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Chronic intermittent ethanol exposure leads to alterations in brain-derived neurotrophic factor within the frontal cortex and impaired behavioral flexibility in both adolescent and adult rats

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Abstract

Chronic intermittent exposure to ethanol (EtOH; CIE) that produces binge-like levels of intoxication has been associated with age-dependent deficits in cognitive functioning. Male Sprague-Dawley rats were exposed to CIE (5 g/kg, 25% EtOH, 13 intragastric gavages) beginning at three ages: early adolescence (postnatal day [PD] 28), mid-adolescence (PD35) and adulthood (PD72). In experiment 1, rats were behaviorally tested following CIE. Spatial memory was not affected by CIE, but adult CIE rats were impaired at acquiring a non-spatial discrimination task and subsequent reversal tasks. Rats exposed to CIE during early or mid-adolescence were impaired on the first reversal, demonstrating transient impairment in behavioral flexibility. Blood EtOH concentrations negatively correlated with performance on reversal tasks. Experiment 2 examined changes in brain derived neurotrophic factor (BDNF) levels within the frontal cortex (FC) and hippocampus (HPC) at four time points: during intoxication, 24-hrs after the final EtOH exposure (acute abstinence), 3-weeks following abstinence (recovery) and after behavioral testing. HPC BDNF levels were not affected by CIE at any time point. During intoxication, BDNF was suppressed in the FC, regardless of the age of exposure. However, during acute abstinence, reduced FC BDNF levels persisted in early adolescent CIE rats, whereas adult CIE rats displayed an increase in BDNF levels. Following recovery, neurotrophin levels in all CIE rats recovered. Our results indicate that intermittent binge-like EtOH exposure leads to acute disruptions in FC BDNF levels and long-lasting behavioral deficits. However, the type of cognitive impairment and its duration differ depending on the age of exposure.

Keywords

Chronic Intermittent Ethanol Exposure; Discrimination Learning; Reversal Learning; Adult; Early Adolescence; Mid- Adolescence

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Introduction

Adolescent and early adult alcohol drinking has been linked to development of alcohol use disorders, which can lead cognitive deficits and behavioral problems (Crews et al., 2007; Spear and Swartzwelder, 2014; Risher et al., 2015). Early adolescent ethanol (EtOH) exposure appears to solidify an adolescent-like behavioral phenotype in adulthood, which includes impulsivity, impaired behavioral flexibility, and increased anxiety (Vetreno and Crews, 2012; Semenova, 2012; Risher et al., 2013; Coleman et al., 2014; Gass et al., 2014; Mejia-Toiber et al., 2014). However, mid adolescent and adult chronic intermittent exposure to ethanol (CIE) has also been associated with deficits in attention, reversal learning and extinction learning (Slawecki, 2006; Kuzmin et al., 2012; Broadwater & Spear, 2014; Badanich et al., 2016). Thus, further examination of the long-term effects of EtOH exposure across early adolescence into early adulthood is critical for understanding the unique age-specific effects of CIE on cognition and neural adaption.

Binge-like EtOH exposure, particularly during adolescence, leads to reductions in neurogenesis in the hippocampus, decreased gliogenesis in the frontal cortex, as well as a loss of forebrain cholinergic neurons (Crews and Nixon, 2009; Koss et al., 2012; Broadwater et al., 2014; Vetreno and Crews, 2015). Such pathology is believed to be caused by EtOH mediated induction of neuroimmune genes within the frontal cortex and hippocampus that persist into adulthood (Vetreno and Crews, 2012; Crews et al., 2015). Ethanol-induced activation of proinflammatory signaling in the brain can lead to neurodegeneration through exacerbated oxidative stress and excitotoxicity. As such, damage to both the frontal cortex, such as decreases in myelination, and neural degeneration in the hippocampus, visualized using an amino-culpric silver technique, have been observed following adolescent CIE exposure (Crews et al., 2000; Vargas et al., 2014; Vetreno et al., 2014).

Neurotrophins are key modulators of neurodegeneration associated with aging and disease. It has been shown that prenatal and adult chronic EtOH exposure alters levels of neurotrophin, such as brain derived neurotrophic factor (BDNF), in the frontal cortex and hippocampus (Miller et al., 2002; Davis, 2008; Nixon and McClain, 2010; Mooney and Miller, 2011; Vedder et al., 2015). However, few studies have assessed neurotrophin expression after adolescent CIE and the results are variable (Briones and Woods, 2013; McClain et al., 2014; Sakharkar et al., 2016). One key factor in alcohol-associated neurotrophin dysfunction is the timing or stage of the disease process during which neurotrophin measures are assessed (see Davis, 2008).

Our goal was to determine an ontogenetic profile across early adolescence into early adulthood regarding the effect of binge-like EtOH exposure on hippocampal and frontal cortical neurotrophin adaption. We employed a CIE model in early adolescent, mid-adolescent and young adult rats. In experiment 1, following a 3-week EtOH free recovery period, which matured both early and mid-adolescent rats to adulthood, spontaneous alternation and a non-spatial discrimination task with reversals were conducted to determine deficits in hippocampal-dependent spatial memory and frontocortical-dependent cognitive flexibility. Since BDNF has been shown to modulate neuroadaption, we examined the effects of CIE on mature BDNF levels in the frontal cortex and hippocampus in experiment 2.

BDNF levels were measured at differing time points during CIE: During the final EtOH exposure (intoxication), 24-hours after the final EtOH exposure (acute abstinence), 3-weeks following final EtOH exposure (recovery) and post-behavioral testing.

Methods

Subjects

Early Adolescent (PD28), mid-adolescent (PD35), and adult (PD65-78) male Sprague-Dawley rats were obtained from litters bred at Binghamton University. No more than one rat from each litter was randomly assigned within each treatment condition. Rats were pair housed in a temperature (20°C) and humidity controlled colony under a 12-hour light/dark cycle (onset at 7:00 am). Rats were provided with *ad libitum* access to lab chow and water. During CIE treatment, rats were weighed on each treatment dosing date. After CIE, rats were weighed on a weekly basis to ensure normal weight gain and health. Experimental procedures were in compliance with the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee (IACUC) at the State University of New York at Binghamton.

Experiment 1: Behavioral Testing and BDNF Measurement

Rats at each age range were randomly divided into CIE (Early Adolescent: n=10; Mid Adolescent: n=9; Adult: n=9) and water treated control groups (Early Adolescent: n=10; Early Adolescent: n=11; Adult: n=10). Three weeks following CIE cessation, rats were behaviorally tested. This cohort also served as the time-point 4 (behaviorally tested) cohort in Experiment 2. Figure 1 demonstrates a schematic of the exposure and treatment timeline.

Chronic Intermittent Ethanol Treatment

For both experiments 1 and 2, adolescent and adult rats were subject to 13 intragastric gavages of either 25% EtOH (v/v) or water, administered at a dose of 5 g/kg. The dosing schedule followed a modified 2-day on/off cycle, where animals were dosed once per day for 2 days, followed by a 2-day recovery period until the 12th gavage. The final gavage (#13) was administered 2 days following gavage #12. Blood samples were collected via a small incision in the lateral tail vein 30 minutes to an hour following the first, fifth, and final gavage. Blood collection occurred during the time course when BEC levels would be increasing, but not at peak intoxication (Livy et al., 2003; Quertemont et al., 2003). Plasma was separated using a centrifuge and stored at -20°C until blood ethanol content (BEC) levels were measured using an AM1 Alcohol Analyzer (Analox Instruments, MA). Throughout treatment, all animals gained weight, and there was no significant effect of CIE treatment on animal weights.

Following the cessation of CIE, rats in experiment 1 had a 3-week recovery period, during which they were weighed and handled once per week. Prior to the start of behavioral testing, rats were food restricted to 90% of their free feed weight over the course of 5 days to induce searching and digging motivation. Spontaneous alternation testing occurred first, followed by training in a multiple phase, non-spatial discrimination task. Figure 1 illustrates the exposure protocol.

Spontaneous Alternation

Details for our spontaneous alternation protocol can be found in Fernandez et al., 2016. In brief, rats were tested once for spontaneous alternation behavior in a plus maze (105.5 cm x 14.4 cm x 15 cm) with clear, plastic walls and black, wooden floors. The animal was habituated to the testing room for 20-minutes, after which it was placed on the center of the maze. Each rat explored the maze for 18-minutes. Arm entries were recorded during testing, and percent alternation scores were analyzed. An alternation was defined as entry into four different arms in a successive sequence. Spontaneous alternation scores were corrected to account for significant differences in activity between groups: arm entries were only recorded up to 27 possible arm entries, which was the average number of arm entries made by the lowest activity group (adults). The normalization of percent alternation scores is adapted from Savage, 2012 and Fernandez et al., 2016.

Non-spatial Discrimination Learning and Reversal Task

Details regarding the non-spatial discrimination and reversal task can be found in Fernandez et al., 2016. In brief, the day after spontaneous alternation testing, rats began dig training in their home cage. Ceramic bowls were filled with wood shavings and baited with Cheerios. Rats continued dig training until they reliably retrieved and ate a Cheerio reward from the bottom of the bowl.

Discrimination learning and reversal testing took place in a white, plastic chamber (70.3 cm x 40 cm x 36.4 cm) with black floors. The chamber was sectioned into a start box (16.5 cm x 40 cm) and a testing area (53.8 cm x 40 cm) by a removable divider. Two ceramic bowls filled with digging substrates, referred to as mediums (see table 1), were located against the back wall of the testing area and were divided by a smaller removable partition (19.8 cm x 25.9 cm). Training and testing occurred across 3 phases.

Phase one of training consisted of a 5-minute habituation to the testing chamber. At the start of each trial, the large divider was lifted, and each rat had 2 minutes to approach a bowl and eat a Cheerio reward until criterion was met. Criterion was defined as a rat eating from either baited bowl on 6-consecutive trials. Phase 2 and 3 used the same criterion (a rat eating a reward from the correct bowl on 6 consecutive trials) definition to determine the number of trials required to reach criterion.

During phase 2, the rat learned two simple discriminations: a scent based discrimination and a media based discrimination. Once a rat reached criterion, phase 3 began, which required the rat to perform a series of five discriminations (see Table 1). Discrimination 1: another simple discrimination (SD) that consisted of a single dimension, where a bowl primed with a unique scent or filled with a unique medium was rewarded. Discrimination 2: compound discrimination 1 (CD1) combined both a unique scent and digging medium that were different from those previously utilized in SD. Although each bowl contained both a scent and medium dimension, animals had to discriminate based on a single dimension. Discrimination 3: Reversal 1 (R1) inverted the CD1 rule, so that the opposite stimulus was now rewarded. Discrimination 4: a new set of scent and digging mediums were introduced during compound discrimination 2 (CD2), but the rat was still required to discriminate based

on the same dimension as in CD1 and R1. Discrimination 5: the rewarded stimulus from CD2 was switched in reversal 2 (R2).

Brain Collection

Within seven days following the conclusion of behavioral testing, time-point 4 (T4; behaviorally tested) rats were decapitated and their brains were immediately extracted. Hippocampal and frontal cortical tissue was dissected and stored at -80°C to be used for BDNF ELISA.

Experiment 2: Mature BDNF ELISA

Rats were randomly assigned to 1 of 3 tissue collection time-points. At time-point 1 (T1; intoxication), brain tissue was collected an hour following the final gavage for both CIE and control animals. At time-point 2 (T2; acute abstinence or withdrawal), brain tissue was collected 24-hrs after CIE ended, in an EtOH-free state. At time-point 3 (T3; protracted abstinence or recovered), brain tissue was collected 3- weeks after the cessation of CIE. Rats were randomly assigned to the following treatment onset conditions: Early adolescent control (T1=7, T2=7, T3=7); Mid Adolescent control (T1=6, T2=8; T3=6); Adult control (T1=8, T2=7; T3=8); Early Adolescent CIE (T1= 9; T2=9; T3=10); Mid Adolescent CIE (T1=8, T2=9; T3=7); and Adult CIE (T1= 8; T2=7; T3=8). Tissue from behaviorally tested animals in Experiment 1 was also analyzed (T4). Figure 1 illustrates a schematic of the time-point collection timeline in reference to CIE treatment and behavioral testing.

Brain Collection

An hour following the final gavage, brain tissue was collected for T1 rats; 24- hours following the final gavage, brain tissue was collected for T2 animals; and 3-weeks following the final gavage, brain tissue was collected for T3 animals. Tissue for behaviorally tested, T-4, rats was collected a week following non- spatial discrimination testing. Rats were decapitated and brains were rapidly extracted. Hippocampal and frontal cortical regions were dissected and stored at -80°C to be used for BDNF enzyme-linked immunosorbent assays (ELISA).

Mature BDNF ELISA

The vendor supplied BDNF ELISA protocol (Promega, Madison, WI, USA) was followed for the detection of BDNF. To limit measures to the mature form of BDNF, an acid wash step was not performed on neural tissue. Hippocampal tissue was diluted at a ratio of 1:4, while frontocortical was diluted at a ratio of 1:5 for appropriate detection within a standard curve. Protocol details can be found in Vedder et al., 2015. Homogenate samples (198) were run in duplicate across multiple days, therefore water i.g. brain samples served as internal controls across plates.

Experimental Design and Statistics

Analyses were performed in SPSS (IBM Corporation, Version 22, New York). A 2 (Treatment: CIE vs. Control) x 3 (Age at exposure: early adolescence, mid-adolescence, adult) analysis of variance (ANOVA) assessed spontaneous alternation behavioral measures.

Repeated-measures ANOVAs (RMANOVA), with Treatment and Age as between subject factors, were used to analyze differences in discrimination learning (across tasks). Reported Discrimination task F statistics and degrees of freedom were corrected using Greenhouse-Geisser values due to a significant violation of sphericity, $\chi^2(20)=50.77$, $p<0.05$. Bivariate correlations were analyzed using Pearson's correlation coefficient to compare the relationship between BEC levels and ASST performance. A Bonferroni correction was used to account for multiple correlation comparisons, and significance was defined in these analyses as $p<0.01$. A 2 (Treatment) x 3 (Age) x 4 (Time point: T1, T2, T3, T4) ANOVA assessed BDNF levels in the hippocampus and prefrontal cortex. Error bars represent standard error mean. BDNF values were calculated relative to the percent of change from age- specific control groups (water i.g.).

Results

Blood Ethanol Content

For CIE rats, BEC values after the first gavage were significantly higher than BEC values collected during the middle of treatment ($p<0.01$) and at the end of treatment ($p<0.01$). Figure 2 illustrates average CIE BEC values, which well exceeded binge EtOH benchmarks of 80 mg/dL at all age groups (Spear, 2015).

Experiment 1: Behavioral Testing

The following 3 sections describe the behavioral testing results for spontaneous alternation and non-spatial discrimination tasks. The first section reports the 2-way ANOVA results that analyze the interaction between CIE treatment and age of exposure on percent alternation scores. The same analysis is used to examine differences in activity levels (arm entries) during spontaneous alternation behavior. Due to a significant age-specific difference in arm entries, where adults had significantly less arm entries compared to early and mid adolescents, percent alternation scores were recalculated to a maximum of 27 arm entries, which was the adult arm entry average. The second section describes the repeated measures ANOVA for non-spatial discrimination and reversal tasks, with age of exposure and CIE treatment as between- subject variables. Individual ANOVAs were used to analyze the interaction between age and CIE treatment within each task. Finally, BEC values are correlated with reversal task performance.

Spontaneous Alternation

Analysis of percent alternation scores did not yield any main effects or interaction between Treatment and Age (all F 's[2,53] < 2.0 , p 's > 0.10 ; Figure 3-A). When comparing arm entry differences, CIE early adolescent rats made significantly less arm entries compared to age matched controls ($[F(1,19)=5.17$, $p<0.05$; Figure 3-B). Additionally, there was a main effect of Age on activity, analyzed as total arm entries. Overall, adolescent animals made more arm entries compared to mid- adolescent and adult animals ($F[2,53]= 6.55$, $p< 0.01$; Figure 3-B). Therefore, alternation scores were corrected for overall activity, where percent alternation scores were calculated up to a maximum of 27 arm entries (the average arm entries for adults, which was the age group that demonstrated the lowest activity level). Subsequent

analysis of corrected alternation behavior did not find any significant difference as a function of Treatment or Age or their interaction ($F[2,53] < 1$, $p > 0.10$; Figure 3-C).

Discrimination Learning and Reversal Learning

Only rats that could reach criterion on a given task within 3 days were included in the final analysis (final n 's per group are shown in Table 2). Figure 4-A illustrates the average number of trials required to reach criterion across the discrimination and reversal tasks. The number of trials to reach criterion varied as a function of the discrimination task ($F[4.23,264] = 20.05$, $p < 0.001$). There was a significant interaction between Treatment, Age at time of exposure, and discrimination Task ($F[8.56,264] = 4.57$, $p < 0.001$), so we analyzed separate ANOVAs for each discrimination task. These follow-up analyses revealed a unique pattern of impairment as a function of Age at which CIE was initiated. When rats learned a simple discrimination, CIE rats, on average, required more trials to reach criterion ($F[1, 48] = 6.00$, $p < 0.01$). However, only those exposed to CIE as Adults were significantly different from their age-match controls ($F[2, 48] = 5.82$, $p < 0.01$). In contrast, regardless of the Age at which CIE began, all CIE rats, relative to age matched control rats, required more trials to switch to a successful behavior in the first reversal (R1) learning test ($F[1,44] = 29.95$, $p < 0.001$). It should be noted that adolescent treated control rats required more trials to master R1 than other age control rats ($F[2, 25] = 9.68$, $p < 0.01$). When rats were required to learn a second complex discrimination and that rule was reverse (R2), although all CIE treated rats required more trials ($F[1, 45] = 16.47$, $p < 0.01$), only those exposed to CIE as Adults were significantly impaired compared to their age-match controls ($F[2, 45] = 3.59$, $p < 0.05$). However, the lack of a CIE effect in adolescent rats during R2 may be due to the fact that there was a trend for control-treated early and mid-adolescent rats to require more trials, compared to adult-treated control rats, to reach criterion ($F[2, 26] = 3.06$, $p = 0.07$).

Behavioral Correlations

As shown in Figure 4-B, average BEC levels were significantly correlated with reversal performance at both the first ($r = .50$, $p < 0.001$) and second reversal ($r = .45$, $p < 0.001$; Figure 4-C). Regardless of Age at exposure, animals with higher BEC levels required more trials to adapt their behavior per the new stimulus-reward contingencies.

Experiment 2: BDNF Measurement

The samples from 6 subjects were excluded from the analysis due to insufficient homogenate collection (final n 's per group are shown in Table 2). In the prefrontal cortex, there was a significant effect of CIE Treatment ($F[1, 35] = 7.28$, $p < 0.05$; Figure 5-A) on BDNF levels measured as percent change from control for T1 animals: CIE rats had lower BDNF content in the prefrontal cortex during intoxication. At T2, there was a significant effect of Age ($F[2, 38] = 5.25$, $p < 0.05$) and Age x Treatment interaction ($F[2, 38] = 5.26$, $p < 0.05$) on prefrontal BDNF levels: Adult CIE animals had higher BDNF content compared to age-matched controls ($F[1,12] = 4.8$, $p < 0.05$), whereas early adolescent CIE animals had significantly lower cortical BDNF levels during acute abstinence ($F[1,13] = 6.12$, $p < 0.05$). However, by T3, BDNF levels in the frontal cortex normalized as there were no significant effects of Age, Treatment or Age X Treatment interaction (all F 's < 1 ; p 's > 0.10). T3 and T4

BDNF levels were not significantly different across Age, CIE Treatment, Timepoint, or region (all F 's<1; p 's>0.10).

In the hippocampus, there was no significant effect of Age, Treatment or the interaction of those variables (all F 's<1; p 's>0.10) on BDNF change from control levels during any time point (Figure 5-B). Analysis of total BDNF protein levels did yield a main effect of region of interest ($F[1,46]=183.68$; $p<.001$). Pairwise comparisons indicated that BDNF protein levels in the hippocampus were significantly higher than frontal cortical levels, $p<.001$.

Discussion

Following CIE exposure during early adolescence, mid-adolescence or adulthood, we found that binge-like levels of EtOH exposure lead to reversal learning deficits following a drug-free recovery period. BEC levels are correlated with reversal memory impairments. CIE caused a significant decrease in BDNF levels within the prefrontal cortex, a reduction that was maintained throughout withdrawal in early adolescent CIE animals. Adult treated CIE animals demonstrated a resurgence of BDNF levels with the prefrontal cortex during withdrawal. Frontal cortical BDNF levels stabilized following a protracted drug free period. Within the hippocampus, CIE did not alter BDNF levels. Our results indicate that CIE leads to impaired behavioral flexibility regardless of the age at which EtOH exposure began. However, CIE exposure during early adulthood produced a non-spatial learning deficit and a persistent impairment in behavioral flexibility. Thus, our data reveal that significant cognitive impairment can be observed with binge-like EtOH exposure in early adulthood.

The lack of a CIE exposure effect on spatial working memory is similar to other reported null effects on the radial arm maze (Risher et al., 2013) and Morris Water Maze (Van Skike et al., 2012). Spatial memory is also unaffected following either adolescent or adult CIE in a radial arm maze task, but an acute EtOH challenge did alter path efficiency in both age groups (Swartzwelder et al., 2014). Our findings add to the growing evidence that chronic, binge-like levels of EtOH during adolescence or adulthood does not negatively affect spatial memory when tested in a drug-free state.

Although CIE did not affect spontaneous alternation behavior, we found that early adolescent rats exposed to CIE were less active on the maze compared to their age-matched controls. Previous studies have shown a correlation between decreased activity (total distance travelled and arm entries) and anxiety-like behavior (center time and open arm time) on both open field and elevated plus maze behavior across rodent species (Walf and Frye, 2007; Bailey and Crawley, 2009; Moore et al., 2011; Burke et al., 2016). Other studies using traditional assessments of anxiety (open field, elevated plus maze, light-dark box) have shown that CIE increases anxiety-like behavior, where early adolescent EtOH exposure leads to decreased time spent in the light, open arms or center of a field (Pandey et al., 2006; Coleman et al., 2014; Sakharkar et al., 2016). Increased anxiety-like behavior is a phenotype that develops following adolescent CIE, which can persist into adulthood. Thus, the reduced activity or exploratory behavior that we observed in early adolescent CIE treated rats may be related to a long-lasting increase in anxiety.

We found that BEC levels negatively correlated with performance on both reversal tasks. Our data demonstrate that rats with high levels of metabolic intoxication were more likely to commit reversal task errors. In humans, high blood alcohol content has been linked to impairments in executive functioning and perseveration (Pihl et al., 2003; Lyvers and Tobias-Webb, 2010; Lyvers et al., 2015). Our findings support the hypothesis that behavioral deficits resulting from CIE exposure represent changes in frontal cortical mediated behavioral flexibility (Badanich et al., 2011). Previous work found that although adolescent CIE exposure did not impair the initial detection and memory of a safe, spatial location, such treatment did impair learning the location of a novel safe, spatial location (reversal) on the Morris Water Maze and Barnes Maze (Obernier et al., 2002; Coleman et al., 2011). There are two pathological sequelae that may contribute to impaired behavioral flexibility after CIE. Impairments in reversal learning, such as those seen in our study, are also caused by basal forebrain lesions (Tait and Brown, 2008). CIE, especially during early adolescence, leads to a marked reduction of cholinergic neurons in the forebrain and prefrontal cortex (Vetreno et al., 2014; Boutros et al., 2015). Thus, it is possible that the reversal learning impairments in our EtOH treated animals indicates a disruption of basal forebrain signaling to the prefrontal cortex.

In addition, there is evidence to suggest that reversal learning is dependent on orbitofrontal cortical activity (Floresco et al., 2008; Ghods-Sharifi et al., 2008). Thus, our reversal learning deficits may indicate an orbitofrontal deficit (Chudasama and Robbins, 2003; Xue et al., 2013; Hamilton and Brigman, 2015), which may be a result of disruptions in neurotrophin signaling, coupled with EtOH mediated increases in neuroimmune responses (Vetreno and Crews, 2014). EtOH increases nuclear factor kappa- light- chain enhancer of activated B cells (NF- κ B), which can lead to the induction of proinflammatory cytokines and neuroimmune genes in the orbitofrontal cortex, such as receptors for advanced glycation end products (RAGE) and high-mobility group box 1 (HMGB1), that are related to neurodegeneration (Vetreno et al., 2013; Crews et al., 2015). EtOH also causes a downregulation of cyclic AMP-responsive element binding protein, which is a critical trophic factor involved in the induction of several synaptic plasticity pathways (see Wang and Peng, 2016 for a review). The disruption of these trophic factors ultimately leads to decreases in behavioral control/response inhibition via alterations in the functioning of regulatory neural networks (Vetreno and Crews, 2014).

In all exposure age groups, we found a region-specific suppression of BDNF levels within the frontal cortex ($\approx 25\%$) during EtOH intoxication. However, in the acute abstinence or withdrawal phase, we saw the emergence of age-specific differences: early adolescent rats had a persistent suppression of BDNF levels, whereas young adult rats had a dramatic rise in BDNF levels, and the mid adolescent rats displayed normalized BDNF levels. Neurotrophin levels are altered by chronic alcohol exposure, but in an intoxication dependent manner (Davis, 2008). Both acute and long-term EtOH exposure decreases neurotrophin levels (Huang et al., 2011; Raivio et al., 2012; Boschen et al., 2015; see Crews et al., 2016 for a review). However, during withdrawal, neurotrophin release has been documented to notably increase, and this compensation has been suggested to be a neuroprotective measure that regulates further EtOH consumption and neural damage (Logrip et al., 2015). CIE adult exposed rats displayed this transient increase, but it was not observed in the adolescent CIE

groups. Interestingly, the post withdrawal increase in frontal cortical BDNF in the CIE adult rats did not protect this group from frontal cortical dependent behavioral impairments. It is important to note that there was a 3-week period during which BDNF levels were not assessed for any experimental group. Since this time period encompasses both withdrawal and recovery, it is possible that differential BDNF recovery trajectories fell beyond our detection window.

The CIE paradigm we used did not produce differences in hippocampal BDNF levels across age ranges or EtOH phases. Our findings are consistent with previous studies that do not show differences in hippocampal BDNF levels following a 4-day excessive binge exposure (McClain et al., 2014). However, a drinking in the dark EtOH exposure paradigm throughout adolescence led to a 20% reduction of BDNF protein levels in the hippocampus (Briones & Woods, 2013). Furthermore, lower EtOH CIE doses (20% EtOH w/v; 2 g/kg) during early adolescence did result in a decreased BDNF protein density expression (26%) within CA1 and CA3 regions, but not in the dentate gyrus (Sakharkar et al., 2016). Thus, within the hippocampus, there may be regional sensitivities in how EtOH exposure differentially modulates BDNF expression.

Conclusions

Regardless of the age when CIE begins, intermittent EtOH exposure leads to impairments in behavioral flexibility. Impaired behavioral flexibility can lead to maladaptive behavioral patterns of alcohol seeking and consumption that can ultimately lead to addiction, especially when alcohol exposure begins in adolescence (Verdejo-García et al., 2006; Winstanley et al., 2010; Jansen et al., 2015). By including an early adult age as a comparison to early and mid-adolescent exposure, we found more persistent reversal learning impairments following adult exposure to binge-like levels of EtOH. The specificity of EtOH induced impairments on behavioral flexibility measures in the adult cohort demonstrates that binge-like EtOH exposure is harmful even at later stages of development. Interestingly, our data reveal that mid-adolescent binge EtOH exposure can lead to similar patterns of reversal learning impairments as those seen following early adolescent CIE, and these impairments were related to high BEC levels in all age groups. Changes in BDNF levels within the brain can reflect neural adaption following trauma, toxicity and/or degeneration. Within the frontal cortex, acute changes in BDNF levels occur during intoxication and acute abstinence following CIE that are developmentally unique. Whether these early alterations in frontocortical BDNF levels set the stage for neural adaptations that led to more persistent pathological responses remains to be determined.

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Highlights

- Chronic intermittent exposure to ethanol transiently alter prefrontal cortical Brain Derived Neurotrophic Factor levels
- Adults exposed to chronic intermittent ethanol display impairments in discrimination and reversal learning
- Early and mid adolescent animals exposed to chronic intermittent ethanol only display impairments during reversal learning

Figure 1. CIE protocol and experimental timeline

The schematic illustrates CIE exposure (arrow heads), BEC collection time points (dotted lines), and age ranges for early adolescent (EA), mid adolescent (MA), and adult animals during treatment. An hour after the final gavage, time-point 1 (T1; intoxicated; closed circle) animals were sacrificed for tissue collection. A day after the final EtOH exposure, tissue from time-point 2 (T2) was collected. After the final gavage, time-point 3 (T3) and 4 (T4) animals underwent a 3-week, drug-free recovery period. Tissue was collected for T3 animals at the end of the recovery period, and tissue for T4 animals was collected after behavior testing.

Figure 2. CIE Animals achieved significant blood ethanol concentrations across treatment
Regardless of time point or age, CIE treated animals had significant BEC levels throughout the CIE protocol. BEC levels were highest after the first gavage, compared to BEC levels during the middle and end of treatment (E, $p < 0.05$).

Figure 3. CIE does not lead to impairments in spontaneous alternation

A: All animals alternated at similar rates regardless of age or CIE exposure. **B:** Compared to age-matched controls, early adolescent CIE animals made significantly less arm entries (E, $p < 0.05$). Additionally, early adolescent animals, regardless of treatment, made significantly more arm entries compared to other experimental groups (A, $p < 0.05$). **C:** Due to age specific differences in activity levels, percent alternation behavior was rescored to a maximum of 27 arm entries (lowest activity levels seen in 2-B). Reanalyzed data did not show an effect of CIE treatment or age of exposure on spatial navigation behavior.

Figure 4. CIE leads to impairments in reversal learning

A: Although all CIE rats, regardless of age, were impaired on the first reversal ($E, p's < 0.05$), only the Adult CIE rats required significantly more trials to learn the initial simple discrimination and the second reversal (AXE, $p's < 0.05$). Early adolescent rats required more trials to reach criterion for reversal 1, compared to mid-adolescent and adult rats ($A, p < 0.05$). **B:** Performance on reversal 1 was significantly correlated with average blood ethanol concentrations: Rats with higher BECs required more trials to learn the rule reversal ($p < 0.01$). **C:** Higher BEC levels also significantly correlated with requiring more trials to reach criterion on the second reversal ($p < 0.01$).

Figure 5. In the prefrontal cortex, CIE initially alters BDNF levels, but after a protracted abstinence, BDNF levels normalize

A: All CIE rats had significantly less BDNF content (measured as percent change from control) in the frontal cortex, compared to control animals, while intoxicated (T1; during last gavage; E ; $p < 0.05$). During acute abstinence (T2; 24-hr post gavage), adult CIE rats had significantly higher BDNF content in the frontal cortex, whereas early adolescent CIE rats had significantly lower BDNF content compared to age matched controls (Ax E , $p < 0.05$). Following a protracted recovery (T3; 3 weeks post gavage), and after behavioral testing (T4), BDNF levels in all CIE rats normalized. **B:** Hippocampal BDNF levels were not changed by CIE treatment at any age or at any time point (measured as percent change from controls). Dotted lines represent control value (100%) and solid lines represent controls SEM. Numbers represent CIE protein levels (pg/mL total protein). Total BDNF protein levels were higher in the hippocampus compared to the prefrontal cortex ($p < 0.05$). Average BDNF pg/mL of total protein for control groups was 23.66 (± 2.4) in the frontal cortex and 61.38 (± 3.1) in the hippocampus.

Table 1

Discrimination Task Stimuli Examples

Discrimination Task	Medium-based Cues		Scent-base Cue	
	Bowl 1	Bowl 2	Bowl 1	Bowl 2
Simple	easter grass	shredded paper	clove /bedding	nutmeg/bedding
Compound 1	thyme/ rocks	citronella/tubes	piña colada/confetti	lavender /colored beads
Reversal 1	thyme/rocks	citronella/ tubes	piña colada /confetti	lavender/colored beads
Compound 2	rosemary/sand	cinnamon/ gravel	sweet pea /sand	vanilla/wood chips
Reversal 2	rosemary/ sand	cinnamon/gravel	sweet pea/sand	vanilla /wood chips

Bolded stimuli indicates the rewarded cue.

Table 2

Final subject numbers per experimental conditions. The subject numbers fluctuate due to learning criteria and tissue viability.

Experiment	Group n's					
	Control EA	Control MA	Control Adult	CIE EA	CIE MA	CIE Adult
Blood Ethanol Content	30	33	30	34	33	33
Spontaneous Alternation	10	11	10	10	9	9
Simple Discrimination	10	10	10	7	8	9
Compound 1	10	9	8	7	8	9
Reversal 1	10	8	8	7	8	9
Compound 2	10	9	8	7	8	9
Reversal 2	7	9	8	7	8	9
Prefrontal BDNF T1	7	7	8	7	7	8
Prefrontal BDNF T2	8	5	6	8	5	6
Prefrontal BDNF T3	10	8	8	10	8	8
Prefrontal BDNF T4	9	7	8	9	7	8
Hippocampal BDNF T1	9	8	8	9	8	8
Hippocampal BDNF T2	9	9	7	9	9	7
Hippocampal BDNF T3	9	7	7	9	7	7
Hippocampal BDNF T4	10	9	7	9	7	7

EA: Early Adolescent; MA: Mid- Adolescent; CIE: Chronic Intermittent Ethanol Exposure T1=During intoxication; T2=Acute abstinence; T3=Three-weeks following ethanol exposure; T4=Behavioral tested rats