Hippocampal Cell Proliferation Is Reduced Following Prenatal Ethanol Exposure But Can Be Rescued With Voluntary Exercise

Van A. Redila, Andrea K. Olson, Sarah E. Swann, Gisou Mohades, Alina J. Webber, Joanne Weinberg, and Brian R. Christie

ABSTRACT: The ingestion of ethanol during pregnancy has a number of deleterious consequences for the unborn offspring, producing structural and functional deficits that affect the brain and many other organs into adulthood. The hippocampus is a brain area that is particularly sensitive to ethanol’s adverse effects. In a previous study we showed that voluntary exercise can ameliorate deficits in long-term potentiation and behavior that occur following prenatal ethanol exposure (Eur J Neurosci, 2005, 21, 1719–1726). In the present study, we investigated the effects of prenatal ethanol exposure on neurogenesis in adulthood, and tested the hypothesis that voluntary exercise would ameliorate any deficits observed. Sprague-Dawley females were administered one of three diets throughout gestation: (i) ethanol (E), a liquid diet containing 36.5% ethanol-derived calories; (ii) pair-fed (PF), a liquid control diet, with maltose-dextrin isocalorically substituted for ethanol, in the amount consumed by an E partner (g/kg body wt/day of gestation); and (iii) ad-libitum-fed control (C), normal laboratory chow and water, ad libitum. The offspring were housed individually at postnatal day (PND) 35, and at PND 50 were randomly assigned to cages either with or without an exercise wheel. BrdU (200 mg/kg, I.P.) was injected on PND 57, and animals terminated either 24 h (proliferation) or 4 weeks (neurogenesis) later. Our results demonstrate that prenatal ethanol exposure significantly decreases both cell proliferation and neurogenesis in the adult dentate gyrus. Animals in the PF condition also showed reduced neurogenesis. In contrast, all animals that engaged in voluntary exercise showed a significant increase in cell proliferation and neurogenesis. These results indicate that prenatal ethanol exposure can suppress both cell proliferation and neurogenesis, and that these effects may be, at least in part, nutritionally mediated. Importantly, voluntary exercise appears to have beneficial effects for these long-lasting deficits in hippocampal volume and cell number that have been observed in animals exposed to ethanol in utero. © 2006 Wiley-Liss, Inc.

KEY WORDS: neurogenesis; dentate gyrus; running; BrdU; ethanol

INTRODUCTION

Prenatal exposure to high levels of alcohol can result in growth retardation, neurodevelopmental abnormalities, and a set of characteristic facial anomalies, that together are termed fetal alcohol syndrome (FAS; Jones et al., 1973; Stratton and Battaglia, 1996). Exposure to levels of alcohol that do not produce the full syndrome can result in partial FAS, where only some components of the facial anomalies and growth and neurodevelopmental deficits are present. Even in cases where lower doses of alcohol do not produce any facial anomalies, children may still exhibit neurodevelopmental or behavioral deficits, a condition termed alcohol-related neurodevelopmental disorders, or physical malformations, termed alcohol-related birth defects. Together, these alcohol-related conditions have been grouped under the umbrella term of fetal alcohol spectrum disorder (Sokol et al., 2003). Importantly, prenatal alcohol exposure, even at social drinking levels, has been linked to learning, memory, and behavioral problems that can affect academic and social functioning throughout life (Sokol et al., 2003). Exposure to alcohol in utero has been implicated as the most common cause of mental retardation, and in the United States is the leading preventable cause of birth defects (Stratton and Battaglia, 1996), with estimates of FASD incidence approaching 1 in 100 births (Sampson et al., 1997).

Animal models of FAS have been vital to our understanding of the neural structures and behaviors affected by prenatal ethanol exposure (E); (Berman and Hannigan, 2000). In utero ethanol exposure or neonatal exposure during the brain growth spurt induces numerous cognitive and behavioral deficits. Of relevance to the current study are findings of impaired spatial learning and memory in the offspring (Blanchard et al., 1987; Kelly et al., 1988; Gianoulakis, 1990; Kim et al., 1997; Gabriel et al., 2002; Richardson et al., 2002; Savage et al., 2002; Iqbal et al., 2004; Christie et al., 2005), with animals exhibiting deficits similar to those observed following bilateral lesions of the hippocampus (Morris et al., 1982). In addition, long-term potentiation (LTP), a biological model for learning and memory processes, is also impaired in E animals (Sutherland et al., 1997; Richardson et al., 2002; Savage et al., 2002; Christie et al., 2005). In part, the functional changes in hippocampal synaptic plasticity may reflect the alterations in hippocampal structure induced by prenatal ethanol exposure. Decreased neuronal density in the CA1
and Modified Lieber-DeCarli High Protein Control Liquid Diet (Dyets no. 710109), respectively. Fresh diet was presented daily at 1700–1900 h. This feeding schedule permits maintenance of a relatively normal corticoid rhythm in the PF dams, as the corticoid rhythm in animals receiving a reduced ration, such as the PF animals, re-entains to the time of feeding (Gallo and Weinberg, 1981). Bottles from the previous day were removed and weighed to determine the amount consumed. Experimental diets were continued until gestational day (GD) 22, at which time they were replaced with standard laboratory chow and water, ad libitum. All animal use procedures were in accordance with the Canadian Council on Animal Care and the NIH Guidelines for the Care and Use of Laboratory Animals, and were approved by the University of British Columbia Animal Care Committee.

The pregnant females were weighed on GDs 1, 7, 14, and 21. Forty-eight hours after birth, on postnatal day (PND) 2, the pups and the mother were weighed and the litters were culled to 10 (5 females and 5 males, when possible), to control for any confounding effects of litter size or sex ratio. The pups were weighed on PNDs 2, 8, 15, and 22 and were weaned on PN22, after which the offspring were group housed (3–5 cages) according to litter and sex in clear polycarbonate cages (46 × 24 × 20 cm³), with ad libitum access to standard rat chow and water.

**Experimental Subjects**

Offspring were singly housed on PN 35, and on PN 50 males in each group were randomly assigned to the standard cage condition (STD) or to the voluntary exercise (VEx) condition. A maximum of two animals from any one litter was assigned to any one group. Animals in the VEx group were housed in clear polycarbonate cages equipped with stainless steel running wheels connected to a PC computer (Mini-Mitter Systems Inc., WA). Animals were injected intraperitoneally with BrdU (Sigma; 200 mg/kg at a volume of 20 ml/kg) 1 week after being placed in their experimental cages, and then sacrificed either 24 h (proliferation) or 4 weeks (neurogenesis) later.

**Blood Alcohol Concentration Measurements**

The average ethanol intake for the Ethanol fed dams was 11.48 ± 0.54 g/kg body wt/day. To assess peak blood ethanol concentrations (BECs), tail blood samples were taken on GD 15, 2 h after the presentation of the ethanol diet. Serum was extracted and BECs were measured using an alcohol reagent set (no. A7504–150) obtained from Pointe Scientific Inc (Lincoln Park, MI).

**BrdU Immunocytochemistry**

The thymidine analogue BrdU (5-Bromo-2'-deoxyuridine) immunocytochemistry was used to label dividing cells in the DG as we have previously described (Eadie et al., 2005). At termination, animals were given a lethal dose of sodium pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge Ont.) and perfused transcardially with 60 ml of 0.9% saline, followed by 60 ml of 4% paraformaldehyde. Brains were removed and
stored in 4% paraformaldehyde at 4°C overnight and then transferred into a 30% sucrose solution until saturated. Coronal slices (40 μm) were obtained throughout the entire extent of the hippocampus using a vibratome (Leica, VT1000S). Every 12th section was then used for BrdU immunocytochemistry. Free-floating sections were washed thrice for 5 min each with Tris-buffered saline (TBS, 0.15 M NaCl, 0.1% Tris-HCl, pH 7.5) and treated with 0.6% H2O2 in TBS for 30 min to block endogenous peroxide activity. Sections were then incubated for 2 h in 50% formamide/2× SSC (0.3 M NaCl and 0.3 M sodium nitrate) at 65°C, rinsed in 2× SSC (5 min), incubated in 2 N HCl at 37°C (30 min), and then placed in 0.1 M boric acid (pH 8.5) for 10 min. Sections were then rinsed in TBS 6 times for 15 min each, incubated in TBS ++ (TBS, 0.1% Triton X-100 and 3% normal donkey serum) for 30 min and then incubated in anti-BrdU (Chemicon, 1:100) for 16 h at 4°C. After being rinsed with TBS thrice for 5 min, sections were incubated in an avidin–biotin-peroxidase solution (Vector Laboratories, ABC Elite kit) for 2 h and then visualized with diaminobenzidine (Vector Laboratories, DAB SK-4,100 kit). Finally, tissue was mounted and on the next day cell bodies were visualized with a cresyl violet stain in the following solutions; 100% ethanol (1 min), 95% ethanol (30 s), 70% ethanol (30 s), dH2O (30 s), a 0.2% cresyl violet solution (5 min), dH2O (30 s), 70% ethanol (30 s), differentiator (85% of 100% ethanol, 15% chloroform) for 1–2 min, 95% ethanol (30 s), 100% ethanol (30 s), xylene (twice for 5 min each). Slides were then cover slipped with Permount.

**Immunohistochemistry Quantification and Analysis**

We used a conservative stereological approach to quantify BrdU-labeling in the DG of VEx and STD animals for the E, PF, and C groups. From each animal, every 12th coronal section was assessed from the rostral to caudal end of the hippocampus. In the x–y plane, immunopositive cells limited to the neurogenic subgranular zone of the DG, defined as zero to two cell bodies from the inner edge of the molecular layer, were manually counted with the investigator blinded as to group identity. In the x-plane, a modified optical dissector method was employed, in which cells in the uppermost region of the focal plane were not counted. The DG and hilus in both hemispheres were counted for each section. Split nuclei cells, or cells that have not completed cytokinesis, were counted as a single cell, again to provide a conservative estimate. The mean number of BrdU positive cells per section was then used in statistical analyses. This number was multiplied by 96 to provide an estimate of the total number of BrdU cell per hippocampus.

**Statistical Analyses**

The number of BrdU-positive cells per 40-μm section was calculated as a mean, and then multiplied by 96 to estimate the total number of BrdU cells. The mean number of BrdU-positive cells was calculated for both proliferation and survival. Finally, the mean area (μm²) of the DG was calculated for each hippocampus. Factorial ANOVAs were performed to compare the two test conditions (VEx and STD) and the three prenatal treatments (E, PF, and C). Duncan’s post hoc analyses were performed as warranted. All statistical analyses were performed using Statistica software (Statsoft Inc., Tulsa, OK).

### RESULTS

#### Pregnancy Outcome

All pregnant females gained weight throughout gestation and appeared healthy. The average consumption of the ethanol diet was 221.47 ± 10.43 g/kg body wt/day. The average consumption of the isocaloric control diet (PF condition) was 220.42 ± 7.45 g/kg body wt/day. The mean BEC for the E dams was 183.6 ± 22.54 mg/dl. These values are consistent with previous studies that have employed the same breeding and feeding protocol and reflect a peak BEC level that is known to induce biological and behavioral deficits in the offspring (Keiver and Weinberg, 2003, 2004; Christie et al., 2005). A repeated measures analysis of variance (ANOVA) found no gestational weight by diet interaction throughout gestation ($F_{(2,45)} = 1.116$, $P = 0.3365$, see Table 1). There were no differences in litter size, proportion of male and female offspring, spontaneous resorptions or maternal morbidity. On average, however, E females gave birth ~1.0 day later than that of PF animals and 0.75 days later than that of control animals ($F_{(2,39)} = 13.00$, $P = 0.002$), reflecting the fact that ethanol delays parturition (Keiver and Weinberg, 2003, 2004; Christie et al., 2005). The offspring in the E group initially weighed less than that of both PF and C pups on PND 2, but body weights did not differ among groups by PND 15. Perinatal death occurred in 7 PF pups from one litter due to maternal cannibalism.

#### Prenatal Ethanol Exposure Reduces Cellular Proliferation in the DG, But Exercise Can Rescue This Deficit

One week after being placed in their experimental cages, E, PF, and C animals in both the STD and VEx conditions received a single injection of BrdU (200 mg/kg) and were sacrificed 24 h later. A two-way factorial ANOVA revealed significant main effects of both the adult treatment condition (STD vs. VEx; $F_{(1,24)} = 31.98$, $P = 0.000008$) and prenatal diet group (E, PF, or C; $F_{(2,24)} = 5.58$, $P = 0.0108$) (Fig. 1). Post hoc analyses revealed that in the standard housing condition, significantly fewer DG cells were labeled with BrdU in E (1,436 ± 218, $n = 5$) than that in C (3,021 ± 685, $n = 5$) animals ($P = 0.02040$), whereas PF animals (2,238 ± 201, $n = 5$) did not differ significantly from either E ($P = 0.2010$) or C ($P = 0.2116$) animals. In contrast, in the VEx condition, PF animals (3,477.12 ± 326.6; $n = 5$) had significantly fewer labeled cells than those of C animals (5,093 ± 504, $n = 5$) ($P = 0.0182$), but neither PF nor C was significantly different.
from E animals (3,928 ± 874 cells, n = 5). In addition, both E (P = 0.0005) and C (P = 0.004) animals in the VEx condition had significantly more labeled cells than those of their STD counterparts, whereas VEx-PF animals showed only a marginally significant increase in cell proliferation following voluntary exercise (P = 0.0653). Thus, while prenatal ethanol exposure adversely affects cellular proliferation in the DG, exposure to voluntary exercise can ameliorate these deficits, increasing the level of cell proliferation so that it is not significantly different from that of C animals. In contrast, the PF animals showed a marginal increase in proliferation following exercise when compared with that of STD-PF animals. Thus, exercise may not attenuate dietary- or pair-feeding-induced deficiencies in cellular proliferation.

Effects of Exercise on DG Neurogenesis Following Prenatal Ethanol Exposure

Because voluntary exercise differentially altered cell proliferation in E, PF, and C animals, we also examined the extent to which neurogenesis was affected by prenatal administration of ethanol. These experiments were conducted in a separate cohort of animals, administered their initial BrdU injection (200 mg/kg) at the same time as the animals in the cell proliferation study, but were sacrificed 4 weeks later rather than 24 h later. These animals spent the intervening 4-week period in their respective caging conditions (STD or VEx).

A factorial ANOVA indicated significant main effects for both housing condition ($F_{(1,1)} = 43.16$, $P = 0.000001$) and prenatal treatment ($F_{(1,2)} = 4.42$, $P = 0.023155$; Fig. 2). Post hoc analyses demonstrated that in the STD housing condition,

![FIGURE 1. Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be recovered by exposing animals to voluntary exercise. Bar graphs represent the mean ± SEM number of BrdU-positive cells in the DG for the offspring of animals in the ethanol (E), pair-fed (PF), and control (C) groups. White bars indicate animals in the STD caging condition, while black bars indicate animals given access to voluntary exercise. Animals in the STD-E group showed significantly less cell proliferation than that of STD-C animals. In both the E and C groups, voluntary exercise significantly increased the number of BrdU-positive cells. Asterisk indicates $P < 0.05$.](image-url)
The data from this study demonstrate that prenatal ethanol exposure can cause long-lasting reductions in hippocampal cellular proliferation and neurogenesis that can be observed in adult animals. More importantly, we also show for the first time that voluntary exercise can increase both cell proliferation and neurogenesis in E animals that exercised for a period of at least 1 week. Remarkably, the rates of cellular proliferation and survival of the E animals that exercised were not significantly different from those of control animals. Our data indicate that ethanol exposure during gestation induces a structural deficit that can result in reduced cell populations in the DG of the hippocampus. Indeed, reduced DG granule cell numbers have been reported in previous studies (Wigal and Amsel, 1990), although this is the first time the rate of cell proliferation and neurogenesis has been examined in adults following prenatal ethanol exposure. What is striking is that both cell proliferation and neurogenesis were inhibited months after the animals were initially exposed to ethanol in utero, suggesting that the neural stem-cell or progenitor-cell populations that give rise to new hippocampal granule cells and glia are impaired early on. It remains unclear whether this deficit represents a slowing of the cell cycle, or if there are just fewer DG progenitor cells available to engage in mitotic activity. However it does appear that these effects are at least in part mediated by the nutritional effects of prenatal ethanol exposure, as PF animals did not differ significantly from E animals in either neurogenesis or cell survival.

The mechanism(s) responsible for ethanol’s effects on neurogenesis remain unclear, however there are several possibilities. Ethanol is a known NMDA receptor antagonist, but paradoxically, previous studies have shown that the acute administration of NMDA receptor antagonists enhances cell proliferation in the DG (Cameron et al., 1995; Cameron et al., 1998). In contrast, long-term ethanol consumption in adult mice was accompanied by reduced cell proliferation (Crews et al., 2004). These results may suggest that acute NMDA receptor blockade is akin to some form of injury response (Parent, 2003), but that long-term blockade is needed to produce a reduction in neurogenesis. Also, the effects of ethanol at NMDA receptors must occur in concert with other events to produce a long-lasting change in neurogenesis.

In the present study, we found that cell survival was similarly impaired in E and PF animals, indicating that nutritional effects of ethanol may play a role in mediating impairments in progenitor cell populations. Furthermore, adverse effects of pair-feeding itself were observed. That is, for PF animals, exercise only marginally increased cell proliferation over that in animals (P = 0.066), whereas in the VEx condition, C animals (886.1 ± 119.8, n = 5) had more BrdU labeled cells than those of both the E (150.7 ± 45.0, n = 5) and PF (165.7 ± 12.9, n = 5) animals (P = 0.006), whereas in the VEx condition, C animals (418.8 ± 120.3, n = 5) had more BrdU labeled cells than those of both the E (150.7 ± 45.0, n = 5) and PF (165.7 ± 12.9, n = 5) animals (P = 0.006), whereas in the VEx condition, C animals (886.1 ± 119.8, n = 5) had marginally more BrdU-labeled cells than those of PF animals (639.7 ± 119.8, n = 5; P = 0.088) but were not significantly different from E animals (701.7 ± 46.7, n = 5; P = 0.173). E and PF animals did not differ from each other (P = 0.642). Importantly, animals in all prenatal groups showed significant increases in neurogenesis with exercise (ps < 0.003). These data extend our previous findings (Farmer et al., 2004), indicating that C animals that had access to exercise had significantly more labeled cells than those of C animals in the STD caging condition.

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It is also intriguing that exercise has been shown to benefit neurogenesis following long-term ethanol administration in adult animals (Crews et al., 2004). In the present study, exercise enhanced cell proliferation in both the E and C groups, and neurogenesis in all three groups. These findings indicate the robust ability of exercise to modulate mitotic activity in the DG, even in animals that have undergone adverse in utero experiences. Indeed, our previous studies have shown that exercise can in fact ameliorate behavioral deficits in E animals, as well as restore LTP of synaptic efficacy back to control levels (Christie et al., 2005). The present results, together with those of Crews et al. (2004) indicate that exercise has a substantial capacity to enhance brain function following teratogen exposure.

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