Published in final edited form as:
Alcohol Clin Exp Res. 2013 September ; 37(9): 1466–1475. doi:10.1111/acer.12125.

Periadolescent ethanol vapor exposure produces reductions in hippocampal volume that are correlated with deficits in prepulse inhibition of the startle

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Abstract

BACKGROUND—Epidemiological studies suggest that excessive alcohol consumption is prevalent among adolescents and may have lasting neurobehavioral consequences. The use of animal models allows for the separation of the effects of adolescent ethanol exposure from genetic background and other environmental insults. In the present study the effects of moderate ethanol vapor exposure, during adolescence, on structural diffusion tensor imaging (DTI) and behavioral measures were evaluated in adulthood.

METHODS—A total of 53 Wistar rats were received at postnatal day (PD) 21, and were randomly assigned to ethanol vapor (14 hrs on/10 hrs off/day) or air exposure for 35 days from PD 23-PD 58 (average blood ethanol concentration (BEC): 169 mg%). Animals were received in two groups that were subsequently sacrificed at two time points following withdrawal from ethanol vapor: (1) at 72 days of age, 2 weeks following withdrawal or (2) at day 128, 10 weeks following withdrawal. In the second group, behavior in the light/dark box and prepulse inhibition of the startle (PPI) were also evaluated. Fifteen animals in each group were scanned, post mortem, for structural DTI.

RESULTS—There were no significant differences in body weight between ethanol and control animals. Volumetric data, demonstrated that total brain, hippocampal, corpus callosum but not ventricular volume was significantly larger in the 128 day sacrificed animals as compared to the 72 day animals. The hippocampus was smaller and the ventricles larger at 128 days as compared to 72 days, in the ethanol exposed animals, leading to a significant group × time effect. Ethanol exposed animals sacrificed at 128 days also had diminished PPI and more rears in the light box that were significantly correlated with hippocampal size.

CONCLUSIONS—These studies demonstrate that DTI volumetric measures of hippocampus are significantly impacted by age and periadolescent ethanol exposure and withdrawal in Wistar rats.

Keywords

Adolescent; ethanol exposure; MRI; PPI; Light/Dark box

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INTRODUCTION

Adolescence is a critical stage of brain development when humans are initially exposed to a number of potentially toxic external stimuli such as ethanol and other drugs of abuse (Clark et al., 2008; Johnston, 1995; Squeglia et al., 2009). Given that the brain continues to develop before and throughout the adolescent period into early adulthood (Markus and Petit, 1987; Pfefferbaum et al., 1994; Sowell et al., 1999a,b), ethanol exposure during that time period may have unique deleterious consequences including changes in brain morphology.

Several studies in humans have provided data showing that heavy alcohol use over the lifespan can be associated with changes in brain morphology. In adults with chronic alcohol dependence, studies using magnetic resonance imaging (MRI) have demonstrated that several brain areas are reduced in volume and that the cerebral ventricles are enlarged (for review see Oscar-Berman and Marinkovic, 2007; Sullivan and Pfefferbaum, 2005). MRI studies in adolescent alcohol users have revealed that hippocampal volume decreases can be observed in several studies (see DeBellis et al., 2000; Medina et al., 2007; Nagel et al., 2005). Additionally, prefrontal white matter volumes also appear to be smaller in adolescent heavy alcohol users (DeBellis et al., 2005), but perhaps only in females (Medina et al., 2008). However, it is still not clear whether all such deficits are the result of alcohol usage or reflect pre-existing differences in high risk groups (Nagel et al., 2005).

The use of animal models of adolescent and adult ethanol exposure allows for the control necessary to evaluate the effects of ethanol on the brain and separate such effects from genetic background. During adolescent development alterations in neurobiological organization and behavior are seen that have been notably conserved during evolution with a number of similarities seen across mammalian species (Spear, 2000). However, few studies have evaluated the effects of ethanol on brain and ventricular volume in adult or adolescent animal models using MRI technology. In one study, 3 MRIs were obtained in adult Wistar rats before (MRI 1) and after exposure to high levels of ethanol vapor for 16 weeks which resulted in blood alcohol concentrations (BACs) of about 200 mg/dL (MRI 2), and then after 8 weeks of exposure at levels of vapor that produced BACs of about 400 mg/dL (MRI 3) (Pfefferbaum et al., 2008). A comparison of the scans from MRI 1 to MRI 2, in that study, revealed a significant increase in alcohol induced cerebroventricular volumes, whereas comparison of MRI 2 to MRI 3 showed an alcohol induced shrinking of the corpus callosum. In another study, MRIs were collected in adult Wistar rats that were selectively bred for preference for alcohol (P rats) (Pfefferbaum et al., 2006). In that study, P rats voluntarily consumed alcohol to BACs of about 125 mg/dL, and the results indicated that the greatest demonstrable effect was an attenuated growth of the corpus callosum. The authors of these studies concluded that ethanol treatment of adult rats only induces modest changes in brain morphology.

Since the adolescent brain is going through a period of significant reorganization it is possible that excessive ethanol exposure during peri-adolescence may impact brain structures and behaviors that are developing over that time period. Therefore, the present study evaluated whether the effects of moderate levels of ethanol vapor exposure during adolescence and early adulthood might have an impact on measures of brain structural volume in adult rats. We assessed brain morphology in adulthood 2 weeks and 10 weeks after withdrawal from peri-adolescent ethanol vapor exposure. Based on previous studies in adolescent humans (see DeBellis et al., 2000; Medina et al., 2007; Nagel et al., 2005), and vapor studies in adult rats (Pfefferbaum et al., 2006; 2008), we predicted that hippocampal and corpus callosum volumes would be decreased and ventricular volumes would be increased. To reduce multiple comparisons, our analyses focused on these brain areas. In

addition, behavioral measures of arousal and sensory gating (prepulse inhibition of the startle, activity in the light/dark box), that have been demonstrated previously to be sensitive to periadolescent ethanol vapor exposure using this model (Ehlers et al., 2011; Slawecki and Ehlers, 2005; Slawecki et al., 2004), were also tested in this sample and correlations made to the MRI analyses.

MATERIAL AND METHODS

Subjects

Two shipments of male Wistar rats and their dams were received at postnatal day (PD) 21 (total n = 53, Charles River, USA) were used in this study. The adolescent animals (PD 21) were housed 3 per cage respectively in standard cages until P70 when they were housed 2 per cage for the duration of the experiment. Animals were kept in a light/dark (12 hrs light/ 12 hrs dark, lights on at 06:00 a.m.) and temperature-controlled environment. Food and water were available ad libitum throughout the experiment. All experimental protocols were approved by the Institutional Animal Care and Use Committee at The Scripps Research Institute and were consistent with the guidelines of the NIH Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996).

Ethanol vapor exposure

The ethanol vapor inhalation procedure and the chambers used in this study were previously described (see O'Dell et al., 2004; Roberts et al., 1996, 2000; Rogers et al., 1979; Slawecki et al., 2001; Zahr et al., 2011). Ethanol vapor chambers were calibrated to produce high to moderate BECs between 150–225 mg/dL. In brief, juvenile animals were acclimated to their environment by being placed in standard rat cages on arrival for 36 hours, then they were randomly divided into two groups each (ethanol-exposed group (n=29), control group (n=24)) (see figure 1). Ethanol-exposed rats were housed in sealed cages that were infused with vaporized 95% ethanol from 6 p.m. to 8 a.m. Control animals were in the same room and in similar but unsealed cages exposed only to ambient air. The fact that the control animals were in unsealed cages whereas the vapor animals were in sealed cages is a potential limitation of this study. At the start of the ethanol exposure, juvenile rats were 23 days old and the exposure continued until they were 58 days old. Blood samples were collected from the tip of the tail approximately 8 times during the 5 week exposure period in order to assess BECs (target: 150 to 200 mg/dl). Control animals also had blood removed from the tail at the same time points. BECs were determined in the ethanol-exposed animals using the Analox micro-statGM7 (Analox Instr. Ltd., Lunenberg, MA). Following the 5 week exposure animals were transferred to standard vivarium cages for the duration of the experiments.

Prepulse inhibition of the startle response (PPI)

Prepulse inhibition of the acoustic startle response (PPI) has been previously demonstrated to be sensitive to adolescent ethanol exposure (Ehlers et al., 2011; Slawecki and Ehlers, 2005). In the present study PPI was assessed 2 months after ethanol exposure (PD 120/121) in the second group of animals (n=38, 22 ethanol-exposed, 16 controls) that were sacrificed at 128 days. Acoustic startle responses were measured in SR LAB Startle chambers (San Diego Instruments, San Diego, CA). The session contained 30 trials consisting of 15 pulse trials (120 dB auditory (white noise) pulse burst for 40 msec) and 15 prepulse + pulse trials (120 dB auditory pulse burst which was preceded 10 msec by a 85 dB auditory prepulse (white noise) burst for 20 msec duration) presented randomly throughout the session. Background white noise was presented for the entire session at a level of 70 dB.

The outcome variable was the amount of prepulse inhibition of the startle response:

$$\left(\left(\frac{(AmplitudePrePulseStartleResponse - AmplitudeStartleResponse)}{AmplitudeStartleResponse}\right) \times 100\right)$$

The order of assessment on the test day was counterbalanced across treatment groups to minimize any potential influence of time of day during testing.

Behavior in the light-dark box

At PD 106–107 behavior in the light/dark box was assessed, as previously described (Slawecki, 2005), 49 days after the end of vapor exposure in group 2 (n=38, 22 ethanol-exposed, 16 controls) which were sacrificed at 128 days. One chamber was painted black and covered with a black lid (i.e. the dark box). The other chamber was opaque and remained uncovered during the test session (i.e. the light box). A light (60 lux) was positioned 165 cm above the light box. Inside the dark box there was no appreciable illumination (i.e. < 2 lux). On the test day, between 10 a.m. and 2 p.m., the rats were placed individually in the center of the light box and allowed to freely explore the entire apparatus for 5 min. Behaviors assessed included: latency to exit the light, and the latency to return back to the light box, as well as rears in the light box.

Perfusion, brain preparation and DTI analyses

Rats in the two shipment groups were sacrificed after different elapsed time periods from withdrawal following ethanol vapor. The first group was sacrificed on postnatal day 71 and 72, 13 and 14 days following termination of vapor exposure. The second group was sacrificed on postnatal day 128, 70 days after the termination of vapor exposure. They were first anesthetized with pentobarbital (100 mg/kg, intraperitoneal) and then euthanized by perfusion as described previously (Crews et al., 2004). The animals were perfused transcardially with 0.1 M phosphate buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in PBS. The ears and skin were then removed from the head and shipped in PBS to the University of North Carolina (UNC). Rat skulls with the brains intact were then submerged in a 1% PFA/PBS solution at 4 degrees until the MRI session.

Image acquisition

Fixed heads were sent to UNC for MRI using diffusion tensor imaging (DTI). This allowed for the collection of both structural volumetric and diffusion data. Fifteen animals from each sacrifice group at 72 days (n= 15, 7 ethanol, 8 control) and at 128 days (n=15, 8 ethanol, 7 controls) were selected for DTI. Whole rat skulls were then scanned blindly at the UNC Biomedical Research Imaging Center (BRIC) using a dedicated 9.4T Bruker small animal scanner. Isotropic DTI images (0.16mm × 0.16mm × 0.16mm) were acquired using a diffusion-weighted 3D RARE sequence with 12 quasi-uniformly distributed diffusion encoding gradient directions and 2 baseline images. The total scan time was approximately 13 hours per subject.

It should be noted that, in most human DTI studies, image acquisition time is extremely limited and the rapid switching on/off of the gradients create strong eddy currents, which cause considerable geometric distortion in the acquired images. Therefore, structural (T1- or T2-weighted) MRI is often preferred to diffusion MRI for volumetric measurements in human studies. However, in the postmortem rodent imaging setting, the eddy current distortion is negligible and therefore diffusion MRI scans are a valid tool for measuring volume in addition to the tissue's diffusion properties.

Image processing

The acquired MRI data were processed using our fully automatic pipeline (Lee et al., 2009) with thorough quality control checks after each step. After bias correction of the raw data, diffusion tensors were estimated and diffusion property maps, including Fractional Anisotropy (FA), were computed for each subject. These FA maps were rigidly aligned (translation and rotation) with a pre-existing atlas using mutual information. The entire tensor field (i.e. the DTI) was transformed using these rigid transforms using Log-Euclidean interpolation (Arsigny et al., 2006) and the diffusion property maps were re-computed in this atlas space.

Next, these rigidly registered FA images were used for skull-stripping the images using an atlas-based classification method, as described in detail elsewhere (Oguz et al., 2011). For each age group, the skull-stripped FA maps were deformably registered together in a group-wise manner to compute an unbiased population average (Joshi et al., 2004). The pre-existing atlas was registered to these two age-specific population averages, and the accompanying segmentation into regions of interest (ROI's) was propagated along these computed deformation fields, first to the population average and then to the individual subjects.

The prolonged postmortem scan allows the acquisition of isotropic DTI data with a high resolution both in-plane and out-of-plane. Therefore, we were able to align and segment the images in 3D and compute the volume of each structure in each subject. Additionally, the atlas-based segmentation of the entire brain at once allowed for smooth and accurate boundaries between neighboring regions, compared to a region-by-region basis that may have biased the analysis based on the order of processing.

We have used the individual segmentations computed in the previous step to determine the volume of each ROI for each subject. Based on previous studies in adolescent humans, and vapor studies in rats, we predicted that hippocampal and corpus callosum volumes would be decreased and ventricular volumes would be increased (Pfefferbaum et al., 2006; 2008). Therefore to reduce multiple comparisons our volumetric measures focused on these brain areas measured on the FA map.

Data Analysis

Statistical analyses were performed using: Statistical Package for the Social Sciences (IBM Corp., Armonk, NY). Analysis of variance (ANOVA) was used to determine the effects of chronic ethanol exposure on body weight, BECs and behavior in the startle and light/dark box tests. Independent ANOVAs (2-way, group: control vs. ethanol and time: time 1 vs. time 2) were also used to assess the effects of ethanol exposure on DTI brain volumes. Correlations between the behavioral measures and the structural DTI volumes were made using Pearson correlations. Significance was taken at $p<0.05$.

RESULTS

Body weight and BECs

As seen in figure 2, all rats gained weight over the course of the experiment. The 15 rats in the 72 day sacrifice group who had DTI data grew from about 50 g at PD 22 to about 370 g being 373 (± 37) and 366 (± 35) g for control and ethanol at PD 72 respectively. No significant overall differences in body weight were seen between the ethanol and control animals over the 7 week experiment in a group × time analysis (group effect: $F=0.32$, $df=1,29$, $p>0.5$). The 15 rats in the 128 day sacrifice group who had DTI data also grew, from about 50 g at PD 22 to about 550 g being 558 (± 49) and 557 (± 38) g for control and

ethanol at PD 128 respectively. No significant overall differences in body weight between ethanol and control animals were found over the 15 week experiment in a group × time analysis (group effect: F=0.72, df=1,29, p>0.7). Additionally, there were no significant differences in body weight between the DTI animals in the 72 day sacrifice group and the 128 day sacrifice group over the 5 weeks of ethanol exposure (F=0.12, df=1,29, p>0.7). Also, the 23 rats with only behavioral data in the 128 day sacrifice group also did not differ from the DTI animals in their overall body weights (F=0.97, df=1,47, p>0.3).

Blood ethanol concentrations (BECs) were measured 8 times during the 5 week vapor exposure period and the values for each week are presented in figure 3. Mean blood ethanol concentrations (±S.E.) over the 5 week exposure period were 168.2 (± 22.2) mg/dL for group 1 (72 day sacrifice) and 171.3 (± 25.7) mg/dL for group 2 (128 day sacrifice). A group × time analysis revealed that there was no significant overall difference between the two groups in BEC over the 5 week exposure period (group effect: F=0.21, df=1,13, p>0.6). In addition, the 23 rats with only behavioral data in the 128 day sacrifice group also did not differ from the DTI animals in their blood ethanol concentrations (F=0.44, df=1,22, p>0.5). These blood ethanol levels are consistent with this protocol being a model of blood ethanol levels observed during adolescent binge drinking (Donovan, 2009).

Imaging findings

To investigate brain structure in adults in rats that had received vapor or control treatments as adolescents, structural DTI data from postmortem adult rat brains were analyzed. A 2 × 2 analysis that compared treatment group (ethanol vs. control rats) × time after ethanol/air exposure to sacrifice (72 day sacrifice vs. 128 day sacrifice) on each of the brain areas predicted to change (hippocampus, ventricle, corpus callosum) was conducted. Representative brain images for each treatment, age, and brain region are presented in figure 4. A comparison of the total brain volumes for the ethanol and control condition at both the 72 day, and 128 day time points was also computed, and is presented in figure 5.

Total brain volume was significantly larger in the 128 day sacrifice animals as compared to the 72 day animals (F=28.62, df=1,29, p<0.0001), as seen in figure 5. However, there was no overall ethanol group effect (ethanol vs. control: F=0.028, df=1,29, p>0.8) or a group × time of sacrifice effect (F=3.09, df=1,29, p>0.1). In contrast, the ventricular volume was not significantly different in size between the 128 day sacrifice animals as compared to the 72 day sacrifice animals (F=2.8, df=1,29, p>0.10). There was a significant group × time of sacrifice effect for the ventricle (F=4.9, df=1,29, p<0.036) but no overall ethanol effect (F=0.01, df=1,29, p>0.9). Evaluation of the DTI volumetric data for the corpus callosum revealed that there was a significant age effect with the 128 day sacrifice animals having significantly larger corpus callosums compared to the 72 day sacrifice animals (F=7.22, df=1,29, p<0.012). However, there was not a significant overall ethanol effect (F=0.16, df=1,29, p>0.6), or a group × time of sacrifice effect (F=0.65, df=1,29, p>0.43). Evaluation of the DTI volumetric data for the hippocampus revealed that there was a significant age effect with the 128 day sacrifice animals having significantly larger hippocampi compared to the 72 day sacrifice animals (F=4.55, df=1,29, p<0.042). However, there was not a significant overall ethanol effect (F=2.29, df=1,29, p>0.1), but there was a significant group × time of sacrifice effect (F=10.53, df=1,29, p<0.003). An exploratory post hoc analysis also revealed that the hippocampus was non-significantly larger in the ethanol treated animals as compared to the controls at day 72 (F=3.6, df=1,14, p<0.08); whereas, it was significantly smaller in the ethanol animals as compared to the controls at day 128 (F=7.15, df=1,15, p<0.019). This same trend was also found for corpus callosum, as seen in figure 5. These data suggest that the hippocampal areas are not significantly different 2 weeks following withdrawal from 5 weeks of ethanol exposure but by 10 weeks following ethanol

withdrawal, the hippocampus of ethanol exposed animals has not increased in size to the same extent as the control animals.

Behavior in the startle paradigm and the light/dark box

Prepulse inhibition of the startle (PPI) was assessed 2 months after ethanol exposure (PD 120/121) in the second group of animals (n=38, 22 ethanol exposed, 16 controls) that were sacrificed at 128 days. One way ANOVA revealed that the ethanol exposed animals had significantly disrupted prepulse inhibition of the startle as compared to the control animals seen in figure 6 (ethanol group:16.7%, controls: 34.4%; ($F=7.7$, $df=1,36$, $p<0.008$). Reductions in the percent of PPI in this test suggest that the ethanol exposed animals may have less sensory gating produced by the prepulse inhibition stimuli. Additionally, this reduction in prepulse inhibition of the startle was significantly correlated with hippocampal size in those animals who had both behavior and volumetric analyses conducted (Pearson correlation: −.701, $df=1,14$, $p<0.005$), as seen in figure 7. Thus, these data suggest that ethanol exposure reduces sensory gating and this reduction is correlated with smaller hippocampal size.

Behavior in the light/dark box was assessed 49 days after the end of vapor exposure, in group 2, (n=38, 22 ethanol-exposed, 16 controls) that were sacrificed at 128 days at PD 106–107. Ethanol-exposed animals did not differ from controls in the amount of time they spent initially in the light box before entering the dark box (ethanol group: 57.8 ± 6.5, control group: 88.9 ± 18.0 sec; $F=3.3$, $df=1,37$, $p<0.08$). However, as seen in figure 6, they spent a significantly shorter time in the dark box before returning to the light box (ethanol group: 31.6 ± 3.9, controls 48.2 ± 5.6 sec; $F=6.05$, $df=1,35$, $p<0.019$). Additionally, they exhibited significantly more rears while in the light box (ethanol group: 19.4 ± 1.6, control group: 13.3 ± 1.5 sec; $F=6.8$, $df=1,37$, $p<0.013$). Shorter latencies to enter the light box and more rears in the light box in the light/dark box test suggest that the ethanol exposed animals may be more aroused and may be displaying more “disinhibitory” behaviors or less “anxiety-like” behaviors as compared to control animals. In addition, the increased rearing in the light box was found to be significantly correlated with hippocampal size in those animals who had both behavior and volumetric analyses conducted (Pearson correlation: −.651, $df=1,14$, $p<0.05$), and is shown in figure 7. Thus, these data suggest that ethanol exposure enhances disinhibitory behaviors and this reduction is correlated with smaller hippocampal size.

DISCUSSION

In the present study, rats were exposed to ethanol vapors during the periadolescent period in order to examine ethanol's effects on neuromorphology in whole brain, ventricle, hippocampus and corpus callosum and its relationship to behavior in adulthood. There were no significant differences in body weight between ethanol and control animals or blood ethanol concentrations or body weight between the two groups sacrificed at 72 days vs. 128 days when they were equivalent days of age. However, some of the volumetric data, measured on the fractional anisotropy (FA) map were significant. Total brain, hippocampal, and corpus callosum volume was significantly larger in the animals sacrificed at 128 days as compared to those sacrificed at 72 days. Increases in whole brain volume over adult development, which reached maximal levels by about 450 days, has been reported previously in MRI studied conducted in adult P rats (Sullivan et al., 2006). Increases in total brain volume have also been reported in C57BL/6J mice evaluated from age 4 to 24 months (von Kienlin et al., 2005). While increases in whole brain volume were found in the present study no age related differences were found in the size of the ventricle. These data are also consistent with what was reported for adult P rats where no age related changes were seen in the size of the ventricle (Sullivan et al., 2006).

A number of studies have demonstrated that the hippocampus and corpus callosum are especially vulnerable to the effects of alcohol both during early development and over the lifespan (see Hommer et al., 1996; Sullivan and Pfefferbaum, 2005; Yang et al., 2012). In human adolescent alcohol users MRI studies have demonstrated that hippocampal volume decreases can be observed (see DeBellis et al., 2000; Medina et al., 2007; Nagel et al., 2005). However, it is still not clear whether all such deficits are the result of alcohol usage or represent pre-existing conditions (Nagel et al., 2005). In the present study, the hippocampus was smaller and the ventricle larger in the ethanol vapor exposed animals at 128 days as compared to 72 days resulting in a significant group × time effect. In a previous study, in which adult Wistar rats were exposed to alcohol vapor at high levels (up to 445 mg/dl) for long exposures (8 and 24 weeks), an increase in ventricular volume and shrinkage of the corpus callosum was found (Pfefferbaum et al., 2008). Additionally, another study found that adult P rats who were drinking alcohol over a period of a year, also demonstrated reductions in corpus callosum as compared to water drinking animals. In the present study a trend for shrinkage of the corpus callosum was found in the ethanol-exposed animals but it did not reach significance. Taken together, these studies suggest that changes in corpus callosum may be more likely to occur in adult vs. adolescent ethanol-exposed animals and/or that either longer exposures or higher blood ethanol levels are required to produce deleterious effects on that brain structure. Additionally, these data suggest that hippocampal volume may be selectively sensitive to adolescent alcohol exposure in both humans and rats. Interestingly, our data suggest that hippocampal volume decreases are not present following 2 weeks of withdrawal from 5 weeks of ethanol exposure but are seen at 10 weeks following exposure. It has been suggested that reductions in neurogenesis may participate in the process that leads to reduced hippocampal size and neurodegeneration (Nixon et al., 2010; Winner et al., 2011). Although the mechanisms of decreased hippocampal volume are most likely include many factors, it is possible that the cumulative effect of a persistent loss of neurogenesis combined with cell death over a long period of time may contribute to hippocampal volume loss that was observed in 128 day sacrifice as compared to the 72 day animals. Alternatively, the reduction in hippocampal size may represent an insufficient growth trajectory that becomes significant over a longer time period.

Several authors have posited that acute and chronic effects of alcohol may cause toxic effects on adolescent developing brain systems that result in an increase in affective, impulsive and or disinhibitory behaviors, which in turn may facilitate further alcohol use (Crews and Boettiger, 2009; de Wit, 2009; White et al., 2011). In the present study ethanol-exposed animals that were sacrificed at 128 days, spent a significantly shorter time in the dark box before returning to the light box and exhibited significantly more rears while in the light box than controls. This suggests that the ethanol vapor exposed animals may be less anxious or more disinihibited in the light/dark box test. These data are also consistent with findings from previous studies, using this alcohol vapor exposure paradigm, where more disinhibitory behavior was found in the ethanol vapor exposed animals two weeks following withdrawal of the vapor exposure using an open field conflict test (Ehlers et al., 2012). Thus it appears that adolescent ethanol exposure may result in an increase in disinhibitory behaviors that persists long after withdrawal from ethanol.

In the present study, prepulse inhibition of the startle (PPI) was also assessed 2 months after ethanol exposure in the animals that were sacrificed at 128 days. Ethanol-exposed animals had significantly diminished PPI as compared to the control animals that was also significantly correlated with hippocampal size. These data are consistent with studies conducted in both humans and rodents. In a study of structural brain correlates of PPI in healthy humans, significant positive correlations were obtained between PPI measures and grey matter volume obtained from the hippocampus and extended brain structures (Kumari et al., 2005). Additionally, correlations between PPI responses and hippocampal volumes

have been demonstrated in MRI volumetric studies conducted in normally developing rats (Schubert et al., 2009). These studies suggest that the hippocampus may be an important substrate for the expression of PPI in both humans and rodents.

Studies characterizing the cellular and molecular mechanisms underlying the enhanced vulnerability to ethanol exposure during adolescence have frequently focused on studying glutamatergic neurotransmission (see Crews et al., 2002; Fadda and Rossetti, 1998), and in particular the N-methyl-D-aspartate (NMDA) type of glutamate receptor. In rats treated with the NMDA receptor blocker MK-801, during postnatal day 7, a reduction in volume and neuronal number within the hippocampus as well as altered hippocampal NMDA receptor (NR1 subunit) expression and PPI deficits(in females) were seen when the animals reached adulthood (Harris et al., 2003). We have also demonstrated previously that two weeks of ethanol vapor exposure during adolescence as compared to adulthood results in decreased NR1 and NR2A subunit expression in hippocampus in the adolescents whereas no such effects were seen in the ethanol-exposed adults (Pian et al., 2010). Substantial loss of synapses, especially the excitatory glutamatergic inputs to the forebrain, occurs during adolescence (Huttenlocher, 1984; Zecevic et al., 1989) and may be vulnerable to ethanol exposure. In the hippocampus, the prolific outgrowth of excitatory axon collaterals and synapses that occur earlier during young ages are morphologically remodeled and branches within dendritic arbors are pruned during adolescent maturation with most synaptic pruning involving glutamatergic receptors (Swann et al., 1999). Taken together these studies suggest that hippocampal structure and function may be particularly vulnerable to insult by ethanol during adolescence and that insult may result in deficits in sensory gating as assessed by PPI, perhaps through changes in glutaminergic or other less well characterized neurotransmitter mechanisms (see Miller et al., 2010; Swerdlow et al., 2012).

Thus our data support the hypothesis that adolescent ethanol exposure can have significant effects on brain and behavior in an animal model where control of ethanol exposure can help delineate environmental effects from genetic background. Our studies demonstrate that DTI volumetric measures of hippocampus are significantly impacted by age and time following periadolescent ethanol exposure and withdrawal in Wistar rats. Also, reductions in the size of the hippocampus, associated with peri-adolescent ethanol exposure and withdrawal, are correlated with reduced sensory gating and increases in disinhibitory behavior. However, the model of ethanol exposure used in the present study, 14 hours of daily vapor exposure, does not mimic the typical pattern of alcohol drinking in human adolescents who are more likely to experience intermittent binge drinking at weekly or monthly intervals. However, adolescence in the human may span a 10 year period whereas in the rat periadolescence is condensed into a period of 35 days. This exposure period, although not directly translatable to humans was selected to ensure that the animals were exposed during the entire extended periadolescent period (Spear, 2000). However, additional studies will be necessary to determine whether shorter intermittent periods of exposure produce similar effects, and to test whether such effects are persistent farther into adulthood.

Acknowledgments

This study was supported in part by the NADIA Initiative Project of the NIH National Institute on Alcoholism and Alcohol Abuse grants AA019969 (CLE) and AA020022, AA020023 and AA020024 (FTC), HD03110 (IO) and the UNC Bowles Center for Alcohol Studies. The authors thank Jose Criado and Greta Berg for their assistance in data collection and Shirley Sanchez for assistance in editing the manuscript.

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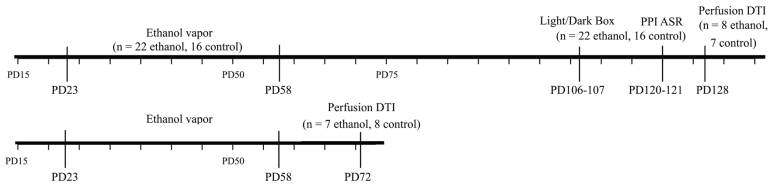

Figure 1.

Timelines representing two groups of animals used in this study. The first group was sacrificed at P72 and DTI measures were collected subsequently, the second group had behavioral measure from P106 to P121 and DTI measures after sacrifice at P128. Shown with each timeline is the number (n) of subjects included in each analysis.

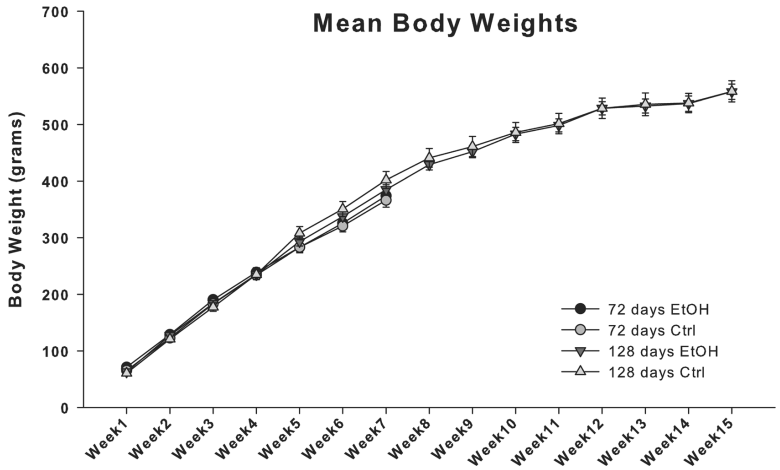

Figure 2.

Weekly averages of body weights shown for rats with DTI data over the course of the experimental protocol. Animals sacrificed at PD72 (n=15, 7 ethanol exposed, 8 controls) and at PD128 (n=15, 8 ethanol exposed, 7 control) show parallel increases in weight at equivalent ages. There were no significant differences between ethanol exposed and control rats over the course of the experiment and at sacrifice. Error Bars = S.E.M

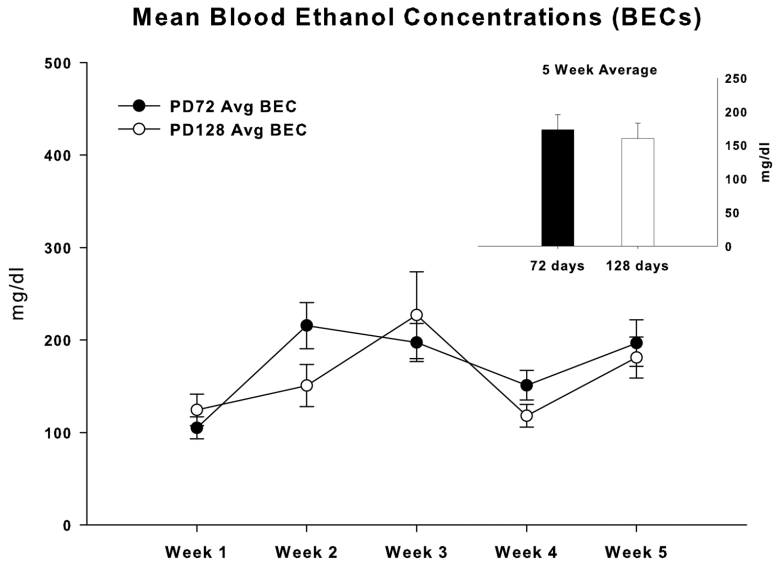

Figure 3.

Blood ethanol concentrations (BECs) graphed over the five week ethanol exposure for rats with DTI data. No significant difference was found between ethanol animals in the PD72 group (n=7) and PD128 (n=8) over the 5 week exposure period. The overall average BAC for the five week exposure is shown in the top right of the figure. Error Bars = S.E.M

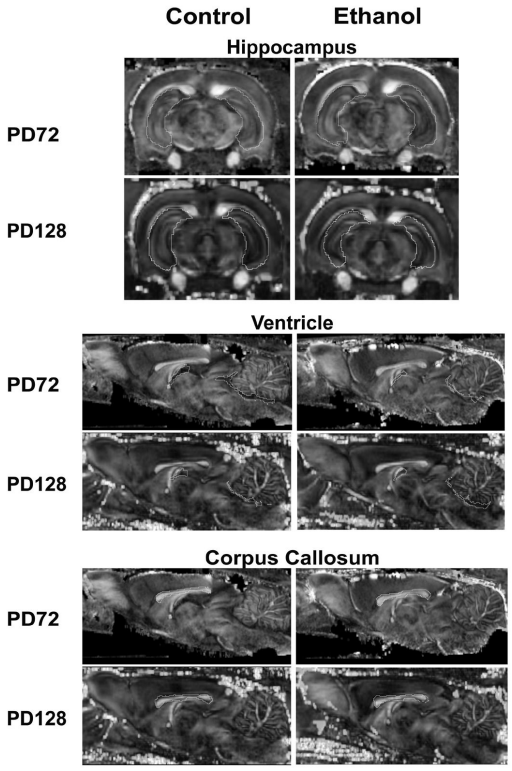

Figure 4.

DTI images (160 micron isotropic) of ethanol vapor treated animals and their respective controls at each age (PD72, PD128). Representative coronal and sagittal slices shown for hippocampus, corpus callosum and ventricle with volumes outlined. There was no significant difference between ethanol treated animals and controls in corpus callosum or ventricle at both ages, and hippocampal volumes at PD72. At PD128 the hippocampal volume of ethanol treated animals was significantly smaller ($p<0.019$) than their controls (data shown in figure 5).

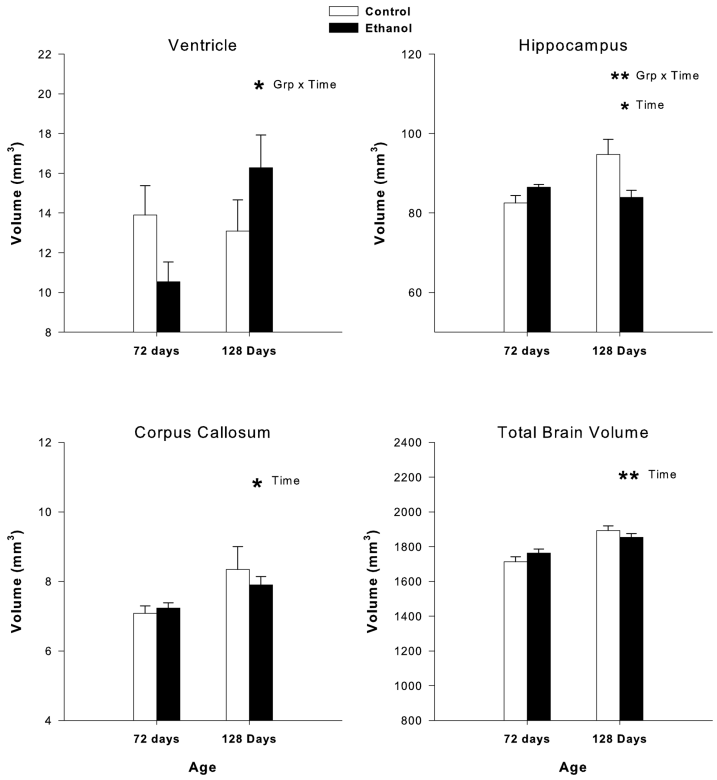

Figure 5.

Effects of adolescent ethanol vapor exposure on DTI measures for animals sacrificed at PD72 (n=15, 7 ethanol exposed, 8 controls) and PD128 (n=15, 8 ethanol exposed, 7 controls). (Top left) There was a significant ethanol treatment × time effect for the ventricle ($p<0.036$). (Top right) Hippocampus volume showed an age effect ($p<0.042$) between those sacrificed at PD72 and those at PD128 as well as a treatment × time effect ($p<0.003$). (Bottom left) Corpus callosum also revealed a significant age effect ($p<0.012$) between the age of sacrifice. (Bottom right) Total brain volume is significantly larger overall in the 128 day sacrificed animals ($p<0.001$) but no ethanol or ethanol × time effect was seen. * indicates $p<0.05$ ** indicates $p<0.01$ Error Bars = S.E.M

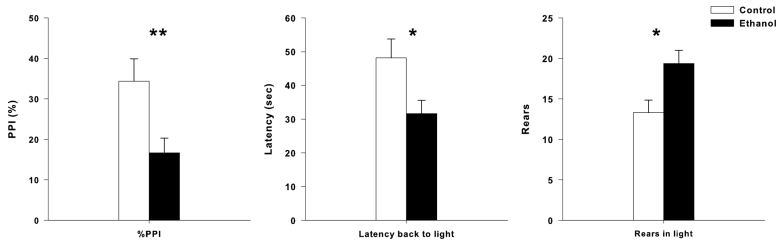

Figure 6.

Effects of peri-adolescent ethanol exposure on the prepulse inhibition of the acoustic startle response (PPI) and light/dark box behaviors shown for the rats sacrificed at PD128. (Left) Ethanol treated rats (n=22) showed significantly less inhibition of the startle from the prepulse tone compared to controls (n=16). (Center) A comparison of the duration of first entry into the dark box revealed that the ethanol treated rats spent significantly less time in the dark box prior to returning to the light box than control rats. (Right) Additionally, ethanol exposure significantly increased the number of rears while inside the light box. * indicates $p<0.05$. ** indicates $p<0.01$. Error Bars = S.E.M

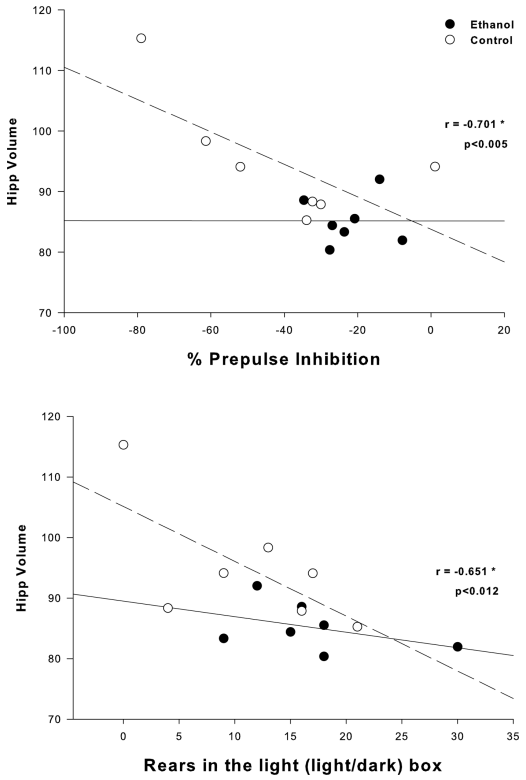

Figure 7.

Effect of adolescent alcohol exposure on hippocampal volume and behavior in rats sacrificed at PD128. Pearson correlation r value shown with trend lines plotted for each treatment group. Reductions in hippocampal volume were found to significantly correlate with lower prepulse inhibition of the acoustic startle response as well as increased numbers of rears in the light/dark box.