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Chronic ethanol during adolescence impacts corticolimbic dendritic spines and behavior

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Abstract

Background—Risk for alcohol use disorders (AUDs) is linked to alcohol drinking during adolescence, but understanding of the neural and behavioral consequences of alcohol exposure during adolescence remains incomplete. Here, we examined the neurobehavioral impact of adolescent chronic intermittent EtOH (CIE) vapor exposure in mice.

Methods—C57BL/6J-background Thy1-EGFP mice were CIE-exposed during adolescence or adulthood and examined, as adults, for alterations in the density and morphology of dendritic spines in infralimbic cortex (IL), prelimbic cortex (PL) and basolateral amygdala (BLA). In parallel, adolescent- and adult-exposed C57BL/6J mice were tested as adults for two-bottle EtOH drinking, sensitivity to EtOH intoxication (loss of righting reflex, LORR), blood EtOH clearance, and measures of operant responding for food reward.

Results—CIE during adolescence decreased IL neuronal spine density and increased the head-width of relatively wide-head IL and BLA spines, whereas CIE decreased head-width of relatively narrow-head BLA spines. Adolescents had higher EtOH consumption prior to CIE than adults, while CIE during adulthood, but not adolescence, increased EtOH consumption relative to pre-CIE baseline. CIE produced a tolerance-like decrease in LORR sensitivity to EtOH challenge, irrespective of the age at which mice received CIE exposure. Mice exposed to CIE during adolescence, but not adulthood, required more sessions than AIR controls to reliably respond for food reward on a fixed-ratio (FR1), but not subsequent FR3, reinforcement schedule. On a progressive ratio reinforcement schedule, breakpoint responding was higher in the adolescent- than the adult-exposed mice, regardless of CIE. Finally, footshock-punishment markedly suppressed responding for reward in all groups.

Conclusions—Exposure to CIE during adolescence altered dendritic spine density and morphology in IL and BLA neurons, in parallel with a limited set of behavioral alterations. Together, these data add to growing evidence that key corticolimbic circuits are vulnerable to the

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effects of alcohol during adolescence, with lasting, potentially detrimental, consequences for behavior.

Keywords

alcohol; underage drinking; neural circuit; amygdala; prefrontal

Introduction

Adolescence through early adulthood is widely regarded as a period of heightened vulnerability to addictions, including alcohol use disorders (AUDs). Individuals that begin drinking alcohol during this period are more likely to present with an AUD in adulthood (Nixon and McClain, 2010). This increased risk has been attributed to factors ranging from genetic predisposition, poor impulse control to a heightened susceptibility of the still-developing brain to drug-related toxicity (Spear, 2000). One possibility is that damage to brain regions mediating higher-order cognitive and executive behaviors in heavy-drinking adolescents (Pfefferbaum et al., 1996) weakens the ability to regulate drinking later in life.

To better understand the link between adolescence and increased risk for AUDs, prior studies have examined how adolescent rodents compare to adults in their responses to ethanol (EtOH). This work has revealed how adolescent rats and mice differ from adults, both in their propensity to drink EtOH and in their behavioral responses to acute EtOH challenge. For example, adolescent rats tend to drink more than adults and display attenuated behavioral and physiological (e.g., ataxic, hypothermic, anxiolytic-like, sedative/hypnotic) responses to EtOH (reviewed in Spear and Varlinskaya, 2005). In C57BL/6J mice, adolescents have been shown to display attenuated taste aversion and, in some but not other cases, sedative/hypnotic responses (Hefner and Holmes, 2007, Moore et al., 2013, Holstein et al., 2011, Linsenhardt et al., 2009), but stronger locomotor-stimulant, ataxic and anxiolytic-like responses to EtOH (Hefner and Holmes, 2007, Linsenhardt et al., 2009). The drinking phenotype of adolescent C57BL/6J mice is also somewhat variable across studies, perhaps due to variations in the methods of testing used, with reports of increases (Holstein et al., 2011, Agoglia et al., 2016, Agoglia et al., 2015, Quoilin and Boehm, 2016, Moore et al., 2010), decreases (Ho et al., 1989) and no difference (Hefner and Holmes, 2007, Melon et al., 2013) in consumption, relative to adults.

A number of prior studies have also investigated the lasting consequences of exposing adolescent rodents to EtOH. A series of studies by Crews and colleagues using a high-dose intermittent intragastric EtOH dosing model, has shown that chronic adolescent EtOH exposure results in volume reductions in prefrontal cortex (PFC), hippocampus and corpus callosum in rats assessed in adulthood (Coleman et al., 2012, Vetreno et al., 2015, Vetreno et al., 2016, Ehlers et al., 2013b, Ehlers et al., 2013a). In addition, these and other laboratories have reported myelin-related alterations, synaptic remodeling and functional hypoactivation in the PFC of rats and C57BL/6J mice chronically exposed to EtOH in adolescence (Montesinos et al., 2015a, Liu and Crews, 2015).

The potential behavioral impact of these neural alterations has, however, not yet been comprehensively described. Using their adolescent intermittent intragastric model, Crews

and colleagues have observed deficits in object-recognition in adults rats (Vetreno and Crews, 2015, Vetreno et al., 2015) and Gass and coworkers reported poor extinction of EtOH-seeking signs in rats that had been intermittently exposed to EtOH vapors during adolescence (Gass et al., 2014). The impact of various forms of adolescent EtOH exposure on EtOH consumption in rats are mixed (Gilpin et al., 2012, Alaux-Cantin et al., 2013, Vetter et al., 2007, Slawecki and Betancourt, 2002, Fabio et al., 2014). However, C57BL/6J mice given limited-access drinking or intermittent vapor-exposure in adolescence were found to consume elevated amounts of EtOH during adolescence and, in the case of the limited access model, continue to drink more into adulthood relative to mice without adolescent drinking experience (Moore et al., 2010, Carrara-Nascimento et al., 2013) (but see Melon et al., 2013). Such sustained drinking has also been seen in sub-strains of BALB/c mice, which normally show a strong aversion to EtOH (Kakihana and McClearn, 1963, Blizard et al., 2004). Also of note is the finding that lasting behavioral effects of adolescent exposure appear to generalize across sexes. Female C57BL/6J mice given repeated EtOH injections during adolescence showed later increases in EtOH drinking and EtOH place-preference, as well as impairments in object-recognition and passive avoidance (Montesinos et al., 2015b, Montesinos et al., 2015a).

The aim of the current study was to extend the literature by examining neural and behavioral sequelae of chronic adolescent EtOH exposure in mice. To this end, male C57BL/6J-background Thy1-EGFP reporter mice were exposed to chronic intermittent EtOH vapors (CIE) throughout the adolescent period or, for comparison, during adulthood. Mice were subsequently assessed, as adults, for CIE-induced alterations in the density of dendritic spines, subcategorized based on head-width, in prefrontal cortical (PFC) and basolateral amygdala (BLA) neurons. To examine potential behavioral correlates of these neural adaptations, male C57BL/6J mice were assessed for two-bottle EtOH drinking, sensitivity to the acute intoxicating effects of EtOH (loss of righting reflex, LORR), effects of CIE on EtOH metabolism, and for operant food-reward self-administration under continuous and progressive-ratio schedules of reinforcement, as well as after footshock-punishment.

Material and Methods

Subjects

For the dendritic spine density and morphometry experiments, subjects were male Thy-1 mice backcrossed to C57BL/6J for an undetermined number of generations (Tg(Thy1-EGFP)MJrs/J, JAX stock 007788). Male Thy-1 mice were obtained from The Jackson Laboratory (Bar Harbor, Me, USA) and bred to female C57BL/6J mice to produce hemizygous mutants for experimentation. For behavioral experiments, mice were male C57BL/6J mice, delivered at either 21 ± 4 days (adolescent group) or 49 ± 4 days (adult group) of age from The Jackson Laboratory (Bar Harbor, Me, USA). Adolescent mice were 4–6 weeks and adult mice were 8–10 weeks of age at the beginning of CIE exposure, with the exception of the EtOH drinking experiment, where mice were 4–6 (adolescent) and 8–10 (adult) weeks old in order to accommodate a 2-week pre-CIE baseline drinking period. For all experiments, the age and CIE exposure groups were examined in parallel, in a counterbalanced order.

Mice were housed 2/cage in a temperature- ($72 \pm 5^\circ \text{F}$) and humidity- ($45 \pm 15\%$) controlled vivarium under a 12 hour light/dark cycle (lights on at 0600 h), and acclimated to the vivarium for at least 1 week prior to experimentation. The numbers of mice used in each experiment are given in the figure legends. Experimental procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee and followed the US National Institutes of Health guidelines outlined in *Using Animals in Intramural Research*.

Chronic intermittent EtOH (CIE) exposure

Chronic EtOH exposure was achieved via vapor inhalation, as previously described (DePoy et al., 2015, Lopez et al., 2014, Jury et al., 2016, Lopez et al., 2016). Mice were placed in standard mouse cages in Plexiglas vapor chambers ($60 \times 36 \times 60 \text{ cm}$, PlasLabs, Lansing, MI, USA) and exposed to EtOH volatized by passing air through a vaporization stone submerged in EtOH (95%) and mixed with fresh air to deliver 19–22 mg EtOH/L of air at a rate of ~10 L per minute. EtOH delivery parameters were designed to produce blood EtOH concentrations (BECs) of $175 \pm 25 \text{ mg/dL}$ (unless otherwise specified) - confirmed weekly via blood samples taken from age-matched 'sentinel' mice. BECs were measured using the Analox AM1 alcohol analyzer (Analox Instruments USA, Lunenburg, MA, USA). In order to induce intoxication and stabilize BECs, the EtOH group received intraperitoneal (i.p.) injections of 71.6 mg/kg of the alcohol dehydrogenase inhibitor pyrazole (Sigma, St. Louis, MO, USA) combined with 1.5 g/kg 20% (v/v) EtOH, in a volume of 10 mL/kg body weight, prior to placement in the chambers. Air controls received an injection of 68.1 mg/kg pyrazole and were placed in dedicated chambers (located adjacent to the EtOH chambers) in which air was exchanged at a rate of ~10 L/minute. Each CIE and air exposure lasted 16 hours per day (in at 1700 h, 1 hour before the start of the 12-hr circadian dark phase, out at 0900 h), followed by an 8-hour withdrawal. There were 4 consecutive days of exposure (Monday-Friday) followed by a longer, 80-hour, withdrawal (Friday–Monday). This was repeated for a total of 4 cycles.

CIE effects on PFC and BLA spine density and morphology

To visualize Thy-1 expression at the completion of testing, mice were then given CIE exposure (adolescent-exposed from 4 weeks of age, adult-exposed from 8 weeks of age). At 3 days after the completion of CIE exposure (adolescent-exposed now 8 weeks of age, adult-exposed now 12 weeks of age, (for schematic of experimental design, see Figure 1A), mice were terminally overdosed with ketamine/xylazine and transcardially perfused with phosphate-buffered saline (PBS), then 4% paraformaldehyde (PFA). After suspension in 4% PFA overnight and in 0.1 M phosphate buffer at 4°C , 50- μm coronal sections were cut with a vibratome (Classic 1000 model, Vibratome, Bannockburn, IL, USA). Sections were mounted and coverslipped with Vectashield HardSet mounting medium with 4',6-diamidino-2-phenylindole (Vector Laboratories Inc, Burlingame, CA, USA). The sections were imaged with a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

Dendritic segments from cells located in the BLA (Bregma: -0.70 to -2.06 mm) and the infralimbic (IL) and prelimbic (PL) subregions of the PFC (Bregma: 1.34 to 1.98 mm) were

identified under a 10X/1.2 NA objective (Zeiss Model LSM 700 microscope). Segments that met all of the following criteria were selected for analyses: (1) were >150- μ m from the soma, (2) showed clear spine protrusions, (3) were not obscured by other dendrites, and (4) were parallel or near parallel with the coronal plane of the section. All confocal stacks comprised ± 5 - μ m above and below the extent of the dendritic segment. Segments were imaged under a 63X/1.4 NA oil-immersion objective, using voxel dimensions $0.08 \times 0.08 \times 0.6$ - μ m³ and 2.5 $\times$ zoom at a resolution of 300×512 pixels by an individual blinded to the treatment groups. Z series were obtained by imaging serial confocal planes at 0.20- μ m intervals. Settings for pinhole size (1 airy disc) and gain (600) were optimized initially and remained constant throughout imaging to ensure images were digitized under consistent illumination. These parameters resulted in dendritic segments ~30–80- μ m in length. Data were obtained from 10–12 cells per brain section. Dendritic spines were sampled from an equivalent location between neurons at approximately 150 μ m from the soma on the primary apical dendritic tree of layer II/III pyramidal neurons in the IL and PL. Sampling of dendritic spines was restricted to secondary or higher branch orders on principal neurons from the BLA (apical versus basilar dendritic distinctions were not attempted for BLA neurons).

Spines were designated manually from gamma-corrected digital confocal image stacks using NeuronStudio software. Spine density was calculated as the number of spines divided by dendritic segment length. Spine head-width was defined as the maximum diameter of the spine head, and spines were segregated into narrow and wide head-width spines using a median split, in order to ensure all spines were accounted for one or the other subcategory (Ruszczycki et al., 2012), based on previously described procedures (Fitzgerald et al., 2014, Inaba et al., 2016). For examples of narrow-head and wide-head spines, see Figure 1B. Spine density and head-width data were expressed as cumulative distribution plots (Ruszczycki et al., 2012). As in previous studies (Horner and Arbuthnott, 1991, Sargin et al., 2013), dendritic protrusions >4.0- μ m in length were considered to be probable filopodia, rather than spines, and were excluded from the analysis.

Effects of CIE on EtOH drinking

As in prior studies from our laboratory and others (DePoy et al., 2015, Lopez et al., 2014, Jury et al., 2016, Lopez et al., 2016), mice were first given (a 2-week) period of EtOH drinking prior to CIE (or air) exposure in order to establish baseline EtOH consumption and provide a reference with which to compare post-CIE drinking. Drinking was measured using a 24-hour access 2-bottle choice procedure, as previously described (Boyce-Rustay et al., 2007). Mice were individually housed in ‘Space Saver’ cages (Model 1145T with Model 1145T482SUDB Polysulfone cage-top, Tecniplast, Buguggiate, Italy) and offered 2 bottles: 1 containing 15% (v/v) EtOH in water and the other containing tap water. Every 2 days, mice were weighed and EtOH and water consumption measured, correcting for evaporation and spillage measured from empty ‘dummy’ cages adjacent to the test cages, and the left/right position of the bottles was switched to control for side bias. Food was available *ad libitum*. Beginning 3 days after the completion of CIE exposure, post-CIE 2-bottle drinking was measured, using the same 2-bottle procedure, over a 2-week period (for schematic of experimental design, see Figure 2A).

Effects of CIE on sensitivity to acute EtOH intoxication

Sensitivity to the acute intoxicating effects of EtOH was assayed using the loss of righting reflex (LORR) test the day after the completion of CIE exposure. Mice were first given CIE exposure for four weeks (for schematic of experimental design, see Figure 3A). LORR was tested at 1 day after the completion of CIE exposure. This post-CIE time point was chosen based on prior data showing significant reductions in LORR sensitivity (i.e., tolerance) 1 day but not 3 days after CIE in adult male C57BL/6J mice (Daut et al., 2015).

LORR was tested by intraperitoneally injecting mice with 3.5 g/kg 20% (v/v) EtOH and placing them into the supine position in a 'V'-shaped chamber. LORR duration was measured as the time from injection to the time when the mouse was able to self-right onto all 4 paws twice within 30 seconds.

Effects of CIE on EtOH metabolism

To examine potential effects of CIE on EtOH metabolism, EtOH clearance from the blood was measured. Mice were first given CIE exposure for four weeks (for schematic of experimental design, see Figure 3A). One day after the completion of CIE exposure, mice were intraperitoneally injected with 3.5 g/kg EtOH 20% (v/v). Blood samples were taken from the submandibular vein at 5, 30, and 60 minutes and from the trunk (after rapid cervical dislocation and rapid decapitation) at 240 minutes. The samples were centrifuged in a microcentrifuge at 4 degrees Celsius for 30 minutes at a speed of 13,000 rpm and then analyzed for BECs using the Analox AM1 alcohol analyzer.

CIE effects on operant responding for food reward

Multiple measures of operant responding for food reward were assessed following CIE. Mice were first given CIE exposure for 4 weeks and operant testing began 3 days after the completion of CIE exposure (for schematic of experimental design, see Figure 3A) and continued for 5–6 weeks using methods previously described (Radke et al., 2015a, Radke et al., 2015b, Halladay et al., 2016). Mice were maintained at ~85% of their free-feeding body weight throughout testing to motivate performance. Testing was conducted in an operant chamber (Model ENV-307W, Med Associates, St. Albans, VT, USA) housed in a sound- and light-attenuating box (Med Associates, St. Albans, VT, USA). The grid floor of the chamber was covered with solid Plexiglas to facilitate movement. A magazine delivering reward was located between 2 continuously available levers. Mice were trained to press the left (=active) lever for a 14 mg food pellet (F05684, BioServ, Frenchtown, NJ, USA) on a fixed-ratio (FR) 1 schedule of reinforcement (1 active lever press = 1 food pellet), and subsequently on an FR3 schedule (3 consecutive active lever presses = 1 food pellet). Upon reward-delivery, a 65-dB tone was emitted for 2 seconds to support the reward-lever association. Presses on the right lever (=inactive) had no programmed consequences. Sixty-minute training sessions were blocked into 2 × 20-minute reward-available (active lever=reward) and 10-minute reward-unavailable periods, with reward-availability signaled by illumination of the magazine. Training continued until mice achieved >35 presses of the reward lever during the available sessions. The schedule was then changed to FR3 and continued until responding on the active lever during reward-available periods was stable (<20% coefficient of variation in

responding) across 3 consecutive sessions. The total number of sessions taken to reach (FR3) criterion, from the beginning of training, was measured.

Next, to test for motivation to work for the food-reward, mice were given a 60-minute test under a progressive ratio (PR) schedule, in which an increasing number of lever presses (5, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, 219, 268, 328, 402, 492, 606, 737) were required to obtain a single reward. The maximum number of presses made (breakpoint) was measured.

Following PR testing, the effects of punishment on operant responding were next tested on a 40-minute session during which reward-lever press outcomes were organized into the following sequence: press 1 illuminated the house light and delivered no reward; press 2 delivered a 2-second 0.4 mA footshock through the grid floor in the proximity of the levers/magazine and no reward; press 3 delivered a reward (Radke et al., 2017). This sequence was repeated throughout the session. Punished-suppression of responding was measured by comparing reward-lever presses on the punishment session with presses on the final session of training.

Statistical analysis

Levene's test was used to check the equality of variance for all groups. For the dendritic spine analysis, the effects of age-at-exposure and CIE exposure were analyzed using analysis of variance (ANOVA). Cumulative distribution plots of spine density and head-width were analyzed for simple effects using a Kolmogorov-Smirnov test (Ruszczycki et al., 2012). The effects of age at CIE exposure and pre- versus post-CIE time point, time post-injection (in the metabolism experiment) and punishment stage were analyzed using two-factor ANOVA, with repeated measures for time point and punishment stage, followed by Sidak's multiple comparison *post hoc* analysis. In the metabolism experiment the rate of EtOH clearance was determined by comparing the slopes of the curves using regression analysis. The statistical threshold was set at $P < .05$. All statistical analyses were performed using Graph Pad Prism version 7.01.

Results

CIE effects on cortical and amygdaloid dendritic spines

We first examined the effects of CIE exposure during adolescence or adulthood on the density of dendritic spines in neurons in the BLA nucleus of the amygdala and the IL and PL subcortices of the vmPFC (for schematic of experimental design, see Figure 1A).

For overall dendritic spine density in BLA neurons, there was a significant effect of the age-at-CIE exposure ($F_{1,135}=46.95$, $P < .001$), but no significant effect of CIE and no CIE $\times$ age-at-exposure interaction, reflecting the greater density in the exposed-at-adolescence group (Figure 1D–E). Similarly, there were no differences in spines density when spines were subcategorized based on narrow or wide head-width (data not shown). However, considering the head-width of the spines in BLA neurons of the exposed-at-adolescence mice, there was a significant interaction between the CIE exposure and spine subcategory ($F_{1,67}=18.87$, $P < .001$). CIE exposure during adolescence was associated with significantly smaller spine head-

width in the narrow spines (*post hoc* test: $P < .01$), but significantly greater head-width in the wide, but not narrow, spines (*post hoc* test: $P < .05$), relative to air-exposed controls, when spines were examined in the now adult mice (Figure 1F–G). By contrast, CIE exposure during adulthood had no effects on spine head-width of every narrow or wide BLA spines.

For overall spine density in IL neurons, there was a significant interaction between age-at-exposure and CIE ($F_{1,125}=4.98$, $P=.028$). Density was significantly reduced by CIE, relative to air, in the exposed-at-adolescence group (*post hoc* test: $P < .01$), but not in adult-exposed counterparts. Density was lower, irrespective of CIE exposure, in the adult-exposed than the adolescent-exposed group ($F_{1,126}=38.06$, $P<.001$) (Figure 1I–J). However, when spines were subcategorized as narrow or wide, there were no differences between any of the groups in density of IL neurons (data not shown). Next, when considering the head-width of narrow and wide spines, there was a significant interaction between CIE exposure and spine subcategory in IL neurons of exposed-at-adolescence mice ($F_{1,72}=9.75$, $P=.003$). The head-width of wide (but not narrow) spines was significantly increased by CIE exposure during adolescence, as compared to air-exposed controls (*post hoc* test: $P < .05$) (Figure 1K–L). Conversely, CIE exposure during adulthood had no effects on spine head-width for either narrow or wide IL neuronal spines.

Finally, for PL neurons, overall spine density was not affected by CIE exposure, but was significantly lower in the adult-exposed, than the adolescent-exposed, group ($F_{1,141}=15.23$, $P<.001$) (Figure 1N–O). There were no group differences in density when spines were subcategorized as having either narrow or wide heads (data not shown). There was, however, a significant effect of CIE exposure ($F_{1,61}=4.83$, $P<.001$) and spine subcategory ($F_{1,61}=88.05$, $P<.001$), but no interaction, for head-width in adult-exposed mice. The head-width of narrow spines was lesser following CIE, relative to air, exposure in adolescent mice (*post hoc* test: $P < .05$) (Figure 1P–Q). CIE exposure during adulthood, by contrast, increased the head-width of wide spines (*post hoc* test: $P < .05$).

Collectively, these analyses show that CIE during adolescence produces a complex set of changes in the dendritic and morphology of dendritic spines in key nodes within corticolimbic brain circuits.

CIE effects on EtOH drinking

Next, to test for potential behavioral alterations caused by CIE exposure that may have occurred in tandem with the changes observed in dendritic spine density and morphology, we began by assessing 2-bottle EtOH drinking.

For EtOH consumption prior to CIE exposure, there was a significant effect of age group ($F_{1,14}=6.08$, $P=.027$), but not of CIE exposure ($P>.05$) and no age $\times$ CIE interaction ($P>.05$). There was significantly higher EtOH consumption in the adolescents, as compared to the adults (Figure 2B). Following CIE exposure, there was a significant effect of CIE exposure ($F_{1,14}=9.79$, $P=.007$) but not age-at-exposure ($P>.05$), but no interaction ($P>.05$) for EtOH consumption. CIE exposure led to a significant increase in EtOH consumption in mice exposed as adults, relative to air-exposed counterparts (*post hoc* test: $P < .05$), but did not alter consumption in mice that had been exposed during adolescence (Figure 2B).

Relative preference for the EtOH solution over water did not differ between age of CIE groups, either during the pre-CIE or post-CIE testing periods (all effects: $P>.05$), though there was significantly higher preference post-CIE, relative to pre-CIE, regardless of group ($F_{1,28}=64.89$, $P<.001$) (Figure 2C).

These data indicate higher basal EtOH consumption in adolescent mice and the absence of a CIE-induced increase in consumption in mice exposed during the adolescent period.

CIE effects on sensitivity to EtOH challenge

We next tested for potential differences between mice exposed to CIE during adolescence or adulthood on a behavioral measure of sensitivity to acute EtOH challenge (LORR). There was a significant effect of CIE exposure ($F_{1,36}=18.14$, $P<.001$], but not of age-at-exposure ($P>.05$), and no significant CIE $\times$ age interaction ($P>.05$). CIE exposure decreased LORR duration, relative to air controls, irrespective of the age at which mice were exposed (Figure 3B).

CIE effects on EtOH metabolism

To determine whether differential behavioral responses to CIE exposure were associated with alterations in EtOH metabolism, we quantified the rate of BEC clearance following a 3.5 g/kg i.p. EtOH challenge. Results indicated a significant effect of time post-injection ($F_{3,60}=34.01$, $P<.001$), but not of either age or CIE group and no interaction between factors. BEC decreases over time did not vary as a function of CIE exposure or age-at-exposure (Figure 3C). Regression analysis also found no effect of either age-at-exposure or CIE on the slope of BEC decay over time ($F_{3,68}=0.53$, $P=0.67$).

CIE effects on operant responding for food reward

In our next set of behavioral analyses, we tested for CIE effects on multiple tests of operant responding for food reward. For sessions to reach criterion levels of responding for reward on a FR1 schedule of reinforcement, there was a significant effect of CIE exposure ($F_{1,33}=6.76$, $P=0.013$), but not of age-at-exposure and no CIE $\times$ age-at-exposure interaction. Mice exposed to CIE during adolescence required significantly more sessions to reach criterion than air-exposed counterparts (*post hoc* test: $P<.05$) (Figure 3D). There were no significant differences between the age-at-exposure or CIE exposure groups for responses on the unrewarded-lever during this or any other phase of testing (data not shown). There was also no significant effect of CIE or age-at-exposure for sessions to criterion when mice were tested on a FR3 schedule of reinforcement (all effects: $P>.05$) (Figure 3D). However, neither response rate nor the number of rewards earned differed between groups during FR1 or FR3 training (Table 1).

In the next phase of operant testing, progressive ratio breakpoint was assayed as a measure of motivation for reward. There was a significant effect of age-at-exposure group ($F_{1,34}=4.78$, $P=.036$), but not CIE exposure ($P>.05$) and no interaction between the variables ($P>.05$), for the maximum number of reward-lever presses made on the progressive ratio reinforcement schedule. The mice exposed at adolescence did have significantly higher

breakpoints (but did not earn more rewards in total) than mice exposed as adults, but this was independent of CIE exposure (Figure 3E, Table 1).

The final test assayed behavior during punished responding. There was a significant effect of punishment ($F_{1,68}=155.50$, $P<.001$), but not of CIE exposure ($P>.05$) or age-at-exposure and no significant interactions between the factors ($P>.05$). Reward-lever pressing was significantly reduced when associated with an intermittent footshock, as compared to rates of unpunished pressing, irrespective of CIE exposure or age-at-exposure group (Figure 3F).

The results of these operant tests showed that CIE exposure during adolescence retarded initial instrumental learning but, thereafter, did not impair learning, motivation or a measure of 'compulsive-like' responding for food reward.

Discussion

The main findings of the current study were that chronic exposure to EtOH vapors during adolescence produced significant, persistent and highly regionally specific alterations in neuronal dendritic spine density that wholly differed from the pattern of changes produced by EtOH exposure in adult mice. Behaviorally, adolescent EtOH exposure failed to increase EtOH consumption, possibly due to heightened baseline drinking levels, and produced a transient deficit in rewarded operant learning. Other behavioral measures, including tolerance to acute EtOH challenge, motivation to respond for food reward (measured on progressive ratio schedule) and punished-suppression of reward-seeking were equally affected by CIE exposure during adolescence and adulthood.

Prior work from our laboratory has not examined CIE-related changes in spines, but has found increased dendritic material in medium spiny neurons of the dorsal striatum (DePoy et al., 2013) and a shift in the distribution of material in pyramidal cells of the PL, three days following CIE (Holmes et al., 2012). A series of elegant studies by Mullholland, Chandler and their colleagues has examined the effects of CIE exposure on dendritic spines in multiple brain regions of adult C57BL/6J mice (Mullholland and Chandler, 2007). For example, Kroener and colleagues found an increase in the density of relatively wide-head ('mushroom') spines in the PL was detected after CIE exposure, when spines were examined immediately or one week later (Kroener et al., 2012). Persistent increases in spines have also been reported in the lateral orbitofrontal cortex, at seven, but not zero, days after adult CIE exposure (McGuier et al., 2015a) (for similar data in rats, see (Kim et al., 2015)). However, another recent study found that CIE exposure led to an increase in neuronal spine density in the nucleus accumbens when measured immediately after exposure, but a loss of density of relatively wide-head ('mushroom') spines at three days post-CIE (Uys et al., 2016). A similar trend for increased overall spine density was also detected in the NAc of C57BL/6J-backcrossed (wild type) mice examined right after repeated intraperitoneal EtOH injections (McGuier et al., 2015b). Thus, the effects of chronic EtOH exposure during adulthood appear not only to be vary dynamically after the cessation of exposure, but to differ profoundly across brain regions.

In the current analysis, CIE exposure during adolescence caused a shift from spines with narrow to wide heads in BLA neurons, as well as an overabundance of wide head spines in the IL that was coupled with an overall loss of spine density. These changes observed in the current study were extant three days after CIE exposure, when mice had reached adulthood, and were not evident in mice that had been given the same regimen of EtOH exposure as adults. This persistent overabundance of wide spines in the IL and BLA after adolescent CIE exposure could reflect a deficiency in the normal pruning that occurs, across species, during the transition into adulthood (Gourley et al., 2012), consonant with the view that adolescent EtOH exposure delays the maturation of the PFC. We also found that for spines with relatively wide heads in the PL, width was increased by CIE exposure during adulthood, but reduced by CIE during adolescent. This observation in the PL echoes to some extent the preponderance of immature-like spines recently found in rats exposed to CIE during adolescence (Trantham-Davidson et al., 2016); though this study also observed increased overall spine density in these animals, which we did not see in mice. Nonetheless, taken together with the current findings in mice, we conclude that CIE exposure has a potent and lasting effect on dendritic spine density or morphology in a manner that varies as function of brain region, species and, critically, the age at exposure.

An important issue arising from these structural alterations in response to CIE is the identification of the molecular mechanisms driving them. One obvious candidate is alterations in NMDA receptor and glutamate signaling, which has been repeatedly linked to CIE-induced changes in behavior and spine dynamics (Mulholland et al., 2016). Another noteworthy finding given our spine changes in the BLA, is that of decreased CaMKII phosphorylation in the amygdala of adolescent, but not adult, C57BL/6J mice after binge-drinking (Agoglia et al., 2015). Beyond the glutamate system, Uys and colleagues have found, using proteomic analyses, CIE-induced spine changes are associated a suite of changes in the expression of scaffolding proteins, kinases and other molecules located in the postsynaptic density, including PSD-95 (Uys et al., 2016). Multiple gene expression changes have also been reported in adolescent EtOH-exposed alcohol-preferring P rats, including a number of genes encoding GABAA receptors, a finding of particular interest considering the important role of these receptors in AUDs and other drug addictions (McBride et al., 2014, McClintick et al., 2015, Centanni et al., 2014). Clearly there are many interesting molecular candidates to be examined in future work in this area.

Another corollary question is the potential impact of spine abnormalities on behavior. Increases in the density and head-width of dendritic spines have been linked to synaptic strengthening in a variety of brain regions, through processes of *de novo* spinogenesis and enlargement of existing spines (Gipson and Olive, 2016). However, in adult-exposed rodents, CIE-induced increases in, for example mPFC neuronal spine density and dendritic material, are concomitant with behavioral (e.g., cognitive flexibility, fear extinction) disturbances, rather than performance enhancements (Kroener et al., 2012, Holmes et al., 2012). Given the importance of dendritic spines as a critical locus for synaptic function and the fine-tuning of neuronal coordination between regions, it is not surprising that there is not a simple one-to-one relationship between (more) spines and (better) learning. Indeed, it remains to be determined precisely how the spine abnormalities resulting from adolescent CIE exposure transfer to deficits in behavior.

Providing preliminary insight into this question, we did observe differences in the effects of CIE exposure in adults and adolescents in a limited set of behavioral paradigms. Replicating an observation made in multiple previous studies, male C57BL/6J mice exposed to CIE as adults drank more EtOH than air-exposed controls (McCool and Chappell, 2015, Becker and Lopez, 2004, Griffin et al., 2009, Lopez and Becker, 2005, Holmes et al., 2012, Finn et al., 2007, Carrara-Nascimento et al., 2013). This increase in drinking was absent in mice that received CIE exposure during adolescence (when six-ten weeks of age, as opposed to four-eight weeks as in other experiments, in order to accommodate a pre-CIE drinking period) and were tested as adults. One interpretation of these data is that adolescent mice are resistant to the lasting effects of CIE exposure on drinking. It should also be noted, however, that this lack of elevation in drinking was seen in the context of higher drinking in these mice prior to CIE exposure, and in the group that had been air-exposed during adolescence. Thus, there may simply be limited range to detect CIE-induced increases in drinking in adolescents when compared to already elevated baselines. If these behavioral differences are indeed the result of resistance to the CIE-induced EtOH-dependence in adolescent-exposed group, it is difficult to determine to what extent they may not relate to the spine abnormalities present in these mice; especially as the neural circuitry mediating two-bottle EtOH drinking is poorly defined. Nonetheless, given the important roles attributed to the IL and BLA in reward processing and control over alcohol and drug-seeking (Everitt and Robbins, 2005), it is tempting to speculate that spine disturbances in the regions could contribute to altered EtOH drinking in adolescent-exposed mice (Gipson and Olive, 2016).

Age-related differences in CIE-induced EtOH drinking were not accompanied by differential behavioral or metabolic responses to acute EtOH challenge. BECs showed a peak and decay profile after injection with a 3.5 g/kg dose of EtOH that did not differ as a function of CIE or age at exposure. Though this finding indicates a lack of CIE-induced pharmacokinetic adaptation to CIE, the same challenge EtOH dose did produce lesser LORR in mice that had CIE exposure – demonstrating tolerance to this behavioral measure of intoxication; in line with LORR previously reported for rats and C57BL/6J mice (Walls et al., 2012, Daut et al., 2015). Of particular note in the context of the goals of the current study, the effect of CIE on LORR was evident regardless of the age at which mice had undergone exposure: i.e., tolerance was apparent whether mice had been CIE-exposed as adults or adolescents. It is also worth pointing out that there was not an overall difference in LORR responses between the groups exposed to air or CIE at different ages. While this is perhaps not unexpected given all groups were tested when they had reached adulthood, it does contrast with previously reported age-related differences in sensitivity to this or other responses to acute EtOH challenge when mice are tested *as* adolescents and adults (Hefner and Holmes, 2007, Linsenhardt et al., 2009).

There was little indication that the age of CIE exposure affected a number of measures of operant responding for a food reward. One exception was that mice exposed to CIE during adolescence required more sessions to reach the criterion for operant responding on a FR1 reinforcement schedule, suggesting there may have been a modest or transient deficit in learning after CIE exposure in this age group. There are currently very few studies examining the effects of adolescent EtOH on measures of cognition, an earlier study of CIE-exposure in adolescent rats also found an impairment in operant performance, albeit one that

was specific to a deficit in behavioral flexibility (set-shifting) (Gass et al., 2014). Of further note, though they were tested in adulthood, mice that had been exposed to *either air or CIE* during adolescence, higher breakpoints when tested on PR schedule than adult-exposed counterparts, suggesting a higher level of motivation for reward in these mice. This could be due to the slightly younger age (2 weeks younger) of the adolescent-exposed group at testing, but more likely reflects lasting effects of the general exposure procedure during adolescence; stemming, for example, from the extensive handling involved. As already noted, however, any such lasting effects did not generalize to other operant behaviors, including the marked suppression of operant responding following footshock punishment, which was evident irrespective of the age at mice received exposure or whether they had been exposed to air or CIE.

In summary, the main findings of the current study were that exposure to chronic intermittent EtOH vapors throughout most of the adolescent period produced a significant loss of dendritic spines on neurons in the IL subregion of the medial PFC, as well as a shift towards wider, more mature spine head-width in IL and BLA, neurons. These neural alterations were not evident in mice exposed to the same regimen of EtOH during adulthood. Moreover, they were selective for this brain region, with no changes found in the PL PFC subregion or the BLA, irrespective of the age at which mice were chronically exposed to EtOH. Assessment of a range of behavioral measures found that mice drank more when adolescent, and on subsequent testing as adults following chronic exposure to EtOH during the adolescent period, failed to show an increase in EtOH drinking due to the EtOH-exposure, unlike mice that had been EtOH-exposed as adults. By contrast, chronic EtOH-exposure did not differentially affect adolescent and adult exposed mice in terms of either the LORR response or blood EtOH clearance following acute EtOH challenge. With the exception of slower initial acquisition, adolescent EtOH exposure also had no demonstrable influence on various measures of operant responding for a food reward.

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Figure 1. Effects of adolescent CIE exposure on dendritic spine density in the prefrontal cortex and basolateral amygdala

(A) Schematic of experimental design for testing the effects of CIE exposure on dendritic spine density and morphometry in adolescent- and adult-exposed mice. (B) Representative examples of EGFP-labeled narrow-head and wide-head spines in the infralimbic cortex (IL) (scale bar = 1 μ m). (C) Representative examples of EGFP-labeled neurons in the basolateral amygdala (BLA) of Thy-1 mutant mice (scale bar = 15 μ m). Cumulative distribution plot (D) and population averages (E) showing CIE exposure did not alter overall spine density in BLA neurons, irrespective of age-at-exposure, while density was higher in the adolescent-exposed group than the adult-exposed group (n=24–39 neurons per group from n=3–5 mice per group). Cumulative distribution plot (solid lines = narrow head-width spines, dashed lines = wide head-width spines) (F) and population averages (G) showing CIE exposure during adolescent, not adulthood, decreased the head-width of narrow-head spines, and increased the head-width of wide-head spines, in BLA neurons, relative to air-exposure (n=12–20 neurons per group from n=4–6 mice per group). (H) Representative example of EGFP-labeled neurons in the IL of Thy-1 mutant mice (scale bar = 15 μ m). Cumulative

distribution plot (**I**) and population averages (**J**) showing CIE exposure decreased overall spine density in IL neurons from adolescent-exposed, but not adult-exposed, mice (n=20–37 neurons per group from n=3–5 mice per group). Cumulative distribution plot (solid lines = narrow head-width spines, dashed lines = wide head-width spines) (**K**) and population averages (**L**) showing CIE exposure increased the head-width of wide-head spines, as compared to air-exposure, in mice exposed at adolescence, but not adulthood (n=9–20 neurons per group from n=3–5 mice per group). (**M**) Representative example of EGFP-labeled neurons in the prelimbic (PL) cortex of Thy-1 mutant mice (scale bar = 15 μ m). Cumulative distribution plot (**N**) and population averages (**O**) showing adolescent-exposed group had higher spine density than adult-exposed counterparts, regardless of CIE exposure (n=25–39 neurons per group from n=3–5 mice per group). Cumulative distribution plot (solid lines = narrow head-width spines, dashed lines = wide head-width spines) (**P**) and population averages (**Q**) showing CIE exposure increased the head-width of narrow-head spines, as compared to air-exposure, in mice exposed in adulthood (n=12–22 neurons per group from n=3–5 mice per group). Data are means $\pm$ SEM. * P <.05, ** P <0.01, *** P <0.001

Figure 2. Effects of adolescent CIE exposure on EtOH drinking

(A) Schematic of experimental design for testing the effects of CIE exposure on 2-bottle EtOH consumption in adolescent- and adult-exposed mice. (B) Mice exposed to CIE during adulthood, but not adolescence, showed a CIE-induced increase in EtOH consumption. The adolescent-exposed group drank more EtOH than the adult-exposed group during the pre- and post-CIE stages ($n=8$ per group). (C) No differences in EtOH preference were observed in either the adolescent or adult group during baseline and irrespective of CIE-exposure, though preference was higher post-CIE relative to pre-CIE, regardless of exposure-group ($n=8$ per group). Data are means $\pm$ SEM. * $P<.05$, *** $P<.001$

Figure 3. Effects of adolescent CIE exposure on sensitivity to acute EtOH challenge and operant responding for food reward

(A) Schematic of experimental design for testing the effects of CIE exposure on acute EtOH-induced loss of righting reflex (LORR), EtOH clearance from blood after acute EtOH-challenge and multiple measures of operant responding for food reward, in adolescent- and adult-exposed mice. (B) Exposure to CIE decreased LORR duration, regardless of age at exposure (n=8–12 per group). (C) BECs decreased with time after EtOH injection at a similar rate in the age groups and irrespective to CIE exposure (n=4–6 per group). (D) The adolescent CIE-exposed group required more sessions to reach criterion on a food-reward operant task when compared to AIR-exposed controls, but this measure was unaffected the adult group (n=8–12). (E) PR breakpoints were higher in the adolescent-exposed group than the adult-exposed group, regardless of CIE-exposure (n=8–12). (F) Punishment decreased reward-lever responding to a similar degree across the age and CIE exposure groups (n=8–12). Data are means $\pm$ SEM. * $P < .05$, ** $P < .01$.

Table 1

Measures of performance during operant testing

Neither response rate (rewarded-lever presses per minute) nor total reward earned differed between groups during operant training on a FR1 or FR3 schedule of reinforcement. Total rewards earned on a PR schedule of reinforcement also did not differ between groups. n=8–12 per group. Data are means ± SEM.

		FR1		FR3		PR	
		Response rate	Rewards	Response rate	Rewards	Rewards	Rewards
Adolescent	AIR	0.5 ± 0.05	16 ± 1.8	4.7 ± 0.7	63 ± 9.5	20 ± 1.1	
	CIE	0.4 ± 0.05	14 ± 1.6	5.0 ± 0.6	67 ± 7.4	20 ± 1.2	
Adult	AIR	0.6 ± 0.08	21 ± 2.7	4.2 ± 0.7	55 ± 9.2	18 ± 0.9	
	CIE	0.4 ± 0.06	14 ± 2.1	5.6 ± 0.9	74 ± 12.4	19 ± 0.6	