling before the initiation of experiments. Different groups of rats from the same breeding cohort were used for the behavioral and neurochemical assessments.

Apparatus. Modified force-plate actometers (Fowler et al., 2001) were used to assess response to environmental spatial change as described in detail previously (Fowler et al., 2010). The modified Plexiglas actometer chamber consisted of a “main” compartment (28×42 cm) and a small “alcove” (14×14 cm) separated from the main compartment area by a removable guillotine-type door. Movement of the rat in the main compartment and alcove was automatically quantified by the x - y coordinates and Fz forces derived from the force plate. Spatial resolution of the actometer was less than 2 mm and temporal resolution was 0.02 s.

FA-NA Task Procedures. All testing occurred between 10:00 AM and 1:00 PM and was conducted under dim, indirect lighting conditions. Rats were transported to the testing room in their home cages 1 h before testing. The FA-NA task assessed activity and response to spatial change in the environment in a three-stage, 90-min session in the modified force-plate actometer chambers (Fig. 1). Rats were injected with methylphenidate HCl (2.5 or 5.0 mg/kg s.c. in a volume of 1 ml/kg; Sigma-Aldrich, St. Louis, MO) or saline vehicle (1 ml/kg) and returned to the home cage. These doses induced locomotion but not stereotypies in previous studies (Gupta et al., 1971; Costall and Naylor, 1975; Fowler et al., 2010). Fifteen minutes after injection, each rat was placed in the main compartment (period 1, habituation) with the door to the alcove closed. After 30 min, the rat was removed from the main compartment, placed in the home cage for 10 min, and then returned to the unchanged main compartment (period 2, return to familiar environment) for 20 min. After period 2, the rat was again removed from the main compartment to the home cage for 10 min and then returned to the main compartment with the door to the alcove open (period 3, novel stimulus in familiar environment) for 20 min.

Methylphenidate Enzyme-Linked Immunosorbent Assay. Serum and brain methylphenidate concentrations were determined using a methylphenidate direct enzyme-linked immunosorbent assay kit (Immunalysis, Pomona, CA) according to the manufacturer's instructions and incorporating a methylphenidate standard curve. To determine drug concentrations during the test procedure, rats used in the behavior studies were treated with a second injection of the same dose of methylphenidate 48 h after the first dose, a sufficient interval to allow for clearance of the initial dose ($t_{1/2} = 52$ min) (Thai et al., 1999). Rats were euthanized by decapitation 20 min later. Brains were rapidly removed and frozen on dry ice and then extracted in phosphate-buffered saline. Serum was prepared by cen-

Fig. 1. Schematic diagram of the experimental apparatus (A) and test protocol (B) used in the FA-NA task.

trifugation from trunk blood. All samples were stored at -80°C until assayed. A randomly selected subset of the collected samples (9–10/group) was assayed.

Measurement of Monoamine Neurotransmitters and Metabolites. Rats were euthanized by decapitation, and brains were rapidly removed, frozen, and stored at -80°C until assayed. Regions of interest were isolated by free-hand dissection on ice. Concentrations of dopamine, dihydroxyphenylacetic acid, and homovanillic acid were then quantified using an isocratic high-performance liquid chromatography with electrochemical detection system (Coulochem III; ESA—A Dionex Company, Chelmsford, MA) coupled to a Coulochem III dual-channel electrochemical array detector (model 5100A, $E_1 -150\text{ mV}$ and $E_2 +275\text{ mV}$ using a 5011 dual analytical cell; ESA—A Dionex Company) as described previously (Levant et al., 2008). Tissues were extracted in perchloric acid and diluted with the mobile phase, and analytes were separated using a C18 reverse-phase column (HR-80, $4.6 \times 80\text{ mm}$, $3\text{-}\mu\text{m}$ particles; ESA—A Dionex Company) with a citrate-acetate mobile phase containing 6.0% methanol and 0.35 mM 1-octane-sulfonic acid (pH 4.1). The flow rate was 1.8 ml/min. The internal standard was 3,4-dihydroxybenzylamine. Protein concentrations of the extracted tissues were determined by the BCA method (Pierce Chemical, Rockford, IL). Monoamine concentrations are expressed as nanograms per milligram of protein. Neurotransmitter turnover was calculated as the ratio of the metabolites to the neurotransmitter.

Receptor Binding Assays. Specific brain regions were isolated as described for the HPLC analysis above. The affinity and density of D_1 -like dopamine receptors were assessed by Scatchard analysis using four concentrations of [N -methyl- ^3H]7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol (SCH 23390) (87 Ci/mmol, 0.4–1.5 nM; GE Healthcare, Little Chalfont, Buckinghamshire, UK) in an equilibrium filtration assay as described previously (Levant, 2003). The assay buffer was 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , and 1 mM MgCl_2 , pH 7.4, at 23°C . Nonspecific binding was defined in the presence of 1 μM (+)-butaclamol. Membrane protein concentrations were determined by the BCA method (Pierce Chemical) and were $\sim 50\text{ }\mu\text{g/tube}$. Assay tubes were incubated at 23°C for 90 min. The reaction was terminated by rapid vacuum filtration. Specific binding is expressed as femtomoles per milligram of protein and analyzed for K_D and B_{max} using SigmaPlot (version 8.0.2; Systat Software, Inc., San Jose, CA).

The affinity and density of D_2 -like dopamine receptors were assessed using [^3H]spiperone (21 Ci/mmol, 0.03–1.2 nM; GE Healthcare) as described previously (Levant, 2003). Assays were performed as described for [^3H]SCH 23390 except that the assay buffer was 50 mM Tris, 5 mM KCl, 2 mM CaCl_2 , and 1 mM MgCl_2 , pH 7.4, at 23°C . In the frontal cortex, D_2 -like binding was determined in the presence of 10 μM ketanserin to block the binding of [^3H]spiperone to serotonin (5-HT $_2$) receptors.

The affinity and density of the dopamine transporter were determined using five concentrations of [N -methyl- ^3H](–)-2- β -carbomethoxy-3- β -(4-fluorophenyl)tropane 1,5-naphthalenedisulfonate monohydrate (WIN 35,428) [specific activity = 83.6 Ci/mmol, diluted with unlabeled WIN 35,428 (Sigma-Aldrich) to a specific activity of 30 Ci/mmol, 0.6–18 nM; PerkinElmer Life and Analytical Sciences, Waltham, MA] as described previously (Wilson et al., 1994). Assays were performed as described above except that the assay buffer was 25 mM NaPO_4 , pH 7.7; nonspecific binding was defined by 50 μM cocaine; and the incubation time was 2 h.

Data Analysis. For the behavioral procedure, the dependent variables were distance traveled (meters) and time spent in the alcove (expressed as the proportion of the observation period). Data were analyzed in 5-min time blocks. Between-time block comparisons of interest were within-period habituation, recovery of activity upon replacement in the test chamber in period 2 and stimulation of activity by the novel spatial stimulus in period 3. To control for differences in baseline activity (Leussis and Bolivar, 2006), these between-time block parameters were calculated as the relative ac-

tivity in the first and last time blocks of the relevant behavior period(s) for each rat. Accordingly, within-period habituation was defined as the ratio of the distances traveled in last and first time blocks of the period, minus 1 [for example, for period 1, habituation = (block 6/block 1) – 1, to indicate the relative decrease in activity]. Period 2, recovery of activity, was the ratio of distances traveled in time blocks 9 and 6. Period 3, stimulation of activity, was the ratio of distances traveled in time blocks 15 and 12.

Data were analyzed for statistically significant effects using various analysis of variance (ANOVA) procedures followed by the Tukey test (Systat version 12; Systat Software, Inc.). Outliers identified by Systat were removed. Correlations were determined using Pearson's r (InStat3; GraphPad Software, Inc., San Diego, CA). Differences between slopes were determined by assessment of the confidence interval between regression coefficients (Cohen et al., 2003). Differences between groups were considered to be statistically significant at $P < 0.05$.

Results

Serum and Brain Methylphenidate Concentrations.

For serum methylphenidate concentrations (Table 1), two-way ANOVA indicated significant main effects of dose [$F(1,53) = 23.54$, $P < 0.0001$] and age [$F(2,53) = 14.01$, $P < 0.0001$] but no significant interaction. Post hoc analysis of the main effects indicated that the serum methylphenidate concentration in rats treated with 5.0 mg/kg was 192% of those treated with 2.5 mg/kg ($P < 0.0001$). Serum drug concentrations were also different at each age such that the concentrations at P70 and P42 were 253 and 185%, respectively, of the concentration at P35 ($P < 0.0001$ and $P < 0.05$, respectively) and were also different from each other ($P < 0.05$).

For brain methylphenidate concentrations, two-way ANOVA indicated significant main effects of dose [$F(1,52) = 46.85$, $P < 0.0001$] and age [$F(2,52) = 18.01$, $P < 0.0001$] but no significant interaction. Post hoc analysis of the main effects indicated that brain methylphenidate concentration in rats treated with 5.0 mg/kg was 173% of the concentration in those treated with 2.5 mg/kg ($P < 0.0001$). Brain drug concentrations at P70 and P42 were 182 and 155% of the concentration at P35 (both $P < 0.0001$).

Brain and serum methylphenidate concentrations were correlated for each age group ($r = 0.5016$ for P35, 0.5477 for P42, and 0.6416 for P70; all $P < 0.05$) (Fig. 2). Regression slopes were $4.99 \pm 2.22\text{ ml/g}$ for P35, $3.55 \pm 1.40\text{ ml/g}$ for

TABLE 1

Serum and brain methylphenidate concentrations

Data are presented as the mean $\pm$ S.E.M. ($n = 9$ –10/group, randomly selected from the total sample). Methylphenidate concentrations were determined 20 min after administration. For serum concentrations, there were significant main effects of dose ($P < 0.0001$) and age ($P < 0.0001$) with all age groups different from each other ($P < 0.05$) but no significant interaction. One outlier was removed from the P70 5 mg/kg group. For brain concentrations, there were significant main effects of dose ($P < 0.0001$) and age ($P < 0.0001$) with concentrations higher at P70 and P42 than at P35 (both $P < 0.001$) but no significant interaction. One outlier was removed from the P35 2.5 mg/kg group.

Age (Postnatal Day)	Methylphenidate Dose	
	2.5 mg/kg	5.0 mg/kg
Serum (ng/ml)		
35	29 ± 5.9	51 ± 6.5
42	48 ± 11	100 ± 14
70	70 ± 8.2	136 ± 21
Brain (ng/g wet wt.)		
35	277 ± 61	646 ± 49
42	504 ± 98	985 ± 44
70	713 ± 91	1006 ± 32

Fig. 2. Relationship between serum and brain methylphenidate concentrations. Sample sizes were $n = 17$ for P35, $n = 17$ for P42, and $n = 17$ for P70. This sample size reflects all animals at each age in the three dose groups (0, 2.5, and 5.0 mg/kg) for which there were data on both serum and brain methylphenidate concentrations. Regression slopes were 4.99 ± 2.22 ml/g for P35, 3.55 ± 1.40 ml/g for P42, and 4.19 ± 1.29 ml/g for P70 and were not different among groups as determined by assessment of the confidence interval between regression coefficients. r^2 values are the coefficient of determination for each age group calculated from the Pearson correlation coefficient (r).

P42, and 4.19 ± 1.29 ml/g for P70 and were not different between groups.

Dose-Response Effects of Methylphenidate on Behavior. Methylphenidate produced distinct dose-response profiles in periadolescent and young adult rats in the FA-NA task (Figs. 3 and 4).

Period 1 (habituation) distance traveled. All rats exhibited exploratory activity when initially placed in the test chamber and at least some degree of habituation (Fig. 3). Analysis of distance traveled by repeated-measures ANOVA with factors of age, dose, and time block revealed significant main effects of dose [$F(2,108) = 166.35$, $P < 0.0001$], age [$F(2,108) = 17.39$, $P < 0.0001$], and time block [$F(5,540) = 290.15$, $P < 0.0001$], as well as interactions of dose with time block [$F(10,540) = 5.90$, $P < 0.0001$] and of dose and age with time block [$F(20,540) = 2.93$, $P < 0.0001$]. The interaction of dose with age was not quite significant [$F(4,108) = 2.39$, $P = 0.056$].

Total distance traveled in period 1 by vehicle-treated rats was significantly greater at P35 than at P70 ($P < 0.01$) (Fig. 4), in agreement with previous studies (Levant et al., 2010). In rats treated with methylphenidate (5.0 mg/kg), total distance traveled was also greater at P35 and P42 than at P70 (both $P < 0.01$); however, when viewed as the relative increase in activity over baseline (0 mg/kg), P35 rats exhibited the smallest increases in activity when treated with methylphenidate (66 and 110% over baseline at 2.5 and 5.0 mg/kg, respectively), whereas P70 rats exhibited the largest increases (158 and 164% over baseline at 2.5 and 5.0 mg/kg, respectively), with P42 rats exhibiting an intermediate response. Interestingly, whereas the effects of methylphenidate on total distance traveled were clearly dose-related at P35 and P42, this was not the case at P70 where the 2.5 and 5.0 mg/kg doses resulted in similar total distances traveled (Fig. 4, period 1).

Period 2 (return to familiar environment) distance traveled. Analysis of distance traveled by repeated-measures ANOVA

with factors of dose, age, and time block indicated significant main effects of dose [$F(2,108) = 109.66$, $P < 0.0001$] and time block [$F(3,324) = 21.08$, $P < 0.0001$], as well as interactions of dose with age [$F(4,108) = 2.60$, $P < 0.05$] and dose with time block [$F(6,540) = 6.77$, $P < 0.0001$] (Fig. 3). Total activity in period 2 was increased in methylphenidate-treated rats in a dose-related manner; however, the 2.5 and 5.0 mg/kg dose groups were significantly different only at P42 (Fig. 4).

Period 3 (novel stimulus in familiar environment) distance traveled. Analysis of distance traveled by repeated-measures ANOVA with factors of dose, age, and time block indicated significant main effects of dose [$F(2,108) = 59.75$, $P < 0.0001$], age [$F(2,108) = 3.41$, $P < 0.05$], and time block [$F(3,324) = 245.04$, $P < 0.0001$] and interactions of dose with age [$F(4,108) = 12.01$, $P < 0.0001$], age with time block [$F(6,324) = 4.00$, $P < 0.001$], and dose with time block [$F(6,324) = 22.17$, $P < 0.0001$] (Fig. 3). Methylphenidate increased total activity during period 3 in a clearly dose-related manner at P70 but not at P35, with an intermediate response at P42 (Fig. 4).

Period 3 alcove time. Alcove time exhibited significant main effects of dose [$F(2,108) = 6.61$, $P < 0.01$], age [$F(2,108) = 8.65$, $P < 0.0001$], and time block [$F(3,324) = 29.94$, $P < 0.0001$], as well as interactions of dose with age [$F(4,108) = 2.72$, $P < 0.05$], age with time block [$F(6,324) = 4.44$, $P < 0.0001$], and dose with time block [$F(6,324) = 2.21$, $P < 0.05$]. As previously shown (Levant et al., 2010), all vehicle-treated rats spent increasing amounts of time in the alcove across time blocks during period 3 (Fig. 3). Likewise, methylphenidate disrupted the tendency of vehicle-treated rats to spend increasingly more time in the alcove at P70, as shown previously (Fowler et al., 2010); however, this disruption of alcove occupancy was not observed at P35 or P42 (Fig. 5).

Habituation, recovery of activity, and stimulation of activity by the alcove. Habituation (the ratio of the distances traveled in first and last time blocks of the period minus 1) in test periods 1 and 3 exhibited significant main effects of both dose and age. Habituation in period 2, recovery of activity in period 2 (the ratio the distances traveled in blocks 9 and 6), and stimulation of activity by the introduction of the alcove (the ratio of the distances traveled in time blocks 15 and 12) exhibited a significant main effect of dose. However, none of these parameters exhibited significant interactions of dose and age (Supplemental Table 1).

Relationship between Drug Concentration and Activity. Correlation analysis indicated that activity during time blocks 4 to 6 of period 1 (which was shortly after drug concentrations were measured but less influenced by exploratory behavior) increased with brain methylphenidate concentration for all age groups (Fig. 6). Regression slopes were 0.07798 ± 0.009193 m/ng $\cdot$ g $^{-1}$ for P35, 0.04826 ± 0.007028 m/ng $\cdot$ g $^{-1}$ for P42, and 0.03951 ± 0.005176 m/ng $\cdot$ g $^{-1}$ for P70. At P35, the regression slope was significantly greater than at P42 or P70 ($P < 0.05$, for both comparisons). Regression slopes at P42 and P70 were not different from each other.

Developmental Changes in Regional Dopaminergic Neurochemistry. Several dopaminergic neurochemical parameters exhibited developmental differences during the period between periadolescence and young adulthood. In agreement with previous studies (Noisin and Thomas,

Fig. 3. Effects of methylphenidate on distance traveled across periods 1, 2, and 3 and on period 3 alcove time as a function of 5-min time blocks. Data are presented as the mean $\pm$ S.E.M. Sample sizes were as follows: P35: 0 mg/kg, $n = 16$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 13$; P42: 0 mg/kg, $n = 11$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 12$; and P70: 0 mg/kg, $n = 13$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 13$.

1988; Rao et al., 1991), regional dopamine content increased 38 to 58% between P35 and P70 in the caudate-putamen, nucleus accumbens, and frontal cortex ($P < 0.05$) but did not exhibit developmental changes in concentration in the substantia nigra/ventral tegmental area (Fig. 7). Dopamine turnover in the caudate-putamen was also decreased at P70 compared with that at P35 ($P < 0.05$) but did not vary with age in the other brain regions examined (data not shown).

The density of dopamine transporters in the frontal cortex at P70 was 34 to 41% lower than that at P35 and P42, respectively, but this difference was not quite significant ($P = 0.069$) (Table 2). In contrast, densities of the dopamine transporter were near adult levels in the caudate-putamen and nucleus accumbens at P35 in concordance with previous reports (Galineau et al., 2004).

Also in agreement with previous studies of postnatal ontogeny of dopamine receptors (Noisin and Thomas, 1988; Leslie et al., 1991; Rao et al., 1991), the densities of D_1 -like dopamine receptors at P35 were not different from P70 levels

in the caudate-putamen and nucleus accumbens; however, the density of D_1 -like receptors in the nucleus accumbens was lower at P70 than at P42 ($P < 0.05$) and was accompanied by a small, but significant, increase in affinity. In addition, the densities of D_1 -like receptors in the frontal cortex were similar at P35 and P42 but were 28% lower at P70 than in the younger rats ($P < 0.05$) (Table 3). D_2 -like dopamine receptors in the caudate-putamen and nucleus accumbens were present in similar densities at all ages and were not detectable in frontal cortex (Table 4).

Discussion

Although many aspects of the behavioral effects of methylphenidate have been well characterized (for review, see Askenasy et al., 2007), few studies have compared its effects in periadolescent and adult animals. The FA-NA task assesses elementary forms of behavioral plasticity involving learning, memory, arousal level, and attention (Berlyne, 1969; Leussis and Bolivar, 2006), which are highly relevant to ADHD and

Fig. 4. Effects of methylphenidate on total distance traveled in periods 1, 2, and 3. Data are presented as the mean $\pm$ S.E.M. Sample sizes were as follows: P35: 0 mg/kg, $n = 16$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 13$; P42: 0 mg/kg, $n = 11$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 12$; and P70: 0 mg/kg, $n = 13$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 13$. Different from a, 0 mg/kg, same age; b, 2.5 mg/kg, same age; *, P35, same dose; †, P42, same dose; by ANOVA and Tukey test ($P < 0.05$).

other developmental disorders. In particular, the test assessed 1) within-period “habituation” involving gaining familiarity with a novel environment, 2) adaptation in the form of between-period habituation involving memory of the prior period (Leussis and Bolivar, 2006), and 3) response to spatial change. Our previous studies demonstrated the utility of the FA-NA task to discriminate developmental differences in rats’ behavior, with younger rats exhibiting higher activity, less habituation, and less stimulation of activity induced by spatial novelty (Levant et al., 2010), as well as its ability to discriminate dose-related effects of methylphenidate in adult rats (Fowler et al., 2010).

In this study, nonstereotypy-inducing doses of the methyl-

Fig. 5. Effects of methylphenidate on alcove time for the entirety of period 3. Data are presented as the mean $\pm$ S.E.M. Sample sizes were as follows: P35: 0 mg/kg, $n = 16$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 13$; P42: 0 mg/kg, $n = 11$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 12$; and P70: 0 mg/kg, $n = 13$, 2.5 mg/kg, $n = 13$, and 5.0 mg/kg, $n = 13$. Different from a, 0 mg/kg, same age or *, P35, same dose; by ANOVA and Tukey test ($P < 0.05$).

Fig. 6. Relationship between brain methylphenidate concentration and distance traveled. For this analysis, distance traveled during time blocks 4 to 6 of period 1 was used as a measure of activity occurring shortly after drug concentrations were measured that was not confounded by exploratory behavior. Sample sizes were $n = 36$ for P35, $n = 31$ for P42, and $n = 32$ for P70. This sample size reflects all animals at each age in the three dose groups (0, 2.5, and 5.0 mg/kg) for which there were data on brain methylphenidate concentration. Regression slopes were 0.07798 ± 0.009193 m/ng $\cdot$ g $^{-1}$ for P35, 0.04826 ± 0.007028 m/ng $\cdot$ g $^{-1}$ for P42, and 0.03951 ± 0.005176 m/ng $\cdot$ g $^{-1}$ for P70. At P35, the regression slope was significantly higher than at P42 or P70 ($P < 0.05$, for both comparisons) as determined by assessment of the confidence interval between regression coefficients. r^2 values are the coefficient of determination for each age group calculated from the Pearson correlation coefficient (r).

phenidate produced differential effects in periadolescent rats relative to those in young adults. Of note, the effects of methylphenidate on locomotor activity during period 1 were clearly dose-related in the periadolescent rats but not in the young adult rats in which the 2.5 and 5.0 mg/kg doses produced similar increases in locomotion (Figs. 3 and 4). In agreement with the trend for amphetamine to produce smaller increases in locomotor activity relative to baseline in young rats than in adults (Sofia, 1969), assessment of the

Fig. 7. Developmental changes in regional dopamine content. Data are presented as the mean $\pm$ S.E.M. ($n = 9-10$ /group). Different from *, P35 or †, P42; by ANOVA and Tukey test ($P < 0.05$). n., nucleus; SN/VTA, substantia nigra-ventral tegmental area.

TABLE 2

Developmental changes in regional density and affinity of the dopamine transporter

Data are presented as the mean $\pm$ S.E.M. ($n = 5-10$ /group). One outlier was removed from the P35 group for caudate-putamen and nucleus accumbens. No significant effects of age were found by ANOVA.

Age (Postnatal Day)	$[^3\text{H}]\text{WIN 35,428}$ Binding	
	B_{max}	K_D
	<i>fmol/mg protein</i>	<i>nM</i>
Caudate-putamen		
35	631 $\pm$ 65	17 $\pm$ 3.9
42	691 $\pm$ 47	15 $\pm$ 2.1
70	595 $\pm$ 43	14 $\pm$ 2.5
Nucleus accumbens		
35	564 $\pm$ 100	16 $\pm$ 2.9
42	962 $\pm$ 166	26 $\pm$ 5.0
70	677 $\pm$ 147	18 $\pm$ 2.2
Frontal cortex		
35	238 $\pm$ 28	19 $\pm$ 3.4
42	265 $\pm$ 35	21 $\pm$ 3.8
70	156 $\pm$ 16	19 $\pm$ 4.3

TABLE 3

Developmental changes in regional density and affinity of D_1 -like dopamine receptors

Data are presented as the mean $\pm$ S.E.M. ($n = 9-10$ /group). One outlier was removed from the P35 group for caudate-putamen and one was from the P70 group for nucleus accumbens.

Age (Postnatal Day)	$[^3\text{H}]\text{SCH 23390}$ Binding	
	B_{max}	K_D
	<i>fmol/mg protein</i>	<i>nM</i>
Caudate-putamen		
35	871 $\pm$ 40	0.21 $\pm$ 0.013
42	877 $\pm$ 34	0.21 $\pm$ 0.010
70	784 $\pm$ 50	0.18 $\pm$ 0.012
Nucleus accumbens		
35	494 $\pm$ 32	0.41 $\pm$ 0.028
42	583 $\pm$ 32	0.45 $\pm$ 0.033
70	466 $\pm$ 24 ^a	0.34 $\pm$ 0.019 ^a
Frontal cortex		
35	71 $\pm$ 4.6	0.46 $\pm$ 0.040
42	74 $\pm$ 6.7	0.49 $\pm$ 0.046
70	53 $\pm$ 5.7 ^a	0.33 $\pm$ 0.057

^a Different from P42 by ANOVA and Tukey test ($P < 0.05$).

effects of methylphenidate during period 1 of the test procedure indicated that methylphenidate also produced smaller relative increases in locomotion at P35 than at P70 (Fig. 4).

The introduction of the novel spatial stimulus in period 3 also elicited different effects of methylphenidate between the periadolescent and adult rats. In our previous study, the introduction of the novel alcove produced similar effects in all age groups of drug-naïve rats (Levant et al., 2010). In contrast, methylphenidate produced a dose-related disruption of the inclination of rats to spend increasing amounts of time in the alcove across the test period in the young adult but not the periadolescent rats. In particular, P35 rats spent increasing amounts of time in the alcove across the test period at both doses of methylphenidate, whereas P42 and P70 rats spent very little time in the

alcove after being treated with the 5.0 mg/kg dose (Figs. 3 and 4). The methylphenidate-related disruption of the tendency of adult rats to spend time in the alcove after initial exploration appears to result from the reflexively evoked locomotion induced by the drug overriding the rats' propensity to occupy what may be interpreted as a "preferred" space (Fowler et al., 2010). All of these effects were significantly different between P35 and P70 rats, with P42 rats exhibiting intermediate responses. This pattern suggests that the observed differences represent developmental changes in behavior and response to the drug, which could underlie the differential responses to the drug observed between adults and young children with ADHD treated with the drug (Wilens et al., 2002).

TABLE 4

Developmental changes in regional density and affinity of D₂-like dopamine receptors

Data are presented as the mean $\pm$ S.E.M. ($n = 9-10$ /group). [³H]Spiperone binding in the frontal cortex was not consistently detected above background. One outlier was removed from the P35 group for nucleus accumbens. No significant effects of age were found by ANOVA.

Age (Postnatal Day)	[³ H]Spiperone Binding	
	$B_{\max}$	K_D
	<i>fmol/mg protein</i>	<i>nM</i>
Caudate-putamen		
35	341 $\pm$ 20	0.12 $\pm$ 0.009
42	343 $\pm$ 17	0.13 $\pm$ 0.016
70	323 $\pm$ 19	0.13 $\pm$ 0.015
Nucleus accumbens		
35	293 $\pm$ 23	0.76 $\pm$ 0.13
42	260 $\pm$ 15	0.52 $\pm$ 0.069
70	282 $\pm$ 23	0.60 $\pm$ 0.092

Although certain aspects of methylphenidate-elicited behavior exhibited dose by age interactions, other parameters were similar at all of the ages studied. For example, methylphenidate produced dose-related decreases in within-period habituation at all ages tested (Supplemental Table 1). This effect was similar to that produced by apomorphine (Carlsson, 1972) and probably reflects the increased stimulation of D₁-like and D₂-like dopamine receptors produced by both drugs. The smaller magnitude of within-period habituation in the methylphenidate-treated rats probably also reflects the increased levels of arousal and suggests that these rats may have learned less about the environment in period 1 than control rats. In addition, methylphenidate-treated rats exhibited smaller magnitudes of the recovery of activity in period 2 and stimulation of activity by the introduction of the novel spatial stimulus in period 3 (Supplemental Table 1). These effects, which were similar in periadolescent and young adult rats, are also consistent with the decreased habituation produced by methylphenidate.

Differences in pharmacokinetics represent one factor that may contribute to the developmental differences in the behavioral effects of methylphenidate. Serum and brain methylphenidate concentrations differed between periadolescent and young adult rats, with lower concentrations observed at P35 than at P70 and intermediate levels observed at P42. Although the determination of drug concentrations at a single time point cannot adequately elucidate the mechanism underlying this difference, these data suggest that younger rats may have poorer/slower absorption or more rapid metabolism or excretion of the drug. The regression slopes of the relationship between serum and brain methylphenidate concentrations were not different between age groups (Fig. 2), suggesting that central nervous system permeability of the drug was not different. The lower drug concentration in the younger rats may in part explain the smaller percent increase in locomotion over basal activity exhibited by the P35 rats when treated with methylphenidate, compared with the P70 rats, despite their higher absolute levels of methylphenidate-induced and basal activity. In addition, this difference activity may involve a variety of factors including the differential relationship between the size of the rat and the apparatus. However, the steeper regression slope of the relationship between distance traveled and brain methylphenidate concentrations for rats at P35 than at P42 or P70 (Fig. 6) suggests that pharmacodynamic factors also contribute to

the observed age-related behavioral differences. Studies in humans indicated that normal adults and children with ADHD (7–12 years old) exhibited similar pharmacokinetics after a single dose of methylphenidate (Wargin et al., 1983). Even so, considerable individual variation in the dose response to the drug is observed clinically (Kimko et al., 1999). Of interest, compared with men, women exhibited lower plasma methylphenidate levels but great subjective stimulant effects (Patrick et al., 2007), similar to the differences between periadolescent and young adult rats reported here.

Developmental differences in dopaminergic neurochemistry represent a potential underlying contributing factor to the observed pharmacodynamic differences in the effects of methylphenidate between periadolescent and young adult rats. Of note, among its pharmacological effects, methylphenidate blocks the dopamine transporter, consequently increasing synaptic availability of dopamine, which leads to increased stimulation of dopamine receptors (Wilens, 2008). These effects on frontostriatal function are hypothesized to underlie the therapeutic effects of the drug in ADHD (Vaidya et al., 1998; Moll et al., 2000). Neurochemical assessment of rats from the same breeding cohort as those used in the behavioral studies indicated several developmental differences in the central nervous system dopamine systems between P35 and P70. More specifically, dopamine content in the frontal cortex, caudate-putamen, and nucleus accumbens increased with age (Fig. 7). Young adult rats also exhibited decreased densities of cortical D₁ receptors (Table 3) and possibly the dopamine transporter (Table 2) compared with periadolescent rats. This result suggests the potential involvement of these developmental changes in the dopaminergic systems in the differences in basal and methylphenidate-stimulated behavior between periadolescent and young adult rats, although the specific contributions of each of these findings to specific behavioral differences must be established in future studies.

In addition to its effects on the dopamine transporter, methylphenidate blocks the norepinephrine transporter (Kuczenski and Segal, 2002; Han and Gu, 2006). We found no developmental differences in cortical or striatal norepinephrine content in these animals (Supplemental Table 2); however, other developmental changes in the noradrenergic system may also contribute to the observed behavioral differences.

Finally, it must be noted that the data presented here are the effects of methylphenidate in normal rats. The function of the dopamine transporter and consequently the effects of methylphenidate appear to be altered in individuals with ADHD compared with those in individuals without the disorder (Mazei-Robison et al., 2008). Consistent with that finding, self-administration of methylphenidate in humans produced reinforcing effects in normal adults but not in adult subjects with ADHD (Kollins et al., 2009). Further studies must be conducted to determine whether the observed effects of the drug in this study also reflect the drug's effects in ADHD or are limited to the normal brain.

In conclusion, these findings demonstrate that normal periadolescent and young adult rats exhibit different dose-response profiles to methylphenidate with respect to both pharmacokinetics and response in the FA-NA task. Developmental changes in dopaminergic function in the frontal cortex and other regions may contribute to the pharmacodynamic differences. Taken together, these results suggest that

children and adults may respond differently when treated with this drug, particularly in situations involving response to novelty.

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Authorship Contributions

Participated in research design: Levant, Zarccone, and Fowler.

Conducted experiments: Levant, Davis, and Ozias.

Performed data analysis: Levant, Davis, Ozias, and Fowler.

Wrote or contributed to the writing of the manuscript: Levant, Zarccone, and Fowler.

Other: Levant acquired funding for the research.

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