Published in final edited form as:

J Comp Neurol. 2009 May 20; 514(3): 259–271. doi:10.1002/cne.22018.

Binge-like Postnatal Alcohol Exposure Triggers Cortical Gliogenesis in Adolescent Rats

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Abstract

The long-term effects of binge-like alcohol exposure on cell proliferation and differentiation in the adolescent rat neocortex were examined. Unlike the hippocampal dentate gyrus, where proliferation of progenitors results primarily in addition of granule cells in adulthood, the vast majority of newly generated cells in the intact mature rodent neocortex appear to be glial cells. The current study examined cytogenesis in the motor cortex of adolescent and adult rats that were exposed to 5.25 g/kg/day of alcohol on postnatal days (PD) 4–9 in a binge manner. Cytogenesis was examined at PD 50 (through bromodeoxyuridine, BrdU, labeling) and survival of these newly generated cells was evaluated at PD80. At PD50, significantly more BrdU positive cells were present in the motor cortex of alcohol-exposed rats than controls. Confocal analysis revealed that the majority (>60%) of these labeled cells also expressed NG2 chondroitin sulfate proteoglycan (NG2 glia). Additionally, survival of these newly generated cortical cells was affected by neonatal alcohol exposure, based on the greater reduction in the number of BrdU-labeled cells from PD50 to PD80 in the alcohol-exposed animals compared to controls. These findings demonstrate that neonatal alcohol exposure triggers an increase in gliogenesis in the adult motor cortex.

Keywords

Fetal Alcohol Spectrum Disorder; Gliogenesis

INTRODUCTION

Alcohol abuse during pregnancy can produce Fetal Alcohol Syndrome (FAS) or Alcohol-Related Neurodevelopmental Disorders which are collectively referred to as Fetal Alcohol Spectrum Disorders (FASDs) (Bertrand et al., 2005). The reported prevalence of FASDs varies widely and recent estimates approach 1% of live births (Sampson et al., 1997). Epidemiological studies by May and colleagues (May et al., 2004) indicate that mothers that give birth to FASD children typically binge drink. FASD is associated with damage to the developing central nervous system (CNS) including significant neuronal loss, microcephaly in children with FAS, and behavioral deficits that persist into adulthood (Bookstein et al.,

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Associate Editor:

Joseph L. Price, Washington University School of Medicine

2002; Jones, 1975; Mattson et al., 1996; Mattson et al., 2001; McGee et al., 2008; Sowell et al., 2008; Spohr et al., 1993; Spohr et al., 2007; Streissguth and O'Malley, 2000).

Use of animal models allows for the identification of alcohol as teratogen, the determination of specific targets of prenatal alcohol exposure, and detection of the mechanisms of damage. Animal models of developmental alcohol exposure have demonstrated behavioral effects that persist into adulthood, including impairments in learning/memory, motor performance, locomotor activity, and circadian regulation (Allen et al., 2005; Goodlett and Eilers, 1997; Klintsova et al., 2002; Stanton and Goodlett, 1998). In particular, adult rats exposed to alcohol during the neonatal period of rapid brain growth (first two postnatal weeks), the period of brain development comparable to that of the human 3rd trimester, are impaired on motor coordination tasks (Klintsova et al., 1998; Klintsova et al., 2002; Thomas et al., 2000; Thomas et al., 1998). These behavioral effects are accompanied by reductions in brain size and cell loss in several brain structures, including the cerebellum, hippocampus, thalamus, and neocortical areas (Bonthius and West, 1990; Byrnes et al., 2004; Goodlett et al., 1998; Mooney and Napper, 2005; Napper and West, 1995; Thomas et al., 1998).

Prenatal or neonatal alcohol exposure in rodent models can alter brain development through multiple mechanisms, including disrupted cell proliferation, altered migration, and enhanced apoptosis (Dunty et al., 2001; Ikonomidou et al., 2000; Miller, 1995; Miller and Potempa, 1990; Wozniak et al., 2004). Additionally, developmental neurogenesis and gliogenesis (Gressens et al., 1992; Miller, 1986; Rema and Ebner, 1999) as well as cellular migration to the appropriate cortical layer (Miller, 1986; Miller, 1987) are negatively affected by alcohol exposure during development. However, increased levels of stem cell proliferative activity occur after significant cortical damage, such as stroke (Arvidsson et al., 2002; Parent et al., 2002) or lesion (Magavi and Macklis, 2002; Sundholm-Peters et al., 2005), and atypical migration of stem cells toward the site of damage has been described (Magavi and Macklis, 2002). This raises the possibility that persistent effects of developmental exposure to alcohol could potentially alter cytotogenesis in the cortex. Emerging studies using quantitative MRI suggest that prenatal alcohol exposure impacts the organization of cortical regions (thickness and myelination) and that abnormalities may result from ongoing cellular changes (Sowell et al., 2008).

Glial cells are important to the development and function of the CNS. Previous studies have demonstrated that prenatal and postnatal alcohol exposure notably affects glial cells (Goodlett et al., 1993; Goodlett et al., 1997; Guerri et al., 2001; Miller and Robertson, 1993). Acute astrogliosis has been demonstrated in cortical areas after binge-like alcohol exposure in neonatal rats (Goodlett et al., 1993; Goodlett et al., 1997). Additionally, premature transformation of radial glia was shown after prenatal exposure to alcohol (Miller and Robertson, 1993). Low-dose, chronic prenatal alcohol exposure produced astroglial hypertrophy and increased GFAP and S-100 β immunoreactivity in the prefrontal cortex which lasted more than three weeks after birth (Evrard et al., 2003). Persistent neocortical astrogliosis was observed in adult rats following prenatal alcohol exposure (Fakoya, 2005). Patients exposed prenatally to alcohol have decreased white matter density (Archibald et al., 2001; Sowell et al., 2002) and delays in myelination of the white matter (Riikonen et al., 1999). However, it is not currently known whether neonatal alcohol exposure can have long-term effects on the generation and differentiation of glial cells in the mature cortex.

The current study investigated the hypothesis that neonatal alcohol exposure affects cytotogenesis in the adult motor cortex (MC). Additionally, the current study assessed whether early neonatal alcohol exposure had long-term effects on proliferation, survival, and phenotype of newly generating cells in the MC. The MC was chosen because postnatal

alcohol exposure is known to produce long-term deficits in motor performance (Thomas et al., 1996) and produce lasting structural changes in the MC.

MATERIALS AND METHODS

Subjects

Timed-pregnant Long-Evans rat dams (Simonsen, Gilroy, CA) were housed in the AALAC accredited facilities of the Department of Psychology, SUNY Binghamton and of the University of Delaware. Embryonic day 0 (ED0) was the day when vaginal plug was present and ED22 was postnatal day 0 (PD0); most of the dams gave birth on ED22. On PD3, litters were culled to 8 pups (4 males and 4 females when possible) and pup's paws were tattooed with India ink for permanent identification of littermates. On PD4, litters were assigned to either the intubation or suckle control groups. For the intubated litters, 2 male and 2 female pups were assigned within each litter to the **sham intubated (SI)** group and the other 2 males and 2 females were assigned to the **alcohol exposed (AE)** group. Rats from the undisturbed litters constituted the **suckle control (SC)** group and were left undisturbed with the exception of daily weighing from PD4-9 (Fig. 1B). All pups remained with their dam until weaning on PD21-23, at which time they were housed with same-sex littermates. Both male and female rats were used in the study. Care was taken in all procedures to minimize pain and discomfort. All procedures were in accord with the National Institutes of Health animal welfare guide and were approved by the Institutional Animal Care and Use Committee of Binghamton University and the University of Delaware Institutional Animal Care and Use Committee.

Neonatal Treatments

Milk and milk/ethanol treatment formulas were prepared from a base milk formula according to the previously described method (West et al., 1984) and were delivered by gavage via intragastric intubation as previously described (Goodlett and Johnson, 1997). For each round of intubations, the pups were removed from the dam as a litter and kept on a 37° C heating pad. A pre-measured length of PD-10 tubing was lubricated with corn oil and gently inserted down the pup's esophagus into the stomach, and the milk/ethanol formula was infused over a 20-sec period. During the PD 4-9 treatment period, the AE group was given two daily intubations of the milk/ethanol formula containing 11.9% (v/v) ethanol, with a 2-hour interval between intubations. Each intubation delivered 2.625 g/kg of ethanol resulting in a total daily dose of 5.25 g/kg for each animal. On PD4, AE pups received two additional intubations of milk formula alone (two 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 help limit growth deficiency that would otherwise result from the alcohol-induced impairment of suckling behavior. SI pups received intubations alone, without infusion of base milk, since milk infusions are known to abnormally accelerate the growth of SI pups (Goodlett et al., 1998).

Blood Alcohol Concentration

On PD4, 3.5 hours after the first 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. Blood alcohol concentrations (BACs) for AE animals were assayed from the plasma with a GL-5 Alcohol Analyzer (Analox Instruments, Lunenburg, MA). BAC levels were 315±19 mg/dl (mean ± SEM).

BrdU Injections

From PD30-50, each animal received an i.p. injection of the synthetic thymidine analog 5-Bromo-2-deoxyuridine (BrdU, 50 mg/kg in sterile 0.9% saline solution (20 mg/ml), i.p.) every other day between 10–11am. Because the period of interest spanned 20 days, pups were injected every other day to decrease the potential stress of daily injections over this period as well as resultant tissue scarring. On PD50, half of the animals within a given litter were perfused to determine cumulative levels of cytogenesis from PD30-50. The remaining animals were perfused on PD80 to ascertain the long term survival rate of the cells generated between PD30-50 (Fig. 1B).

Tissue Preparation

Animals were deeply anaesthetized (sodium pentobarbital, 100 mg/kg i.p.) then transcardially perfused first with 50 ml of heparinized 0.1M phosphate buffered saline (PBS, pH 7.2) followed by 250 ml 4% paraformaldehyde in 0.1M PBS. Brains were removed and placed in buffered sucrose solutions of increasing (10–30%) concentration until they sank. Serial coronal sections (40 μ m) containing the entire rostro-caudal extent of the motor cortex (MC) were cut on a cryostat microtome. Every section was collected and each section placed in an individual well. The order of the sections was maintained throughout the following procedures. Sections were stored at -20°C in cyroprotectant until use.

Antibody Characterization

The following information on primary antibodies used in this study is summarized in Table 1.

The rat monoclonal antibody directed against BrdU reacts with free BrdU and BrdU in single stranded DNA and detects nuclei of cells that incorporated BrdU into their DNA during the S-phase of the cell cycle (Vanderlaan and Thomas, 1985). This antibody has been used in numerous studies examining proliferation (Cameron and McKay, 2001; Kao et al., 2008). Anti-BrdU did not label tissues unexposed to BrdU (data not shown). To confirm BrdU immunostaining in the cortex, two proliferative zones of the adult rat were examined. BrdU labeling in the rostral hippocampal dentate gyrus (located in the most caudal sections of MC analyzed) and the subventricular zone of the lateral ventricle (located in the rostral sections of the MC analyzed) served as a positive controls. All animals exhibited similar and robust staining for BrdU labeling (Fig. 2). The effect of a binge-like postnatal alcohol exposure on adult neurogenesis in the dentate gyrus was demonstrated in a recent publication by our laboratory (Klintsova et al., 2007).

The mouse monoclonal antibody NeuN reacts with most neuronal cell types throughout the central nervous system of rodents and primarily stains the nucleus of the neurons (Mullen et al., 1992; Sarnat et al., 1998; Wolf et al., 1996). On Western blots, the antibody recognizes two to three bands in the 46–48 kDa range (Mullen et al., 1992; Unal-Cevik et al., 2004); manufacturer's technical information: <http://www.millipore.com/catalogue/item/mab377>). The antibody stained a similar pattern of cellular morphology and distribution in the rat hippocampus as found in previous studies (Bulloch et al., 2008; Cameron and McKay, 2001; Kao et al., 2008; Klintsova et al., 2007).

The rabbit polyclonal antibody Iba-1 was raised against the synthetic peptide corresponding to the Iba1 carboxy- terminal sequence (N'-PTGPPAKKAISELP-C') (Imai et al., 1996; Ito et al., 1998). Iba1 is a 17-kDa EF hand protein that is specifically expressed in macrophages/microglia (Imai et al., 1996). The antibody is specific to humans, mouse, and rat microglia and macrophage protein Iba1 and recognizes a single 17 kD band on Western blot (Imai et

al., 1996; Ito et al., 1998). We found a pattern of staining similar to that described in a previous study that used this antibody (Bullock et al., 2008; Kihira et al., 2007).

The rabbit polyclonal antibody GFAP was used to identify astrocytes in the central nervous system (Geddes et al., 1996; Oh and Prayson, 1999; Reske-Nielsen et al., 1987). GFAP is a 50 kDa intracytoplasmic filamentous protein that constitutes a portion of the cytoskeleton in astrocytes and is the principal intermediate filament of mature astrocytes (Eng et al., 2000; Rutka et al., 1997). The antibody identifies a 51 kDa band in immunoblots of protein extracts from mouse brains, corresponding to GFAP (Wishcamper et al., 2001). We found a pattern of staining similar to those described in previous studies that used this antibody (Castellano et al., 1991; Pecchi et al., 2007).

CNPase is highly expressed in oligodendrocytes in the central nervous system and immunohistochemical staining reveals selective staining of oligodendrocytes in the grey and white matter of rat brains (Sprinkle, 1989; Steiner et al., 2004). The mouse monoclonal antibody directed against CNPase localizes both 46kD (CNP1) and 48kD (CNP2) bands in an immunoblotting assay (Glaser et al., 2005; Sprinkle, 1989). A pattern of staining similar to that described in previous studies using this antibody was detected in our study (Schad et al., 2003; Steiner et al., 2004).

NG2 is found on the surfaces of an unusual class of glial cells within the developing and mature central nervous system (Nishiyama, 2001; Nishiyama et al., 1999). The mouse monoclonal antibody against NG2 reacts with native, recombinant, and core protein from cells which express NG2 (manufacturer's technical information: <http://www.millipore.com/catalogue/item/mab5384#>). The antibody is raised against the truncated (domain 3 D3, amino acids 1592–2222) form of NG2 (Ughrin et al., 2003) and recognizes the >280 kD NG2 protein on Western blots (manufacturer's technical information: <http://www.millipore.com/catalogue/item/mab5384#>). A pattern of staining similar to that described in previous studies that used this antibody was found in our study (Barberi et al., 2003; Dayer et al., 2005; Ohya et al., 2007; Tamura et al., 2007).

Fluorescence Immunohistochemistry

A systematic random sampling procedure was used in selecting the sections for processing. Sections containing the MC were identified using specific anatomical landmarks, including the anterior commissure, corpus callosum, and hippocampus. Within the pool of sections containing MC, every 16th section was placed in an individual well and processed for fluorescent immunohistochemistry.

Sections were stained using a previously published protocol (Dolbeare, 1995; Kuhn et al., 1997). For proliferation, survival, and phenotyping analysis, staining for anti-BrdU (1:1000, Accurate, Westbury, NY, USA) and another antibody was performed as follows: free-floating sections were rinsed in 0.1 M Tris-buffered saline (TBS), incubated for 2 hrs in 50% formamide and 2x SSC (0.3M NaCl, 0.03M sodium citrate) at 65°C, rinsed for 5 min in 2 × SSC, incubated for 30 min in 2N HCl at 37°C, and rinsed for 10 min in 0.1M boric acid in TBS, pH 8.6. Sections were incubated in TDS-TBS (0.5% Triton-X100 and 3% donkey serum in TBS) for 1 hr, followed 72 hrs at 4°C incubation of anti-BrdU and one of the following antibodies in TDS-TBS: anti-NeuN (1:500, Chemicon, Temecula, CA, USA), Iba-1 (microglia, 1:400, Wako, Richmond, VA, USA), GFAP (astrocytes, 1:500, Dako, Glostrup, Denmark), CNPase (mature oligodendrocytes, 1:200, Abcam, Cambridge, MA, USA), and NG2 (oligodendrocyte progenitors, 1:200, Chemicon, Temecula, CA, USA). Sections were rinsed in TBS then incubated in the biotinylated donkey anti-rat (to reveal BrdU labeling, Jackson Immuno Research, West Grove, PA, USA) and Cy3 secondary antibody (to reveal other antibody labeling, Jackson Immuno Research, West Grove, PA,

USA) in TDS-TBS for 2 hrs, and then with Cy2-conjugated streptavidin (to reveal BrdU labeling, Jackson Immuno Research, West Grove, PA, USA) in TDS-TBS for 2 hrs. Sections were mounted on slides and coverslipped with antifade media (ProLong Antifade Media, Molecular Probes/Invitrogen, Carlsbad, CA). Slides were sealed with nail polish and stored at -20°C . Information on the secondary antibodies used in this study is summarized in Table 2.

To assess staining specificity, negative controls were performed by omitting the primary antibodies.

Stereological Counting Procedure

BrdU-positive cell counts, on coded slides by an investigator blind to the treatment condition, were made within a known volume of MC using the optical fractionator probe (Stereo Investigator Version 8.10.1, MicroBrightField Inc., Williston, VT). To delineate the MC, each section from the chosen fraction (1/16) of the series of sections was matched to atlas plates and assigned the corresponding Bregma level (Paxinos, 2004) (Fig. 1A). StereoInvestigator software was used to generate a digital representation of each section and cell and provide an unbiased estimate of the number of labeled cells in the MC. Histological landmarks on low magnification digital images of each section were used to determine the borders of the MC. A digital outline of this border was created using Stereo Investigator and utilized during the cell counting procedure.

The Stereo Investigator software calculates the total volume of the outlined brain region taking into consideration the number of sections (section sampling fraction, $ssf = 1/16$) within the structure of interest and the number of the sampling sites within the cortical area on each section (area sampling fraction, $asf = 1$). In this study, the sampled fraction of the area was equal to 1: the overall small number of BrdU+ cells in the MC necessitated sampling of the entire region within each section. To accomplish this, the grid and counting frame in StereoInvestigator software were set to the same size ($200 \times 175 \mu\text{m}^2$). A guard zone of 2 μm and a dissector height of 20 μm were used. All BrdU+ cells within these parameters and within the outline of the MC borders were counted. For each labeled cell a corresponding digital marker was placed on the digital representation of the appropriate section. The frozen sections were originally cut at the nominal thickness of 40 μm . Immunostaining and mounting in the anti-fading media provided the opportunity for section thickness to change after processing. Section thickness was measured at every 4th counting site. An average section thickness was computed by the software and used to estimate the total volume of the MC sample region and total number of BrdU+ cells (thickness sampling fraction, $tsf = 20 \mu\text{m}/\text{section thickness}$). In this study, the mean measured thickness of the sections was 38.7 μm (range 36.1–38.5). The mean coefficient of error (CE) for the number of cells (between-section and within section variation) did not exceed recommended 0.1.

BrdU+ cells that appeared to be co-labeled with neuronal or glial markers were labeled with a distinct digital marker for later confocal microscopy (LSM 510 confocal microscope, Zeiss, Thornwood, NY). Phenotyping of these cells was performed on the 3D digital reconstructions and orthogonal representations from a series of confocal images taken at 0.5 μm intervals. Cells were identified as co-labeled if an overlap of the Cy2 and Cy3 labels was observed within a given cell in each of the xy-, xz-, and yz-planes in the orthogonal view. Additional assessment of co-labeling was performed on the opposite hemisphere from where the stereological counting was performed. Twenty five BrdU+ cells per animal were analyzed in NeuN stained tissue to further assess the possibility of a mature neuronal phenotype and at least 50 BrdU+ cells per animal were examined in glial stained tissue. This additional assessment was done to prevent underestimation of phenotypes due to the possible fluorescent bleaching that may have occurred during counting. The images in

Figure 2, Figure 4, and Figure 5 were moderately processed with the ‘brightness-contrast’ function in Zeiss LSM Image Browser (Zeiss, Thornwood, NY) to assist observations. Furthermore, image color was converted in Photoshop from red-green to magenta-green.

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 postnatal day as factors. The morphological data (MC volume and number 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

Body Weights

To determine if the neonatal alcohol exposure decreased pup growth, body weights during the exposure period (PD4-9) and on the first (PD30) and last (PD50) days of BrdU injection were compared across the three treatment groups. Repeated measures ANOVA of body weights from P4-9 yielded an interaction of age $\times$ treatment (ANOVA, $F(10,145)=4.27$, $p<0.05$). Newman-Keuls posthoc analysis of this result revealed that sham intubate (SI) animals had higher body weights than suckle control (SC) and alcohol exposed (AE) animals from PD5-P9 ($p<0.05$); the AE and SC groups did not differ significantly from each other. The body weight differences associated with faster growth of the SI group on PD5-9 were no longer evident when the animals reached early adulthood, and repeated measures ANOVA of body weights on PD30 and PD50 yielded no statistically significant differences among the three treatment groups (Table 3).

Effect of Early Postnatal Alcohol on Motor Cortex Cytogenesis Profiles on PD50

A known fraction of sections from animals perfused on PD50 (SC, $n=10$; SI, $n=9$; AE, $n=10$ animals) were immunostained using antibodies against NeuN and BrdU. Cell counts were converted into a percentage of BrdU-labeled cells relative to the average counts of the SC treatment group; the within-batch SC average was set to 100%. The relative number of BrdU + cells (as a percentage of the SC group) differed among the treatment groups (ANOVA, $F(2,24)=11.51$, $p<0.0005$; Fig. 3). Alcohol exposure increased the percentage of BrdU+ cells compared to the SC and SI groups (Newman Keuls, $p<0.002$; Fig. 3). The difference between SC and SI groups was not statistically significant.

Although care was taken to ascertain the borders of the motor cortex (MC) during analysis, the possibility existed that equivalent-sized areas were not outlined for each treatment group. To ascertain whether the differences in BrdU labeling may have reflected variations in establishing the borders of the MC, the volume of the MC within the anatomical boundaries was compared among the treatment groups, and no significant difference was found (ANOVA, $F(2,26)=0.43$, $p=0.65$).

Phenotype of BrdU Labeled Cells

To determine whether BrdU+ cells were neuronal, each BrdU+ cell that appeared double-labeled with NeuN during the initial analysis under epifluorescence was identified and later examined using confocal microscopy. A total of 10 BrdU+ cells across all groups appeared double-labeled under regular fluorescence. However, confocal analysis revealed that no BrdU+ cell examined in adult MC was also NeuN+, that is, neuronal (Fig. 4A). To assure this outcome, an additional 25 BrdU+ cells, from the opposite hemisphere from that where BrdU cell counts were obtained, were phenotyped and none of them co-labeled with NeuN.

In addition, quantitative assessment of sections from each treatment group was performed for BrdU co-labeling with glial markers: microglia (Iba-1, Fig. 4B), astrocytes (GFAP, Fig. 4C), and oligodendrocytes (CNPase, Fig. 4 D; NG2, Fig. 4 E and Fig. 5). Less than 1% of BrdU+ cells were positive for GFAP, approximately 1–2% were positive for Iba-1, approximately 10% were positive for CNPase, and 61% were positive for NG2 (Fig. 4F). These distributions did not differ statistically across treatment groups.

Effect of Early Postnatal Alcohol on the Survival of New Cells in Adult MC at PD80

Extended survival of the new MC cells that were proliferating during the BrdU injection period (PD30-50) was assessed in rats perfused on PD80, 30 days after the final BrdU injection. In contrast to the group differences evident on PD50, the three neonatal treatment groups (SC, n=6; SI, n=6; AE, n=5 animals) did not differ significantly from each other in the number of BrdU-labeled cells (ANOVA, $F(2,14)=0.90$, $p=0.43$; Fig. 6A). This suggested that the neonatal alcohol exposure group had a decreased survival rate of adult-generated cells. No significant differences were found in the MC volume among the treatment groups (ANOVA, $F(2,14)=1.32$, $p=0.30$). To examine the phenotype of these surviving cells in the MC, quantitative assessment of sections from each treatment group was performed for BrdU co-labeling with glial marker NG2. Analysis revealed that 78% of BrdU+ cells in the control animals and 80% of BrdU+ cells in alcohol-exposed group co-expressed NG2. Thus this data suggests that neonatal alcohol exposure decrease the rate of survival in newly generated adult glial cells.

Direct Comparison of Early Postnatal Alcohol Effects on MC Cytogenesis at PD50 and PD80

Given that the number of BrdU-labeled cells (generated during the PD30-50 labeling period) did not differ among the SC, SI, and AE groups at PD80, together with the observed increase in BrdU+ cells in AE animals at PD50, it appeared that the percentage of newly generated cells surviving to PD80 in AE animals was significantly lower than in controls. To test this, a separate set of sections from male SI and AE animals at PD50 and PD80 were stained and analyzed concurrently to allow for direct comparison among the four groups. Sixty percent of the animals included in this comparison were also used in the initial analyses. The total number of MC cells that were proliferating during the BrdU injection period was assessed in this cohort of PD50 and PD80 rats (SI, n=3; AE, n=3). A two-way factorial ANOVA on postnatal treatment (SI, AE) $\times$ age (PD50, PD80) yielded a significant interaction [$F(1,8)=9.40$, $p<0.05$; Fig. 6B]. Posthoc analysis revealed a significant decrease in the number of BrdU+ cells at PD80 compared to PD50 in the AE group (Newman-Keuls, $p<0.05$), but not in the SI group. No significant differences were found in MC volume between the SI and AE groups at the two ages.

DISCUSSION

The current study demonstrates that binge alcohol exposure during the early postnatal period in rodents, a period of accelerated brain growth comparable to the processes and growth during the human third trimester, has enduring effects on adult motor cortex (MC) gliogenesis. Alcohol exposure on postnatal day (PD) 4–9 increased the cumulative number of neocortical cells generated and surviving between PD30-50 as demonstrated by the greater number of BrdU+ cells in the alcohol exposed (AE) group at PD50. After an additional 30 days of survival (at PD80), a significantly greater decrease in BrdU+ cells was found in the AE animals than in controls, suggesting that early postnatal alcohol exposure interfered with conditions that promote the long-term survival of these newly generated cells. Confocal microscopy revealed that the majority of these newly generated and surviving MC cells possessed a glial phenotype, predominately NG2-positive glia. A small

number of these newly generated cells expressed CNPase (mature oligodendrocyte marker). These results demonstrate that alcohol exposure during the neonatal period has long-term consequences for adult cortical gliogenesis.

Gliogenesis is known to continue in the adolescent and adult rat neocortex and the majority of generated cells express the oligodendrocyte-specific phenotype and markers (Levison et al., 1999; Ling and Leblond, 1973; Reynolds and Hardy, 1997). The number of oligodendrocytes in the rat neocortex increases 3.5 times between PD30 and 5 months of age while the number of microglia and astrocytes do not change significantly (Ling and Leblond, 1973). Although data on the ratio between neurons and glia in rat MC is not readily available, parietal and occipital cortices are estimated to have two times more neurons than glial cells on PD10 (Mooney and Napper, 2005). This ratio is decreased significantly by PD115 due to the increase in glial cell number (not a decrease in neuronal counts) with age (Mooney and Napper, 2005; Peinado et al., 1997).

The BrdU dose used in our study was convincingly demonstrated to produce labeling only in dividing cells and not sufficient to identify the DNA repair (Cameron and McKay, 2001; Cooper-Kuhn and Kuhn, 2002). Unfortunately, the cause for the transient increase in gliogenesis detected in our study is not known. It is possible that such an increase could be an enduring compensatory response to the widespread degeneration in the CNS due to the neonatal exposure to alcohol (Hansen et al., 2008; Ikonomidou et al., 2000; Kuhn and Cooper-Kuhn, 2007).

Similar to our findings, studies by Kodama and colleagues (Kodama et al., 2004) and Czeh and colleagues (Czeh et al., 2007) reported increased glial cell genesis in cortical areas following chronic antipsychotic administration or chronic stress treatments. Also consistent with previous studies (Ehninger and Kempermann, 2003; Koketsu et al., 2003; Kornack and Rakic, 2001; Magavi et al., 2000), we did not observe any newborn cells in the MC that acquired a mature neuronal phenotype (BrdU+/NeuN+ cells). However, it would be premature to claim a complete absence of neurogenesis in adult MC, since we did not test various markers for interneurons (Dayer et al., 2005).

Previous studies investigating the effects of developmental exposure to alcohol on adult cytotogenesis have examined the hippocampal dentate gyrus and reported a decrease either in proliferation or survival after prenatal alcohol exposure (Choi et al., 2005; Redila et al., 2006). Following postnatal exposure increases in progenitor proliferation and decreases in new cell survival have been demonstrated (Klintsova et al., 2007; Miller, 1995; Zharkovsky et al., 2003). However, such alterations in cytotogenesis may not reflect a generalized response across brain regions to alcohol insult since it has been shown that alcohol does not alter cytotogenesis in adult olfactory bulb (Herrera et al., 2003). Some caution must be exercised in comparing the effect of alcohol on adult neocortical and hippocampal cytotogenesis. The effect of developmental alcohol exposure on a brain region depends, in part, on the developmental stage of the given region at the time of exposure. The MC and hippocampal dentate gyrus possess markedly different developmental profiles. During rat development, neocortical neurogenesis commences at embryonic day (E)13 and reaches completion by E19 (Bayer et al., 1993). In contrast, developmental neurogenesis in the dentate gyrus peaks at PD0 and continues to PD21 and beyond (Bayer et al., 1993). In adulthood, development-like neuronal proliferation continues in the hippocampal dentate gyrus, but is absent in the rat neocortex under normal conditions (Altman and Das, 1965; Bayer et al., 1993; Cameron et al., 1993; Magavi et al., 2000). Thus, due to the differences in the developmental timetables and adult neurogenic capacity between the hippocampus and cortex, alcohol exposure at a given age would impair distinctly different processes in these brain regions both during development and in adulthood. Although alcohol exposure

results in dysregulated adult cytogenesis across these brain regions, the specific nature of the impairment as well as the mechanism of action may differ between regions.

To our knowledge, no previous report has examined the effect of postnatal alcohol exposure on adult cytogenesis in the neocortex. However, other studies have determined the effect of early postnatal brain injury, via hypoxic and/or ischemic insult, on adult cortical cytogenesis and provide for a useful comparison. Our findings demonstrate that early postnatal alcohol exposure increased cumulative cytogenesis in adolescence and early adulthood. Consistent with this finding, mice reared in a low oxygen environment from PD3-11 and injected with BrdU on PD18 possessed 40% more BrdU+ cells in the cerebral cortex one month after injury than those reared under normal conditions (Fagel et al., 2006). Additionally, 80% of BrdU+ cells in both the experimental and control animals possessed a glial phenotype (Fagel et al., 2006). Similarly, rats subjected to hypoxic-ischemic injury at PD7 and injected with BrdU on PD12-14 showed increased numbers of BrdU+ cells in the subventricular zone at PD35 and these cells possessed glial phenotypes (Ong et al., 2005). Together, these results and ours suggest that the alcohol-induced increase in adult cortical cytogenesis may be part of a generalized, persisting response of the cortex to injury that has occurred during the early postnatal period.

In addition to alterations in adult glial genesis, our data suggests that alcohol exposure during the brain growth spurt may affect the survival of newly generated glial cells. We tested this directly by staining tissue concurrently from a cohort of sham intubated (SI) and AE animals sacrificed at PD50 or PD80. AE, but not SI, animals showed a significant decrease in the number of BrdU+ cells at PD80 compared to PD50. This decrease from PD50 to PD80 in the AE group may be a response to the augmentation of BrdU+ cells seen at PD50. However, whether this represents a beneficial adaptation, rather than a response to limitations of metabolic resources and space for growth, is not clear from our current data. Alcohol was shown to elongate the cell cycle resulting in a lower rate of turnover of proliferating cells (Siegenthaler and Miller, 2005). Thus, the significant decreases of BrdU+ cells in AE animals after 30 days of survival cannot be attributed to the faster dilution of the BrdU labeling if AE cells proliferate at a faster rate.

Newly generated cells in the adult brain are derived from multipotent progenitor cells which can differentiate into either glia or neurons (Emsley et al., 2005; Ming and Song, 2005). In the current study, most of the MC BrdU+ cells possessed a glial phenotype and none possessed a neuronal (NeuN+) phenotype, suggesting that the enhancement of adult MC cell proliferation induced by postnatal alcohol exposure corresponds to an increase in glia. The majority of alcohol studies examining gliogenesis have focused on the acute effect of pre- or postnatal alcohol rather than on long-term consequences later in adulthood, although longer term events may have more salient implications for therapeutic intervention. Prenatal alcohol exposure results in diminished GFAP expression at PD4 (Guerri et al., 2001; Rubert et al., 2006; Valles et al., 1997), decreased radial glia progenitor pool and its transformation into neurons and astrocytes (Rubert et al., 2006), and accelerated early postnatal transformation of cortical radial glia (Miller and Robertson, 1993). In contrast, alcohol exposure from PD4-9, via artificial rearing or intragastric intubation, transiently increases GFAP expression in the cortex from PD10-15 associated with a transient reactive astrogliosis (Goodlett et al., 1993; Goodlett et al., 1997). These studies suggest that alcohol acutely disrupts gliogenesis and development in the cortex both in the short term and on a continuing basis.

Glial cells are vulnerable to prenatal alcohol exposure (reviewed in (Guerri et al., 2001)). Prenatal alcohol exposure has been shown to induce persistent neocortical astrogliosis, as demonstrated by GFAP immunohistochemistry, in adult rats (Fakoya, 2005). Quantitative

assessment of various glial markers in our study revealed that the majority of BrdU+ cells in the MC possess a glial phenotype, predominantly expressing antigens specific for mature (CNPase) oligodendrocytes and their progenitors (NG2). The overall level of cell proliferation was increased by 90% in the AE animals across PD30-50, immediately after completion of BrdU injections. Sixty percent of these newly generated cells expressed NG2 chondroitin sulfate proteoglycan, thus the majority of the newly generated cells in adult MC are NG2 glia. Presence of NG2+ cells in the population of adult-generated cells in MC becomes even more pronounced 30 days after the last BrdU injection: about 80% of BrdU+ cells on PD80 express NG2 immunoreactivity. This finding is important because some NG2+ cells can express neuronal markers and may serve as resident precursors of GABAergic cortical interneurons (Dayer et al., 2005; Sellers and Horner, 2005). NG2-expressing cells in adult brain can represent oligodendrocyte (and some protoplasmic astrocytes) progenitor cells (Aguirre and Gallo, 2004; Horner et al., 2002; Nishiyama et al., 2002; Zhu et al., 2008) or mature NG2 glia (called the fourth type of glia, polydendrocytes or synantocytes) (Berry et al., 2002; Butt et al., 2002; Peters, 2004; Peters and Sethares, 2004), thus comprising a heterogeneous population of cells. These cells are abundant in both gray and white matter of the adult central nervous system (Horner et al., 2002; Shenton et al., 2001). NG2 glia is a highly reactive cell type that responds to various CNS injuries (physical, excitotoxic, chemical, or X-irradiation) (reviewed in (Levine et al., 2001). NG2 glia responds rapidly to injury through increased proliferation and outgrowth of processes (Butt et al., 2002; Dehn et al., 2006). Expression of these cells remains elevated for days to weeks after the insult (Dehn et al., 2006; Hampton et al., 2004), suggesting that the decrease in survival of BrdU+ cells seen in the current study is related either to discontinuation of this elevation or to a continuing relatively high turnover of these cells. The results from the current study suggest that alcohol exposure during the brain growth spurt induces changes in MC NG2 glia genesis that persist into adolescence. Alterations in NG2 glia genesis following postnatal alcohol exposure may be indicative of cortical recovery.

Several lines of evidence suggest that altered adult cortical gliogenesis, rather than neurogenesis, may be a likely potential consequence of postnatal alcohol exposure. Whereas adult neocortical neurogenesis is either lacking (Koketsu et al., 2003; Kornack and Rakic, 2001; Rakic, 2002) or transient (Gould et al., 2001), there is strong evidence for cortical gliogenesis in the adult (Gensert and Goldman, 1996; Gensert and Goldman, 2001; Levison et al., 1999). As discussed previously, neonatal hypoxia enhanced subsequent cortical cytogenesis in which 80% of the BrdU+ cells possessed a glial phenotype (Fagel et al., 2006). Taken together, these studies suggest that enhanced cortical gliogenesis in adulthood may be a likely response to postnatal cortical damage.

In conclusion, the present study demonstrates a persistent dysregulation in the cytogenesis and long-term survival of cells, particularly those possessing a NG2 glial phenotype, in the adult MC after alcohol exposure during the brain growth spurt. These findings suggest that dysregulated cortical cytogenesis in adulthood may be an additional consequence of (or compensatory response to) the enduring effects of neonatal alcohol-induced brain damage and may even play a role in the persisting behavioral deficits evident in Fetal Alcohol Syndrome. The mechanisms associated with failed survival in the alcohol-exposed groups and the consequences of dysregulated cytogenesis on neuronal signaling and synaptic formation need to be addressed by future studies. Additionally, these results raise the possibility that treatment strategies targeting adult cortical cytogenesis may be one approach towards ameliorating the effects of developmental alcohol exposure.

Acknowledgments

The authors would like to thank Lee Whitcher for his technical assistance with the PD80 animals and Kimberly Edgar for assistance in tissue processing.

Grant sponsor: National Institutes of Health; **Grant number:** AA09838

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Figure 1. Schematic of motor cortex region analyzed and experimental design. A) Representative atlas plates from Bregma 0.48 ± 0.3 through -2.12 ± 0.3 used in analyzing the motor cortex. Drawing adapted from Paxinos and Watson (2004). B) Schematic of experimental design described in the Methods section.

Figure 2.

Confocal images of BrdU labeling in the adult rat brain. Animals were sacrificed 2 hours after the last BrdU injection. A) NeuN (magenta) labeling B) BrdU (green) labeling and C) the merged image of the labeling in the motor cortex. D) NeuN (magenta) labeling E) BrdU (green) labeling and F) the merged image of the labeling in the hippocampal dentate gyrus. G) NeuN (magenta) labeling H) BrdU (green) labeling and I) the merged image of the labeling in the lateral ventricle. Numerous BrdU-labeled cells were observed throughout the dentate gyrus and lateral ventricle. BrdU-labeled cells were sparse in the motor cortex as compared to the dentate gyrus and lateral ventricle. The effect of a binge-like postnatal alcohol exposure on the cell proliferation and adult neurogenesis in the dentate gyrus was demonstrated in a recent publication by our laboratory (Klintsova et al., 2007). Scale bars = 50 μ m in A, B, C, G, H, I; 100 μ m in D, E, F. BrdU, bromodeoxyuridine; NeuN, neuronal nuclei.

Figure 3.

Postnatal alcohol exposure increased the number of BrdU+ cells on PD 50 in the motor cortex in the AE group compared to SC and SI groups. No difference was found between the SC and SI groups. PD, postnatal day; SC, suckle control; SI, sham intubated; AE, alcohol-exposed. Data expressed as mean $\pm$ SEM. * $p < 0.05$ relative to both control groups.

Figure 4.

Analyses of BrdU phenotype in the motor cortex. Sections of MC stained with BrdU (green) and A) NeuN (magenta), B) Iba1 (magenta), C) GFAP (magenta), D) CNPase (magenta), and E) NG2 (magenta). F) Percentages of BrdU phenotypes in the motor cortex at PD 50. Analysis of BrdU+ cell phenotypes revealed that the majority of these cells expressed the glial marker NG2. We found no BrdU+ cells in adult motor cortex in any group that expressed NeuN+, that is, was of a neuronal phenotype. Scale bars = 10 μ m for A – E. BrdU, bromodeoxyuridine; NeuN, neuronal nuclei; PD, postnatal day; GFAP, glial fibrillary acidic protein.

Figure 5.

BrdU and NG2 co-localizes in the motor cortex of adult rats. A) Confocal image of BrdU (green) and NG2 (magenta) labeling. Arrows indicate co-localization and the cells displayed in B–E). B) NG2 (magenta) labeling, C) BrdU (green) labeling and D) the merged (demonstrating co-localization) image of the two labels. For verification of co-localization, the x-, y-, and z- planes of the confocal image were analyzed by orthogonal view. E) Orthogonal view of BrdU (green) and NG2 (magenta) co-localization. Scale bars = 10 μm. BrdU, bromodeoxyuridine.

Figure 6.

A) Alcohol exposure on PD 4–9 did not alter the number of surviving BrdU+ cells at PD80 in the adult motor cortex. There were no significant group differences in the number of BrdU+ cells. PD, postnatal day; SC, suckle control; SI, sham intubated; AE, alcohol-exposed. Data expressed as mean $\pm$ SEM. B) Alcohol exposure decreases the number of surviving BrdU+ cells at PD80 relative to PD50 in the adult motor cortex. PD, postnatal day; SI, sham intubated; AE, alcohol-exposed. Data expressed as mean $\pm$ SEM. * $p < 0.05$.

Table 1
Primary Antibodies Used in Rat Tissue

Antibody	Immunogen	Host	Dilution	Source
Bromodeoxyuridine (BrdU) monoclonal	Purified BrdU	Rat	1:1000	OBT0030; Accurate, Westbury, New York
Neuronal nuclei (NeuN) monoclonal	Purified cell nuclei from mouse brain	Mouse	1:500	MAB377; Chemicon, Temecula, California
Iba1 polyclonal	Synthetic peptide corresponding to C-terminus of Iba-1	Rabbit	1:400	019-19741; Wako, Richmond, Virginia
Glial fibrillary acidic protein (GFAP) polyclonal	GFAP isolated from cow spinal cord	Rabbit	1:500	Z0334; Dako, Glostrup, Denmark
CNPase monoclonal	Purified full length native protein	Mouse	1:200	ab6319; Abcam, Cambridge, Massachusetts
NG2 chondroitin sulfate proteoglycan monoclonal	Cell line expressing a truncated form of NG2	Mouse	1:200	MAB5284; Chemicon, Temecula, California

Table 2
Secondary Antibodies Used in Rat Tissue¹

Antiserum	Dilution	Code Number
Biotin-SP donkey anti-rat IgG	1:1000	712-065-150
Streptavidin Cy2	1:588	016-220-084
Donkey anti-mouse IgG Cy3	1:200 or 1:250	715-165-151
Donkey anti-rabbit IgG Cy3	1:250	711-165-152

¹ All secondary antibodies were purchased from Jackson Immuno Research (West Grove, PA, USA). Cy2, cyanine; Cy3, indocarbocyanine.

Table 3

Weights (grams $\pm$ SEM) of Animals

Group	Postnatal Day									
	4	5	6	7	8	9	30	50		
SC (n=11)	9.5 $\pm$ 0.3	10.6 $\pm$ 0.4	12.0 $\pm$ 0.5	13.4 $\pm$ 0.5	15.1 $\pm$ 0.5	16.7 $\pm$ 0.6	103.6 $\pm$ 4.6	225.3 $\pm$ 5.9		
SI (n=11)	9.9 $\pm$ 0.3	11.4 $\pm$ 0.4 ^a	12.9 $\pm$ 0.4 ^a	14.8 $\pm$ 0.5 ^a	16.5 $\pm$ 0.6 ^a	18.5 $\pm$ 0.6 ^a	106.8 $\pm$ 3.6	240.5 $\pm$ 5.6		
AE (n=10)	9.9 $\pm$ 0.1	10.8 $\pm$ 0.3	11.9 $\pm$ 0.4	13.2 $\pm$ 0.5	14.8 $\pm$ 0.5	16.7 $\pm$ 0.6	96.6 $\pm$ 2.6	227.8 $\pm$ 6.6		

Differences in weight were found from PD5-PD9. No statistically significant differences were found among treatment groups from PD30 to PD50. SC, suckle control; SI, sham intubated; AE, alcohol exposed. Repeated Measures ANOVA, F(10,145)=4.27, Age $\times$ Treatment Interaction; Neuman-Keuls post-hoc,

^a p<0.05 different from the other two treatment groups.