Juvenile Administration of Methylphenidate Attenuates Adult Hippocampal Neurogenesis

Diane C. Lagace, Jessica K. Yee, Carlos A. Bolaños, and Amelia J. Eisch

**Background:** The neural consequences of early-life exposure to methylphenidate (MPH, Ritalin) are of great interest given the widespread, and sometimes inappropriate, use in children. Here we examine the impact of juvenile MPH exposure on adult hippocampal neurogenesis.

**Methods:** Rats received MPH (2.0 mg/kg, intraperitoneal, twice daily) or saline (SAL) during preadolescence (postnatal days 20–35). Hippocampal cell proliferation (Experiment 1), neurogenesis (Experiment 2), and stress-induced changes in cell proliferation (Experiment 3) were assessed at several developmental stages including adulthood.

**Results:** Juvenile exposure to MPH did not alter proliferation at any developmental time point relative to control rats, however, exposure to MPH significantly decreased the long-term survival of newborn cells in adult rats, particularly in the temporal hippocampus. Although MPH-treated rats had higher levels of corticosterone after restraint stress, they did not show the expected greater decrease in hippocampal cell proliferation relative to control animals.

**Conclusions:** Early-life exposure to MPH inhibits the survival of adult-generated neurons in the temporal hippocampus and may reduce progenitor sensitivity to corticosterone-induced decreases in proliferation. These findings suggest that decreased adult neurogenesis is an enduring consequence of early-life exposure to MPH and are discussed for their relevance to humans.

**Key Words:** ADHD, BrdU, development, hippocampus, psychostimulant, Ritalin

Methylphenidate (MPH; Ritalin) is commonly prescribed for the treatment of attention-deficit/hyperactivity disorder (ADHD). Treatment with MPH is generally well tolerated and effective in reducing symptoms associated with ADHD: inattentiveness, impulsivity, and hyperactivity (Abikoff et al. 2004; Jensen et al. 2001; Wilens and Dodson 2004). However, given that the prescription rate of this psychostimulant for children, including preschoolers, is on the rise (Fogelman et al. 2003; Frances et al. 2002; Miller et al. 2001; Olsson et al. 2002; Schmidt-Troschke et al. 2004; Zito et al. 2000), researchers are actively investigating the long-term consequences of preadolescent MPH treatment on brain and behavior (Andersen et al. 2005; Carlezon and Konradi 2004). The urgency of this issue is underscored by the prescription of MPH to children who do not meet full diagnostic criteria for ADHD (Angold et al. 2000).

Fueling speculation that exposure to MPH in early life may be detrimental to brain development, administration of MPH to young rodents produces notable changes in behavior, neurophysiology, and biochemistry in adulthood. Behavioral changes seen after juvenile exposure to MPH (postnatal day [P] 20–35) include decreased response to rewarding stimuli, as well as increases in depressive- and anxiety-like behaviors (Achat-Mendes et al. 2003; Adriani et al. 2005; Andersen et al. 2002; Bolaños et al. 2003; Carlezon et al. 2003; Mague et al. 2005). Physiologic and biochemical changes include decreased activity of dopaminergic neurons (Brandon et al. 2001; Federic et al. 2005), increased levels of cAMP (cyclic adenosine monophosphate) response element-binding (CREB) protein and Homer 1A transcript (Adriani et al. 2005; Andersen et al. 2002), and enhanced corticosterone levels following restraint stress (Bolaños et al. 2003). One interpretation of these studies is that juvenile administration of MPH produces a depressive- or anxiety-like phenotype in adulthood, marked by anhedonia and decreased physiological and behavioral correlates of reward (Andersen and Navalta 2004; Carlezon and Konradi 2004). Although this interpretation deserves careful testing for its validity and its applicability to humans (Hyman 2003; Kuczenski and Segal 2005; Volkow and Insel 2003), clearly these findings urge additional exploration of the long-term consequences of developmental administration of MPH in rodents, particularly in regard to neural substrates linked to affect and reward.

Although the brain's reward neurocircuitry has been examined for adult biochemical and molecular adaptations seen after juvenile MPH exposure (Andersen 2005), the hippocampus has received relatively little attention. The hippocampus plays a prominent role in affective behaviors, drug taking, and relapse (Carlezon et al. 2005; Duman et al. 1997; Nestler et al. 2002), making it a prime target in testing the hypothesis that juvenile exposure to MPH leads to a depressive-like phenotype in adulthood. Moreover, because acute MPH treatment in adolescent rodents dose-dependently increases norepinephrine levels in the hippocampus in the absence of altering dopamine levels in the nucleus accumbens (Kuczenski and Segal 2002), MPH actions in the hippocampus may be central to MPH long-term effects. The hippocampus in turn can influence the brain's reward neurocircuitry and psychostimulant-related behaviors (Charara and Grace 2003; Emerich and Walsh 1990; Mitchell et al. 2000; Won et al. 2003; Yang and Mogenson 1987). Thus, identifying MPH-induced changes in hippocampal physiology or function will be critical in interpreting MPH-induced changes in reward circuitry and behavior.

One aspect of hippocampal plasticity that has received much attention in regard to both depression and addiction is adult neurogenesis, or the ability to give rise to new neurons throughout life (Duman 2004; Eisch 2002). Chronic exposure to antidepressant drugs increases the number of neural progenitors in the rodent subgranular zone (SGZ) of the dentate gyrus (DG) and can block the decrease in neurogenesis induced by stress (Czech et al. 2001; Malberg and Duman 2003; Malberg et al. 2000;
Santarelli et al. 2003). In contrast, numerous drugs of abuse (Eisch et al. 2000; Eisch and Harburg 2006; Opanashuk et al. 2001; Yamaguchi et al. 2004), including psychostimulants such as methamphetamine (Teuchert-Noodt et al. 2000), decrease neurogenesis. Negative early-life experiences can also have a negative impact on adult hippocampal neurogenesis and function (Karten et al. 2005; Lemaire et al. 2000; Mirescu et al. 2004). It is unknown how early-life exposure to MPH affects adult hippocampal neurogenesis. However, given that juvenile MPH exposure produces a depressive-like phenotype in adult rats (Bolanos et al. 2003; Carlezon et al. 2003) and MPH is a psychostimulant, it is reasonable to hypothesize that juvenile MPH exposure will diminish adult hippocampal neurogenesis.

We performed three experiments to assess adult hippocampal neurogenesis after juvenile exposure to MPH. Because adult neurogenesis is a process, not a time point (Kempermann et al. 2004), the first and second experiments explore the impact of juvenile MPH exposure on the proliferation and the survival of newborn cells, respectively. Because early exposure to MPH is also associated with enhanced corticosterone response to stress (Bolanos et al. 2003) and corticosterone inhibits cell proliferation (Gould and Tanapat 1999), the third experiment investigates whether proliferation is altered in MPH rats after stress. Although these studies explore the specific hypothesis that early-life exposure to MPH is detrimental to hippocampal neurogenesis in adulthood, in general they begin to provide much-needed insight into hippocampal neuroadaptations induced after juvenile exposure to MPH.

Methods and Materials

Rats and Drug Treatment

Lactating female Sprague–Dawley rats with their male pups were purchased from Harlan (Indianapolis, Indiana). Pups were at D14 on arrival and were given 4 days of acclimation before weaning at D18. Drug treatment lasted for 16 days (D20–35), during which the rats received intraperitoneal (IP) injections twice a day (12 AM and 6 PM) of either MPH hydrochloride (MPH HCl, 2.0 mg/kg, dissolved in 5% saline as 1 mL/kg, Sigma Laboratories, St. Louis, Missouri) or 5% saline vehicle injections (SAL, 1.0 mg/kg). Rats were weighed daily during drug treatment, and, as previously reported (Bolanos et al. 2003), weights between SAL and MPH rats did not significantly differ during the period of SAL or MPH administration or on the day of sacrifice (data not shown). The animal colony was in an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility and followed a 12–12 light–dark cycle. All rats were provided with food and water ad libitum. Experiments were conducted in compliance with the National Institute of Health and University of Texas Southwestern’s institutional review committee.

Experimental Design

As depicted in Table 1, each treatment group (SAL or MPH) was divided into six subgroups that were named according to the postnatal day of sacrifice: D46 (adolescence), D77 (early adulthood), or D90A, D90B, D90C, and D112 (adulthood).

<table>
<thead>
<tr>
<th>Group</th>
<th>MPH or SAL</th>
<th>Locomotion Testing</th>
<th>Restraint Stress</th>
<th>BrdU Injection</th>
<th>Perfusion</th>
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<tr>
<td></td>
<td>Day 20–35</td>
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<tr>
<td>D46</td>
<td>MPH (n = 6)</td>
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<td>Day 46</td>
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<td></td>
<td>SAL (n = 6)</td>
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<td>D77</td>
<td>MPH (n = 6)</td>
<td>Day 74</td>
<td>—</td>
<td>Day 77</td>
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<td></td>
<td>SAL (n = 6)</td>
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<td>D90A</td>
<td>MPH (n = 4)</td>
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<td>Day 90</td>
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<td>SAL (n = 6)</td>
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<td>D90B</td>
<td>MPH (n = 5)</td>
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<td>Day 90</td>
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<td>SAL (n = 6)</td>
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<td>D90C</td>
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<td>SAL (n = 6)</td>
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<td>D112</td>
<td>MPH (n = 6)</td>
<td>Day 109</td>
<td>—</td>
<td>Day 85</td>
<td>Day 112</td>
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<td>SAL (n = 6)</td>
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Juvenile rats (n = 69) received methylphenidate (MPH; 2.0 mg/kg) or saline (SAL, 1.0 mg/kg) treatment twice daily from postnatal (D) 20 to D35. They were divided into six groups and named occurring to the postnatal day of sacrifice. Locomotor activity in a novel environment was assessed in rats during adolescence (D46), young adulthood (D77), and adulthood (D90A, D90B, D112). Experiment 1 assessed dentate gyrus (DG) cell proliferation during adolescence (D46), young adulthood (D77), and adulthood (D90A) 2 hours after BrdU injection. Experiment 2 assessed DG cell survival in adulthood (D112) 4 weeks after BrdU injection at D85. Experiment 3 assessed DG cell proliferation in adult rats that had undergone restraint stress (D90B). — = not performed or assessed. BrdU, bromodeoxyuridine.

BrdU Injections and Tissue Preparation

To label SGZ progenitors at various developmental stages, rats received an IP injection of BrdU (150 mg/kg; Boehringer Mannheim, Mannheim, Germany) in adolescence (D46), early adulthood (D77), and adulthood (D90A, D90B, D112). To assess levels of SGZ progenitor proliferation or survival, rats were sacrificed either 2 hours (D46, D77, D90A, D90B) or 4 weeks (D112) following BrdU injection. All rats were anesthetized with chloral hydrate and perfused transcardially with cold .1 mmol/mL phosphate buffered saline (PBS) followed by 4% paraformaldehyde in .1 mmol/mL PBS (pH 7.4) at a rate of 7 mL/min and processed as previously described (Eisch et al. 2000; Mandym et al. 2004). Brains were sectioned coronally on a freezing microtome (Leica, Wetzlar, Germany) at 30 μm through the hippocampus. Nine serial sets of sections were stored in .1% NaN₃ in 1X PBS at 4°C until processing.

Immunohistochemistry

To assess levels of cell proliferation, cell survival, and cell fate, BrdU was detected via diaminobenzidine (DAB) or fluorescent immunohistochemistry (IHC; Eisch et al. 2000). Serial sets of sections through the hippocampus were ordered from rostral to caudal, mounted on glass slides (Fisher Superfrost/Plus, Hampton, New

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Hampshire), and dried overnight. Slides were coded to ensure objectivity, and the code was not broken until after data collection was complete. Pretreatment for both types of IHC was as follows: antigen retrieval (.01 mol citric acid, pH 6.0, 95°C, 10 min), membrane permeabilization (.1% trypsin in .1 mol Tris and .1% CaCl₂, 10 min), and DNA denaturation (2NHCl in 1X PBS, 30 min). Following pretreatment, sections for DAB IHC were blocked with 3% normal goat serum (NGS, Vector Laboratories, Burlingame, California) and incubated with mouse-anti-BrdU primary antibody (1:100, Becton-Dickinson Laboratories, San Jose, California) overnight. The following day, sections were incubated with 1.5% NGS and goat-anti-mouse secondary antibody (1:200, Sigma Laboratories, St. Louis, Missouri) for 60 min, .3% hydrogen peroxide (Sigma Laboratories) for 30 min, and avidin biotin complex (Vector Laboratories) for 90 min. Staining of BrdU-labeled cells was visualized using DAB (Pierce, Rockford, Illinois) for 30 min. Nuclear Fast Red (Vector Laboratories) was used as a counterstain. For triple-labeling immunofluorescence, sections were blocked with 3% normal donkey and goat sera (Vector Laboratories) for 60 min before overnight incubation with rat-anti-BrdU (1:100, Accurate Chemical, Westbury, New York), mouse-anti-neuronal nuclei (NeuN, 1:50, Chemicon International, Temecula, CA) and rabbit-anti-glial fibrillary acidic protein (GFAP, 1:500, DAKO, Glostrup, Denmark). Sections were then incubated in fluorescent donkey-anti-rat, donkey-anti-rabbit, and goat-anti-mouse secondary antibodies (1:200, Jackson ImmunoResearch, West Grove, Pennsylvania) for 3 hours, followed by counterstaining with DAPI (1:5000, Roche, Basel, Switzerland). All slides were dehydrated and coverslipped.

**Experiment 1: Juvenile MPH and Adult Hippocampal Cell Proliferation**

To assess whether juvenile MPH exposure alters the number of DG progenitors in the adult hippocampus, BrdU-labeled cells were counted in the DG of adolescent, early adult, and adult rats (D46, D77, D90A, D90B). Four regions of the DG (bregma –1.5 to –6.3 mm) were examined as previously described (Mandyam et al 2004): molecular layer (Mol), SGZ, outer granule cell layer (oGCL), and hilus (H). The habenula was also examined as a nonneurogenic region. Counting of BrdU-labeled cells was done in bright field by an examiner blind to treatment at 400× with an Olympus BX-51 microscope (Olympus, Tokyo). The BrdU-labeled cells counted within the four regions of the dentate gyrus (DG) and the habenula were irregular in shape, dark brown, and commonly found in clusters (Cameron and McKay 2001; Eisch et al 2000). Because counting of cells was conducted on every ninth section of the hippocampus bilaterally, the number of counted cells in each region was multiplied by nine to obtain an estimate of the total number of cells per region (Eisch et al 2000). In addition to estimating the total number of BrdU-labeled cells, the number of cells was also quantified across the longitudinal axis of the hippocampus. For this analysis by bregma, data are presented as total number of cells in the SGZ per section at each septotemporal level analyzed (Paxinos and Watson 1997).

**Experiment 2: Juvenile MPH Exposure and Adult Hippocampal Neurogenesis**

To determine whether juvenile MPH exposure alters hippocampal neurogenesis, tissue from rats sacrificed 4 weeks after BrdU injection (D112) was examined for BrdU-labeled cell counts within the four DG regions and the habenula, as well as across the longitudinal axis of the hippocampus (see Experiment 1). The BrdU-labeled cells from D112 rats displayed characteristics of mature granule cell neurons: large, round, individual (rather than in clusters), with dark or punctate BrdU staining reflecting division since initial BrdU incorporation (Cameron and McKay 2001; Eisch et al 2000). In addition, BrdU-labeled cells were classified as neuronal or glial based on the colocalization of BrdU with the mature neuronal protein NeuN or the astrocyte protein GFAP. Colocalization of immunofluorescence was determined with a confocal microscope (Zeiss Axiom 200 and LSM510-META, Carl Zeiss, Oberkochen, Germany; excitation wavelengths 488, 543, and 633 nm) at 63× using multitrack scanning and an optical section thickness of approximately .5 μm in the Z plane. Approximately 32 ± 3 cells per rat (387 cells SGZ and oGCL) were selected by a blind examiner to assess colocalization of BrdU with either NeuN or GFAP. To guard against false positives (Raff 2003), colocalization was verified by importing stacks of Z images into a three-dimensional reconstruction program, Velocity (Improvision, Lexington, Massachusetts). Three-dimensional renderings were rotated and colocalization was examined from all perspectives. For presentation, images were imported into Photoshop (Adobe Systems 7.0, Carlsbad, California), and adjustments were made only via changes in gamma in the level function.

**Experiment 3: Juvenile MPH Exposure and Restraint Stress-Induced Alterations in Cell Proliferation**

Rats from D90B received restraint stress on D90 to determine whether a previously described MPH-induced increase in stress response (Bolanos et al 2003) corresponded to altered hippocampal cell proliferation. Restraint stress for 20 min was performed as previously described (Bolanos et al 2003). Briefly, tail blood samples were collected at initiation of restraint (t = 0) and near completion of restraint (t = 15 min). The rats were returned to home cage after restraint. Blood samples were collected in heparin-coated tubes and immediately centrifuged (1000 g for 15 min). Plasma was divided into aliquots and frozen at –20°C until assayed for corticosterone levels using the OCTEIA corticosterone competitive enzyme immunoassay (ELA, ALPCO Diagnostics, Windham, New Hampshire). Four hours after restraint stress, rats were injected with BrdU and perfused transcardially 2 hours later for assessment of cell proliferation.

**Statistical Analyses**

The data are reported as mean ± SEM for MPH versus SAL rats. Statistical analyses were performed using a multiple variable analysis of variance (ANOVA) followed by a Bonferroni post hoc test. For repeated measure variables (90 min of locomotion assessment, BrdU-labeled cell counts across bregma levels), a repeated-measures ANOVA was used to assess the effect 1) of the repeated measure (time or bregma), 2) group (SAL vs. MPH), and 3) any interaction effects. Post hoc analyses were performed using the Bonferroni comparison. To assess differences between the number of BrdU-labeled cells colabeled with either NeuN or GFAP, a chi-square analysis was performed. All statistical analyses were performed using either SPSS (version 11.0.2) or Prism software. Statistical significance was defined as p < .05.

**Results**

*Juvenile MPH Decreases Locomotor Response to Novelty in Adulthood*

Locomotor activity in a novel environment was significantly different between SAL and MPH rats (Figure 1A; F(1,40) = 4.3, p < .05), with the D112 MPH rats displaying less locomotion compared with D112 SAL rats (p < .01). During the 90-min test, there was also a significant difference between MPH and SAL rats...
Moreover, across the longitudinal axis of the hippocampus there was a significant difference in BrdU-labeled cells within the SGZ in all three groups [Figure 2B; data from D46, \( F(16,144) = 19.4, p < .001 \); similar results for D77 or D90A, data not shown]. There were a greater number of cells located in the temporal sections, with the greatest number of SGZ BrdU-labeled cells at bregma −5.3 and −5.8. Although these results demonstrate a gradient in proliferation along the axis of the hippocampus with more proliferation occurring in the temporal hippocampus, juvenile exposure to MPH did not result in altered proliferation in later life anywhere along the longitudinal axis of the hippocampus.

**Experiment 2: Juvenile MPH Reduces the Survival of Newly Born Cells in Adulthood**

Having established that neural progenitor proliferation is not altered after juvenile exposure to MPH, we allowed a parallel group of rats injected with BrdU in adulthood (D85) to survive for an additional 4 weeks. Although no gross morphologic differences were evident between BrdU-labeled cells in SAL and MPH rats (Figure 3A), there was a significant difference between the number of BrdU-labeled cells between the regions examined [Figure 3B; \( F(4,50) = 66.72, p < .005 \)], as well as a significant difference between SAL and MPH rats [Figure 3B; \( F(1,50) = 10.08, p < .01 \)]. Post hoc analysis demonstrated MPH rats had significantly fewer BrdU-labeled cells in the SGZ compared with SAL rats \( (p < .05) \), emphasizing the region-specificity of the effects of MPH on survival of cells within the hippocampus.

**Experiment 1: Juvenile MPH Does Not Affect Neural Progenitor Cell Proliferation**

Between MPH and SAL rats, there was no difference in the number of BrdU-labeled cells in the SGZ at any developmental stage (Figure 2A). There was also no significant difference between SAL and MPH rats in the number of newly born cells in the other examined regions (data not shown) or across the longitudinal axis of the hippocampus (Figure 2B). These results show that repeated juvenile MPH treatment does not alter hippocampal progenitor proliferation, as assessed by BrdU-labeled cell counts, in adolescence, early adulthood, or adulthood.

Independent of early-life exposure to MPH or SAL, there was an age-dependent decrease in BrdU-labeled cells [Figure 2A; \( F(2,28) = 31.3, p < .001 \)]. Similar to previous results [Bizon and Gallagher 2003; Heine et al 2004a], we find approximately half the number of BrdU-labeled cells in adult rats compared with adolescent rats (D46 vs. D77 and D46 vs. D90A; \( p < .05 \)). Moreover, across the longitudinal axis of the hippocampus there was a significant difference in BrdU-labeled cells within the SGZ in all three groups [Figure 2B; data from D46, \( F(16,144) = 19.4, p < .001 \); similar results for D77 or D90A, data not shown]. There were a greater number of cells located in the temporal sections, with the greatest number of SGZ BrdU-labeled cells at bregma −5.3 and −5.8. Although these results demonstrate a gradient in proliferation along the axis of the hippocampus with more proliferation occurring in the temporal hippocampus, juvenile exposure to MPH did not result in altered proliferation in later life anywhere along the longitudinal axis of the hippocampus.

**Figure 1.** Juvenile methylphenidate (MPH) exposure reduced locomotor activity in a novel environment when assessed during adulthood. (A) There was a significant difference between saline (SAL) and MPH rats, with MPH rats having significantly less locomotion compared with the SAL rats at day (D)112. Data presented represent the mean ± SEM photocell counts during the 90 min test. (B) Time course of locomotor behavior from D112 group. Movement counts over the course of the 90-min test revealed that MPH rats explored less than SAL rats, with a significant reduction in movement in 15, 20, 25, and 35 min following placement into the chamber. *\( p < .05 \), **\( p < .01 \), \( F(1,9) = 8.1, p < .05 \), with MPH rats displaying less locomotor activity at the beginning of the test (Figure 1B; 15, 20, 25, and 35 min, \( p < .05 \)). These results confirm previous work [Bolanos et al 2003] that juvenile MPH exposure significantly reduces locomotor activity in a novel environment during adulthood. Moreover, this finding demonstrates that juvenile MPH administration does not produce any significant alteration in locomotion in response to novel environment prior to D112.

**Figure 2.** Juvenile methylphenidate (MPH) administration did not affect hippocampal progenitor proliferation in the subgranular zone (SGZ) at any stage of development or alter the distribution of proliferation along the septotemporal axis. (A) Proliferation, as assessed by bromodeoxyuridine (BrdU)-labeled cell counts in groups D46, 77, and 90A, did not differ between MPH and saline (SAL) rats in the SGZ. Other regions of the DG and the habenula were similarly unaffected by MPH treatment (data not shown). Subgranular zone (SGZ): Septotemporal analysis of SGZ proliferation revealed that sections in the temporal hippocampus had more BrdU-labeled cells than the septal hippocampus with no significant difference between MPH and SAL rats. Data shown represented mean BrdU-labeled cell counts per section for D46 SAL and MPH rats. Similar patterns of distribution by bregma were observed in D77 and D90A rats (data not shown).
addition, because there was no difference in the number of labeled proliferating cells (Figure 2), these results show that preadolescent MPH exposure reduces the survival of newly born cells within the SGZ in adulthood, in the absence of a change in cell proliferation.

In addition to finding a decrease in total number of BrdU-labeled cells in the SGZ (Figure 3B), further septotemporal analysis revealed a significant difference in the number of BrdU-labeled cells along the longitudinal axis of the SGZ [Figure 3C; F(17,170) = 14.01, p < .001] and a significant interaction between group (SAL and MPH) and location on the axis [Figure 3C; F(17,170) = 1.67, p < .05]. Post hoc analysis demonstrated that in sections corresponding to bregma –5.3 there were fewer BrdU-labeled cells in MPH rats compared with control rats (p < .05). This shows that juvenile exposure to MPH reduces the survival of cells specifically at the region where the most number of cells are proliferating (Figure 2B) within the temporal hippocampus of the adult rat.

Newborn cells have many potential fates, including differentiation into glia or neuronal cell types (Abrous et al 2005). Having established that juvenile MPH exposure decreases the number of progenitor cells surviving to 4 weeks, we next explored whether it influenced the maturation of BrdU-labeled cells as assessed by expression of a mature neuronal protein. Most BrdU-labeled cells in both SAL and MPH rats expressed a neuronal morphology (Figure 3D–F) and were positive for both BrdU and the mature neuronal protein NeuN (Figure 3D–F). No significant differences were found between SAL and MPH rats in the percentage of BrdU/NeuN, BrdU/GFAP, or BrdU only cells (Figure 3D). Although there were no significant differences in the relative proportion of cells that had differential developmental fate, the reduction in the number of surviving cells (Figure 3B), most of which express a key neuronal protein (Figure 3F), supports the conclusion that early-life exposure to MPH results in decreased survival of new neurons in the adult hippocampus.

Experiment 3: Juvenile MPH-Induced Increase in Response to Stress Does Not Alter Proliferation

Stress decreases proliferation in the hippocampus, and this effect is dependent on circulating levels of corticosterone (Gould and Tanapat 1999; McEwen 2001). Although we saw no difference in basal proliferation in adult rats that had juvenile exposure to MPH (Figure 2), we wondered whether SGZ progenitors would be more sensitive to the previously described enhanced stress response observed in adulthood, as gauged by a heightened corticosterone secretion following restraint stress (Bolanos et al 2003). Therefore, rats that had juvenile SAL or MPH exposure were subjected to restraint stress in adulthood, and serum corticosterone levels and the number of proliferating cells in the DG were assessed. We predicted that MPH rats would have reduced proliferation compared with SAL rats after stress.

SAL and MPH rats did not significantly differ in basal corticosterone levels before stress (SAL: 26.7 ± 7.9 ng/mL; MPH: 10.5 ± 4.3 ng/mL), but similar to previous results (Bolanos et al 2003), MPH rats had 10-fold greater increase in corticosterone relative to SAL rats 15 min following the initiation of restraint (mean [SE] 7.9 ng/mL; MPH: 10.5 ± 4.3 ng/mL). This shows that juvenile SAL or MPH exposure were subjected to restraint stress in adulthood, and serum corticosterone levels and the number of proliferating cells in the DG were assessed. We predicted that MPH rats would have reduced proliferation compared with SAL rats after stress.

Figure 3: Juvenile methylphenidate (MPH) administration decreased hippocampal subgranular zone (SGZ) progenitor survival in the temporal region of the hippocampus in adult rats but did not alter neuronal fate. (A) Morphologies of bromodeoxyuridine (BrdU)-labeled cells from saline (SAL; top) and methylphenidate (MPH; bottom) rats sacrificed 4 weeks after BrdU (day [D]112). At this cell survival time point, BrdU-labeled cells had the nuclear morphology of mature granule cells: round, regular in shape, and rarely in clusters. BrdU staining was speckled, reflecting multiple cell divisional events across regions of the hippocampus and dif. (B) The same field shown in panel C, presented here as an orthogonal analysis of the confocal Z-stack. White arrow indicates BrdU positive cell for the neuronal marker NeuN (white arrow; BrdU [green] and NeuN [red]) and a BrdU-positive cell for the glial marker GFAP (black arrow; BrdU [green] and GFAP [blue]). (E) The same field shown in panel C, presented here as an orthogonal analysis of the confocal Z-stack. White arrow indicates BrdU positive cell for the neuronal marker NeuN (white arrow; BrdU [green] and NeuN [red]) and a BrdU-positive cell for the glial marker GFAP (black arrow; BrdU [green] and GFAP [blue]). (F) The same field shown in panel C, presented here as an orthogonal analysis of the confocal Z-stack. White arrow indicates BrdU positive cell for the neuronal marker NeuN (white arrow; BrdU [green] and NeuN [red]) and a BrdU-positive cell for the glial marker GFAP (black arrow; BrdU [green] and GFAP [blue]). (B) The same field shown in panel C, presented here as an orthogonal analysis of the confocal Z-stack. White arrow indicates BrdU positive cell for the neuronal marker NeuN (white arrow; BrdU [green] and NeuN [red]) and a BrdU-positive cell for the glial marker GFAP (black arrow; BrdU [green] and GFAP [blue]).
Repeated exposure to MPH in early life affects a variety of behavioral and physiological responses in adulthood (Achat-Mendes et al. 2003; Adriani et al. 2005; Andersen et al. 2002; Bolanos et al. 2003; Carlezon et al. 2003; Mague et al. 2005). Given the impact of psychostimulants on brain reward circuitry (Nesler et al. 2005; Nesler and Malenka 2004; Robinson 2004) and the decreased response to rewarding stimuli in adulthood seen after early MPH exposure (Carlezon et al. 2003; Mague et al. 2005), most studies focus on the striatum, nucleus accumbens, and other brain regions related to the reward pathway in exploring the consequences of adolescent MPH exposure (Adriani et al. 2005; Andersen et al. 2002; Branden et al. 2003; Carlezon et al. 2003; Chase et al. 2005a, 2005b; Shen and Choong 2005). Decreased adult hippocampal neurogenesis now joins the growing list of long-term effects of early MPH exposure, and the findings presented here emphasize that MPH-induced neuroadaptations are not limited to the primary sites of reward in the adult brain.

Of the thousands of new cells generated in the rat DG each day (Cameron and McKay 2001), half of SGZ progenitors may die between 6 and 22 days after labeling with BrdU (Dayer et al. 2003). The newborn granule neurons that survive this period of cell death integrate into hippocampal circuitry (Gould et al. 1999). Here we find that juvenile MPH exposure leads to decreased survival of new neurons in the adult hippocampus, without influencing the number of proliferating cells. We speculate that this distinction points to MPH decreasing survival by increasing apoptosis of maturing, not proliferating, neurons. In support of this theory, decreases in survival in the absence of changes in proliferation have been associated with increases in apoptosis (Ambrogini et al. 2005; Kuhn et al. 2005; Sun et al. 2004). Work is ongoing in our laboratory to address the hypothesis that early-life exposure to juvenile MPH increases apoptosis of nascent neurons leading to reduced survival in MPH rats. Alternative possibilities exist to explain the reduced neuronal survival in MPH rats, such as the effect of decreased reactivity to a novel environment or the stress of the locomotor testing. The first possibility is unlikely because reduced reactivity does not alter survival and indeed is correlated with increased, not decreased, proliferation (Lemaire et al. 1999). The second possibility is particularly important to consider because juvenile MPH exposure enhances serum corticosterone levels in response to stress in adulthood (Bolanos et al. 2003; present data), and stress generally decreases neurogenesis (Mirescu and Gould 2006). Unlike proliferation, however, decreases in survival appear to require chronic exposure to a changed environment (Ambrogini et al. 2005; Lee et al. 2006; Lichtenwalner et al. 2006); this is in contrast to the single exposure to a novel environment used here. In addition, exposure to chronic unpredictable stress, which decreases proliferation, does not alter the number of surviving hippocampal cells (Heine et al. 2004b). Taken together with the fact that restraint stress did not influence proliferation differentially between the MPH and control animals (Figure 4), these data suggest that the relatively mild stressor of locomotion testing for 90 min would not alter survival in itself. This evidence therefore suggests the reduced survival in MPH rats is not due to a differential response to the possibly stressful novel environment associated with locomotor testing.

Relatively little is known about factors within the SGZ microen-
vironment that regulate cell survival independent of proliferation (Abrous et al 2005; Ming and Song 2005). Several recent factors have been identified, however, that preferentially influence survival relative to proliferation (Ambrogini et al 2005; Kuhn et al 2005; Lee et al 2006; Lichtenwalner et al 2006; Sun et al 2004). The data presented here add juvenile exposure to MPH to the growing list of factors that alter survival independent of proliferation. Because MPH exposure selectively diminished the number of surviving BrdU cells in the temporal hippocampus, additional studies could build on these findings to examine how the septal versus temporal SGZ microenvironment is altered after juvenile MPH exposure to help elucidate possible mechanisms for the effect of MPH on survival. This work also has tremendous potential to shed light on how experiences, such as maternal care (Bredy et al 2003) and hippocampal-dependent learning (Gould et al 1999), similarly influence the survival of adult generated neurons independent of proliferation.

Given the diverse anatomic connections and functions of the septal versus temporal hippocampus (Amaral 1993; Bannerman et al 2004), it is interesting that less than a dozen studies have evaluated adult neurogenesis along the longitudinal axis of the hippocampus in the rat (Ambrogini et al 2000; Banasr et al, in press; Cameron et al 1995; Kim et al 2005; Rao and Shetty 2004), mouse (Ferland et al 2002; Hayes and Nowakowski 2002), or gerbil (Dawirs et al 1998; Hildebrandt et al 1999; Teuchert-Noodt et al 2000). The data presented here are the first to show proliferation and survival across the entire longitudinal axis as well as regulation across this axis, and it is notable that the decreased survival shown here occurs specifically in the temporal hippocampus. This is in keeping with recent papers showing that the temporal hippocampus is the preferential site of regulation of proliferation by restraint stress (Kim et al 2005) and agomelatine, a newer antidepressant drug (Banasr et al 2006). Taken together with our data demonstrating regulation of survival, these data highlight an underappreciated heterogeneity of the hippocampus in regard to regulation of neurogenesis. The differential response of the septal versus temporal hippocampus may be related to the distinct anatomic connections and functions of these regions. The septal, or rostral, hippocampus is connected to sensory areas and is implicated in spatial memory, whereas the temporal, or caudal, hippocampus is connected to limbic-related areas and is implicated in anxiety- and depression-related behaviors (Bannerman et al 2004). Future studies might therefore examine whether the reduced number of surviving cells in the temporal hippocampus seen after juvenile MPH exposure correlates with anxiety- and depression-like behaviors in the adult. Moreover, as studies identify the molecular heterogeneity along the axis of the rat dentate gyrus, as has recently been done for the mouse hippocampus (Lein et al 2004, 2005; Leonardo et al 2006), proposed mechanisms for the region-specific effects observed in this study could be tested. Although certainly out of the scope of this study, such studies promise to be fruitful in exploring how regional differences in neurogenesis contribute to distinct hippocampal processes.

We found no influence of juvenile MPH on the number of proliferating cells at any developmental time point; however, one aspect of our work suggests that the proliferating cells are perhaps not completely unaffected by juvenile MPH exposure. The reactivity of the HPA axis to restraint stress was greatly increased in MPH rats, replicating previous work (Bolanos et al 2003), and stress decreased the number of proliferating cells (Figure 4A), in agreement with previous studies (Bairn et al 2004). Despite MPH rats having increased corticosterone response to stress, MPH rats did not have the expected greater decrease in number of proliferating cells. This was somewhat surprising because high levels of glucocorticoids, whether resulting from administered hormones (Cameron and Gould 1994; Gould et al 1992) or experimental stressors (Czech et al 2002; Malberg and Duman 2003; Tanapat et al 2001), decrease the number of proliferating cells within the hippocampus. Although the relationship between neurogenesis and corticosterone is complex (Brunson et al 2005; Pham et al 2003), one interpretation of our data is that proliferating cells in MPH rats may have a blunted response to corticosterone. Initial studies in the rat found no corticoid receptors on proliferating cells (Cameron et al 1993), but recent technical advances in receptor detection and progenitor subtype analysis show that a subpopulation of progenitors in the mouse have corticoid receptors (Garcia et al 2004). If future studies do find corticoid receptors on rat progenitor cells, additional work is warranted to examine whether corticoid receptor distribution or function is altered in the SGZ progenitors of rats exposed to juvenile MPH. Important for our results, corticosterone is also a recognized player in cell survival in the adult mouse SGZ. Neurons that express a glucocorticoid receptor during key phases of maturation appear to be targeted for cell death (Garcia et al 2004). Therefore, studies are needed to examine whether altered glucocorticoid receptor expression on maturing progenitor cells may contribute to the diminished survival reported here in MPH rats.

Adult-generated SGZ cells become DG neurons and functionally integrate into hippocampal circuitry (Hastings and Gould 2003; Markakis et al 2004; van Praag et al 2002), raising speculations that these neurons play an important role in hippocampal-dependent behaviors. Correlative studies support this because the addition of new neurons to the adult DG has positive effects on learning and memory, whereas inhibition of new neuron birth contributes to memory loss or lack of clearance of old memories (Abrous et al 2005). Others have shown that early-life experiences such as prenatal stress (Lemaire et al 2000) and perinatal maternal deprivation (Bredy et al 2003) attenuate proliferation and neurogenesis and lead to impairments in hippocampal-related learning and memory tasks in adulthood (Lemaire et al 2000; Liu et al 2000). Because we demonstrate a reduction in the survival of new neurons in MPH rats, it would be of interest to identify whether a decrease in survival occurs throughout the developmental period and whether this coincides with impaired learning of hippocampal-dependent tasks (Shors et al 2002). If adult rats treated with MPH earlier in life have deficits in learning and memory in adulthood, this finding would support that a reduction in survival of newly born cells may have functional effects.

Direct extrapolations of our findings to the human condition are unwarranted given the lack of animal models to assess appropriately psychostimulant exposure and the difficulty in achieving similar pharmacokinetic profiles for psychotropic drugs in rodents (Andersen 2005; Kuczenski and Segal 2005); however, discussion of these results would be incomplete without mention of how the data integrate with three established avenues of research into the long-term consequences of MPH treatment. First, our findings of juvenile exposure to MPH leading to decreased adult hippocampal neurogenesis are in parallel with ongoing studies examining the impact of long-term MPH exposure on human hippocampal volume. While youth treated with MPH appear to not have diminished hippocampal volume, longitudinal follow-up studies are addressing the hypothesis that altered hippocampal volume may not appear until adulthood (Judith Rapoport, personal communication, 2005). Second, because neurogenesis is presumed to contribute to hippocampal function, our findings encourage the assessment of juvenile
MPH exposure in adult rodents and humans on outcomes related to hippocampal function, such as learning and memory. Third, studies implicate adult hippocampal neurogenesis in depression (Duman 2004; Eisch 2002; Henn and Vollmayer 2004), and juvenile MPH produces a depressive-like phenotype in adult rodents (Bolanos et al 2003; Carlezon et al 2005) Therefore, our finding of decreased hippocampal neurogenesis with no obvious impact on proliferation raises the hypothesis that depression is marked by diminished survival of adult-generated neurons independent of an effect on proliferation. Indeed, this hypothesis may explain why depressive-like behavior in animal models does not always correlate with decreased proliferation of hippocampal progenitors (Malberg and Duman 2003; Vollmayer et al 2003). Studies that assess neurogenesis in animal models of depression are encouraged to include measures of survival in addition to proliferation to address this hypothesis further.

In sum, when considered in light of previous work, this study provides additional evidence that early-life exposure to MPH can have complex effects that endure later in life. These findings urge further exploration of hippocampal neuroadaptations after juvenile MPH exposure and emphasize the need for continued appropriate use of MPH in youth.

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