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Persistent Impairment of Hippocampal Neurogenesis in Young Adult Rats Following Early Postnatal Alcohol Exposure

Anna Y. Klintsova, Jennifer L. Helfer, Lyngine H. Calizo, Willie K. Dong, Charles R. Goodlett, and William T. Greenough

Background: Prenatal alcohol exposure can cause damage to the developing fetus with outcomes including growth deficiency, facial dysmorphology, brain damage, and cognitive and behavioral deficits. Smaller brains in children with FASD have been linked both with reduced cell proliferation in the developing CNS and with apoptotic cell loss of postmitotic neurons. Prenatal alcohol exposure in rodents during the period of brain development comparable to that of the first and second trimesters of human pregnancy persistently alters adult neurogenesis. Long-term effects of alcohol exposure during the third trimester equivalent, which occurs postnatally in the rat, on adult neurogenesis have not been previously reported. The goal of this study was to examine the effect of postnatal binge-like alcohol exposure on cell proliferation and neurogenesis in hippocampal dentate gyrus during adolescence and young adulthood.

Methods: Male Long-Evans rat pups were assigned to 3 groups: alcohol-exposed (AE), sham-intubated (SI) or suckle control (SC). AE pups received ethanol in a milk formula in a binge manner (2 feedings, 2 hours apart, total dose 5.25 g/kg/day) on postnatal days (PD) 4–9. BrdU was injected every other day on PD30–50. Animals were perfused either on PD50 to examine cytogenesis and neurogenesis in hippocampal dentate gyrus at the end of BrdU injections or on PD80 to evaluate new cell survival. Dorsal hippocampal sections were immunostained for BrdU, a marker for proliferating cells, Ki67, endogenous marker of proliferation, and NeuN, a marker for mature neurons.

Results: Binge-like alcohol exposure on PD4–9 significantly reduced the number of mature neurons in adult hippocampal dentate gyrus (DG) both on PD50 and PD80, without altering cumulative cytogenesis on PD50. In addition, the number of new neurons, that were generated between PD30 and 50, was further reduced after 30 days of survival in all 3 groups (SC, SI, and AE).

Conclusions: These observations suggest that early postnatal binge alcohol exposure results in long-term deficits of adult hippocampal neurogenesis, providing a potential basis for the deficits of hippocampus-dependent behaviors reported for this model.

Key Words: Adult Neurogenesis, Neonatal Alcohol Exposure, Dentate Gyrus, BrdU, NeuN

PRENATAL EXPOSURE OF the human fetus to alcohol can cause birth defects and enduring CNS damage. The outcomes can range from the full fetal alcohol syndrome (FAS), diagnosed by the constellation of facial dysmorphology, growth deficiency, and CNS damage, to alcohol-related neurodevelopmental disorders (ARND), in which only CNS effects are present (Stratton et al., 1996). This broad range of adverse effects resulting from prenatal alcohol exposure has been termed fetal alcohol spectrum disorder (FASD), and the prevalence of FASD has been estimated to be nearly 1 per 100 live births (Hoyme et al., 2005; Sampson et al., 1997; Streissguth and O'Malley, 2000). The most significant consequences for affected individuals and for society are the CNS damage and neurobehavioral abnormalities, including cognitive, socio-emotional, and motor performance abnormalities (Barr et al., 1990; Clarren et al., 1978; Conry, 1990; Jones and Smith, 1973; Streissguth, 1986; Streissguth et al., 1991). Neuroimaging studies in children with FASD have demonstrated significantly smaller brains (Roebuck et al., 1998) with persistent reduction of the volume of basal ganglia, corpus callosum, cerebellum, and hippocampus (Archibald et al., 2001; Autti-Ramo et al., 2002; Mattson et al., 1996; Riley et al., 1995). This loss of brain volume in FAS has been linked both to reduced CNS cell acquisition due to effects on cell proliferation (Miller, 1988, 1989) and to alcohol-induced apoptotic cell loss of postmitotic neurons (Ikonomidou et al., 2000). Tissue loss in the brains of patients with FASD varies across

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different brain regions, likely related to differences in the quantity, frequency, and timing of alcohol exposure over development (Rosett et al., 1983).

Experimental animal models of developmental alcohol exposure have demonstrated that the type and extent of neuronal loss depends on the developmental timing of exposure (Maier et al., 1996). For example, prenatal alcohol exposure during periods of developmental neurogenesis results in decreased numbers of hippocampal CA1 pyramidal cells (Bartzokis et al., 2003; Miller, 1995), Purkinje cells in cerebellum (Maier and West, 2001; Maier et al., 1999) and cortical neurons (Miller, 1986, 1988). In addition, the brain is also vulnerable to alcohol exposure during the period of the "brain growth spurt," a time of rapid growth and synaptogenesis that occurs over the third trimester of pregnancy in humans, but over the first ten postnatal days in rats. Alcohol administered to neonatal rats during this third trimester equivalent, particularly when it occurs in a binge-like daily episodes that produce relatively high peak blood-alcohol concentrations (BACs), induces significant cell loss in the cerebellum, cerebral cortex, hippocampus and striatum (Goodlett and Eilers, 1997; Ikonomidou et al., 2000; Marcussen et al., 1994; Miller, 1995; Mooney et al., 1996; Tran and Kelly, 2003; West et al., 2001).

Rodent models of either pre- or postnatal alcohol exposure have demonstrated impaired hippocampal-dependent behavior (such as spatial learning and memory) (Berman and Hannigan, 2000; Choi et al., 2005; Goodlett and Peterson, 1995; Goodlett et al., 1987; Johnson and Goodlett, 2002; Kelly et al., 1988; Richardson et al., 2002; Tomlinson et al., 1998; Wozniak et al., 2004). Several studies have demonstrated altered structure of different hippocampal subfields and reductions in hippocampal cell number following alcohol exposure during the brain growth spurt. Livy et al. (2003) reported cell loss in both CA1 and dentate gyrus on postnatal day (PD) 10 in rats after binge-like alcohol exposure given on PD4–9. Miller (1995) reported that effects of neonatal alcohol exposure on cells in hippocampus of 30–35-day-old rats depended on the BAC produced; at high BACs, neuronal number and neuronal generation in the dentate gyrus were decreased. In contrast to these findings, others have found that binge-like alcohol exposure during the third trimester equivalent only produced long-term reductions in neurons in CA1, not in CA3 or in the DG granule cells (Bonthius et al., 2001; Tran and Kelly, 2003).

Dentate gyrus of hippocampus is one of the two distinct areas of the brain where neurogenesis continues through the lifespan (Christie and Cameron, 2006; Emsley et al., 2005). Adult neurogenesis has been shown to be affected by many factors, both intrinsic and extrinsic, including stress, environmental experience, exercise and drug abuse. These effects may be immediate or delayed, temporary or long-lasting, or even permanent.

Recent data suggests that prenatal alcohol exposure results in a long-lasting (into adulthood) effect on the ability to generate new cells from the pool of subgranular zone (SGZ) progenitors (Redila et al., 2006). In that study, in rats from dams drinking an alcohol liquid diet during gestation, both 24-hour and 4-week survival of BrdU-labeled cells were significantly decreased in the adult dentate gyrus under standard housing conditions (compared to ad lib controls), but the number of labeled cells was significantly increased (to control levels) by voluntary exercise. In contrast, in a mouse model of voluntary alcohol consumption during gestation, adult cytogenesis and neurogenesis rates were not affected under standard housing conditions, but persistent deficits in adult hippocampal neurogenesis in response to environmental enrichment were demonstrated (Choi et al., 2005). The effects of alcohol exposure during the third trimester equivalent (first twelve postnatal days in rat) on hippocampal neurogenesis in the mature CNS have not been previously reported, and many questions need to be addressed. For example, does the exposure to binge-like alcohol during the brain growth spurt result in long-lasting changes in proliferative activity in SGZ of hippocampus? If the proliferation rate is unaffected, what are the capabilities of the newly generated cells to survive? Is there a persistent effect of neonatal alcohol exposure on the fate of newly generated cells in the DG in adulthood?

The purpose of the present study was to examine the effects of binge-like exposure to alcohol during the third trimester equivalent (PD4–9 in rats) on cell proliferation and neurogenesis in SGZ of hippocampal DG, using bromodeoxyuridine (BrdU) injections on PD30–50 to label dividing cells. BrdU-labeled cells were assessed at PD50 to establish the number of cells in the DG at the end of the BrdU incorporation period, and also at PD80 to assess the survival of labeled cells.

MATERIALS AND METHODS

Animals

Time-pregnant Long-Evans dams (Simonsen, Gilroy, CA) were housed in the vivarium of the Department of Psychology, SUNY Binghamton and in the University of Delaware animal facility. GD0 was designated as the day of sperm plug generation and GD22 was designated PD0; with rare exceptions, dams gave birth on GD22. On PD2, litters were culled to 8 pups (four males and four females when possible). On PD4, litters were randomly assigned to either the intubation condition or to the undisturbed condition. Using a split-litter design for the intubuated litters, two male and two female pups were randomly assigned within each litter to the sham-intubated (SI) group and the other two males and two females were randomly assigned to the alcohol exposed (AE) group. Rats from the undisturbed litters constituted the suckle control (SC) group. All pups remained with their dam until weaning on PD21–23, at which time they were housed with same-sex littermates. Only males were used in this study (SC, $n = 14$; SI, $n = 16$; AE, $n = 12$ animals) since natural fluctuation in cell proliferation has been reported across the estrous cycle in female rats (Tanapat et al., 1999).

Neonatal Treatments

Milk and milk/ethanol treatment formulas were prepared from a base milk formula prepared according to the previously described method (West et al., 1984) and were delivered via intragastric intubation (Goodlett and Johnson, 1997). On PD4–9 (Fig. 1), pups of the AE group were given 2 daily intubations of the milk/ethanol formula

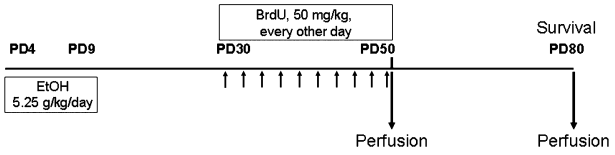

Fig. 1. Experimental time line, ethanol and BrdU administration protocol. Rats received ethanol in a binge-like manner, through the temporary intragastric intubation, twice a day 2 hours apart. BrdU injections were done between PD30 and 50, once a day every other day. Half of the animals were sacrificed on PD50, 2 hours after the last BrdU injection. The other rats were allowed to survive for 30 more days and were sacrificed on PD80.

containing 11.9% (v/v) ethanol, with a 2-hour interval between intubations. Each infusion delivered 2.625 g/kg of alcohol resulting in a total daily dose of 5.25 g/kg for each animal. On PD4, AE pups received 2 additional intragastric intubations of milk formula alone after completion of alcohol treatment (2 hours apart); on PD5–9 the AE pups were given one additional intubation of milk formula alone, 2 hours after the second ethanol/milk formula intubation. These additional feeding intubations were given to AE pups to limit growth deficiency that would otherwise result from the alcohol-induced impairment of suckling behavior. SI pups received intubations alone, without infusion of milk, since multiple daily milk infusions have been shown to abnormally accelerate the growth of SI pups (Goodlett et al., 1998).

Blood-Alcohol Concentration

On PD4, 3.5 hours after the first alcohol intubation, tail clips were made in AE and SI pups and blood samples were collected in heparinized capillary tubes. The tubes were centrifuged and plasma collected and stored at −70°C. BACs for AE animals were assayed from the plasma with an Analox GL-5 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

BrdU-Injections

Beginning on PD30, each animal received an injection of BrdU (50 mg/kg in sterile 0.9% saline solution (20 mg/ml), i.p.) every other day until PD50 (Fig. 1). On PD50, half of the animals within a given litter were transcardially perfused to establish cytogenesis status at the end of the BrdU treatment period. The remainder of the animals were perfused on PD80 to ascertain the survival rate of the cells generated between PD30–50.

Tissue Preparation

Animals were deeply anesthetized (sodium pentobarbital, 100 mg/kg i.p.) then transcardially perfused first with 50 ml of heparinized phosphate-buffered saline (PBS, pH 7.2) followed by 250 ml of 4% paraformaldehyde in PBS. Brains were carefully removed from the skull and placed in buffered sucrose solutions of increasing (10–30%) concentration until they sank. Serial coronal sections (40 μm) were cut on a cryostat and each section was placed into an individual well. The order of the sections was maintained throughout all the following procedures. Sections were stored at −20°C in a cryoprotectant solution containing glycerol and ethylene glycol in Tris-buffer solution.

BrdU and NeuN Immunohistochemistry

A systematic random sampling procedure was used in selecting the sections for processing. Within the pool of sections containing dorsal hippocampal formation (including the dentate gyrus), every fifth section was placed in an individual well and processed for BrdU and mature neuronal marker NeuN immunofluorescent histochemistry.

The following antibodies and final dilutions were used: rat monoclonal anti-BrdU (1:100, Accurate, Westbury, NY), mouse monoclonal anti-NeuN (1:100, Chemicon, Temecula, CA), Cy2 anti-rat IgG (3:500, Jackson Laboratories, West Grove, PA), Cy3 anti-mouse IgG (3:500, Jackson Laboratories,West Grove, PA). Double immunofluorescence for BrdU and NeuN was performed as follows: free-floating sections were rinsed in 0.1 M Tris-buffered saline (TBS), incubated for 2 hours in 50% formamide in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) at 65°C, washed in SSC followed by incubation for 30 min in 2 N HCl at 37°C and rinsed for 10 min in 0.1 M boric acid in TBS, pH 8.6. Sections were then immersed in a blocking solution of TDS-TBS (0.5% Triton X-100 and 3% donkey serum in TBS) at room temperature (RT) for 1 hour, followed by incubation in anti-BrdU primary antibody in TDS-TBS for 72 hours at 4°C. Additional sections not exposed to the primary anti-BrdU antibody but incubated with the rest of the solutions were included as no-primary control. Sections were then rinsed in TDS-TBS, immersed in Cy2-labeled secondary antibody and TDS-TBS at RT for 3 hours, rinsed in TBS and incubated in anti-NeuN primary antibody in TDS-TBS for 48 hours at 4°C. Following washing in TDS-TBS, tissue was immersed in Cy3-labeled secondary antibody in TDS-TBS at RT for 3 hours, rinsed in TDS-TBS and mounted on nongelatin subbed slides. Sections were coverslipped with antifade media (ProLong Antifade Media, Molecular Probes/Invitrogen, Carlsbad, CA). The edges of coverslips were sealed with nail polish after the mounting media dried. Slides were stored at −20°C.

Ki67 Immunohistochemistry

An additional subset of sections (every fifth one, with the first one in series picked randomly within the first 5 sections containing dorsal hippocampus) was immunostained using primary antibody against Ki67 (mouse monoclonal, 1:200, Novocastra, Newcastle, UK), followed by incubation with secondary antibody (goat anti-mouse, 1:1000, Vector) and avidin–biotin-peroxidase complex (Vector) to amplify the signal. Localization of the binding sites was visualized with nickel-enhanced diaminobenzidine as a chromagen. Ki67-labeled sections were lightly counterstained with eosin Y cytoplasmic stain and coverslipped with DPX mounting medium.

Stereological Counting Procedure

Cells that incorporated BrdU were easily identified using immunofluorescence (Fig. 2). All BrdU+ cell counts were made on coded slides by an investigator blind to the treatment group. Cell counts were obtained from unbiased, uniform samples within a known volume of the dorsal hippocampal dentate gyrus using 3D disector measures (MCID Elite and Analysis Software, Imaging Research Inc., St. Catherine's, Ontario, Canada). Disector frames were distributed along the medio-lateral extent of the dentate gyrus in a systematic manner. BrdU+ cell counts were derived from aligned serial sections (z-plane) separated by 3 μm and digitally imaged by fluorescence microscopy for Cy2 label at 200× final magnification. Guard zones (3 μm each) were implemented for the top and bottom sections. When the disector analysis was applied to each z-plane section, it identified only new "cell tops" seen within the sampling frame. To reduce variability in digital imaging and 3D disector analysis across tissue samples, digital camera gain and exposure time were standardized as were the target detection and measurement criteria (e.g., pixel density limits or thresholds). To determine the volume sampled, the area

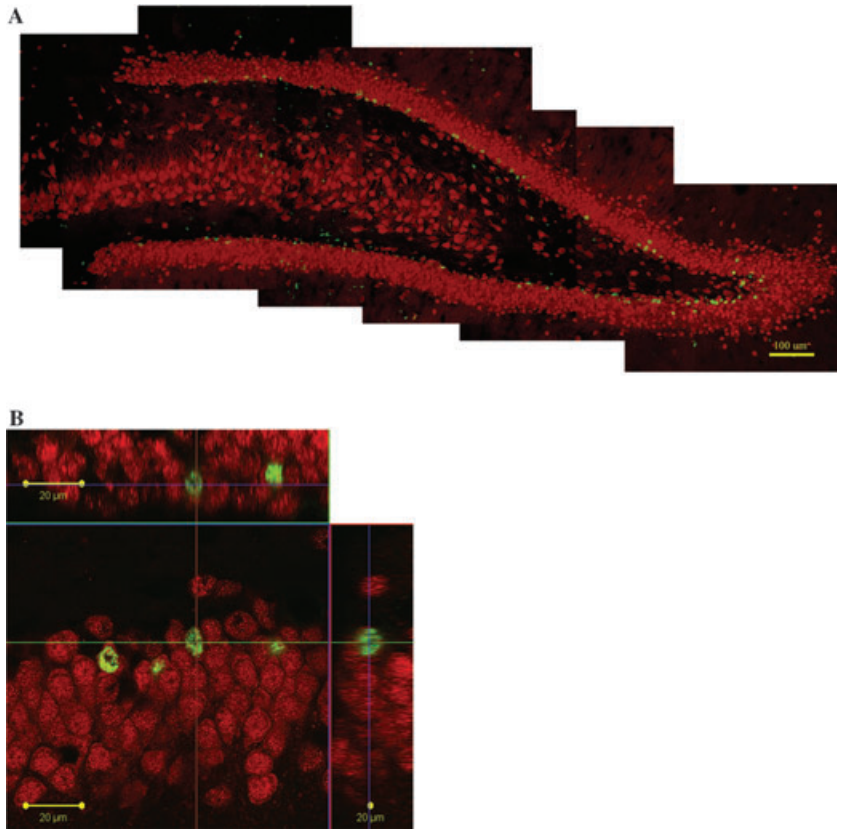

Fig. 2. (**A**) Confocal composite of the dentate gyrus from the SC animal on PD50, after completion of BrdU injections. NeuN positive mature neurons are labeled in red and BrdU-incorporated cells are labeled in green. (**B**) Confocal image (with orthogonal views) of BrdU+ cells (green nuclear staining) and NeuN+ granule cells (red staining) in dentate gyrus. Co-localized labels appear yellow and can be confirmed in all three projections.

occupied by the granule cells in the dentate gyrus was digitally imaged by fluorescence microscopy for Cy3 label at 200× magnification, measured by scanning with MCID software and multiplied by the thickness of the sectioned and unprocessed tissue (40 μm). To ensure comparable sampling of the rostrocaudal extent of the hippocampal dentate gyrus across animals, only sections containing DG within the dorsal hippocampus (between approx. Bregma −2.56 and −3.80) were used in the analysis. There was no significant difference in the mean reference volume analyzed between 3 experimental animal groups.

BrdU + cells in DG and SGZ were inspected further with confocal microscopy (LSM 510 confocal microscope, Zeiss, Thornwood, NY) (Fig. 2). Phenotyping was performed on the DG from the same sections that were used for BrdU counting but from the opposite hemisphere (to avoid underestimation of counts due to photobleaching during stereological counting). Z-stacks of 1 μm thick images (acquired with 63× objective with 1.2 numerical aperture) of the DG granule cell layer were collected from dorsal and ventral blades of DG in a systematic random manner: it was assured that 75 BrdU + cells per animal were selected and phenotyped to confirm or refute BrdU co-localization with the NeuN. BrdU-labeled cells could be identified as neurons if an overlap of the Cy2 and Cy3 labels was observed in a given cell in each of the *xy*-, *xz*-, and *yz*-planes in the orthogonal view (Fig. 2*B*).

Ki67 cells were counted using optical fractionator method in StereoInvestigator software (MBF Bioscience, Williston, VT). Counts were obtained from every fifth section of dorsal hippocampus (5 to 6 sections per animal) using the counting frame 100 × 100 μm^2 and the grid size of the same dimensions. The guard zone was set on 2 μm from the surface of the section and *z*-dimension of the counting frame was 6 μm. Total number of Ki67 cells in the dorsal hippocampus was estimated using formula:

$$N_V = \sum Q^- \left(\frac{1}{SSF}\right)\left(\frac{1}{ASF}\right)\left(\frac{1}{TSF}\right)$$

where ΣQ^- is the sum of labeled cell counts; SSF is the section sampling fraction (5); ASF is the area of the counting frame relative to the sampling area (1 in this particular study, such that 100% of the outlined DG was sampled); TSF is the sampled fraction of the section thickness (ratio between disector height, 6 μm, and measured section thickness, 11.2 μm on average).

Statistical Analysis

Data were analyzed using Statistica software (StatSoft Inc., Tulsa, OK). Body weights were analyzed with a 2-way ANOVA, with treatment group (SC, SI, and AE) and age (PD50 and PD80) as factors. The morphological data (DG volume and percentage of BrdU + cells) were analyzed with a one-way ANOVA, with treatment group (SC, SI, and AE) as the factor. Post hoc analyses were performed using the Newman–Keuls test.

RESULTS

Blood-Alcohol Concentrations and Body Weight

The delivery of alcohol in two consecutive feedings resulted in an average peak blood-alcohol concentration of

Table 1. Weights (g) of SC, SI, and AE Animals on PD9, PD30, and PD50

	PD9	PD30	PD50
SC (n = 11)	18.66 ± 0.46	111.82 ± 5.65	241.64 ± 5.47
SI (n = 11)	19.81 ± 0.36	114.79 ± 6.74	238.86 ± 7.04
AE (n = 9)	17.16 ± 0.53 *	112.29 ± 9.71	240.86 ± 12.29

SC, suckle control; SI, sham-intubated; AE, alcohol-exposed. Data expressed as mean ± s.e.m. *p = 0.001.

315 ± 19 mg/dl. While AE pups had significantly lower weights after completion of alcohol treatment on PD9 [effect of postnatal treatment, $F_{(2,29)} = 8.24$, $p = 0.001$], animal body weights did not differ significantly at the beginning of the BrdU injections (PD30) or at the end of the BrdU injection procedure (PD50) (see Table 1).

General Description of Immunohistochemical Labeling

Sections were stained in two separate batches containing all three postnatal treatment groups and two adult time points (PD50 and PD80). No significant difference was found between the data from the different batches within the same treatment/condition. Thus, the data from the two batches were combined and analyzed together. Cells that incorporated BrdU were easily identified using immunofluorescence (Fig. 2). The number of BrdU+ nuclei within the SGZ (defined as two nuclei diameter into the hilus) and dentate gyrus cell layer was estimated. Numerous cell nuclei throughout dentate gyrus appeared to be double-labeled with the mature neuronal marker NeuN, and phenotyping of BrdU+ cells was performed as a next step using confocal microscopy.

Alcohol Exposure on PD4–9 Did Not Alter Cumulative Cytogenesis at PD50 But Did Decrease Long-Term Survival of Adult-Generated Cells

The volume of the dorsal hippocampal dentate gyrus used for the cell count sampling did not differ among the 3 treatments (SC, SI, and AE) groups (ANOVA, $F_{(2,37)} = 0.359$, $p = 0.701$). Consequently, all data are presented as a volume density (cell number per unit volume of the DG).

Two-way ANOVA with postnatal TREATMENT (AE, SI, SC) and AGE (PD50, PD80) as factors revealed significant effects of AGE on the number of BrdU+ cells per unit volume [$F_{(1,32)} = 14.32$, $p < 0.01$] (Fig. 3*A*). Neither the TREATMENT main effect nor the interaction was significant. The density of labeled cells was significantly lower on PD80 (30 day survival post-treatment) than on PD50 for all three treatment groups, confirmed by post hoc comparisons of BrdU+ cells at PD50 and at PD80 within each group [SC_{PD50} vs. SC_{PD80}, $p < 0.05$; SI_{PD50} vs. SI_{PD80}, $p < 0.05$; AE_{PD50} vs. AE_{PD80}, $p < 0.01$]. The percentage of labeled (surviving) cells on PD80 relative to PD50 was 37% for the AE group and 55% for the SI and SC groups. Separate one-way ANOVAs were also conducted on the density of labeled cells at each age. On PD80, a significant effect of TREATMENT was confirmed [$F_{(1,9)} = 10.28$, $p = 0.012$], due to fewer surviving (labeled) cells in the AE group ($p < 0.05$). A one-way ANOVA on the PD50 counts indicated no significant group differences related to TREATMENT. Immunohistochemical staining for endogenous proliferating marker Ki67 on PD50 and stereological estimate of the number of proliferating cells in the DG of dorsal hippocampus confirmed the findings from BrdU labeling study: the number of Ki67+ cells did not differ across treatment groups (Fig. 3*B*).

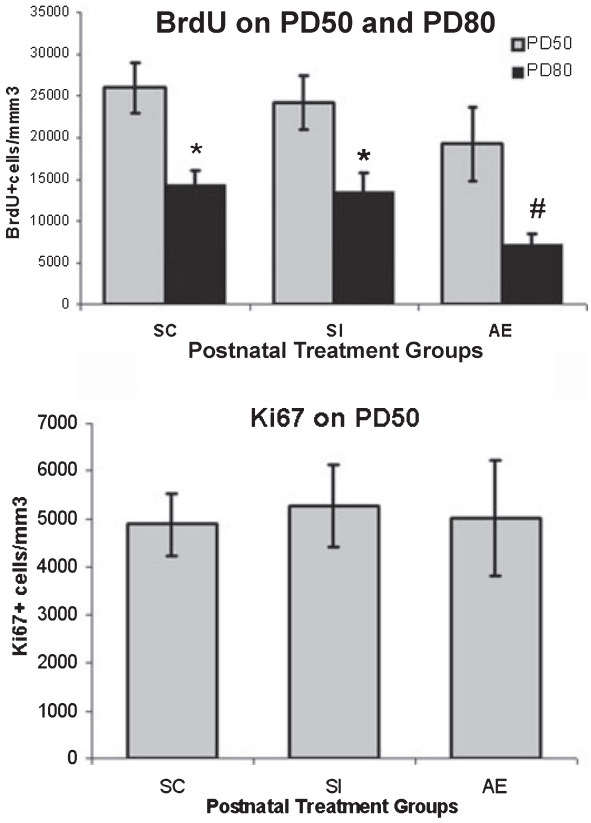

Fig. 3. Volume densities of BrdU+ cells in the DG on PD50 and PD80 (top panel). Alcohol exposure during the third trimester equivalent does not affect the number of proliferating (BrdU+) cells in hippocampal DG as estimated on PD50. Survival of new cells (PD80) is affected by neonatal exposure to alcohol. The AE group had significantly fewer labeled cells compared to the SC and SI rats. No difference in Ki67 + cell density was found on PD50. SC, suckle control; SI, sham-intubated; AE, alcohol exposed. Data expressed as mean ± SEM. *$p < 0.05$, #$p < 0.01$.

Alcohol Exposure on PD4–9 Did Not Alter Fate Specification of Newly Generated Cells at PD50 or PD80

To assess the effect of binge-like alcohol exposure on phenotypic fate of adult-generated cells in DG, we determined the co-localization of BrdU+ and NeuN+ labeling in 50 randomly selected, BrdU-labeled cells in the DG of each animal in the study, using confocal microscopy. The percentage of BrdU+ cells double-labeled with NeuN did not differ significantly among the SC, SI, and EtOH groups at P50 or at P80 (Fig. 4). This suggests that early postnatal EtOH exposure did

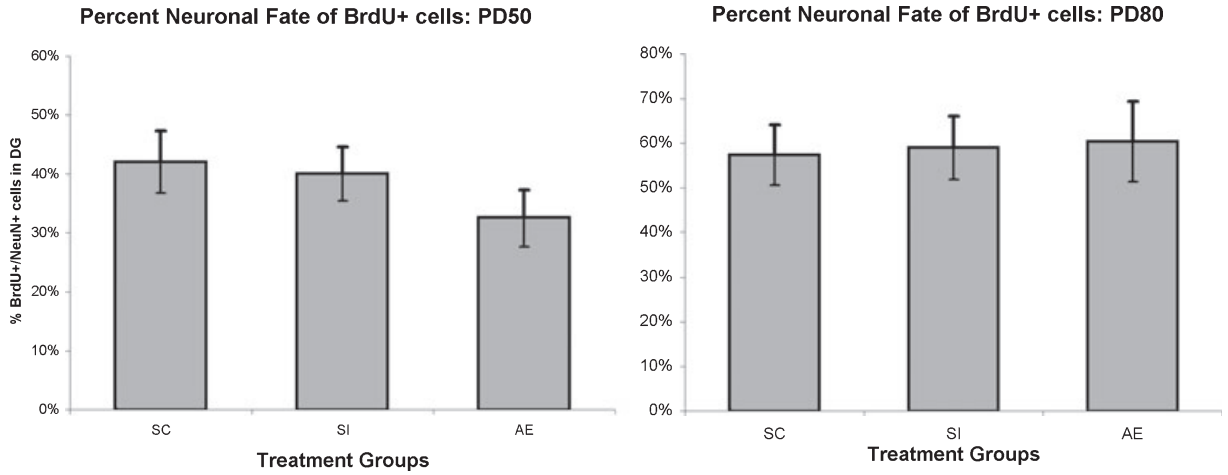

Fig. 4. Percent of neuronal fate of BrdU+ cells in hippocampal dentate gyrus on PD50 (left panel) and after 30 days survival interval, on PD80 (right panel). A minimum of 50 BrdU+ cells per animal were evaluated for colocalization with NeuN marker; all animals in the study were phenotyped. There was no significant difference in neuronal fate of proliferating progenitors.

not alter fate specification of newly generated cells labeled during the BrdU treatment period. Only 32 to 45% of all BrdU+ cells were double-labeled with the mature neuronal marker NeuN at any given condition on PD50; these numbers were higher on PD80—55 to 60%.

Alcohol Exposure on PD4–9 Altered the Number of BrdU-Labeled Neurons at PD50 and Their Survival at PD80

To evaluate the consequences of binge-like alcohol exposure during the third trimester equivalent on the acquisition of new neurons in DG, we converted the number of BrdU-positive cells per unit volume into an estimate of the number of new NeuN-positive cells per unit volume by multiplying the BrdU-positive cell density by the percentage of NeuN+ cells among BrdU+ cells.

Two-way ANOVA with TREATMENT and AGE as factors revealed significant effects of both factors on the estimated volume density of BrdU+/NeuN+ cells in DG [$F_{(2,32)} = 5.46$, $p < 0.01$ for TREATMENT; $F_{(1,32)} = 5.14$, $p < 0.05$ for AGE] (Fig. 5). The interaction was not significant. On PD50, there were significantly fewer double-labeled neurons per unit volume in the AE animals than in both SC and SI animals ($p < 0.05$). On PD80, AE animals had significantly lower densities of double-labeled neurons (BrdU+/-NeuN+) than both SC and SI rats ($p < 0.05$).

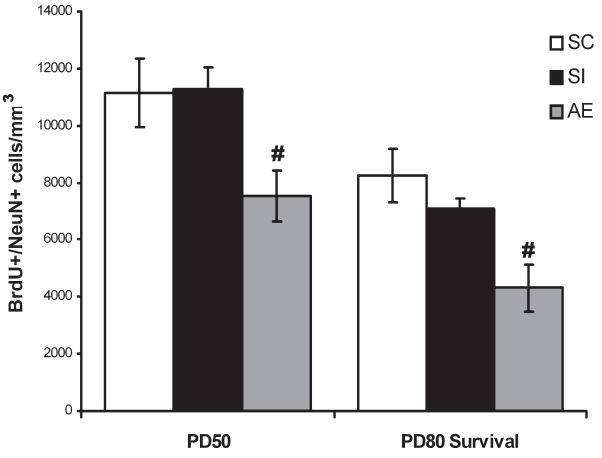

Fig. 5. Volume densities of double-labeled neurons in the DG on PD50 and PD80. The density of neurons was significantly decreased in the AE group relative to the SC and SI groups on PD50, and significantly decreased relative to both control groups on PD80. Survival rate for new neurons was 70% in control groups and only 55% in AE animals. Densities for neurogenesis were obtained by multiplying the percentage of BrdU+/NeuN+ cells by the total number of BrdU+ cells in the dentate gyrus. SC, suckle control; SI, sham-intubated; AE, alcohol exposed. Data expressed as mean ± SEM. #$p < 0.05$, comparison between postnatal treatments.

DISCUSSION

This is the first study to demonstrate that binge-like exposure to alcohol during the neonatal brain growth spurt in rats can produce persistent effects on adult hippocampal neurogenesis. The alcohol treatments on PD4–9 reduced number of mature neurons in adolescent/young adult hippocampal DG derived from cells generated between PD30 and PD50. This treatment models heavy binge drinking during the third trimester, a pattern of drinking typically observed in women who abuse alcohol throughout pregnancy (Rosett et al., 1983) and which can produce similarly high BACs in human binge drinkers (Urso et al., 1981) and in cases of binge drinking in near-term pregnancies (Church and Gerkin, 1988). The outcomes of this neonatal rat model suggest that heavy binge drinking during the 3rd trimester can persistently interfere with adult hippocampal neurogenesis.

Previous studies examining the effect of developmental alcohol exposure on hippocampal cell proliferation and adult neurogenesis have yielded different outcomes. Redila and colleagues (Redila et al., 2006) reported a significant decrease in cell proliferation in adult rat DG after prenatal alcohol

exposure via liquid diet (35.5% ethanol-derived calories) that produced peak BACs of 184 mg/dl. Neurogenesis in these alcohol-exposed animals was inhibited in comparison with control (but not pair-fed) rats, when measured 4 weeks after BrdU injection. Redila et al. (2006) pointed at the nutritional effect of alcohol and pair-feeding procedure since pair-fed animals in their study has impairment in cell survival similar to alcohol-exposed rats. In our study, the effect of sham-intubation was not as obvious and the difference in cell proliferation or neurogenesis between SC and SI was never significant.

In contrast, Choi and colleagues (Choi et al., 2005), using voluntary consumption of sweetened 10% alcohol in pregnant C57BL/6 mice that produced peak BACs of 121 mg/dl, did not find any change in proliferative or neurogenic activity in the DG of the adult mice, but found that the prenatal alcohol exposure severely impaired the neurogenic response to environmental enrichment or complexity. Differences in the outcomes of these 2 studies might be explained by the higher BAC in the Redila et al. (2006) study, by procedural differences in the BrdU labeling (single injection vs. cumulative labeling), by species differences (rats vs. mice), or by the differences related to forced versus voluntary consumption of the alcohol. Nevertheless, both studies concluded that prenatal alcohol exposure produces a long-lasting effect on the progenitor cell pool in the SGZ of hippocampal DG.

In the current study, in which alcohol exposure modeled maternal binge drinking during the third trimester, no significant changes were found in the number of BrdU + cells in the young-adult hippocampus of alcohol-exposed rats in comparison with suckle controls or sham-intubated controls. This suggests that the neonatal alcohol exposure had no long-term effect on the ability of progenitor cells to proliferate in adulthood. In contrast, the number of new mature neurons (BrdU-positive, co-labeled with the mature neuronal marker NeuN) was significantly reduced after 20 days of BrdU injections in AE rats. This finding suggests that the neuronal precursors may be particularly vulnerable in adolescent/young adult AE animals, due either to a reduction of their proliferative activity (by increasing the cell cycle length), to a shift in their differentiation more towards a glial phenotype, or to a slower development into mature neurons. The two latter possibilities suggest a long-term effect of developmental alcohol exposure on the hippocampal SGZ that controls neurogenesis. The neurogenic fate of progenitor cells in the adult hippocampal SGZ is regulated primarily by astrocytes that has been recently recognized as the major component of the neurogenic niche [for review, see Campos (2005) and (Lledo et al., 2006)], along with the vasculature (Palmer et al., 2000) and basal lamina (Campos, 2005). Local astrocytes in SGZ of the dentate gyrus express and secrete factors (e.g., neurogenesin I) that promote differentiation of adult neural progenitors and prevent differentiation into glial cells or gliogenesis (Ueki et al., 2003). To the best of our knowledge, the effect of alcohol exposure (i.e., developmental, adult, acute, or chronic) on the expression of neurogenesin I has not been studied. Prenatal exposure to alcohol is known to reduce the levels of another proliferative agent, i.e., fibroblast growth factor receptor 2 (Bartlett et al., 1994; Rubert et al., 2006).

The effects of alcohol on cell proliferation have been extensively studied in cell and tissue cultures. Given in physiologically relevant doses, alcohol significantly decreases the number of viable cells in neocortical tissue culture by slowing cell proliferation, increasing their cell cycle length and increasing the incidence of cell death (Jacobs and Miller, 2001; Miller and Nowakowski, 1991). Exposure to alcohol during embryogenesis reduces the number of radial astrocytes and disrupts their transformation into neurons and mature astrocytes in telencephalic cultures obtained from alcohol-treated rats (Rubert et al., 2006), consistent with effects seen in vivo (Miller and Robertson, 1993). Also, alcohol inhibits neuronal differentiation of neural stem cells in culture at concentrations that are significantly lower than the ones that compromise viability (Tateno et al., 2005). Taken together, these findings suggest that alcohol significantly compromises the neural progenitor pool, although the long-term effects of such alterations have not been studied in culture preparations.

A single alcohol dose administered as a subcutaneous injection on PD7 (5 g/kg) results in decrease in both cell proliferation and neurogenesis in adult mice hippocampus (Ieraci and Herrera, 2007). While the BAC was not reported in that study, similar regiment of alcohol delivery on PD7 (Ikonomidou et al., 2000) produced extremely high alcohol level in blood (around 500 mg/dl) lasting for more than 7–8 hours and resulting in significant degenerative changes in cortical areas and hippocampus. In contrast, when alcohol was administered as a single dose on PD10 (i.p. injection of 3 g/kg, resulting in a high BAC around 400 mg/dl) the outcome was an increase in both cell death and proliferation in hippocampal dentate gyrus 1 and 3 weeks after alcohol exposure (Zharkovsky et al., 2003). In alcohol-exposed animals, a significantly higher percentage of new BrdU-labeled cells co-expressed PSA-NCAM or Tuj1 (markers of young postmitotic neurons), as well as the astroglia marker glial fibrillary acidic protein. Similar to our study, Zharkovsky et al. (2003) reported a decrease in the percentage of new mature neurons (BrdU-labeled/calbindin-colabeled) 3 weeks after alcohol exposure. It should be noted that in Ieraci and Herrera (2007) study, the effect of postnatal alcohol exposure was evaluated in adult (5-months old) animals, and it is generally accepted that in younger animals the effect of a 1-time alcohol exposure might not be detected until a certain age as the generation of new neurons occurs at a high level in adolescent/young adult animals but the process slows with age (Kuhn et al., 1996).

Binge alcohol exposure in adulthood results in a significant decrease of neural progenitor cells proliferation and survival of newly dividing cells (Nixon and Crews, 2002) without inducing robust apoptosis in the hippocampal DG. Cell cycle elongation and delay in entering the synthetic S-phase was suggested as one possible mechanism for reduction of BrdU + cells. A more recent study (Crews et al., 2006) demonstrated an even greater vulnerability of progenitor cells in the

hippocampal SGZ to alcohol exposure during adolescence. The number of BrdU+ cells was reduced by 78% (when the alcohol dose was 5.0 g/kg), and the reduction of labeled progenitors in the rostral migratory stream and subventricular zone was even more robust—up to 99%.

Alcohol is not unique in its effect on the progenitor cell pool in the brain. Cyto- and neurogenesis in adult dentate gyrus is vulnerable to the effects of other drugs and biologically active substances, such as opiates (Eisch et al., 2000), methamphetamine (Teuchert-Noodt et al., 2000) and steroids (Cameron et al., 1998b). These substances seem to affect stem cell proliferation by direct action on the major neurotransmitter sites or indirect action on the local growth factors (Cameron et al., 1998a; Lledo et al., 2006; Powrozek et al., 2004). It should also be mentioned that recent study by Perera et al. (2007) demonstrated that electroconvulsive shock robustly increased precursor cell proliferation. They estimated that the percentage of precursor cells that matured into neurons was between 55 and 60%, with endothelial cells being the second most numerous proliferating cell type (about 30%), and that electroconvulsive shock did not affect the maturational fate of proliferating cells, resulting in the increased net neurogenesis in hippocampal dentate gyrus.

Behavioral outcomes, particularly hippocampal-dependent behaviors, of binge-like alcohol exposure during the third trimester equivalent are well-documented. Spatial learning ability is reduced (Tomlinson et al., 1998) or severely impaired (Goodlett and Johnson, 1997; Johnson and Goodlett, 2002; Wozniak et al., 2004) depending on the alcohol dose; short-term memory is impaired (Girard et al., 2000), an effect that has been related to a decrease of c-*Fos* expression in the hippocampus (Clements et al., 2005). These behavioral changes after third trimester alcohol exposure coincide with permanent reductions of CA1 neurons and transient changes in granule cell number in the DG. Recent studies of adult neurogenesis have demonstrated the importance of neurogenesis in the adult dentate gyrus for hippocampal-dependent memory formation (Gould et al., 1999), and experimental inhibition of neurogenesis in adult rats has been shown to impair hippocampal-dependent forms of associative memory (Shors et al., 2001). There is also indirect evidence that adult neurogenesis is required for learning; experience-induced changes that affect neurogenesis (stress and environmental enrichment) also have an effect on learning (Cameron et al., 1998b; Gould et al., 1997; Karten et al., 2005; Kempermann et al., 1997; van Praag et al., 1999). Consequently, if developmental exposure to alcohol interferes with the normal processes of adult neurogenesis, these effects may be implicated in the deficits on several hippocampal-dependent cognitive tasks reported in FAS children (Hamilton et al., 2003; Uecker and Nadel, 1998).

Our results have demonstrated persistent impairment in adult neurogenesis after neonatal alcohol exposure in an animal model of third trimester binge drinking and suggest that learning deficits reported in children suffering from FASD could be at least partially because of the decreased ability to generate new neurons. Studies are now underway to determine if exercise—a condition known to significantly increase neurogenesis in the dentate gyrus of hippocampus—is capable of ameliorating the reduced level of neurogenesis in the alcohol-damaged brain.

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