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ADOLESCENT BINGE ALCOHOL EXPOSURE ALTERS HIPPOCAMPAL PROGENITOR CELL PROLIFERATION IN RATS: EFFECTS ON CELL CYCLE KINETICS

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Abstract

Binge alcohol exposure in adolescent rats potentially inhibits adult hippocampal neurogenesis by altering neural progenitor cell (NPC) proliferation and survival; however, it is not clear whether alcohol results in an increase or decrease in net proliferation. Thus, the effects of alcohol on hippocampal NPC cell cycle phase distribution and kinetics were assessed in an adolescent rat model of an alcohol use disorder. Cell cycle distribution was measured using a combination of markers (Ki-67, bromo-deoxy-uridine incorporation, and phospho-histone H3) to determine the proportion of NPCs within G1, S, and G2/M phases of the cell cycle. Cell cycle kinetics were calculated using a cumulative bromo-deoxy-uridine injection protocol to determine the effect of alcohol on cell cycle length and S-phase duration. Binge alcohol exposure reduced the proportion of NPCs in S-phase, but had no effect on G1 or G2/M phases, indicating that alcohol specifically targets S-phase of the cell cycle. Cell cycle kinetics studies revealed that alcohol reduced NPC cell cycle duration by 36% and shortened S-phase by 62%, suggesting that binge alcohol exposure accelerates progression through the cell cycle. This effect would be expected to increase NPC proliferation, which was supported by a slight, but significant increase in the number of Sox-2+ NPCs residing in the hippocampal subgranular zone following binge alcohol exposure. These studies suggest the mechanism of alcohol inhibition of neurogenesis but also reveal the earliest evidence of the compensatory neurogenesis reaction that has been observed a week after binge alcohol exposure.

Keywords

adult neurogenesis; alcoholism; ethanol; neural stem cell; neurodegeneration

INTRODUCTION

Alcohol use disorders (AUDs) are a pervasive part of our society with over 17 million Americans meeting the diagnostic criteria for an AUD at any moment (Hasin et al., 2007). Initiation of alcohol consumption during adolescence greatly increases the probability that an individual will develop an AUD (Grant and Dawson, 1997). When this is coupled with the alarmingly high alcohol use rate among adolescents (Johnston et al., 2007), it becomes clear that alcohol's interaction with the developing adolescent brain holds important clues as to how AUDs develop. More than half of 12–17 year olds who consume alcohol do so in a binge pattern (>5/4 drinks for males and females, respectively), which is associated with greater risk for alcohol-induced neurodegeneration and has been hypothesized to be a key

step in the development of addiction (Koob and Moal, 1997; Crews, 1999; Johnston et al., 2007; Crews and Nixon, 2009).

The adolescent brain responds differently to alcohol than the adult brain likely due to its unique developmental state (Spear, 2000; Bava et al., 2010). Compared to adults, adolescents are resistant to the motor impairing and sedative effects of alcohol, but more sensitive to its rewarding and reinforcing properties (Little et al., 1996; Doremus-Fitzwater et al., 2010), a combination that may drive excessive alcohol consumption to neurotoxic levels. In addition, the adolescent limbic system is more susceptible to alcohol-induced damage and alcohol's memory-impairing properties, which suggest a particular vulnerability of the adolescent hippocampus (Crews et al., 2000; White and Swartzwelder, 2004; 2005). Indeed, MRI studies have consistently reported reductions in hippocampal volume in binge drinking and alcohol dependent adolescents, while functional studies have suggested potential impairments in hippocampal-dependent learning and memory processes (De Bellis et al., 2000; Nagel et al., 2005; Medina et al. 2007; Schulteis et al., 2008; Schweinsburg et al., 2010). These findings have been extended to adolescent animal models which show alcohol-induced deficits in spatial memory acquisition, trace fear conditioning, and contextual fear conditioning, all of which depend on hippocampal function (Markwiese et al., 1998; Gould, 2003; Weitemier and Ryabinin, 2003; Sircar and Sircar, 2005; Hunt et al., 2009; Sircar et al., 2009).

The hippocampus is one of two brain regions in which neurogenesis persists throughout life. Here, neural progenitor cells (NPCs) located along the subgranular zone (SGZ) generate new granule neurons found in the hippocampal dentate gyrus (Kuhn et al., 1996; Zhao et al., 2008). The continuous generation of new neurons results in a steady increase in the size of the dentate gyrus granule layer, demonstrating the important contribution of adult neurogenesis in shaping hippocampal structure (Imayoshi et al., 2008). Although the function of adult hippocampal neurogenesis has not been firmly established, accumulating evidence indicates that these new neurons play a role in hippocampal-dependent learning and memory. This is supported by studies demonstrating learning-dependent regulation of adult neurogenesis and the development of specific learning deficits following ablation of neurogenesis (Deng et al., 2010). In addition, hippocampal neurogenesis may regulate drug reward and drug seeking behavior, potentially linking neurogenesis to the development of addiction (Eisch et al., 2008; Noonan et al., 2010). Given the structural and functional importance of adult neurogenesis, alcohol's effects on this process could be an important factor contributing to alcohol-induced disruption of hippocampal structure and function in adolescents (Nixon, 2006; Nixon et al., 2010). In an adult rat binge model, alcohol intoxication inhibits neurogenesis by reducing the number of proliferating NPCs and survival of newly born granule neurons (Nixon and Crews, 2002). In adolescents, alcohol also potentially attenuates new neuron survival, but its effects on NPC proliferation appear more complex (Morris et al., 2010a). For instance, binge alcohol intoxication decreased bromo-deoxy-uridine (BrdU) incorporation without affecting the overall number of proliferating NPCs, suggesting that alcohol may target cell cycle progression in adolescents.

Therefore, the current study examined the effects of alcohol on hippocampal NPC cell cycle progression in adolescent rats. This was accomplished with two stereology-based anatomical studies: the first sought to determine whether binge alcohol exposure altered the distribution of dividing NPCs within the different phases of the cell cycle; the second aimed to measure cell cycle and S-phase duration following binge alcohol intoxication.

MATERIALS AND METHODS

Subjects

Sixty adolescent (postnatal day 30) male Sprague-Dawley rats (Charles River Laboratories, Portage, MI) were used for these experiments. Rats were individually housed and maintained on a 12h light/dark cycle with food and water available *ad libitum*. Rats were acclimated to the vivarium for 5 days prior to the start of ethanol administration. All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996) and were approved by the University of Kentucky's Institutional Animal Care and Use Committee.

Adolescent Rat AUD Model

Binge ethanol administration followed a procedure modified from Majchrowicz (1975) and began at postnatal day 35 as described previously (Morris et al., 2010a; b). Food was removed immediately prior to the start of the experiment, while water remained available throughout. Nutritionally complete ethanol (25% ethanol w/v in Vanilla Ensure Plus) or isocaloric dextrose diet was delivered intragastrically using stainless steel gavage needles (Popper and Sons, New Hyde Park, NY) 3 times a day for 4 days with doses spaced out over 8h intervals. The initial ethanol dose was 5g/kg with subsequent doses titrated based on the following 6-point intoxication behavior scale: 0-normal rat (5g/kg), 1-hypoactive (4g/kg), 2-ataxic (3g/kg), 3-delayed righting reflex (2g/kg), 4-loss of righting reflex (1g/kg), and 5-loss of eye blink reflex (0g/kg). This paradigm results in rapid development of alcohol dependence as evidenced by increased ethanol tolerance and the appearance of symptoms consistent with severe withdrawal once ethanol is cleared.

Blood Ethanol Concentration

Tail blood samples were collected 90 min following the first ethanol dose on the third binge day. Samples were centrifuged for 5 min at $1800 \times g$ and then were stored at -20°C . Blood ethanol concentrations were determined in serum and performed in triplicate using a GM7 Alcohol Analyzer calibrated with a 300mg/dl external standard (Analox, Lunenburg, MA).

Tissue Preparation

Rats were euthanized with a pentobarbital overdose (Nembutal®, MWI Veterinary Supply, Nampa, ID) followed by transcardial perfusion with 0.1M phosphate buffered saline (PBS; pH 7.4) and 4% paraformaldehyde (in 0.1M phosphate buffer, pH 7.4). Brains were extracted, post-fixed for 24h in 4% paraformaldehyde, and then stored in 0.1M PBS at 4°C . Free-floating, 40 μm coronal sections were obtained from the entire length of the cerebral cortex in a 1:12 series with a vibrating microtome (Leica Microsystems, Wetzlar, Germany). This collection procedure results in tissue sections from the entire rostral to caudal extent of the hippocampus. Sections were stored in cryoprotectant at -20°C .

Antibody Characterization

Antibodies used for immunohistochemistry are listed in Table 1. Mouse monoclonal anti-Ki-67 clone MM1 (Vector Laboratories, Burlingame, CA) was generated against a prokaryotic recombinant fusion protein corresponding to amino acids 1159–1522 of the human Ki-67 sequence (Komitova et al., 2009). According to the manufacturer, the MM1 clone recognizes bands of 345 and 395 kDa on western blots of MCF-7 cell lysates, matching reports using other Ki-67 clones (Key et al., 1993). Within the rat SGZ, the Ki-67 antibody only labels dividing cells as was shown in a cumulative BrdU study in which Ki-67 expressing cells eventually all become co-labeled with BrdU (Olariu et al., 2007). Similarly, the Ki-67 antibody stained clusters of cells along the SGZ in the current study.

Mouse monoclonal anti-BrdU clone AH4H7-1/131-14871 (Millipore, Bellerica, MA) labels only cells that have incorporated BrdU into their DNA and does not recognize any endogenous proteins or other cellular components. No labeling is observed with this BrdU antibody in brain sections obtained from rats that were not injected with BrdU, and we have used this antibody previously to assess NPC cell proliferation within the SGZ following binge ethanol intoxication in adolescent rats (Morris et al., 2010a).

Rabbit polyclonal anti-phospho-histone H3 (pHis-H3; Millipore, Bellerica, MA) was generated from a KLH-conjugated peptide (ARKpSTGGKAPRKQLC) corresponding to amino acids 7–20 of S10 phosphorylated human histone H3. According to the manufacturer, this antibody recognizes a single band of protein at 17kDa in colcemid treated HeLa cells. In determining mitotic indices, results obtained with this anti-pHis-H3 antibody strongly correlated with results obtained from H & E staining, showing that this antibody reliably labels proliferating cells (Brenner et al., 2003). Consistent with its specificity for labeling proliferating cells, this antibody detects clusters of cells within the neurogenic regions of the brain, similar to staining patterns for Ki-67 and BrdU (Ackman et al., 2007).

Affinity purified rabbit polyclonal anti-Sox-2 (Millipore, Bellerica, MA) was produced from a synthetic peptide with the sequence SSSPPVVTSSSHSRAPC, which corresponds to amino acids 249–265 of the C-terminal region of human Sox-2. In the NT2/D1 human embryonic stem cell line, this antibody recognizes a 34 kDa protein present only in nuclear extracts, matching the predicted location and size of Sox-2. This antibody labeled cells along the SGZ following lentiviral transduction with a GFP/cre fusion protein, whose expression was driven by the Sox-2 promoter, confirming that the antibody specifically labels Sox-2 expressing cells (Suh et al., 2007). Importantly, fate tracing studies showed that Sox-2 expressing cells in the SGZ could differentiate into neurons and astrocytes, indicating that these cells are multi-potent NPCs (Suh et al., 2007). In addition, a small proportion of Sox-2 positive cells along the SGZ co-localize with Ki-67 and BrdU (Mathews et al., 2010).

Biotinylated horse anti-mouse rat absorbed IgG and goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) were used as secondary antibodies. The manufacturer states that both secondary antibodies show less than 1% cross-reactivity with rat immunoglobulins. For all antibodies, optimal antibody concentrations were determined from dilution curves, while specificity of antibody immunoreactivity was confirmed with control tissue in which either the primary or secondary antibody was omitted.

Immunohistochemistry

For all antibodies, tissue sections were washed 2×5 min in 1x tris-buffered saline (TBS, pH 7.5) to remove traces of the cryoprotectant. In general, sections were incubated in 0.6–1.0% H_2O_2 for 30 min to quench endogenous peroxidase activity. For Ki-67 and pHis-H3 detection, an antigen-retrieval step in sodium citrate buffer for 1h at 65°C was added following the H_2O_2 step. Sections were washed in 1x TBS and blocked in 3–10% normal serum (species as appropriate; 0.1% Triton X-100 in 1x TBS) for 30 min prior to overnight or 2 day incubation in primary antibody at 4°C . BrdU (1:6 series) was detected following methods modified from Kuhn (1996) as reported in Nixon and Crews (2002). Following the H_2O_2 step, sections were washed in 1x TBS, incubated in 50% formamide (prepared in sodium citrate buffer) for 2h at 65°C , washed 2×5 min in sodium citrate buffer, and then incubated in 2N HCl for 1h at 37°C . Sections were neutralized in 0.1M boric acid (pH 8.5), washed in 1x TBS, and then blocked in 3% normal horse serum for 30 min. After blocking, sections were rinsed in 0.02% MgCl₂, incubated in 100U/mL bovine pancreatic DNase I (Sigma-Aldrich, St. Louis, MO) for 1h, rinsed with blocking buffer, and then incubated overnight in mouse anti-BrdU (Nixon and Crews, 2002).

After the primary antibody step, sections were washed 3×10 min in blocking buffer and incubated in the appropriate biotinylated secondary antibody for 1h (Vector Laboratories, Burlingame, CA). This was followed by 3×10 min TBS washes and incubation in avidin-biotin-peroxidase complex for 1h (ABC Elite Kit, Vector Laboratories). Immunoreactivity was visualized using nickel-enhanced diaminobenzidine (0.5% 3,3-diaminobenzidine tetrahydrochloride containing 0.2mM CoCl₂, 0.005% nickel ammonium sulfate, and 0.006% H₂O₂; Polysciences, Waltham, MA). Sections were mounted to glass slides and counterstained and/or coverslipped in Cytoseal® (Richard-Allen Scientific, Kalamazoo, MI). Ki-67 and BrdU-stained tissue was lightly counterstained with neutral red or cresyl violet, respectively. Slides were coded to keep the experimenter blind to treatment conditions during quantification. Representative images were collected with a DP70 digital camera. Images were converted to grayscale and figures compiled using Photoshop CS2 software (Adobe Systems Inc., San Jose, CA). Images were cropped and resized to include only the area of interest. Calibration lines placed on figure images were obtained in Photoshop by drawing a rectangle covering the desired distance on a micrometer image taken at the appropriate magnification. Brightness/Contrast were not altered in any image.

Fluoro-Jade B

Tissue (1:12) was processed for Fluoro-Jade B (FJB) staining identical to published methods (Schmued and Hopkins, 2000; Leasure and Nixon, 2010). Sections were mounted onto Superfrost® Plus microscope slides (Fisher Scientific, Pittsburgh, PA), dried overnight, and then sequentially incubated in 1% NaOH/80% ethanol (5 min), 70% ethanol (2 min), DI H₂O (2 min), 0.06% potassium permanganate (10 min), DI H₂O (2 min), 0.004% FJB (20 min in the dark; Millipore, Bellerica, MA), and DI H₂O (3×1 min). Slides were dried and coverslipped in Cytoseal® (Richard-Allen Scientific, Kalamazoo, MI). Slides were coded to keep the experimenter blind to treatment conditions during quantification.

Stereology

Design-based stereology employing the optical fractionator method was used to quantify the number of immunoreactive cells within the hippocampal SGZ for each cell cycle marker. Equipment consisted of an Olympus BX51 microscope equipped with a ProScan II motorized stage, microcator, and DP70 digital camera (Olympus, Center Valley, PA) coupled to newCAST™ Stereology System software (Visiopharm, Hoersholm, Denmark) that was installed on a Dell Precision 380 workstation. The SGZ was defined as an approximate 30µm thick ribbon of tissue between the granular layer and hilus. Because the previous studies and effects were described in the dorsal hippocampus, only sections (1:12 series for Sox-2, Ki-67, and pHis-3; 1:6 series for BrdU) containing the dorsal hippocampus were quantified (Bregma -1.8 to -5.52; Paxinos and Watson, 2009). The SGZ was traced at 100x and section thickness was assessed at 600x using a 60x oil immersion lens (Olympus PlanApoN, numerical aperture = 1.42) and was averaged from three measurements taken at different locations along the SGZ. For Sox-2, random sampling was conducted using a 20µm × 20µm counting frame with a 120µm × y step length. After tissue processing, section thickness was approximately 20µm; therefore, a dissector height of 14µm with 3µm guard zones was used. All cell counts were made from live images at 1200x projected onto the computer monitor showing the overlaying counting frame. The left and bottom borders of the counting frame were defined as the exclusion lines. Total Sox-2+ cells were calculated with the following equation (West et al., 1991):

$$N = \sum Q \times \frac{1}{asf} \times \frac{1}{tsf} \times \frac{1}{ssf}$$

where Q is the number of cells counted, asf is the area sampling fraction (the counting frame: x,y step ratio), tsf is the thickness sampling fraction (dissector height:section thickness ratio), and ssf is the section sampling fraction (the fraction of sections examined e.g. 1:6 or 1:12; West et al., 1991). For Ki-67, BrdU, and pHis-H3-stained tissue, a 100% asf was chosen due to the limited number and non-homogenous distribution of the immunoreactive cells. For all stereological quantification, coefficient of error ranged from 0.013 to 0.020 (Table 2; Gundersen et al., 1999). Additionally, complete antibody penetration of the tissue was confirmed by comparing profile counts in the top 1/3 and middle 1/3 of the section (Supplemental figure 1).

Experiment 1: Cell Cycle Distribution

The goal of the first experiment was to determine the effect of binge ethanol exposure on the distribution of dividing NPCs within the different stages of the cell cycle. To calculate the distribution of NPCs within G1, S, and G2/M phases of the cell cycle, a combination of cell cycle markers was measured (Figure 1A). In this experiment, 14 rats were administered a saturating dose of BrdU (300mg/kg, i.p., Fluka, Buchs, Switzerland; Cameron and McKay, 2001) immediately following the last ethanol or control dose and then were euthanized 2h later (Figure 1B). One control and two ethanol rats were omitted from analysis due to complete absence of BrdU staining in the subgranular and subventricular zones. Ki-67, expressed during all stages of the cell cycle, was measured to determine the number of actively dividing NPCs in the SGZ, while BrdU, which is incorporated into the DNA during DNA synthesis, was used to quantify cells in S-phase (Nowakowski et al., 1989; Scholzen and Gerdes, 2000). pHis-H3 was measured to quantify the number of cells in G2 and M, since histone H3 becomes highly phosphorylated during these two phases of the cell cycle (Prigent and Dimitrov, 2003). Data from these 3 cell cycle markers were used to estimate the population of dividing NPCs in G1 by subtracting the total number of pHis-H3+ and BrdU+ cells from the number of Ki-67+ cells.

Experiment 2: Cell Cycle Kinetics

Forty-six rats were used for experiment 2 to measure the effect of adolescent binge ethanol exposure on cell cycle length and S-phase duration. This was accomplished by combining a cumulative BrdU injection method with Ki-67 and Sox-2 quantification (Nowakowski et al., 1989; Mooney and Miller, 2007; 2010). The first BrdU injection was made 90 min after the last ethanol gavage to ensure that rats remained intoxicated throughout the entire BrdU injection time course. BrdU was injected once every 2h for a total of 4h, with an ethanol and control group euthanized 30 minutes after each injection; thus, animals are injected at 0, 2, and 4h and groups are sacrificed at 0.5, 2.5, and 4.5h (Figure 1C; n = 6–8 per group). A saturating dose of BrdU (300mg/kg, i.p.) was used for each injection. In addition, Ki-67 was measured to determine the number of dividing cells, and the NPC marker Sox-2 was quantified to determine the size of the NPC pool.

From Ki-67, Sox-2, and cumulative BrdU stereological data, cell cycle length (T_c) and S-phase duration (T_s) were calculated (Nowakowski et al., 1989; Mooney and Miller, 2007; 2010). To do this, the growth fraction is calculated which is equal to the Ki-67 labeling index ($Ki-67/Sox-2 = \text{growth fraction}$). Similarly, the S-phase fraction is calculated which is equal to the BrdU labeling index ($BrdU/Sox-2 = \text{S-phase fraction}$). The S-phase fraction approaches the growth fraction over time as more dividing cells enter S-phase allowing T_c and T_s to be calculated using a linear regression model (Nowakowski et al., 1989). T_c and T_s were calculated with the following equations (Nowakowski et al., 1989; Mooney and Miller, 2007; 2010):

$$T_c = \text{Growth Fraction} / \text{slope}$$

$$T_s = T_c (y - \text{intercept} / \text{Growth Fraction})$$

To determine if cell cycle data was influenced by ethanol-induced degeneration of hippocampal NPCs, cell death following 4-day binge ethanol exposure was measured with FJB staining. FJB+ cells within the dorsal dentate gyrus were examined in the 4.5 h group using an Olympus BX1 microscope equipped with a 488λ epifluorescent cube. FJB stained cells within the SGZ and granule cell layers were counted on sections spanning the dorsal dentate gyrus. This method was chosen over unbiased stereology due to the low number of cells within each section that are FJB+ and the low background staining inherent with this method that makes it difficult to accurately assess section thickness. Furthermore, we have shown previously that our profile counting methodology generates the same percent difference between experimental groups as stereological methodologies (Crews et al., 2004).

Statistical Analysis

Statistical tests were performed using GraphPad Prism version 5.02 for Windows (GraphPad Prism, San Diego, CA). Blood ethanol concentrations and daily ethanol intake were compared using one-way ANOVA followed by Tukey's posthoc test. Intoxication scores were analyzed with Kruskal-Wallis non-parametric analysis of variance. All comparisons in experiment 1 were analyzed by t-test. Sox-2 stereology and BrdU labeling index data was compared using two-way ANOVA with time and diet serving as the dependent variables. FJB data was analyzed with the nonparametric Mann-Whitney test due to unequal variance between groups (verified by significant F-test). For linear regression analysis, control and ethanol regression line slope and intercepts were compared. All other two group comparisons were made with two-tailed, unpaired t-tests. Data is presented as mean ± standard error of the mean and differences were considered significant at $p < 0.05$.

RESULTS

Experiment 1: Cell Cycle Distribution

The effect of adolescent binge ethanol exposure on the distribution of hippocampal NPCs across the cell cycle was examined. In this experiment, the ethanol group had a mean intoxication score of 0.9 ± 0.1 , was administered 12.0 ± 0.2 g/kg/d of ethanol, and had a mean blood ethanol concentration of 297 ± 20.3 mg/dL. In both treatment groups, Ki-67+, pHis-H3+, and BrdU+ NPCs were easily identified within cell clusters located along the SGZ of the dentate gyrus (Figure 2A–F). Rarely, immunoreactive endothelial cells were observed around blood vessels supplying the SGZ, while occasional cell clusters were observed within the hilus and granule neuron/molecular layer border; however, these cells were omitted from analysis. Ki-67, which labels cells in all active phases of the cell cycle, was unaltered by binge ethanol treatment ($p = 0.41$; Figure 2G). Similarly, the number of pHis-H3+ NPCs was not affected by ethanol ($p = 0.98$; Figure 2H). In contrast, binge ethanol exposure reduced the number of BrdU+ NPCs by 44% ($p = 0.03$; Figure 2I). As shown in Figure 2J, the size of the dividing NPC population residing in G1 was calculated by subtracting the total number of BrdU+ and pHis-H3+ cells from the number of Ki-67+ cells, which revealed that ethanol had no effect on G1 cell number ($p = 0.36$). The proportion of actively cycling hippocampal NPCs within G1, S, and G2/M was calculated to determine the effect of alcohol on NPC cell cycle distribution. This demonstrated that in adolescent rats, binge ethanol exposure preferentially reduces the proportion of hippocampal NPCs within S-phase ($p = 0.04$; Figure 2K), with no observed effect on the proportion of NPCs in G1 or G2/M.

Experiment 2: Cell Cycle Kinetics

Due to the observed changes in NPC cell cycle distribution, the effect of ethanol on NPC cell cycle kinetics was examined. This required the measurement of BrdU accumulation over 4.5h following 3 BrdU injections; Sox-2 and Ki-67 were also quantified to provide an estimate of the size of the NPC population and the number of actively dividing cells. The number of Sox-2+ cells was used to calculate the BrdU and Ki-67 labeling index, allowing T_c and T_s to be calculated. There were no differences in behavioral intoxication, daily ethanol dose, or blood ethanol concentrations between the three ethanol groups used in this study (Table 3). The effect of time and diet on BrdU labeling index was analyzed by two-way ANOVA (Figure 3A). As expected, the population of NPCs labeled with BrdU increased significantly with time as cells entering S-phase were labeled following subsequent BrdU injections ($F_{(2, 40)} = 8.1$, $p = 0.001$). In addition, binge ethanol exposure decreased the BrdU labeling index ($F_{(1, 40)} = 13.4$, $p < 0.001$); however, there was no time by diet interaction ($F_{(2, 40)} = 0.32$, $p = 0.73$), although post-hoc tests showed that BrdU labeling was significantly reduced by ethanol at the 0.5h ($p = 0.01$) and 2.5h ($p = 0.01$) time points, where labeling was decreased by 38.1% and 37.6%, respectively. In agreement with the previous cell cycle distribution study, Ki-67 labeling index (measured at 4.5h), which represents the NPC growth fraction, was not affected by 4-day binge ethanol treatment ($p = 0.60$; Figure 3B).

Linear regression analysis of BrdU labeling index combined with the growth fraction was used to calculate T_c and T_s as described in the experimental procedures. Binge ethanol exposure had no effect on the regression slope ($F_{(1, 42)} = 0.39$, $p = 0.54$); however, ethanol significantly decreased the y-intercept ($F_{(1, 43)} = 13.4$, $p < 0.001$), indicating that the lines are different (Figure 4). Control T_c (28.0 ± 2.6 h) and T_s (9.6 ± 0.34 h) were comparable to previously reported studies for juvenile to young rats (Cameron and McKay, 2001; Olariu et al., 2007; Varodayan et al., 2009). Binge ethanol exposure shortened T_c by 36% to 17.9 ± 1.9 h ($p = 0.01$). A large portion of this decrease was due to a 62% reduction in S-phase duration, in which T_s was shortened to 3.6 ± 0.36 h ($p < 0.001$).

The abbreviated T_c and T_s suggest that binge ethanol exposure in adolescent rats accelerates progression through the cell cycle leading to an overall *increase* in NPC proliferation. This was examined further by analyzing the Sox-2 stereology data using two-way ANOVA. A dense population of Sox-2+ cells was observed within the SGZ of the dentate gyrus, although Sox-2+ cells were found scattered throughout the hilus, molecular layer, and even the granule neuron layer (Figure 5B). There was no main effect of time ($F_{(2, 40)} = 0.49$, $p = 0.61$), which was not surprising given the short duration time course used for the cell cycle kinetics measurements. However, in support for an ethanol-induced increase in NPC proliferation, there was a significant main effect of diet ($F_{(1, 40)} = 5.1$, $p = 0.03$), in which 4-day binge ethanol exposure increased the number of Sox-2+ cells in the SGZ by 19% (Figure 5C). There was no time by diet interaction ($F_{(2, 40)} = 0.14$, $p = 0.87$).

To rule out the possibility that ethanol-induced degeneration of hippocampal NPCs is responsible for ethanol's measured effects on cell cycle distribution and kinetics, FJB staining within the granule cell layer and along the SGZ was analyzed. Consistent with previous studies on alcohol-induced degeneration in this model (Crews et al., 2000; Leasure and Nixon, 2010; Morris et al., 2010a), binge ethanol exposed rats had only a few FJB+ cells in the dorsal dentate gyrus, while only a single FJB+ cell was found in one control rat ($t = 1.8$, $p = 0.03$; control = 0.125 ± 0.125 cells/dentate gyrus; ethanol = 2.6 ± 1.4 cells/dentate gyrus). The relatively low level of FJB staining confirms that cell death is not likely responsible for the large decrease seen in BrdU staining or changes in cell cycle kinetics following binge ethanol exposure.

DISCUSSION

Alcohol intoxication, whether during development or in adulthood, detrimentally impacts neurogenesis. Most recently, we have extended this to adolescence where four-day binge exposure results in decreased adult neurogenesis primarily through effects on cell proliferation (Morris et al., 2010a). Although we initially hypothesized that the apparent disconnect between ethanol's effects on Ki-67 and BrdU were due to cell cycle arrest (Morris et al., 2010a), the current data provides strong evidence that binge ethanol exposure during adolescence *accelerates* rather than arrests cell cycle progression. First, we demonstrated that ethanol alters the distribution of NPCs within the phases of the cell cycle by specifically reducing the proportion of NPCs in S-phase without altering the proportion in G1 or G2/M phase. This result led us to assess cell cycle kinetics using the cumulative BrdU labeling method (Mooney and Miller, 2010) which indicated that the ethanol-induced decrease in the proportion of cells in S-phase following a single BrdU pulse results from a significant reduction in the length of S-phase and total cell cycle length. Thus, after four-day binge ethanol exposure, cell cycle length is shorter by 10 hours (36% decrease) versus control rats. NPC cycle acceleration implies that binge ethanol exposure results in an overall *increase* in NPC proliferation during intoxication. In support of this effect, ethanol slightly, but significantly (main effect of diet) increased the number of Sox-2 expressing NPCs within the adolescent SGZ.

This study emphasizes the importance of using multiple cell cycle markers to examine cell proliferation associated with hippocampal neurogenesis. Binge ethanol exposure significantly decreased BrdU incorporation from a single i.p. injection, an effect that could have been interpreted in many different ways unless additional cell cycle markers were studied. Decreased BrdU incorporation can be caused by cell cycle exit, cell death, or accelerated progression through S-phase (Salomoni and Calegari, 2010). Although binge ethanol exposure decreased BrdU labeling, there was no effect on Ki-67 which indicates that ethanol does not decrease the size of the dividing NPC population or result in NPC cell cycle exit. It is also unlikely that cells specifically within S-phase undergo cell death. This conclusion is supported by several lines of evidence. First, ethanol had no effect on the number of Ki-67+ or pHis-H3+ cells. If cells in S-phase were targeted for cell death, we would have anticipated a corresponding decrease in these two cell cycle markers. Second, the low amount of cell death in the dorsal dentate gyrus observed with FJB here as well as in our previous report (Morris et al., 2010a) suggest that effects on cell death are not sufficient to account for the 44% reduction of NPCs in S-phase. FJB staining showed that binge ethanol exposure results in no more than a few dozen dead cells per dorsal dentate gyrus, while BrdU staining demonstrated that the ethanol-induced effect on S-phase involves thousands of cells. Thus, the ethanol-induced decrease in BrdU combined with the lack of an effect on Ki-67 or evidence of cell death in the proliferating cell population provides additional strong support for an important effect of ethanol on NPC cell cycle kinetics.

To our knowledge, this is the first study to examine hippocampal NPC cell cycle kinetics in adolescent rats following binge ethanol exposure. At first glance, it may seem that an accelerated cell cycle or increased NPC proliferation would not be consistent with alcohol-induced decrease in neurogenesis (Morris et al., 2010a); however, the assumption that an increase in proliferation leads to an increase in neurogenesis ignores potentially important effects on other aspects of neurogenesis such as regulation of immature neuron survival, differentiation, and new cell incorporation into the existing hippocampal circuitry. In fact, effects on hippocampal proliferation do not always predict overall effect on neurogenesis. For example, binge 3,4-methylenedioxymethamphetamine (MDMA) exposure in adult rats has no effect on hippocampal NPC proliferation, but significantly decreases the survival of newborn neurons (Hernandez-Rabaza et al., 2006). Interestingly, intermittent MDMA

exposure in adolescent rats increased hippocampal NPC proliferation but an overall decrease in neurogenesis was found due to MDMA's detrimental effects on newborn neuron survival (Catlow et al., 2010).

There are two potential mechanisms that could explain how binge ethanol-mediated cell cycle acceleration results in decreased hippocampal neurogenesis. First, cell cycle acceleration could result in significant damage to daughter cells, triggering activation of apoptotic or other cell death pathways, leading to decreased new neuron survival. Similar results have been described for other drugs of abuse. For example, chronic morphine administration in adult mice increased caspase-3 activation and reduced BrdU incorporation within the dentate gyrus SGZ, an effect correlated with accelerated cell cycle progression from S-phase through G2/M that resulted in premature mitosis (Mandyam et al., 2004; Arguello et al., 2008). Whether a link exists between alcohol-induced cell cycle acceleration and survival of newborn neurons remains to be determined; however, the low level of cell death detected within the adolescent dorsal dentate gyrus following 4-day binge exposure is not sufficient to account for alcohol's inhibition of neurogenesis (see results and Morris et al., 2010a).

A second potential mechanism that could explain how alcohol-induced NPC cell cycle acceleration leads to decreased neurogenesis in adolescents involves effects on cell fate. Hippocampal NPCs are multipotent, meaning they can differentiate into multiple cell types, including astrocytes. Cell phenotype analysis measured 4 weeks after binge alcohol exposure resulted in no change in the proportion of newborn cells that differentiated into neurons or astrocytes, indicating that cell cycle acceleration likely does not affect cell type once the cell has committed to differentiate (Morris et al., 2010a). However, this does not preclude effects on a newborn cell's decision to differentiate or remain undifferentiated. Alcohol-mediated cell cycle acceleration could promote NPC population expansion and maintenance of the undifferentiated state, an effect supported by our observation of increased Sox-2+ cells and supported in models of fetal alcohol syndrome (Santillano et al., 2005). Several studies have shown that cell cycle length regulates a cell's decision to differentiate with longer cell cycle times associated with differentiation and shorter cell cycle times linked to maintenance of the undifferentiated state (Salomoni and Calegari, 2010). For example, G1 length appears particularly important in regulating differentiation of mouse cortical progenitors, since accelerating G1 progression with overexpression of cdk4/cyclinD1 inhibited neurogenesis, while cdk4/cyclinD1 RNA interference lengthened G1 and promoted early differentiation (Lange et al., 2009). Recent work with human embryonic stem cells also demonstrated that inhibition of Notch signaling, an important pathway for stem cell maintenance, delayed G1 to S-phase transition, which was associated with increased neuronal differentiation (Borghese et al., 2010). Others have found an important role for S-phase in differentiation showing that guanosine triphosphate treatment lengthened S-phase in SH-SY5Y cells, which corresponded to enhanced neurite formation and expression of neuronal differentiation markers (Guarnieri et al., 2009). Based on these studies, ethanol-mediated shortening of hippocampal NPC cell cycle in adolescent rats may promote NPC population expansion and maintenance of the undifferentiated state. Such a mechanism would result in a short-term reduction in the generation of new granule cells during binge intoxication, matching our earlier observations that binge ethanol exposure inhibits hippocampal neurogenesis (Morris et al., 2010a). These data also suggest that the shortened cell cycle time and emerging increase in the progenitor pool reflect a compensatory response to repeated alcohol inhibition of neurogenesis or to alcohol-induced dentate gyrus degeneration. Either mechanism could cause sufficient degeneration of the dentate gyrus to initiate a reactive neurogenic response such as that described in adult rats following a 4-day binge (Nixon and Crews, 2004). Other models of degeneration show a similar response. For example, SGZ progenitors have a 20% shorter cell cycle within hours

following seizure in juvenile rats, and subventricular zone progenitor cell cycle length drastically shortens two days after middle cerebral artery occlusion (Varodayan et al., 2009; Zhang et al., 2008). Consistent with other reports on NPC cell cycle following brain insult, the shorter cell cycle was associated with retention of NPCs within the cell cycle and NPC population expansion (Zhang et al., 2008). Importantly, these effects preceded increased neurogenesis observed weeks after the insult (Varodayan et al., 2009; Zhang et al., 2008). Based on these studies, the decrease in cell cycle time in adolescent rats could have significant consequences on neurogenesis after binge ethanol-induced damage and may represent the early stages of compensatory neurogenesis. Indeed, unpublished observations from the same adolescent AUD model revealed a burst in the number of proliferating cells (Ki-67+) along the SGZ 7 days after binge ethanol exposure, an effect that resulted in increased granule cell neurogenesis (Morris, McClain, and Nixon, personal communication).

Similar to many of ethanol's teratogenic effects, the effect of ethanol on adult NPC cell cycle appears to be dependent on the NPC type as well as the dose, duration and developmental timing of ethanol exposure (Goodlett et al., 2005). For example, the results of the current study differ somewhat from those recently reported in a non-human primate model of adolescent alcohol consumption (Taffe et al., 2010). Our results agree with those of Taffe and colleagues (2010) in that ethanol decreases BrdU labeling along the dentate gyrus SGZ; however, in the non-human primate model, Ki-67 labeling was also decreased suggesting that ethanol suppresses NPC proliferation by reducing the number of NPCs that are actively dividing. Notably, this decrease in proliferation was measured 2 months after a much longer duration of binge like exposure, 11 months, and was due to a loss of progenitors in this model. In a fetal alcohol spectrum disorder (FASD) model, fetal alcohol exposure shortens cell cycle length and S-phase duration of NPCs responsible for postnatal generation of ventrobasal thalamic neurons (Mooney and Miller, 2010), while fetal cortical progenitors in the ventricular zone lengthen their cell cycle in response to ethanol (Miller and Nowakowski, 1991; Miller and Kuhn, 1995; Siegenthaler and Miller, 2005).

Many studies have investigated the effects of addictive drugs on hippocampal granule cell neurogenesis (Eisch, 2000; Domínguez-Escribà et al., 2006; Arguello et al., 2008; Mandyam et al., 2008); however, most work has focused on exposure in adulthood, rather than in adolescence, the period during which experimentation with drugs of abuse is most often initiated. Given that granule cell neurogenesis occurs on a greater scale during adolescence compared to adulthood (Altman and Das, 1965; He and Crews, 2007), the effects of drugs, such as alcohol, on this process may have more profound consequences when introduced to the vulnerable adolescent hippocampus. In addition to the effects already described, a recent study demonstrated that repeated exposure to 3,4-methylenedioxymethamphetamine (MDMA) during adolescence dose-dependently increased NPC proliferation, but inhibited survival of differentiating neurons 2-weeks after cell birth (Catlow et al., 2010). Importantly, in both cases, alcohol and MDMA's effects on neurogenesis differ in adolescent rodents compared to adults. In adult rats, binge alcohol exposure reduces the number of proliferating NPCs and significantly inhibits survival of newborn neurons (Crews et al., 2006a; Nixon and Crews, 2002). While alcohol attenuates newborn neuron survival in adolescents, alcohol's effects on NPC proliferation involve no change in dividing NPC number (Morris et al., 2010a) and an accelerated NPC cell cycle, as shown here. MDMA exposure in adult rats reduces neuronal survival, but, unlike with adolescents, MDMA has no effect on NPC proliferation (Hernández-Rabaza et al., 2006). Results from our adolescent binge alcohol model combined with those described for chronic morphine exposure (Mandyam et al., 2004) and FASD (Mooney and Miller, 2010) indicate that hippocampal NPC cell cycle regulation is a significant target of action for drugs of abuse. Furthermore, these differences underscore the important need for understanding how drugs of abuse interact with the developing adolescent brain.

Elucidating the mechanisms by which alcohol interferes with hippocampal structure may hold important clues as to how alcohol-mediated cognitive and behavioral deficits arise and contribute to the downward spiral towards the development of AUDs (Koob and Moal, 1997; Crews, 1999). Indeed, reduced hippocampal adult neurogenesis has recently been reported as a vulnerability factor for cocaine addiction, though the role of adult neurogenesis in alcohol context memory or relapse to alcohol seeking has not been described (Noonan et al., 2010). However, hippocampal degeneration has been much more widely observed in adolescent AUDs as opposed to other drugs of abuse (De Bellis et al., 2000; Nagel et al., 2005) and our work in animal models continues to support that this degeneration is due primarily to alcohol-inhibition of adult neurogenesis (Morris et al., 2010a). Here, we demonstrate that binge ethanol exposure during this vulnerable period of development targets S-phase of the cell cycle resulting in accelerated cell cycle progression, adding to the growing list of effects of ethanol on hippocampal granule cell neurogenesis. The long-term neurogenic consequences of accelerated cell cycle progression of hippocampal NPCs are unknown, but may drive a compensatory burst in neurogenesis that is often observed after brain insult. Given the important role of neurogenesis in hippocampal learning and memory function, further studies are needed to determine if these changes in neurogenesis contribute to ethanol-induced hippocampal volume loss and cognitive deficits in adolescents with AUDs (De Bellis et al., 2000; Nagel et al., 2005; Schulteis et al., 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Cell Cycle Markers and Experimental Timeline. A) Cell cycle diagram showing the stages of the cell cycle labeled by Ki-67, BrdU, and pHis-H3. BrdU labels cells in S-phase, pHis-H3 labels cells in G2 and M phase, and Ki-67 labels actively dividing cells regardless of the stage they are in. From these markers, the size of the G1 population can be calculated by subtracting total BrdU and pHis-H3 cells from Ki-67 cells. B) Time line for cell cycle distribution study (experiment 1). Adolescent rats were subjected to 4-day ethanol binge. Rats were injected with BrdU 90 min after last ethanol or control dose and then sacrificed 2h later. The distribution of actively dividing NPCs within the cell cycle phases were then determined by measuring Ki-67, BrdU, and pHis-H3. C) Time line for cell cycle kinetics study (experiment 2). Adolescent rats were subjected to 4-day ethanol binge. Cumulative BrdU injections were made every 2h for 4h beginning 1.5h after the last ethanol dose, with a control and ethanol group sacrificed 0.5h after each injection. BrdU = Bromo-deoxy-uridine, pHis-H3 = phospho-histone H3.

Figure 2.

Binge ethanol exposure during adolescence alters the cell cycle distribution of SGZ NPCs. A–F) Representative images from sections stained for Ki-67, BrdU, and pHis-H3. In each case, immunoreactive cells were found in clusters concentrated along the SGZ. Arrows denote area represented in the inset. Scale bars = 100 μ m; inset 20 μ m. G) Ki-67 stereology data shows that 4-day binge ethanol exposure has no effect on the number of actively dividing cells in the SGZ. H) pHis-H3 stereology data shows that the number of dividing cells in G2 and M phases of the cell cycle is not altered by binge ethanol exposure. I) BrdU stereology data demonstrates that 4-day binge ethanol exposure significantly decreases the number of dividing cells in S-phase of the cell cycle. J) Calculated number of cells in G1 obtained by subtracting the number of BrdU+ and pHis-H3+ cells from the number of Ki-67+ cells. 4-day binge alcohol exposure had no effect on the number of dividing cells in G1 phase. K) Calculated distribution of dividing NPCs within each phase of the cell cycle showing that binge ethanol exposure specifically reduces the proportion of cells in S-phase. BrdU = Bromo-deoxy-uridine, GCL = granule cell layer, pHis-H3 = phospho-histone H3.

Figure 3.

BrdU and Ki-67 Labeling Indices. A) BrdU labeling index represents the S-phase fraction and was calculated as $100 \times (\text{BrdU}^+ \text{ cells} / \text{Sox-2}^+ \text{ cells})$. For both ethanol and control groups, BrdU labeling index increased with time; however, binge ethanol treatment reduced the BrdU labeling index compared to the control group, matching earlier results obtained with BrdU stereology. B) Ki-67 labeling index represents the growth fraction calculated as $100 \times (\text{Ki-67}^+ \text{ cells} / \text{Sox-2}^+ \text{ cells})$. As expected and reported previously, binge ethanol treatment had no effect of the Ki-67 labeling index (Morris et al., 2010a). BrdU = Bromo-deoxy-uridine, Con = control, EtOH = ethanol.

Figure 4.

The effect of adolescent binge alcohol exposure on SGZ NPC cell cycle kinetics. Shown are the linear regression lines for the control (solid line) and ethanol (dashed line) groups obtained from BrdU labeling index data using a least squares fit model. The regression lines were extrapolated to the point where they intersect the horizontal line that corresponds to the growth fraction, which represents the time needed to reach the maximum BrdU labeling index. The slopes of the two regression lines were not different, but binge ethanol significantly reduced the y-intercept. From the regression equations, T_c and T_s were calculated as described in the methods. Binge ethanol exposure during adolescence significantly shortened cell cycle length by decreasing the length of time spent in S-phase. GF = growth fraction.

Figure 5.

The effect of binge ethanol exposure during adolescence on the number of SGZ NPCs. A–B) Representative images from sections stained for Sox-2. Sox-2+ cells are found throughout the dentate gyrus, hippocampus, and other cortical regions; however, there is a large concentration of Sox-2+ cells located specifically within the SGZ. Arrows denote area represented in the inset. Scale bars = 100 μ m; inset 20 μ m. C) Sox-2 stereology data. Two-way ANOVA demonstrated that there was no effect of time; however, a main effect of diet was detected indicating that the 4-day binge paradigm used in this study slightly, but significantly increased the number of Sox-2+ cells in the SGZ.

Table 1

Primary Antibody Details

Antibody	Host Species	Titer	Time in Primary	Company, Catalog No.	Lot No.	Immunogen
BrdU	Mouse	1:5000	16h	Millipore, MAB3424	NM1645458	Bromodeoxyuridine-bovine serum albumin
Ki-67	Mouse	1:200	41–43h	Vector, VP-K452	L111855, L111560	Recombinant protein corresponding to amino acids 1159–1522 of human Ki-67
pHis-H3	Rabbit	1:1000	16h	Millipore, 06–570	DAM1545035	Peptide corresponding to amino acids 7–20 of human S10 phosphorylated histone H3
Sox-2	Rabbit	1:1000	16h	Millipore, AB5603	LV1622755	Synthetic peptide corresponding to amino acids 249–265 of human Sox-2

Table 2

Stereology Coefficient of Error

Marker	Control	Ethanol
<u>Experiment 1</u>		
Ki-67	0.014 ± 0.001	0.016 ± 0.001
pHis-H3	0.020 ± 0.002	0.020 ± 0.004
BrdU	0.016 ± 0.002	0.013 ± 0.001
<u>Experiment 2</u>		
BrdU	0.013 ± 0.001	0.014 ± 0.001
Ki-67	0.013 ± 0.001	0.014 ± 0.001
Sox-2	0.018 ± 0.001	0.018 ± 0.001

Table 3

Binge Data-Cell Cycle Kinetics Experiment

Time Point	Intoxication Score	EtOH Dose (g/kg/d)	BEC (mg/dL)
0.5h	0.9 ± 0.1	12.4 ± 0.29	316 ± 13.5
2.5h	1.1 ± 0.08	11.7 ± 0.25	378 ± 17.6
4.5h	1.2 ± 0.08	11.4 ± 0.25	348 ± 19.4

EtOH = Ethanol; BEC = Blood Ethanol Concentration