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The Effects of Exercise on Adolescent Hippocampal Neurogenesis in a Rat Model of Binge Alcohol Exposure During the Brain Growth Spurt

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Abstract

Exposure to alcohol during the brain growth spurt results in impaired cognition and learning in adulthood. This impairment is accompanied by permanent structural changes in the hippocampal formation. Exercise improves performance on hippocampal-dependent learning and memory tasks and increases adult neurogenesis in the rat hippocampal dentate gyrus. The present study examined the effects of wheel running during adolescence on dentate gyrus cell proliferation and neurogenesis after postnatal binge-like alcohol exposure. On postnatal days (PD) 4–9, pups were either intubated with alcohol in a binge-like manner, sham-intubated, or reared normally. On PD30–42 all animals were randomly assigned to two adolescent conditions: wheel running or inactive control. Animals were injected with BrdU every day between PD32–42 and perfused on PD42 or PD72. In inactive control animals at both PD42 and 72, cell proliferation and neurogenesis did not differ between postnatal treatment groups. Wheel running significantly increased the number of BrdU labeled cells on PD42 in all three postnatal treatments. On PD72, only the normal controls showed significant increases in survival of newly generated cells resulting from the wheel running. These results indicate that adolescent wheel running can induce comparable increases in cell proliferation and neurogenesis in alcohol-exposed and control rats, but the long-term survival of those newly-generated cells is impaired relative normal controls. Exercise may provide a means to stimulate neurogenesis, with implications for amelioration of hippocampal-dependent learning impairments associated with alcohol exposure. However, benefits requiring long-lasting survival of the newly generated cells will depend on identifying ways to promote survival.

Keywords

Fetal Alcohol Spectrum Disorder; Neurogenesis

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Section: Nervous System Development, Regeneration, and Aging

1. Introduction

Alcohol exposure during pregnancy can lead to various birth defects in the central nervous system (CNS), craniofacial structure, or other organ systems. The range of effects associated with fetal alcohol exposure is referred to collectively as fetal alcohol spectrum disorders (FASD) (Streissguth and O'Malley, 2000). One in one-hundred live births are estimated to be affected by FASD (May and Gossage, 2001). The most serious of these disorders, fetal alcohol syndrome (FAS), is diagnosed by the presence of growth deficits, facial dysmorphology, and CNS abnormalities due to heavy maternal alcohol consumption during pregnancy (Jones and Smith, 1973). Children exposed prenatally to alcohol who do not meet the diagnostic criteria for FAS may still manifest significant cognitive, behavioral, motor, and growth impairments (Coles et al., 1991; Hamilton et al., 2003; Maier et al., 1996; Mattson et al., 1996; Mattson et al., 2001).

Alcohol insult during pregnancy can cause selective brain damage in the offspring including damage to the cerebellum, corpus callosum, and hippocampus (Archibald et al., 2001; Autti-Ramo et al., 2002; Bookstein et al., 2007; Mattson et al., 1996; Riley et al., 1995). Animal models of FASD have demonstrated that the type and extent of the brain tissue damage, including neuronal loss, largely depends on the dose and developmental timing of exposure (Maier et al., 1996). The brain growth spurt, a period characterized by cellular proliferation and differentiation, neuronal migration, axonal growth, and synaptogenesis, occurs during the third trimester of pregnancy in humans and in the first two weeks after birth in rats (Dobbing and Sands, 1979; West et al., 1987). It is a period of vulnerability in which alcohol induces structural damage in the cerebellum, hippocampus, and other cortical regions (Bonhithus et al., 2001; Goodlett and Lundahl, 1996; Goodlett et al., 1997; Goodlett et al., 1998; Livy et al., 2003; Miller, 1995; Tran and Kelly, 2003). Previous studies demonstrated that binge-like alcohol exposure during the third trimester equivalent in rodent models resulted in decreased cell density and cell number in hippocampal dentate gyrus, CA1, and CA3 immediately following alcohol exposure (Bonhithus and West, 1990; Livy et al., 2003; West, 1986). However, Miller (1995) demonstrated an increase in neuronal number in the dentate gyrus and CA1 and in neuronal generation in the dentate gyrus when exposure doses were moderate, whereas others reported reductions in neuronal number in the CA1 hippocampal subfield but not in the dentate gyrus with binge-like exposures (Bonhithus et al., 2001; Tran and Kelly, 2003).

The hippocampal dentate gyrus is one of two neurogenic regions in the adult mammalian CNS (Altman and Das, 1965; Kuhn et al., 1996; Lois and Alvarez-Buylla, 1993). Adult neurogenesis in the dentate gyrus can be regulated by numerous intrinsic and extrinsic factors including genetic background, age, neurotransmitters (dopamine, serotonin), behavior, stress, and drugs (Baker et al., 2004; Gould et al., 1997; Kempermann et al., 1997a; Kempermann et al., 1997b; Kuhn et al., 1996; Malberg et al., 2000; Nacher et al., 2001; Nixon and Crews, 2002; Tanapat et al., 1999; van Praag et al., 1999). There are a limited number of studies examining whether alcohol exposure during the brain growth spurt produces lasting effects on adult neurogenesis. While both Ieraci and Herrera (2007) and Klintsova et al. (2007) found impairments in neurogenesis, Wozniak et al. (2004) did not.

Behavioral studies have also demonstrated that alcohol exposure during the brain growth spurt in rats produces impairments in performance on hippocampal-dependent learning and memory tasks corresponding to those seen in humans with FASD (Bonhithus et al., 2001; Goodlett and Peterson, 1995; Goodlett, 1987; Hamilton et al., 2003; Johnson and Goodlett, 2002; Mattson and Riley, 1999; Pauli et al., 1995; Thomas et al., 2008; Uecker and Nadel, 1998;). Voluntary exercise (wheel running) has been shown to increase cell proliferation and adult neurogenesis in the dentate gyrus and enhance learning and memory performance (Adlard et al., 2004; Eadie

et al., 2005; Holmes et al., 2004; van Praag et al., 1999; Vaynman et al., 2004). Redila and colleagues (2006) demonstrated that, in rats exposed to alcohol prenatally, voluntary exercise in adulthood increased cell genesis in the hippocampal dentate gyrus, rescuing the observed decrease in BrdU-labeled cells. Furthermore, extensive wheel running experience eliminated deficits on hippocampal-dependent behavioral tasks typically induced by early postnatal alcohol exposure in rats (Thomas et al., 2008).

The present study addresses the issue of whether postnatal alcohol exposure during the brain growth spurt affects cell proliferation, differentiation, neurogenesis, and survival in the hippocampal dentate gyrus of young rats. The primary purpose of this study was to test the hypothesis that voluntary exercise during adolescence can stimulate neuro- and gliogenesis in the hippocampal dentate gyrus in rats given a binge-like alcohol exposure during the postnatal brain growth spurt.

2. Results

2.1 Weights

The effects of binge-like postnatal alcohol exposure on body weight at the onset and termination of treatment periods are shown in Table 1. All animals continued to gain weight throughout treatments. A multivariate ANOVA with postnatal treatment by wheel running revealed no significant interaction, but yielded a main effect of postnatal treatment on postnatal (PD) 4 ($F_{(2, 82)} = 8.07$, $p < 0.05$), PD9 ($F_{(2, 82)} = 22.12$, $p < 0.01$), and PD32 ($F_{(2, 82)} = 4.12$, $p < 0.05$). Suckle control (SC) animals weights were significantly lower than alcohol exposed (AE) and sham intubated (SI) animals on PD4 ($p < 0.01$). On PD9 and PD32, AE animals weighed significantly less than SI animals ($p < 0.05$). By PD 42, weights did not differ among postnatal treatment groups. Furthermore, wheel running had no effect on body weight on PD42.

2.2 Blood Alcohol Concentrations

The mean peak BAC ($\pm$ SEM) measured on PD4 was 330.03 ± 6.9 mg/dl. This comparable to previously reported studies using similar alcohol dosages (Goodlett and Johnson, 1997; Helfer et al., 2009; Klintsova et al., 2007; Tran and Kelly, 2003).

2.3 Wheel Running Activity

On average, the number of wheel revolutions ($\pm$ SEM) per twenty-four hour period (per day) was 4392 ± 150 , approximately 4.9 km. When possible, animals were housed with like postnatal treatment groups. This allowed for analyses of the effects of postnatal treatment on activity. One-way ANOVA revealed that postnatal treatment did not significantly affect daily wheel running activity (Fig. 1). A Pearson correlation coefficient was computed to assess the relationship between daily running distance (rotations) and neurogenesis measures. There were no correlations between running distance and PD42 BrdU+ ($r = -0.1261$, $n = 23$, $p = 0.57$) or PD42 BrdU/NeuN+ ($r = -0.075$, $n = 23$, $p = 0.73$). However, there were correlations between running distance and PD72 BrdU+ ($r = 0.4448$, $n = 24$, $p = 0.03$) and PD72 BrdU/NeuN+ ($r = 0.4761$, $n = 24$, $p = 0.02$).

2.4 Proliferation and Neurogenesis at PD42

To ascertain whether postnatal alcohol exposure affected cell proliferation and neurogenesis and whether physical activity had the same effect on hippocampal neurogenesis in AE as in controls, rats were exposed to twelve days of 24-hour voluntary wheel running or inactive control housing. All animals received daily injections of BrdU (50mg/kg) for 10 consecutive days and were perfused on PD42 within 2-4 hours after the last injection. BrdU+ cell labeling was observed in all hippocampal sections with similar distribution in the dentate gyrus (DG).

Fig. 2 presents the effects of postnatal alcohol exposure on cell proliferation and neurogenesis at PD42. A two-way ANOVA on DG BrdU+ cells revealed a significant main effect of postnatal treatment ($F_{(2,37)} = 4.47$, $p < 0.05$) and wheel running ($F_{(1,37)} = 24.44$, $p < 0.01$); the interaction term was not significant. Post-hoc comparisons revealed that there were more BrdU labeled cells in the DG of AE animals than SI animals ($p < 0.05$). Wheel running significantly increased the number of BrdU+ cells compared to inactive controls in each of the three postnatal treatment groups [SC ($t_{11} = 2.95$, $p < 0.05$), SI ($t_{15} = 2.63$, $p < 0.05$), and AE ($t_{11} = 2.99$, $p < 0.05$)] (Fig. 2A). Fig. 3 (A,B) presents confocal images of BrdU labeling in the DG.

To determine the phenotype of the BrdU+ labeled cells in the DG, the percentage of co-labeling with NeuN (mature neuronal marker), DCX (immature neuronal marker), S-100B (astrocyte marker), CNPase (oligodendrocyte marker), Iba-1 (microglia) was analyzed. Fig. 3 (B-G) presents confocal images of these markers and represents co-labeling of these markers with BrdU in the DG. BrdU/S100B+, BrdU/CNPase+, or BrdU/Iba-1+ labeling was less than 1% (data not shown).

The percent of BrdU/DCX co-labeling found was $69 \pm 3\%$ ($\pm$ SEM). There was no significant main effect of postnatal treatment or wheel running on percent of BrdU/DCX+ co-labeling. The total numbers of BrdU+/DCX+ cells was calculated by multiplying the number of BrdU+ cells by the percentage of BrdU+ and DCX+ co-localization (Fig. 2B). A two-way ANOVA on BrdU/DCX+ cell numbers revealed significant main effects of both postnatal treatment ($F_{(2,37)} = 6.77$, $p < 0.01$) and wheel running ($F_{(1,37)} = 20.44$, $p < 0.01$) while the interaction was not significant. Based on Tukey HSD post-hoc comparisons AE animals expressed a higher number of BrdU/DCX labeled cells than did the SI animals ($p < 0.01$). Wheel running increased the number of BrdU/DCX+ cells the AE group ($t_{11} = 3.52$, $p < 0.01$); however the increase did not reach significance in either the SC ($t_{11} = 2.06$, $p = 0.064$) or the SI ($t_{15} = 1.89$, $p = 0.078$) groups.

The percent of BrdU/NeuN co-labeling was $42 \pm 2\%$ ($\pm$ SEM) for AE, $41 \pm 2\%$ ($\pm$ SEM) for SC, and $35 \pm 2\%$ ($\pm$ SEM) for SI. The percentage of BrdU+ cells expressing mature neuronal marker NeuN was significantly affected by the postnatal treatment condition ($F_{(2,37)} = 3.66$, $p < 0.05$). Based on post-hoc comparisons, AE animals had a significantly higher percent of BrdU/NeuN+ labeling than SI animals ($p < 0.05$). After transforming these percentages into BrdU/NeuN+ cell number, two-way ANOVA analysis on the number of BrdU/NeuN+ labeled cells revealed significant main effects of postnatal treatment ($F_{(2,37)} = 14.60$, $p < 0.01$) and wheel running ($F_{(1,37)} = 15.64$, $p < 0.01$); the interaction term was not significant. Post-hoc comparison revealed that there were more BrdU/NeuN labeled cells in the AE animals compared to the SI animals ($p < 0.01$). Comparison between wheel running and inactive control conditions within each postnatal treatment group revealed that AE ($t_{11} = 2.23$, $p < 0.05$) and SI ($t_{15} = 2.61$, $p < 0.05$) animals showed a significant increase in neurogenesis in the wheel running condition (Fig. 2C), but the SC group showed only a non-significant trend for increased numbers in the wheel running condition.

2.5 Survival of Newly Generated Cells and Neurons

To determine whether postnatal alcohol exposure affects survival of newly generated cells and neurons and if exposure to exercise during the period of new cells maturation affects their ability of post-exercise survival, rats were housed in groups in standard cage conditions for a thirty day survival period following the wheel running paradigm and were perfused on PD72. There were no main or interactive effects of postnatal treatment and wheel running on BrdU+ cell survival (Fig. 4A presents these findings). Planned comparisons between the wheel running and inactive control conditions within each postnatal treatment group revealed that wheel running significantly increased the survival of BrdU+ labeled cells in only in the SC group ($t_{12} = 2.23$, $p < 0.05$). Cell phenotype analyses revealed that $87 \pm 1\%$ ($\pm$ SEM) of BrdU

+ cell co-labeled with NeuN on PD72. There were no main or interactive effects of postnatal treatment or wheel running on the percentage of co-labeling. After transforming these percentages into BrdU/NeuN+ numbers (Fig. 4B), ANOVA indicated that neither postnatal treatment nor wheel running significantly affected survival of BrdU/NeuN labeled cells. Planned comparisons between the wheel running and inactive control conditions within each postnatal treatment group revealed only a non-significant trend for more double-labeled neurons in the SC group ($p=0.072$).

2.6 Dentate Gyrus Volume

The volume of the DG was compared across treatment groups to assess the possibility that variations in volume of the DG existed. There were no main or interactive effects of postnatal treatment or wheel running on DG volume for the PD 42 groups. However, there was a significant main effect of postnatal treatment on volume in the PD72 data ($F_{(2,39)} = 3.36$, $p < 0.05$), but post-hoc Tukey comparisons revealed no significant differences between the postnatal treatment groups ($\text{mm}^3 \pm \text{SEM}$; SC-IC: 0.23 ± 0.01 ; SC-WR: 0.23 ± 0.01 ; SI-IC: 0.24 ± 0.01 ; SI-WR: 0.22 ± 0.01 ; AE-IC: 0.21 ± 0.01 ; AE-WR: 0.21 ± 0.01).

3. Discussion

The results of the present study demonstrate that voluntary exercise (wheel running) during adolescence significantly enhanced cell proliferation and neurogenesis in the dentate gyrus (DG) of rats exposed to a binge like alcohol exposure (AE) during the postnatal brain growth spurt in a manner similar to that of the postnatal treatment controls. However, when assessed one month after the termination of wheel running, only the suckle control group showed significant increases in BrdU-labeled cells. In addition, the difference in the number of surviving newly generated neurons between wheel running and inactive control conditions did not reach significance for any postnatal treatment group. We conclude that postnatal alcohol exposure did not affect cell proliferation (bromodeoxyuridine, BrdU+ cells) or neurogenesis (BrdU+ cells co-labeled with DCX or NeuN) in the adolescent DG. Given the lack of a significant effect of voluntary exercise in the AE or in the sham intubated (SI) groups after a 30-day survival period, it appears that the increase in neurogenesis afforded by the voluntary exercise was not sustained in these groups. This study also demonstrated that postnatal AE did not influence differentiation of progenitor cells in the DG, given that the large majority of BrdU + cells expressed neuronal markers, with comparable percentages across survival groups.

3.1 BrdU Labeling

In this study, BrdU was used to identify and monitor proliferating cells in the adolescent hippocampal dentate gyrus. BrdU was administered by intraperitoneal injection daily for ten consecutive days during wheel running treatment. This injection protocol was utilized to identify the pool of proliferating cells that were influenced by physical activity, similar to previous studies (Lie et al., 2002; van Praag et al., 1999). Due to the use of a multiple injection procedure and the potential toxicity of BrdU, a dose of 50 mg/kg was chosen. Multiple injections of this dose support the labeling of many cells undergoing division over a period of time and provide identification of cell proliferation and adult neurogenesis (Cooper-Kuhn and Kuhn, 2002; Kuhn et al., 1996; Palmer et al., 2000; Taupin, 2007; Wojtowicz and Kee, 2006). Studies using similar injection paradigms produce no physiological side effects in the animals or obvious toxic effects on dividing cell (Cameron and McKay, 2001; Cooper-Kuhn and Kuhn, 2002). However, multiple injections do decrease the specificity of the survival time (Cameron and McKay, 1999; Cameron and McKay, 2001) and could be the cause of the high variability in the cell counts seen in this current study.

Exercise not only affects neurogenesis and learning and memory, it also increases cerebral blood flow and blood-brain barrier permeability (Sharma et al., 1991; Yancey et al., 1993). Because BrdU is an exogenous marker, the increases in blood flow and blood-brain barrier permeability resulting from wheel running may alter the bioavailability of BrdU. However, in previous studies utilizing both BrdU and endogenous marks of cell proliferation and neurogenesis (such as Ki67, pH3, and DCX), wheel running was shown to increase proliferation and neurogenesis regardless of the marker used (Koehl et al., 2008; Van der Borgh et al., 2009).

3.2 BrdU Labeled Cells and Newly Generated Neurons in Alcohol Exposed and Control Animals

Using the same alcohol exposure paradigm as in the current study (but with a longer period of BrdU injections, 10 vs. 20 days), we (Klintsova et al., 2007) reported no difference in the number of BrdU+ cells when measured after the last BrdU injection on PD50. These findings are similar to those in the current study, that postnatal alcohol does not affect the number of BrdU+ cells after the injection period. However, these two studies do differ in the outcomes of the survival. Klintsova et al. (2007) found a decrease in survival of newly generated cells and neurons, while the current study found no effect. These differences may be due to the longer BrdU exposure in the previous study (Klintsova et al., 2007), thus resulting in different population of cells labeled and different age at which survival was assessed (PD72 vs. PD80). In the previous study (as suggested by the authors), the newly generated cells had a chance not only to mature but also die at a higher rate in the alcohol exposed animals (Klintsova et al., 2007).

The few other reports investigating the impact of early postnatal alcohol exposure on adult neurogenesis render inconsistent findings. Wozniak and colleagues (2004) reported that a single day of alcohol exposure during the postnatal period produced no effect on DG neurogenesis (BrdU/NeuN+) in young mice (P54). However, another study showed that a single dose of alcohol on PD7 decreased dorsal but not ventral dentate gyrus cell proliferation (BrdU+) and immature neuronal number (DCX+) in older adult mice (P147) (Ieraci and Herrera, 2007). In the latter study, multiple BrdU injections across 7 days were given to 4-month old mice and BrdU+ cell number was evaluated 3 weeks later (assessment of survival of newly generated cells).

There are several differences among these studies including alcohol and BrdU administration, species of rodent used, and age when neurogenesis was studied that may account for the various results. Differences in age may possibly explain why this current study and Wozniak and colleagues (2004) do not see decreases in cell proliferation and adult neurogenesis like those observed in Ieraci and Herrera's (2007) and Klintsova et al.'s (2007) studies. Hippocampal dentate gyrus neurogenesis has been shown to decline considerably with age, especially during the time from adolescence to adulthood (He and Crews, 2007; Heine et al., 2004; Kuhn et al., 1996). As Ieraci and Herrera (2007) suggested, the effects of postnatal alcohol exposure on neurogenesis may not be detectable until a certain age is reached. Interestingly, it was found that a single dose of alcohol during the postnatal period increased cell proliferation in the dentate gyrus of adolescent rats and increased the percentage of newly generated immature neurons (Zharkovsky et al., 2003). Thus, it is possible that postnatal alcohol exposure increases neurogenesis during development and adolescence (perhaps as a way to compensate for the alcohol insult), but as the maturation and aging process continues, the effect of postnatal alcohol exposure becomes more obvious with the enhancement of aging and overall decline of neurogenesis. While age alone does not account for the differences between Wozniak et al.'s (2004) and our previous study (Klintsova et al., 2007) findings, alcohol administration may also play a key role here. Wozniak et al. (2004) utilized a single day exposure paradigm in

mice while Klintsova et al. (2007) administered alcohol for six consecutive days in rats. The binge-like multiple day exposure to alcohol delivered a greater insult to the brain indicating that the effects of postnatal alcohol exposure could depend on the duration of exposure. Another important note is that different species (mice vs. rats) were used in these two studies.

Similarly, there are inconsistencies in studies of cell proliferation in which alcohol exposure occurs earlier during prenatal development in rodents. It has been reported that the level of cell proliferation in adult rodents was decreased (Redila et al., 2006) or not affected (Choi et al., 2005) following prenatal alcohol exposure.

3.3 Influence of Wheel Running

The present study examined the effects of wheel running (voluntary exercise) on cell proliferation and neurogenesis in adolescent rats, particularly in those exposed to alcohol postnatally. The current study shows that after twelve days of wheel running, the number of BrdU+ cells was increased in the DG of all treatment groups, including the AE rats. These findings are the first to demonstrate that wheel running stimulates cell proliferation or neurogenesis in the DG of rats given early postnatal alcohol exposure to a degree comparable to normal rats. However, following a 30-day post-exercise treatment period, there was a significant increase in cell survival only in the SC group, but not in AE or SI rats. This implies that if neurogenesis induced by voluntary exercise is to be a target in potential therapeutic intervention for FASD, it likely will have to be accompanied by additional treatments that could promote survival of the newly-generated neurons.

Postnatal alcohol exposure has been associated with deficits in hippocampal-dependent behavioral task performance. Human FASD patients exhibit deficits on explicit memory tasks and are impaired in place learning (Hamilton et al., 2003; Mattson and Riley, 1999; Uecker and Nadel, 1998). Deficits in spatial working memory and place learning in the Morris water maze have been demonstrated in numerous studies utilizing the postnatal rat model of alcohol exposure (Goodlett and Peterson, 1995; Johnson and Goodlett, 2002; Pauli et al., 1995; Thomas et al., 2008; Wozniak et al., 2004). A number of reports have demonstrated an association between adult neurogenesis and learning and memory in recent years (Hernandez-Rabaza et al., 2009; Saxe et al., 2006; Shors et al., 2001; Shors et al., 2002; Winocur et al., 2006; Wojtowicz et al., 2008). Wheel running has been shown to increase adult neurogenesis in the dentate gyrus and has also been shown to enhance performance on hippocampal-dependent tasks (Adlard et al., 2004; Eadie et al., 2005; Holmes et al., 2004; van Praag et al., 1999). However, improvements in behavioral performance may also be related to exercise effects on growth factor signaling cascades, LTP, dendritic complexity, and spine density (Cotman et al., 2007; Eadie et al., 2005; van Praag et al., 1999b).

Voluntary exercise has been shown to improve performance deficits on water-maze spatial learning tasks following prenatal alcohol exposure in mice (Redila et al., 2006). In a recent study, rats exposed postnatally to alcohol were impaired in the Morris water maze performance and overactive in the open field (Thomas et al., 2008). Following voluntary exercise during adolescence, alcohol exposed animals were no longer impaired in these behaviors (Thomas et al., 2008). These results together with the current study's findings suggest that voluntary exercise ameliorative effects on behavioral deficits resulting from postnatal alcohol exposure could be the result of increased cell proliferation. It still needs to be determined if this behavioral amelioration is temporary or permanent, since in the current study we did not find enhanced long-term survival of newly generated cells and neurons.

Voluntary exercise (wheel running) has been demonstrated to rescue dentate gyrus cell proliferation decreased by the prenatal alcohol exposure (Redila et al., 2006) or by alcohol consumption (Crews et al., 2004). Our results confirm the exercise effect on proliferative

activity of progenitors in the DG subgranular zone. These findings suggest that exercise (voluntary running) is more powerful intervention than exposure to environmental complexity, which failed to bring up the number of dividing progenitors in DG after prenatal alcohol exposure (Choi et al., 2005).

It is well known that exercise increases cell proliferation and neurogenesis in adult animals (Brown, 2003; van Praag et al., 1999). However, there are a few reports of these effects in adolescent animals (Kim et al., 2004; Lou et al., 2008). Adolescence is characterized by brain growth and maturation. Select structures, such as the prefrontal cortex and limbic regions (including hippocampal DG), undergo dynamic development throughout adolescence (Chambers et al., 2003; Spear, 2000). During this time, there are high levels of neurochemical and neuronanatomical remodeling occurring (Andersen et al., 2000; Andersen and Teicher, 2004; Cooke and Woolley, 2005). There is a likely possibility that this ongoing development in the DG affects exercise-induced cell proliferation and neurogenesis. It has been demonstrated that the effects of exercise on cell proliferation are age dependent (Kim et al., 2004), in that treadmill exercise increased cell proliferation in male 4-week-old, 8-week-old, and 62-week old rats (Kim et al., 2004). However, the largest enhancement was observed in the 8-week-old group (Kim et al., 2004). In another treadmill exercise study, it was demonstrated that cell proliferation and neurogenesis were increased following a week of low intensity treadmill exercise in 35 day old rats (Lou et al., 2008). These effects were not observed in the moderate or high intensity groups, suggesting that the effects of exercise on adolescent neurogenesis depend on activity's intensity (Lou et al., 2008). The results from these previous studies suggest that age and intensity of running significantly and differentially affect neurogenesis. This can also explain the outcomes of our adolescent study where the effect of wheel running on cell proliferation and neurogenesis is less pronounced than that seen in the adult wheel running studies.

3.4 Conclusion

In summary, the present study demonstrates that postnatal alcohol exposure does not affect cell proliferation, differentiation, or neurogenesis in the DG of adolescent male rats. In addition, alcohol-exposed rats have a proliferation and neurogenesis response to exercise. However, exercise does not promote the survival of these newly generated cells in these animals. If enhancing neurogenesis is to be a target of therapeutic intervention for FASD, exercise will likely have to be accompanied by additional treatments that will promote the survival of these newly generated cells. Future studies are necessary to determine the extent of the therapeutic possibilities of exercise in animal models of FASD.

4. Experimental Procedure

4.1 Animals

The experimental design (Fig. 5) and procedures were reviewed and approved by the University of Delaware Institutional Animal Care and Use Committee (IACUC). Adult Long Evans time-pregnant dams were obtained from the University of Delaware Animal Care Facility, individually housed, maintained on a 12-hour light/dark cycle, and given free access to food and water. On postnatal day (PD) 3, litters were culled to eight to ten pups and paw marked. On PD4, litters were assigned to one of two conditions: suckle control (SC) litters that received no intubation or intubation litters. A split litter design was implemented for the intubated litters such that pups within these litters were randomly assigned to either sham intubated (SI) or alcohol exposed (AE) groups.

4.2 Postnatal Treatment

The intubation treatments were administered from PD4 to PD9. Milk and milk/ethanol solutions were prepared from a base milk formula according to the previously described method (West et al., 1984) and were delivered by gastric intubation as previously described (Goodlett and Johnson, 1997; Helfer et al., 2009). AE pups were intubated twice daily, two hours apart, with a milk/ethanol solution containing 11.9% (v/v) ethanol. Thus a total of 5.25g/kg of ethanol was delivered to each AE pup daily. All animals were weighed daily throughout treatment.

4.3 Blood Alcohol Concentrations

To assess peak blood alcohol concentrations (BACs), blood samples were obtained from a tail clip on PD4, ninety minutes after the last milk/ethanol intubation. The blood was centrifuged and plasma was collected and stored at -20°C until assay. BACs were analyzed using an Analox GL-5 Alcohol Analyzer which measures oxygen consumption during oxidation of ethanol (Analox Instruments, Lunenburg, MA).

4.4 Wheel Running

After weaning on PD23, male offspring were group-housed three per cage. On PD30, with group-housing assignments maintained, animals were randomly assigned to one of two conditions: standard cage (IC — inactive control) or voluntary exercise (WR — wheel running) condition. Wheel running animals were housed in cages attached to stainless steel running wheels and had twenty-four hour voluntary access to these wheels (multiple rats could run in the same wheel). The running wheels were equipped with counters that recorded each rotation of the wheel. Wheel running activity (wheel revolutions) was recorded every twelve hours, on the light onset and offset.

4.5 BrdU Treatment

On PD32, after two days of acclimation to the running wheels, all animals were injected intraperitoneally with the synthetic thymidine analog 5-Bromo-2-deoxyuridine (BrdU; Sigma Aldrich; 50mg/kg, in sterile 0.9% saline) every day at the onset of the light cycle until PD42. On PD42, half of the animals were perfused while the other half was housed in the standard cage condition (no running wheel access) until perfusions on PD72.

4.6 Tissue Preparation

On the day of perfusions, animals were given an overdose of a ketamine/xylazine mixture and transcardially perfused with heparinized 0.1M phosphate buffered saline (PBS, pH 7.2) followed by 4% paraformaldehyde in PBS (pH 7.2). The brains were carefully removed from the skull and stored in 4% paraformaldehyde for two days then transferred to 30% sucrose in 4% paraformaldehyde. Brains were coronally sectioned at 40 μm on a cryostat and serial sections were collected maintaining order throughout the dorsal hippocampus. Sections were stored at -20°C in cyroprotectant solution containing glycerol and ethylene glycol in Tris-buffered solution (TBS).

4.7 Immunohistochemistry

A systematic random sampling procedure was used in selecting the sections for processing. Starting around bregma -2.56 , every 5th section (4 sections per animal) was used for immunohistochemistry (previously described in (Helfer et al., 2009). Table 2 summarizes the primary antibodies and their final dilutions.

4.8 Stereological Counting Procedure

Quantification of BrdU+ cells in a one-in-five series of sections throughout the dorsal hippocampal dentate gyrus, suprapyramidal and infrapyramidal blades, approximately bregma -2.56 through -3.80 was performed. All BrdU+ cell counts were made on coded slides by an investigator blind to the treatment conditions. Counts were made in an unbiased manner within a known volume of the dentate gyrus using the optical fractionator probe (Stereoinvestigator, Micro Bright Field Inc., Williston, VT). The Stereoinvestigator software calculates the total volume of the outlined brain region taking into consideration the number of sections (section sampling fraction, $ssf = 1/5$) within the structure of interest and the number of the sampling sites within the dentate gyrus on each section (area sampling fraction, $asf = 2500/17500$). The grid frame was set to $100 \times 175 \mu\text{m}$ and the counting frame set to $50 \times 50 \mu\text{m}$. A guard zone of $2 \mu\text{m}$ and a dissector height of $35 \mu\text{m}$ were used. The frozen sections were originally cut at a nominal thickness of $40 \mu\text{m}$. Immunostaining and mounting in the antifading media provided the opportunity for section thickness to change after processing. Section thickness was measured at every fourth counting site. An average section thickness was computed by the software and used to estimate the total volume of the DG sample region and total number of BrdU+ cells (thickness sampling fraction, $tsf = 35 \mu\text{m}/\text{section thickness}$). In this study, the mean measured thickness of the sections was $39.5 \mu\text{m}$ (range $37.6 - 44.6 \mu\text{m}$). The mean coefficient of error (CE) for the number of cells (between-section and within-section variation) did not exceed the recommended 0.1 .

4.9 Phenotype Analysis

For cell phenotype analysis, all animals were assessed for BrdU/NeuN co-labeling while a subset of animals (chosen randomly), in separate series of sections, were assessed for BrdU/DCX, BrdU/CNPase, BrdU/Iba-1, and BrdU/S100B co-labeling. At least 50 BrdU+ cells per animal were analyzed for the co-labeling of NeuN (PD42 and PD72 tissue) and at least 25 BrdU+ cells were analyzed for co-labeling of DCX, Iba-1, CNPase, and S100B (PD42 tissue only). Due to only performing double labeling, the percentages of BrdU/DCX and BrdU/NeuN labeling in the PD42 group equal over 100%. There is a known overlap in the expression of these two markers during neuronal maturation (between the 7th and 21st days after proliferation) (Kempermann et al, 2003). Z-stack images from the dentate gyrus were taken with confocal microscopy (LSM 510 confocal microscope, Zeiss, Thornwood, NY) and analyzed at the xy-, xz-, and yz- planes for co-expression. Percentages of BrdU+ labeled cells that were also labeled with the previously mentioned markers were calculated. To determine total number of co-labeled cells, these percentages were multiplied by the counted BrdU+ numbers.

Within a given section stereological counting was done on one half of the hippocampus, while cell phenotypes were determined on the other. These precautions were taken to prevent underestimation of phenotypes due to the fluorescent bleaching during counting. The images in Fig. 3 were moderately processed with the 'brightness-contrast' function in Zeiss LSM Image Browser (Zeiss, Thornwood, NY) and Photoshop (Adobe, San Jose, CA) to assist observations.

4.10 Statistical Analysis

Data were analyzed using SPSS software (Chicago, IL). All independent variables were analyzed in a total of 43 male Long Evans rats for the PD42 cohort and in 45 males for the PD72 cohort. Data were analyzed with analysis of variance (ANOVA), and post hoc comparisons among postnatal treatment groups were performed with Tukey HSD post-hoc tests. *A priori* comparisons of wheel running effects within each postnatal treatment group, testing the prediction that wheel running increased the number of labeled cells in the DG, were evaluated using two-tailed t-tests. A Pearson two-tailed correlation coefficient was used to

assess the correlation between average daily distance run and neurogenesis measures. The significance level was set at $p < 0.05$.

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References

- Adlard PA, Perreau VM, Engesser-Cesar C, Cotman CW. The timecourse of induction of brain-derived neurotrophic factor mRNA and protein in the rat hippocampus following voluntary exercise. *Neurosci Lett* 2004;363:43–8. [PubMed: 15157993]
- Altman J, Das GD. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J Comp Neurol* 1965;124:319–35. [PubMed: 5861717]
- Andersen SL, Thompson AT, Rutstein M, Hostetter JC, Teicher MH. Dopamine receptor pruning in prefrontal cortex during the periadolescent period in rats. *Synapse* 2000;37:167–9. [PubMed: 10881038]
- Andersen SL, Teicher MH. Delayed effects of early stress on hippocampal development. *Neuropsychopharmacology* 2004;29:1988–93. [PubMed: 15316569]
- Archibald SL, Fennema-Notestine C, Gamst A, Riley EP, Mattson SN, Jernigan TL. Brain dysmorphology in individuals with severe prenatal alcohol exposure. *Dev Med Child Neurol* 2001;43:148–54. [PubMed: 11263683]
- Autti-Ramo I, Autti T, Korkman M, Kettunen S, Salonen O, Valanne L. MRI findings in children with school problems who had been exposed prenatally to alcohol. *Dev Med Child Neurol* 2002;44:98–106. [PubMed: 11848116]
- Baker SA, Baker KA, Hagg T. Dopaminergic nigrostriatal projections regulate neural precursor proliferation in the adult mouse subventricular zone. *Eur J Neurosci* 2004;20:575–9. [PubMed: 15233767]
- Bonthius DJ, West JR. Alcohol-induced neuronal loss in developing rats: increased brain damage with binge exposure. *Alcohol Clin Exp Res* 1990;14:107–18. [PubMed: 1689970]
- Bonthius DJ, Pantazis NJ, Karacay B, Bonthius NE, Taggard Da, Lothman EW. Alcohol exposure during the brain growth spurt promotes hippocampal seizures, rapid kindling, and spreading depression. *Alcohol Clin Exp Res* 2001;25:734–45. [PubMed: 11371723]
- Bookstein FL, Connor PD, Huggins JE, Barr HM, Pimentel KD, Streissguth AP. Many infants prenatally exposed to high levels of alcohol show one particular anomaly of the corpus callosum. *Alcohol Clin Exp Res* 2007;31:868–79. [PubMed: 17386071]
- Brown J, Cooper-Kuhn CM, Kempermann G, van Praag H, Winkler J, Gage FH, Kuhn HG. Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur J Neurosci* 2003;20:2042–2046. [PubMed: 12786970]
- Cameron HA, McKay RD. Restoring production of hippocampal neurons in old age. *Nat Neurosci* 1999;2:894–7. [PubMed: 10491610]
- Cameron HA, McKay RD. Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. *J Comp Neurol* 2001;435:406–17. [PubMed: 11406822]
- Chambers RA, Taylor JR, Potenza MN. Developmental neurocircuitry of motivation in adolescence: a critical period of addiction vulnerability. *Am J Psychiatry* 2003;160:1041–52. [PubMed: 12777258]
- Choi IY, Allan AM, Cunningham LA. Moderate fetal alcohol exposure impairs the neurogenic response to an enriched environment in adult mice. *Alcohol Clin Exp Res* 2005;29:2053–62. [PubMed: 16340464]
- Coles CD, Brown RT, Smith IE, Platzman KA, Erickson S, Falek A. Effects of prenatal alcohol exposure at school age. I. Physical and cognitive development. *Neurotoxicol Teratol* 1991;13:357–67. [PubMed: 1921915]
- Cooke BM, Woolley CS. Gonadal hormone modulation of dendrites in the mammalian CNS. *J Neurobiol* 2005;64:34–46. [PubMed: 15884004]

- Cooper-Kuhn CM, Kuhn HG. Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. *Brain Res Dev Brain Res* 2002;134:13–21.
- Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum Dev* 1979;3:79–83. [PubMed: 118862]
- Cotman CW, Berchtold NC, Christie L. Exercise builds brain health: key roles of growth factor cascades and inflammation. *Trends in Neuro* 2007;30:464–72.
- Eadie BD, Redila VA, Christie BR. Voluntary exercise alters the cytoarchitecture of the adult dentate gyrus by increasing cellular proliferation, dendritic complexity, and spine density. *J Comp Neurol* 2005;486:39–47. [PubMed: 15834963]
- Goodlett CR, Peterson SD. Sex differences in vulnerability to developmental spatial learning deficits induced by limited binge alcohol exposure in neonatal rats. *Neurobiol Learn Mem* 1995;64:265–75. [PubMed: 8564380]
- Goodlett CR, Lundahl KR. Temporal determinants of neonatal alcohol-induced cerebellar damage and motor performance deficits. *Pharmacol Biochem Behav* 1996;55:531–40. [PubMed: 8981583]
- Goodlett CR, Johnson TB. Neonatal binge ethanol exposure using intubation: timing and dose effects on place learning. *Neurotoxicol Teratol* 1997;19:435–46. [PubMed: 9392779]
- Goodlett CR, Peterson SD, Lundahl KR, Pearlman AD. Binge-like alcohol exposure of neonatal rats via intragastric intubation induces both Purkinje cell loss and cortical astrogliosis. *Alcohol Clin Exp Res* 1997;21:1010–7. [PubMed: 9309310]
- Goodlett CR, Pearlman AD, Lundahl KR. Binge neonatal alcohol intubations induce dose-dependent loss of Purkinje cells. *Neurotoxicol Teratol* 1998;20:285–92. [PubMed: 9638686]
- Goodlett CR, Kelly SJ, West JR. Early postnatal alcohol exposure that produces high blood alcohol levels impairs development of spatial learning. *Psychobiology* 1987;15:64–74.
- Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E. Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. *J Neurosci* 1997;17:2492–8. [PubMed: 9065509]
- Crews FT, Nixon K, Wilkie MT. Exercise reverses ethanol inhibition of neural stem cell proliferation. *Alcohol* 2004;33:63–71. [PubMed: 15353174]
- Hamilton DA, Kodituwakku P, Sutherland RJ, Savage DD. Children with Fetal Alcohol Syndrome are impaired at place learning but not cued-navigation in a virtual Morris water task. *Behav Brain Res* 2003;143:85–94. [PubMed: 12842299]
- He J, Crews FT. Neurogenesis decreases during brain maturation from adolescence to adulthood. *Pharmacol Biochem Behav* 2007;86:327–33. [PubMed: 17169417]
- Heine VM, Maslam S, Joels M, Lucassen PJ. Prominent decline of newborn cell proliferation, differentiation, and apoptosis in the aging dentate gyrus, in absence of an age-related hypothalamus-pituitary-adrenal axis activation. *Neurobiol Aging* 2004;25:361–75. [PubMed: 15123342]
- Helfer JL, Calizo LH, Dong WK, Goodlett CR, Greenough WT, Klintsova AY. Binge-like postnatal alcohol exposure triggers cortical gliogenesis in adolescent rats. *J Comp Neurol* 2009;514:259–71. [PubMed: 19296475]
- Hernandez-Rabaza V, Llorens-Martin M, Velazquez-Sanchez C, Ferragud A, Arcusa A, Gumus HG, Gomez-Pinedo U, Perez-Villalba A, Rosello J, Trejo JL, Barcia JA, Canales JJ. Inhibition of adult hippocampal neurogenesis disrupts contextual learning but spares spatial working memory, long-term conditional rule retention and spatial reversal. *Neuroscience* 2009;159:59–68. [PubMed: 19138728]
- Holmes MM, Galea LA, Mistlberger RE, Kempermann G. Adult hippocampal neurogenesis and voluntary running activity: circadian and dose-dependent effects. *J Neurosci Res* 2004;76:216–22. [PubMed: 15048919]
- Ieraci A, Herrera DG. Single alcohol exposure in early life damages hippocampal stem/progenitor cells and reduces adult neurogenesis. *Neurobiol Dis* 2007;26:597–605. [PubMed: 17490887]
- Johnson TB, Goodlett CR. Selective and enduring deficits in spatial learning after limited neonatal binge alcohol exposure in male rats. *Alcohol Clin Exp Res* 2002;26:83–93. [PubMed: 11821658]
- Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. *Lancet* 1973;2:999–1001. [PubMed: 4127281]

- Kempermann G, Kuhn HG, Gage FH. Genetic influence on neurogenesis in the dentate gyrus of adult mice. *Proc Natl Acad Sci U S A* 1997a;94:10409–14. [PubMed: 9294224]
- Kempermann G, Kuhn HG, Gage FH. More hippocampal neurons in adult mice living in an enriched environment. *Nature* 1997b;386:493–5. [PubMed: 9087407]
- Kempermann G, Gast D, Kronenberg G, Yamaguchi M, Gage FH. Early determination and long-term persistence of adult-generated new neurons in the hippocampus of mice. *Development* 2003;130:391–99. [PubMed: 12466205]
- Kim YP, Kim H, Shin MS, Chang HK, Jang MH, Shin MC, Lee SJ, Lee HH, Yoon JH, Jeong IG, Kim CJ. Age-dependence of the effect of treadmill exercise on cell proliferation in the dentate gyrus of rats. *Neurosci Lett* 2004;355:152–4. [PubMed: 14729257]
- Klintsova AY, Helfer JL, Calizo LH, Dong WK, Goodlett CR, Greenough WT. Persistent impairment of hippocampal neurogenesis in young adult rats following early postnatal alcohol exposure. *Alcohol Clin Exp Res* 2007;31:2073–82. [PubMed: 17949464]
- Koehl M, Meerlo P, Gonzales D, Rontal A, Turek FW, Abrous DN. Exercise-induced promotion of hippocampal cell proliferation requires β -endorphin. *FASEB* 22:2253–62.
- Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* 1996;16:2027–33. [PubMed: 8604047]
- Lie DC, Dziewczapolski G, Willhoite AR, Kaspar BK, Shults CW, Gage FH. The adult substantia nigra contains progenitor cells with neurogenic potential. *J Neurosci* 2002;22:6639–49. [PubMed: 12151543]
- Livy DJ, Miller EK, Maier SE, West JR. Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure on the developing rat hippocampus. *Neurotoxicol Teratol* 2003;25:447–58. [PubMed: 12798962]
- Lois C, Alvarez-Buylla A. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. *Proc Natl Acad Sci U S A* 1993;90:2074–7. [PubMed: 8446631]
- Lou SJ, Liu JY, Chang H, Chen PJ. Hippocampal neurogenesis and gene expression depend on exercise intensity in juvenile rats. *Brain Res* 2008;1210:48–55. [PubMed: 18423578]
- Maier SE, Chen WJ, West JR. Prenatal binge-like alcohol exposure alters neurochemical profiles in fetal rat brain. *Pharmacol Biochem Behav* 1996;55:521–9. [PubMed: 8981582]
- Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 2000;20:9104–10. [PubMed: 11124987]
- Mattson SN, Riley EP, Delis DC, Stern C, Jones KL. Verbal learning and memory in children with fetal alcohol syndrome. *Alcohol Clin Exp Res* 1996;20:810–6. [PubMed: 8865953]
- Mattson SN, Riley EP. Implicit and explicit memory functioning in children with heavy prenatal alcohol exposure. *J Int Neuropsychol Soc* 1999;5:462–71. [PubMed: 10439591]
- Mattson SN, Schoenfeld AM, Riley EP. Teratogenic effects of alcohol on brain and behavior. *Alcohol Res Health* 2001;25:185–91. [PubMed: 11810956]
- May PA, Gossage JP. Estimating the prevalence of fetal alcohol syndrome. A summary. *Alcohol Res Health* 2001;25:159–67. [PubMed: 11810953]
- Miller MW. Generation of neurons in the rat dentate gyrus and hippocampus: effects of prenatal and postnatal treatment with ethanol. *Alcohol Clin Exp Res* 1995;19:1500–9. [PubMed: 8749817]
- Nacher J, Rosell DR, Alonso-Llosa G, McEwen BS. NMDA receptor antagonist treatment induces a long-lasting increase in the number of proliferating cells, PSA-NCAM-immunoreactive granule neurons and radial glia in the adult rat dentate gyrus. *Eur J Neurosci* 2001;13:512–20. [PubMed: 11168558]
- Nixon K, Crews FT. Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J Neurochem* 2002;83:1087–93. [PubMed: 12437579]
- Palmer TD, Willhoite AR, Gage FH. Vascular niche for adult hippocampal neurogenesis. *J Comp Neurol* 2000;425:479–94. [PubMed: 10975875]
- Pauli J, Wilce P, Bedi KS. Spatial learning ability of rats following acute exposure to alcohol during early postnatal life. *Physiol Behav* 1995;58:1013–20. [PubMed: 8577871]

- Redila VA, Olson AK, Swann SE, Mohades G, Webber AJ, Weinberg J, Christie BR. Hippocampal cell proliferation is reduced following prenatal ethanol exposure but can be rescued with voluntary exercise. *Hippocampus* 2006;16:305–11. [PubMed: 16425237]
- Riley EP, Mattson SN, Sowell ER, Jernigan TL, Sobel DF, Jones KL. Abnormalities of the corpus callosum in children prenatally exposed to alcohol. *Alcohol Clin Exp Res* 1995;19:1198–202. [PubMed: 8561290]
- Saxe MD, Battaglia F, Wang JW, Malleret G, David DJ, Monckton JE, Garcia AD, Sofroniew MV, Kandel ER, Santarelli L, Hen R, Drew MR. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proc Natl Acad Sci U S A* 2006;103:17501–6. [PubMed: 17088541]
- Sharma HS, Cervos-Navarro J, Dey PK. Increased blood-brain barrier permeability following acute short-term swimming exercise in conscious normotensive young rats. *Neurosci Res* 1991;10:211–21. [PubMed: 1650437]
- Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E. Neurogenesis in the adult is involved in the formation of trace memories. *Nature* 2001;410:372–6. [PubMed: 11268214]
- Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E. Neurogenesis may relate to some but not all types of hippocampal-dependent learning. *Hippocampus* 2002;12:578–84. [PubMed: 12440573]
- Spear L. Modeling adolescent development and alcohol use in animals. *Alcohol Res Health* 2000;24:115–23. [PubMed: 11199278]
- Streissguth AP, O'Malley K. Neuropsychiatric implications and long-term consequences of fetal alcohol spectrum disorders. *Semin Clin Neuropsychiatry* 2000;5:177–90. [PubMed: 11291013]
- Tanapat P, Hastings NB, Reeves AJ, Gould E. Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *J Neurosci* 1999;19:5792–801. [PubMed: 10407020]
- Taupin P. Protocols for studying adult neurogenesis: insights and recent developments. *Regen Med* 2007;2:51–62. [PubMed: 17465775]
- Thomas JD, Sather TM, Whinery LA. Voluntary exercise influences behavioral development in rats exposed to alcohol during the neonatal brain growth spurt. *Behav Neurosci* 2008;122:1264–73. [PubMed: 19045946]
- Tran TD, Kelly SJ. Critical periods for ethanol-induced cell loss in the hippocampal formation. *Neurotoxicol Teratol* 2003;25:519–28. [PubMed: 12972065]
- Uecker A, Nadel L. Spatial but not object memory impairments in children with fetal alcohol syndrome. *Am J Ment Retard* 1998;103:12–8. [PubMed: 9678226]
- Van der Borght K, Kobor-Nyakas DE, Klauke K, Eggen BJL, Nyakas C, Van der Zee EA, Meerlo P. Physical exercise leads to rapid adaptations in hippocampal vasculature: temporal dynamics and relationship to cell proliferation and neurogenesis. *Hippocampus*. 2009Epub ahead of print
- van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci* 1999a;2:266–70. [PubMed: 10195220]
- van Praag H, Christie BR, Sejnowski TJ, Gage FH. Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc Natl Acad Sci* 1999b;96:13427–31. [PubMed: 10557337]
- Vaynman S, Ying Z, Gomez-Pinilla F. Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. *Eur J Neurosci* 2004;20:2580–90. [PubMed: 15548201]
- West JR, Hamre KM, Pierce DR. Delay in brain growth induced by alcohol in artificially reared rat pups. *Alcohol* 1984;1:213–22. [PubMed: 6536287]
- West JR. Long-term effects of developmental exposure to alcohol. *Neurotoxicology* 1986;7:245–56. [PubMed: 3785751]
- West JR, Goodlett CR, Kelly SJ. Alcohol and brain development. *NIDA Res Monogr* 1987;78:45–60. [PubMed: 3123990]
- Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S. Inhibition of neurogenesis interferes with hippocampus-dependent memory function. *Hippocampus* 2006;16:296–304. [PubMed: 16411241]
- Wojtowicz JM, Kee N. BrdU assay for neurogenesis in rodents. *Nat Protoc* 2006;1:1399–405. [PubMed: 17406427]

- Wojtowicz JM, Askew ML, Winocur G. The effects of running and of inhibiting adult neurogenesis on learning and memory in rats. *Eur J Neurosci* 2008;27:1494–502. [PubMed: 18364025]
- Wozniak DF, Hartman RE, Boyle MP, Vogt SK, Brooks AR, Tenkova T, Young C, Olney JW, Muglia LJ. Apoptotic neurodegeneration induced by ethanol in neonatal mice is associated with profound learning/memory deficits in juveniles followed by progressive functional recovery in adults. *Neurobiol Dis* 2004;17:403–14. [PubMed: 15571976]
- Yancey SL, Overton JM. Cardiovascular responses to voluntary and treadmill exercise in rats. *J Appl Physiol* 1993;75:1334–40. [PubMed: 8226548]
- Zharkovsky T, Kaasik A, Jaako K, Zharkovsky A. Neurodegeneration and production of the new cells in the dentate gyrus of juvenile rat hippocampus after a single administration of ethanol. *Brain Res* 2003;978:115–23. [PubMed: 12834905]

Fig. 1.
Daily average of wheel running activity (rotations per twenty-four hours). SC, suckle control; SI, sham-intubated; AE, alcohol exposed. Data expressed as mean $\pm$ SEM.

Fig. 2. Effects of postnatal alcohol exposure on cell proliferation and neurogenesis at postnatal day 42. (A) Mean number of BrdU+ cells. (B) Mean number of BrdU/DCX+ cells. (C) Mean number of BrdU/NeuN+ cells. SC, suckle control; SI, sham-intubated; AE, alcohol exposed. Data expressed as mean ± SEM. * indicates $P < 0.050$.

Fig. 3.

Analysis of BrdU phenotype using multiple cellular markers in the adolescent hippocampal DG. (A) Confocal projection image of the DG stained with anti-bodies against BrdU (newly generated cell; green) and NeuN (mature neurons; red) labeling. Sections of DG stained demonstrating co-labeling of the antibodies against BrdU (green) and (B) NeuN (red), (C) DCX (immature neurons; red), (D) S-100B (astrocytes; red), (E) CNPase (oligodendrocytes; red), and (F) Iba-1 (microglia; red). Phenotyping of BrdU+ cells revealed that the majority of these cells expressed neuronal markers. BrdU, bromodeoxyuridine; DG, dentate gyrus; NeuN, neuronal nuclei. Scale bars = 20 μ m in A and 5 μ m in B-F.

Fig. 4. Effects of postnatal alcohol exposure on the survival of newly generated cells and neurons. (A) Mean number of BrdU+ cells at PD72. (B) Mean number of BrdU/NeuN+ cells at PD72. PD, postnatal day; SC, suckle control; SI, sham-intubated; AE, alcohol exposed. Data expressed as mean $\pm$ SEM. * indicates $P < 0.050$.

Fig. 5.
Schematic of experimental design described in Experimental Procedure.

Table 1

Effect of postnatal alcohol exposure on weight^{*l*}

Outcome Variable	Postnatal Treatment		
	AE	SI	SC
Weight (g)			
PD4	10.9 ± 0.2	10.7 ± 0.2	9.8 ± 0.2 ^{<i>b c</i>}
PD9	16.3 ± 0.4 ^{<i>b</i>}	20.0 ± 0.4 ^{<i>c</i>}	17.6 ± 0.4
PD32	106 ± 2 ^{<i>b</i>}	114 ± 2 ^{<i>c</i>}	109 ± 2
PD42	176 ± 3	185 ± 3	180 ± 3
IC	174 ± 5	186 ± 5	179 ± 5
WR	176 ± 4	183 ± 4	180 ± 5

^{*l*} PD, postnatal day; AE, alcohol exposed; SI, sham intubated; SC, suckle control; IC, inactive control; WR, wheel running. The weights are reported as group means ± SEM.

^{*b*} $P < 0.05$ compared to that of SI group.

^{*c*} $P < 0.05$ compared to that of AE group.

Table 2
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
CNPase monoclonal	Purified full length native protein	Mouse	1:200	ab6319; Abcam, Cambridge, Massachusetts
S-100B	S100B polypeptide from purified bovine brain	Rabbit	1:5000	37A; Swant, Bellinoza, Switzerland
Iba1 polyclonal	Synthetic peptide corresponding to C-terminus of Iba-1	Rabbit	1:400	019-19741; Wako, Richmond, Virginia