NEUROGENESIS IN ADOLESCENT BRAIN IS POTENTLY INHIBITED BY ETHANOL

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Abstract—Adolescence is a period of progressive changes in brain that likely contribute to the maturation of behavior. Human adolescents consume large amounts of ethanol. To investigate the effects of ethanol on adolescent neural progenitor cells, male rats (35–40 days old) were treated with an acute dose of ethanol (1.0, 2.5 or 5.0 g/kg, i.g.) or vehicle that resulted in peak blood levels of 33, 72, and 131 mg/dl, respectively. Bromodeoxyuridine (300 mg/kg i.p.) was administered to label dividing cells and rats were killed at 5 h to assess proliferation or at 28 days to assess cell survival and differentiation. After 5 h, bromodeoxyuridine-immunoreactivity was reduced by 63, 97 and 99% in the rostral migratory stream and 34, 71 and 99% in the subventricular zone by 1.0, 2.5 and 5.0 g/kg of ethanol respectively. In the dentate gyrus, ethanol reduced bromodeoxyuridine-immunoreactivity by 29, 40, and 78% at the three doses respectively. The density of doublecortin immunoreactivity was decreased after 3 days and the number of bromodeoxyuridine+ cells remained decreased at 28 days when most hippocampal bromodeoxyuridine+ cells coexpressed neuronal nuclei, a neuronal marker. These studies indicate that the adolescent brain is very sensitive to acute ethanol inhibition of neurogenesis. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: alcohol, youth, stem cells, nervous system.

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The adolescent brain is in a unique state of transition as it undergoes maturation. A variety of systems are changing in the adolescent brain ranging from the molecular composition to sensitivity of neurotransmitter receptors such as the N-methyl-D-aspartate (NMDA) receptor (Williams et al., 1993) to more global changes such as the absolute volume of prefrontal cortex which declines during adolescent ontogeny in both humans (Jernigan et al., 1991) and rats (van Eden et al., 1990). As the brain matures from adolescent to adult, there are also changes in synapse number (Huttenlocher, 1984; Zecevic et al., 1989), the levels of cholinergic (Gould et al., 1991) and dopaminergic innervation (Rosenberg and Lewis, 1994; Kalsbeek et al., 1988) as well as other changes in a variety of brain regions (Choi and Kellogg, 1992; Wolfer and Lipp, 1995; Choi et al., 1997; Dumas and Foster, 1998). These findings suggest that the adolescent brain is somewhat unique from the adult brain.

Adolescents differ in their response to ethanol. Adolescent rats are less sensitive to the sedative effects of ethanol and have a rapid acute tolerance and/or an innate low response (Silveri and Spear, 1998; Swartzwelder et al., 1998; Grieve and Littleton, 1979). In contrast to this reduced sedative response, ethanol more potently inhibits NMDA-mediated excitation and stimulus-induced long-term potentiation in hippocampal slices from early adolescent rats versus slices from adult hippocampus (Swartzwelder et al., 1995a,b). In addition, adolescent rats are more impaired by ethanol than adult rats in a spatial memory task (Markwiese et al., 1998). As binge drinking-induced brain damage might underlie aspects of cognitive dysfunction (Hunt, 1993), it is disturbing that adolescents also differ in neurodegeneration following binge exposure. Specifically, binge drinking-induced brain damage in forebrain is greater in adolescent than adult rats (Crews et al., 1999). Thus, ethanol has distinct effects on adolescent brain, particularly in pathways involved in learning and memory processes.

Understanding the adolescent brain’s response to ethanol is important as adolescence is a time when individuals begin to drink (Spear, 2000). Although ethanol is an illegal drug for adolescents, adolescent drinking is common (O’Malley et al., 1998), with recent studies suggesting that 20% of all alcohol is consumed by adolescents (Wechsler et al., 2000). One third of high school students and 44% of college students binge drink every two weeks. Among college students, 19% are frequent binge drinkers, having more than three binge drinking episodes per week (Wechsler et al., 1995). Alcohol dependent adolescents report approximately 14 drinks per episode (Deas et al., 2000). Indeed, binge drinking in adolescents is a major public health issue, especially considering the susceptibility of adolescent brain to altered synaptic plasticity and neurodegeneration following ethanol exposure.

Alcohol exposure during adolescence may affect ongoing developmental processes that are occurring in the brain (Spear, 2000). Though neurogenesis is primarily considered a developmental process, it continues into adulthood in specific regions of the brain. Neurogenesis from a population of neural progenitor cells (NPC) occurs in two regions of adult brain: the forebrain subventricular zone (SVZ) and hippocampal dentate gyrus (DG). The major
components of neurogenesis include NPC proliferation, cell migration, cell differentiation and cell survival. Hippocampal neurogenesis may contribute to learning and memory (Shors et al., 2001), as well as mood and affective state (Malberg et al., 2000). Indeed, in disorders of learning, memory, and mood seen in drug abuse, brain damage and stress, neurogenesis is affected. Specifically, adult rat studies have found that binge drinking models reduce hippocampal neurogenesis by inhibiting both NPC proliferation and cell survival (Nixon and Crews, 2002). As the adolescent brain is more sensitive to ethanol disruption of memory and ethanol-induced neurodegeneration, it was hypothesized that adolescent brain might be more sensitive to ethanol inhibition of neurogenesis. We report here for the first time that ethanol potently inhibits both hippocampal and forebrain NPC proliferation in adolescent rat brain.

**EXPERIMENTAL PROCEDURES**

**Ethanol treatment**

Male Sprague-Dawley rats were obtained from Charles River Laboratories (Raleigh, NC, USA). Rats were individually housed and allowed to acclimate for several days with free access to food and water unless otherwise noted. Rats were studied when 35–40 days old and weighing 120–130 g, an age in the middle of the adolescent period for rats (Spear and Brake, 1983). Before alcohol treatment, rats were food deprived for 12 h, and then gavaged with a single dose of ethanol (1.0, 2.5, or 5.0 g/kg, 25% v/v in saline, i.p.) or saline vehicle. Bromodeoxyuridine (BrdU; Sigma, St. Louis, MO, USA), a marker of cell division, was dissolved in saline (20 mg/ml) and administered (300 mg/kg, i.p.) 30 min following ethanol administration. To investigate changes in cell proliferation, one group of rats was killed 5 h after ethanol treatment. In order to examine cell survival and cell phenotype (i.e. neurogenesis), a separate group of rats was injected with BrdU (300 mg/kg, i.p.) 30 min following the 5.0 g/kg acute dose of ethanol, then allowed to survive for 28 days. Blood ethanol concentrations (BEC) were determined at several time points (30 min, 60 min, 90 min, 180 min, and 300 min) from plasma derived from tail blood in a third group of rats. BEC were calculated by a GM7 Analyser (Biometric, Nashville, TN, USA) and a Sony DXC-390 video camera was used to capture images at magnifications of 64× for the DG, and 400× for the SVZ and RMS. Fidelity of data acquisition was ensured by background correction and normalizing to preset light levels. For the DG, the granule cell layer and subgranular zone were circumscribed and the density of immunopositive cells was analyzed by taking a pixel count within the region. The outlined area (in mm²) was determined and staining density was calculated by dividing the pixel count by the overall area (pixels/mm²).

In the SVZ and RMS, immunoreactivity for BrdU was also quantified by image analysis software. For the SVZ, immunopositive staining was measured at a series of three 50 μm boxes along the length of the SVZ as described (Kuhn et al., 1997). Immunoreactivity in the RMS was analyzed in a similar fashion as the SVZ, by using two 50 μm boxes placed along the length of the RMS, similar to Kuhn et al., 1997. For the SVZ and RMS, data were reported in immunopositive area (μm²). In all cases, values were reported as mean ± S.E.M. and analyzed by ANOVA with post hoc tests as noted in each figure.

**Quantification**

BrdU immunoreactivity (BrdU+/IR) and DCX immunoreactivity (DCX+/IR) were quantified by image analysis software similar to that described by Parent et al. (1997). We have previously shown that image analysis software detects percent change identical to stereology or profile counting methodologies (Crews et al., 2004). Briefly, a Bloquant Nova Advanced Image Analysis system (R&M Biometric, Nashville, TN, USA) and a Sony DXC-390 video camera were used to capture images at magnifications of 64× for the DG, and 400× for the SVZ and RMS. Fidelity of data acquisition was ensured by background correction and normalizing to preset light levels. For the DG, the granule cell layer and subgranular zone were circumscribed and the density of immunopositive cells was analyzed by taking a pixel count within the region. The outlined area (in mm²) was determined and staining density was calculated by dividing the pixel count by the overall area (pixels/mm²).

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**Detection of neurodegeneration**

Two histological methods were used to quantify potential neurodegeneration: counting of pyknotic cells and detection of Fluoro-Jade B staining. In the former, a one in 12 series of sections was counterstained with Neutral Red. Pyknotic cells were conservatively defined as nuclei lacking a nuclear membrane or cytoplasm, and specifically had dark Neutral Red-stained spherical chromatins (see Obernier et al., 2002 and He et al., 2005 for detail). Pyknotic cells were counted in each DG and expressed and the number of pyknotic cells per DG. Fluoro-Jade B staining for neurodegeneration was also carried out on five sections from the SVZ and DG and a positive control section as previously described (Schmued and Hopkins, 2000; Obernier et al., 2002).

**Fluorescent immunohistochemistry**

In the 5.0 g/kg ethanol group and controls, triple fluorescence immunohistochemistry was performed on DG sections to determine cell phenotype and differentiation percentages. A one in 12 series of 40 μm coronal sections was used to analyze co-expression of BrdU and neuronal (neuronal nuclei, NeuN) or glial (gliofibrillary acidic protein, GFAP) markers. Triple fluorescent immunohistochemistry followed the methods of Kuhn et al., 1997 as
previously reported (Nixon and Crews, 2004). Briefly, DNA was denatured and sections were blocked in TBS/H11001 followed by incubation overnight at 4 °C in primary antibodies: rat anti-BrdU (1:400, Accurate, Westbury, NY, USA), mouse anti-NeuN (1:500, Chemicon), and rabbit anti-GFAP (1:2500, Dako, Denmark) in TBS/H11001. Sections were incubated for 1 h in fluorescent-conjugated secondary antibodies: Alexa Fluor goat anti-rat 488, Alexa Fluor goat anti-mouse 633, and Alexa Fluor goat anti-rabbit 555 (all from Molecular Probes, Eugene, OR, USA) and mounted in Pro-Long anti-fade mounting medium (Molecular Probes).

Confocal microscopy was performed on a Zeiss laser scanning confocal microscope (510) (Zeiss, Oberkochen, Germany) optimized for thick tissue sections as previously detailed (Nixon and Crews, 2004). A multi-track setting was used and sections were analyzed using a C-Apochromat 40×/1.2 water lens with 2–3× zoom. Fifty BrdU cells were analyzed for co-localization and reported as a percentage of the number of BrdU cells. For each BrdU cell, Z-plane section images were gathered at 1 μm thickness, then analyzed for co-localization in LSM Image Examiner software.

RESULTS

The adolescent brain is undergoing continuous remodeling and development as individuals mature to adults. In adult rats, i.e. older than postnatal day (PND) 60, neurogenesis occurs in the DG and forebrain regions. These regions were investigated in adolescent rats at PND 35–40 in this study. BrdU incorporation was prominent in both the DG and forebrain regions. The DG contained many clustered cells predominantly in the subgranular zone (Fig. 1). In the forebrain SVZ and RMS, BrdU+IR is greater than in the hippocampus of adolescent rats (Fig. 1) suggesting the adolescent forebrain contains many more NPC than the DG.

To investigate the effects of ethanol on neurogenesis in adolescent rats, three intra-gastric doses of alcohol were used: 1.0, 2.5, and 5.0 g/kg. The lowest dose of ethanol studied, 1.0 g/kg, resulted in a peak blood level of 33 mg/dl at 30 min with most of the ethanol eliminated by 90 min (Fig. 2). This moderate amount of ethanol resulted in a 29% decrease in BrdU+IR in the DG (Fig. 3). Ethanol treatment at 2.5 g/kg resulted in blood levels of 72 mg/dl that peaked between 30 and 60 min returning to control levels after approximately 180 min (Fig. 2). This blood level resulted in a 40% decrease in BrdU+IR in the DG (Fig. 3). The highest dose of ethanol, 5.0 g/kg, produced blood levels well above the legal driving limit of 80 mg/dl with...
peak levels of 131 mg/dl found after approximately 90 min with blood levels remaining at 82 mg/dl at 5 h, the time when animals were killed for cell proliferation analysis. BrdU+/IR in the DG was reduced 78% by this high dose. In controls, BrdU+ cells in the DG are clustered, a characteristic of dividing NPC (Fig. 3). In the 5.0 g/kg ethanol tissue, only a few single cells appear without the clusters characteristic in controls after 5 h. Previous studies in adults have found that chronic ethanol increases apoptotic and pyknotic nuclei consistent with inhibition of NPC proliferation being secondary to increased cell death (Herrera et al., 2003; He et al., 2005). We looked for pyknotic nuclei and found 2.4 ± 0.4 and 2.9 ± 0.6 pyknotic nuclei/DG (n = 6) in controls and ethanol-treated rats (5.0 g/kg; n = 5) respectively. In addition, Fluoro-Jade B was used to assess cell death in DG and no positive cells were found. Similarly, DCX staining of new born neurons in the DG was found to be 1818 ± 98 and 1766 ± 54 pixels/mm² (P > 0.05) in control (n = 6) and ethanol-treated (5.0 g/kg; n = 5) rats respectively. This lack of effect is not surprising considering that DCX expression occurs within days (and not hours) of a change in cell proliferation (Brown et al., 2003; Nixon and Crews, 2004). In totality, these findings suggest that acute ethanol is not increasing apoptotic or necrotic cell death of NPC. The mechanisms of ethanol reduced BrdU incorporation require more detailed studies, but these data are consistent with a blockade of the progression through the cell cycle as a mechanism, although enhanced cell death cannot be ruled out. Thus, ethanol potently and dose dependently decreases cell formation in the DG of adolescent rats.

To investigate the effects of ethanol on cell proliferation in adolescent forebrain, BrdU+/IR was quantified in the SVZ and RMS. The SVZ includes cells lining the ventricles and a group of cells that are lateral to the edge of the ventricle that merge with the RMS in more frontal regions. Ethanol (1.0 g/kg) reduced both SVZ and RMS BrdU+/IR by approximately 34% and 63% respectively (Fig. 4 and Fig. 5). The intermediate dose of ethanol, e.g. 2.5 g/kg, decreased BrdU+ cells 71% and 97% in SVZ and RMS respectively. The highest dose studied, e.g. 5.0 g/kg, almost completely eliminated BrdU+ cells in both SVZ and RMS. A comparison of the effects of ethanol on adolescent DG and forebrain suggests that ethanol is more effective at inhibiting NPC proliferation in the forebrain than in the DG. This effect is best seen at the intermediate ethanol dose of 2.5 g/kg where DG neurogenesis is reduced by approximately 40% compared with 70–80% reductions in the forebrain. Thus, forebrain NPC proliferation is dose dependently inhibited by ethanol and appears to be more sensitive to acute ethanol inhibition than hippocampal NPC proliferation.

To investigate the effects of an acute dose of ethanol on adolescent neurogenesis two markers of neurogenesis were studied, DCX, a marker of immature newborn neurons, transiently expressed in NPC destined to become neurons (Brown et al., 2003; Nixon and Crews, 2004). In totality, these findings suggest that acute ethanol is not increasing apoptotic or necrotic cell death of NPC. The mechanisms of ethanol reduced BrdU incorporation require more detailed studies, but these data are consistent with a blockade of the progression through the cell cycle as a mechanism, although enhanced cell death cannot be ruled out. Thus, ethanol potently and dose dependently decreases cell formation in the DG of adolescent rats.
indicated by NeuN expression and remain intact for at least 11 months (Kempermann, 2002) suggesting they have become permanent DG granule cells. At 4 weeks after BrdU injection, most BrdU+ cells (70–80%) with or without ethanol exposure co-localized with the neuron-specific marker NeuN, which indicated that most BrdU+ cells in

Fig. 4. Ethanol dose dependently decreases cell proliferation in the SVZ of adolescent rats. Five hours after acute ethanol, BrdU + IR is significantly decreased after all doses of ethanol [(F(3,17)=21.647, P<0.0001] Fisher’s PLSD post hoc test: P<0.05 (*) less than control. Representative photomicrographs of the SVZ from controls (n=6), and ethanol: E 1.0 g/kg (n=4), E 2.5 g/kg (n=5) and E 5.0 g/kg (n=6) are shown on the right. Scale bar=200 μm.

Fig. 5. Ethanol dose dependently decreases cell proliferation in the RMS of adolescent rats. Five hours after acute ethanol, BrdU + IR is significantly decreased after the 2.5 g/kg and 5.0 g/kg doses of ethanol [(F(3,12)=4.029, P<0.05] Fisher’s PLSD post hoc test: P<0.05 (*) less than control. Representative photomicrographs of the RMS from controls (n=5), and ethanol: E 1.0 g/kg (n=3), E 2.5 g/kg (n=3) and E 5.0 g/kg (n=5) are shown on the right. Scale bar=200 μm.
adolescent brain differentiated into neurons similar to adults. Acute ethanol (5.0 g/kg, i.g.) did not alter differentiation in the subsequent 28 days (Fig. 7). Survival of BrdU-labeled cells for 28 days is slightly reduced by the lowest (1.0 g/kg) and medium (2.5 g/kg) doses of ethanol whereas the highest dose of ethanol (5.0 g/kg) reduced the number of BrdU+ cells by approximately 50% (Fig. 8). Taken together, these findings indicate that acute ethanol decreases NPC proliferation and neurogenesis resulting in fewer new neurons in adolescent hippocampus.

Interestingly, when acute ethanol (5.0 g/kg, 5 h) inhibition of BrdU+ cell formation is compared between adolescent rat hippocampus (PND 35–40; 120–130 g) and adult rats (PND 90; 275–330 g; Nixon and Crews, 2002), ethanol reduces BrdU+IR in adolescent rats by 78±7% whereas the same dose reduces BrdU+ cells in adult hippocampus by 43±3% (Fig. 9). Adolescent BrdU+IR was reduced 40% by the 2.5 g/kg dose of ethanol, a reduction that is strikingly similar to the adult 5.0 g/kg dose. Although both adolescent and adult rats received the same dose of alcohol this comparison is complicated due to different blood alcohol levels and different BrdU dosing. Both ages showed peak blood ethanol levels after 5.0 g/kg, i.g. at 90 min but adolescents were lower (~140 mg/dl) than adults (~233 mg/dl). Thus, the increased sensitivity of adolescents could not be explained by lower blood ethanol levels, although other factors such as BrdU distribution and dosing in these studies could contribute, in theory, to our data that suggest increased sensitivity. Thus, the adolescent brain is sensitive to acute ethanol inhibition of the formation of new cells that results in reduced neurogenesis 4 weeks later.

**DISCUSSION**

In rats, commonly cited times for adolescent onset are PND 30–42 (Spear, 2000), though some signs emerge as early as PND 28 with offsets as late as PND 40–55 in male rats (Ojeda and Urbanski, 1994). We used rats between PND 35–40. Cell proliferation, as assayed by BrdU incorporation, declines between PND 14 and PND 60 in the DG subgranular zone (Dong et al., 2003) and is further decreased during the progression from young adults to middle-aged and senescent rats (Nacher et al., 2003). Rats at PND 40 were found to have approximately twice the BrdU+ cells in hippocampus as young adults, e.g. PND 60 (Dong et al., 2003). We found extensive proliferation in both adolescent hippocampus and forebrain. This is the first report on NPC proliferation in adolescent forebrain, where there are many more BrdU+ cells than in the hip-

Fig. 6. Acute ethanol reduces DCX+IR in adolescent brain. DCX is a marker of adult neurogenesis transiently expressed in NPC between 1 and 7 days as NPC exit cell division and begin development to mature neurons (Brown et al., 2003). DCX+ cells were studied in the subgranular zone of the DG 3 days after an acute dose of ethanol (5 g/kg, i.g.). Left graph: Ethanol treatment decreased DCX+IR \(F(1,13)=47.7, P<0.0001 (***)\). Right: Representative photomicrographs of the DG from controls (C, top, \(n=7\)), and ethanol (E, bottom, \(n=8\)). Scale bar=400 μm for large photo and scale bar=50 μm for inset enlargement.
pocampal DG. Most adolescent BrdU+ cells in the DG became neurons, e.g. 70–80% of BrdU+ cells were also NeuN immunoreactive. The extensive proliferation and survival of adolescent NPC may contribute to the plasticity and maturation of the brain that occurs during the transition from adolescence to adulthood.

This is the first report to find that ethanol dose dependently inhibited NPC proliferation in the adolescent rat DG, and forebrain regions, SVZ and RMS. Hippocampal NPCs have a 24 h cell cycle and our measures of BrdU incorporation at 5 h are well short of the estimated 24 h cell cycle time (Cameron and McKay, 2001), consistent with ethanol inhibition of NPC proliferation. The mechanisms of reduced BrdU incorporation could be due to ethanol-enhanced death of NPC as suggested by previous studies of chronic ethanol treatment (Herrera et al., 2003; He et al., 2005). Although these studies on chronic ethanol treatment did not find any indication of increased cell death with Fluoro-Jade B or histology at the 5 h time point, although these data are also difficult to interpret due to the potential differences in timing of observable apoptotic death and this exposure model.

Although the mechanism of reduced NPC proliferation requires additional experimentation, it is clear that acute ethanol reduces adolescent neurogenesis. This result is supported by both the reduction in DCX immunohistochemistry found 3 days after acute ethanol as well as the reduced number of BrdU+/NeuN+ cells 4 weeks after an acute dose of ethanol. Interestingly, the reduction in formation of new cells at 28 days after acute ethanol was not as robust as that found at 5 h. This may be due to BrdU incorporation occurring at times greater than 5 h, a slowing of cell cycle that then returns when ethanol is cleared, more division during abstinence in the existing BrdU+IR cells (Nixon and Crews, 2004) and/or greater survival of new cells. Adolescents have a high level of neurogenesis that may contribute to adolescent abilities in learning and memory as well as the fluctuations in mood (Eisch et al., 2003). Additional studies will be needed to clearly understand the mechanisms of ethanol-induced inhibition of neurogenesis in both adolescents and long term consequences of reduced neurogenesis.

NPC are not homogeneous and represent a spectrum of stem-progenitor cells with varying potentiality (Seaberg and van der Kooy, 2003) that likely have varying sensitivity to ethanol. Our finding that adolescent forebrain NPC are more sensitive to ethanol than hippocampal NPC supports this idea since the forebrain contains more stem-like NPC and the hippocampus contains more restricted neural progenitors (Seaberg and van der Kooy, 2002). Thus, adolescent brain, and particularly adolescent forebrain NPC are sensitive to low blood ethanol levels.

This is the first report on ethanol inhibition of adolescent neurogenesis. Adolescent humans tend to binge drink often. Alcohol dependent adolescents report approxi-
mately 14 drinks per episode (Deas et al., 2000) and one third of high school students and 44% of college students binge drink every two weeks. Among college students, 19% are frequent binge drinkers, having more than three binge drinking episodes per week (Wechsler et al., 1995). Binge drinking likely achieves blood levels similar to the highest dose studied here that almost completely blocks NPC proliferation. Adult NPC are hypothesized to contribute to learning and memory, as well as affective state and mood (Eisch et al., 2003). Thus, our findings of decreased NPC proliferation and lost neurons one month after a single dose of ethanol suggest that adolescent binge drinking could disrupt learning, affective state and other behaviors undergoing maturation during adolescent brain development.

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