



Binge drinking in male adolescent rats and its relationship to persistent behavioral impairments and elevated proinflammatory/proapoptotic proteins in the cerebellum

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Abstract

Rationale To demonstrate that repeated episodes of binge drinking during the adolescent period can lead to long-term deficits in motor function and memory in adulthood, and increase proteins in the brain involved with inflammation and apoptotic cell death.

Methods Groups of early adolescent (PND 26) and periadolescent (PND 34) Sprague-Dawley rats were exposed to either ethanol or plain air through a vapor chamber apparatus for five consecutive days (2 h per day), achieving a blood ethanol concentration equivalent to 6–8 drinks in the treatment group. Subjects then underwent a series of behavioral tests designed to assess memory, anxiety regulation, and motor function. Brains were collected on PND 94 for subsequent western blot analysis.

Results Behavioral testing using the rota-rod, cage-hang, novel object recognition, light-dark box, and elevated plus maze apparatuses showed significant differences between groups; several of which persisted for up to 60 days after treatment. Western blot testing indicated elevated levels of caspase-3/cleaved caspase-3, NF-κB, and PKC/pPKC proteins in the cerebella of ethanol-treated animals.

Conclusions Differences on anxiety tests indicate a possible failure of behavioral inhibition in the treatment group leading to riskier behavior. Binge drinking also impairs motor coordination and object memory, which involve the cerebellar and hippocampal brain regions, respectively. These experiments indicate the potential dangers of binge drinking while the brain is still developing and indicate the need for future studies in this area.

Keywords Binge drinking · Adolescence · Motor impairment · Memory deficit · Neuroinflammation · Cerebellum

Introduction

Binge drinking among adolescents is an ongoing public health concern among the North American population (Johnston et al. 2018). Although adult humans experience acute consequences from binge drinking ethanol, the adolescent population is significantly more susceptible to aberrant neurological changes as their brains are still undergoing significant

neurodevelopment (Han et al. 2005; Spear 2013). Brain regions particularly susceptible to modulation by ethanol include the hippocampus and cerebellum (Belmeguenai et al. 2008; Nixon and Crews 2002; Urrutia and Gruol 1992). Indeed, adolescent binge drinking alters motor function which is likely due to cerebellar neurodegeneration (Forbes et al. 2013). Even transient exposure of infant rats to ethanol during the period of synaptogenesis triggers widespread neuronal loss in many brain regions (Ikonomidou et al. 2000). Furthermore, people with adolescent-onset alcohol use disorder have decreased cerebellar volumes (De Bellis et al. 2005). This decrease in cerebellar volumes reflects some form of neurodegeneration, which can likely be attributed to neuronal apoptosis due to the involvement of several proteins measured in the current study.

Apoptosis is a term originally devised to describe morphological changes which characterize a specific process of cell death (Wyllie Kerr and Currie 1980). Caspases are a family of cysteine containing zymogens which are integral to the

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apoptotic process. Caspase-3 is known as an effector caspase, meaning that it facilitates the proteolysis leading to apoptosis after the apoptotic signal has been activated by a separate initiator caspase (caspases 8, 9, and 10; Chen and Wang 2002). Activated caspase-3 is a well-validated biomarker of cellular apoptosis in humans (Ward et al. 2008) and is involved in developmental ethanol-induced neurodegeneration (Young et al. 2005). In addition, caspase-3 appears to be a major protease effector protein in this process (Olney et al. 2002a, b). Caspase-3 knockout mice exposed to ethanol displayed an altered time course and cellular morphological features of the apoptotic process compared to wild-type counterparts, but there were no effects in the absolute amount of cell death (Young et al. 2005). Although the above findings show a contribution of caspase-3 in ethanol-mediated neurodegeneration, they also indicate that there must be other cellular pathways involved to induce cell death. Caspase-3 is known to cleave nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) at the p65 subunit inducing apoptosis and upregulating cell death (Kang et al. 2001). NF- κ B is part of a major proinflammatory pathway in cells throughout the body through its transcriptional regulation of cytokine production (Liu et al. 2017). In addition to this immunomodulatory role, NF- κ B also affects the expression of other gene targets, some of which are thought to be involved in addiction (Crews et al. 2011). For example, NF- κ B mediates complex behaviors including learning and memory, stress response, and drug reward which are outside the canonical immune response role (Nennig and Schank 2017). These effects are mediated by the role NF- κ B plays in dendritic spine formation and synaptogenesis (Boersma et al. 2011; Russo et al. 2009). NF- κ B can also be activated by protein kinase C (PKC) *in vitro* through the NK1 receptor (Shirakawa and Mizel 1989).

Given the interconnected relationships between caspase-3, NF- κ B, and PKC, as well as their implication in previous studies of various drugs of abuse made the proteins a clear choice to study in our experimental paradigm (Guerra and Pascual 2010; Nennig and Schank 2017; Russo et al. 2009; Stubbs and Slater 1999; Young et al. 2005). We exposed young rats to ethanol vapors, which has been a well-established model, primarily for chronic alcohol administration (Becker and Hale 1993; McCool and Chappell 2015; Nentwig et al. 2019; Topper and Valenzuela 2014). The intermittent exposure paradigm that we used (2 h a day for 5 days) was meant to mimic binge drinking during the period of adolescent brain development, particularly during the early to mid-adolescent period (Spear 2015). In addition to assaying the above protein expression levels, a battery of behavioral tests was utilized to get a comprehensive overview of the long-term outcomes of adolescent binge drinking into adulthood. Combining both behavioral and biological assays in the same subjects gives a broad perspective of the potential consequences of binge drinking in humans.

Method

Animals

Groups consisted of male Sprague-Dawley rats at two different initial ages (post-natal days; PNDs) to represent approximate early adolescent (PND 26) and periadolescent (PND 34) developmental timepoints (Sengupta 2013). Subjects' ages were determined to reflect a period of neurodevelopment during which the brain is particularly susceptible to damage (Spear and Brake 1983; Spear 2000, 2013). Rats begin to sexually mature after weaning beginning on PND 21 and reach maturity by approximately PND 42 (Adams and Boice 1983).

Animals were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada) and given a 3-day acclimation period after shipping prior to beginning experiments. All experiments were conducted in accordance with the CCAC guidelines and with approval from the Memorial University Institutional Animal Care Committee.

Ethanol administration

Subjects were assigned randomly to either an experimental or control group. The experimental group was exposed to a mixture of ethanol vapor (2 l/min) and plain air (5 l/min) for 2 h in a sealed chamber. Control subjects were also placed in the chamber for 2 h but received only air (5 l/min). Treatment was conducted on PNDs 26–30 for the early adolescent model or PNDs 34–38 for the periadolescent group. Blood samples were collected approximately 30 min after ethanol exposure and blood alcohol concentrations (BACs) were measured using a GM7 Micro-stat biochemical analyzer (Analox Instruments, Ltd., UK). A mean BAC of 172 ± 18 mg/dl was achieved in the treatment group and approximately 0 mg/dl in controls. During the experimental paradigm, ethanol-treated animals were closely monitored for proper weight gain and negative health developments as a result of the exposure (i.e., tremors, piloerection, eye irritation, and respiratory distress).

Behavioral testing

An initial set of experiments analyzed the performance of an early adolescent ($n = 24$) and periadolescent ($n = 24$) group on a fixed-speed rota-rod task during/after undergoing ethanol administration. The majority of the data is from a subsequent full study which was conducted with only a periadolescent group ($n = 24$) using a battery of behavioral tests: rota-rod (PNDs 32–38, 44, 48, 53, and 68), inverted cage-hang (PNDs 46 and 50), open field (OF; PND 76), elevated plus maze (EPM; PND 78), dark/light box (DLB; PND 79), novel object recognition (NOR; PND 82–85), acoustic startle (AS; PND 86), and fear conditioning (FC; PND 89–92). After the

rota-rod and inverted cage-hang tests, subjects were then transferred to a nearby building with facilities necessary for the further behavioral testing and allowed a week to acclimate before they commenced (PNDs 69–76). Therefore experiments were conducted from the periadolescent period through the early adult stage (see Fig. 1 for the experimental timeline).

A limited experiment characterizing withdrawal effects from the ethanol administration paradigm was conducted with a separate group of periadolescent subjects ($n = 24$) after the previous testing was completed. This final group was tested for movement tendencies and speed of movement (both before and after ethanol exposure) in an open arena and response in the light-dark box test the day after the last exposure to ethanol (specifications further in this section). Testing was counterbalanced between groups, and all equipment was rinsed with 70% ethanol between subjects.

Motor testing

The rota-rod task is a well-established measure of balance and cerebellar function (Rustay et al. 2003). The rota-rod (Harvard Apparatus; Barcelona, Spain) consists of a rotating rod divided into four lanes, which then digitally records the time it takes the animals to fall (latency to fall) at various settings of rotations per minute (RPM). Subjects were tested on the rota-rod directly before ethanol/air exposure in the morning on all treatment days. Therefore, during the 5-day ethanol/air exposure period, animals underwent rota-rod testing approximately 21–22 h after the previous exposure to ethanol/air. Additional trials with no exposure were conducted on either PNDs 36, 40, 45, 60 for the early adolescent groups (PND 26) or PNDs 44, 48, 53, 68 for the periadolescent groups (PND 34).

The early adolescent and periadolescent groups from the first set of experiments project were tested only on the fixed-speed rota-rod (FSRR), where the RPMs (5, 10, and 15) are set at the beginning of the 5-min trial and do not change. The periadolescent group from the main study was tested on an accelerating rota-rod (ARR), where the rotations per minute (RPM) of the apparatus increased uniformly from 4 to 35 RPM over a 5-min trial.

The second periadolescent group was also tested with an inverted cage-hang apparatus. In this test, the subject is placed on a metal mesh screen (1.25 cm square spaces) and the screen is then inverted over a pile of soft bedding. The time the animal can remain gripped to the screen when inverted gives a relative indication of muscle tonality/strength (Osmon et al. 2018).

For the withdrawal groups only, subject's general tendency to move about and the speed at which they do so was measured in an open arena. The apparatus was a small wooden box (60 cm × 60 cm × 35 cm) painted in black enamel. Both the total amount of distance covered (cm) and the velocity while this movement took place (cm/s) were measured using

EthoVision® behavioral tracking software (Noldus Information Technology Inc., Wageningen, The Netherlands).

Open field

The open field apparatus was a square wooden box (60 cm × 60 cm × 35 cm) painted with black enamel and taped off into a 3 × 3 grid. Rats were placed in the center of the box to begin the trial and were allowed 5 min to explore while being videotaped. The videos were subsequently scored for total locomotion, time in periphery, and time in center of the open field using the behavioral tracking software. Rats were scored as in the center of the arena when the full body was within the center square demarked by the grid, and in the periphery when all four paws crossed into one of the other squares.

Light-dark box

The light-dark box is a single alley apparatus divided into two chambers of equal size (32 cm long × 10.5 cm wide × 14.5 cm high). Both chambers are covered by a ventilated plexiglass top with the dark side being rendered opaque with a black plastic lining. The floor and sides on the light side are painted white, while the dark side has a metal mesh floor with the walls painted black. This specific light-dark box has been used in previous studies (Kenny et al. 2019; Lau et al. 2016). Testing took place in a dark room with a 100-W lamp positioned 66 cm above the chamber, producing an illumination on the light side of approximately 590 and 20 lx on the dark side. Subjects were placed in the box for a 5-min trial and had their behavior videotaped for future analysis. Behavior measured included time spent on the light side, time spent on the dark side, and the number of crossings between the two (with all four paws). Videos were scored by two individuals blinded to the treatment conditions and the scores averaged.

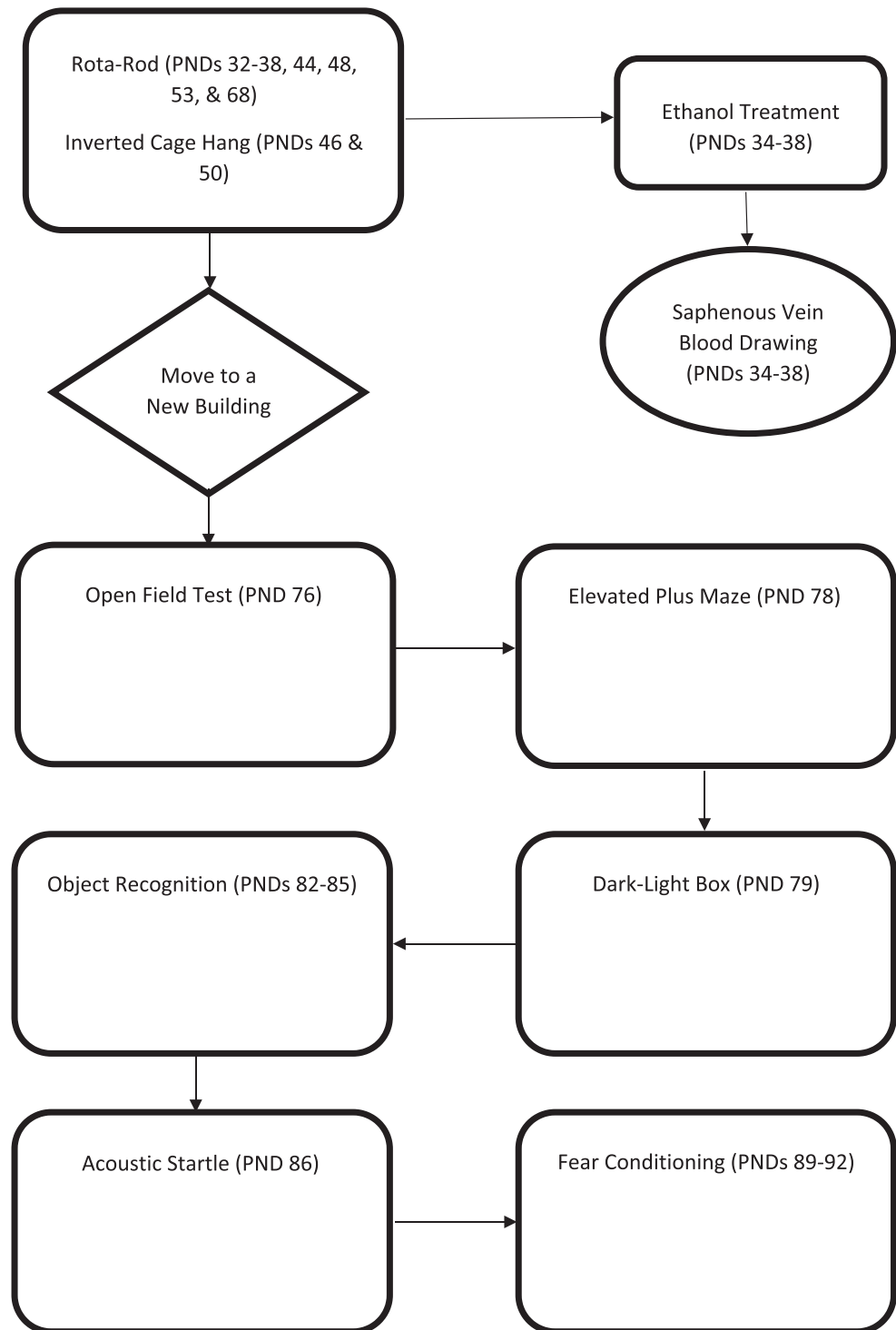
The amount of time spent in the light side versus the dark exploits a natural behavior of the rat in order to get a measure of relative anxiety-like behavior via an unconditioned response. Less time in the light side is indicative of increased anxiety-like behavior as anti-anxiety drugs increase time in the light side (Chaouloff et al. 1996).

Elevated plus maze

The elevated plus maze consists of four arms arranged in the shape of plus sign, with two opposite arms considered open (10 cm wide × 50 cm long) and the other two considered closed (10 cm wide × 50 cm long × 40 cm high). The four arms join at the center of the maze with a 10 cm squared platform. Subjects were placed in the center platform of the maze facing the same open arm for a 5-min trial each. Entry into any arm was defined as having all four paws past the opening. Behaviors scored include time in open arm, time in

Fig. 1 Flowchart for behavioral experiments in the PND 34–38 ethanol treatment group

Experimental Timeline for Ethanol Treated Animals (PNDs 32-92)



closed arm, and the number of entries into each arm. Behavior was scored by two individuals blinded to the treatment conditions. Final data was expressed as percent of total non-center time.

Acoustic startle

Startle testing was conducted in a commercially available standard startle chamber (San Diego Instruments). The

apparatus consists of a cylindrical animal enclosure that sits atop a piezoelectric transducer, the signal from which is recorded by a computer to provide a measure of relative movement intensity by subjects. Rats were first acclimatized to the chamber with a 60-dB white noise for 5 min. During test trials subjects were exposed to 30 pulses (50 ms each) of white noise at 120 dB, rising out of a background of 60 dB white noise with a 30-s inter-trial interval. Startle responses were measured over a 250-ms window and averaged for each subject.

Novel object recognition

The same testing arena from the open field trials was used for the NOR experiments. Rats were first habituated to the area for 10 min over 2 days prior to pre-training and training trials. During pre-training, subjects were allowed to explore two identical objects (A, A) for 3 min. Memory retention was tested 24 h later with one familiar object (A) and one novel objects (B). Objects were placed 12 cm from each wall and 18 cm from each other during the trials. Positions of objects were counterbalanced within the arena and were rinsed with a 70% ethanol solution between trials. Subjects are given an initial trial to explore two identical objects and are then placed back in the apparatus 1 h later with a similar and novel object. Time spent exploring each object was assessed; this included approaching within 2 cm of the object with the face, sniffing, biting, and pawing the object. Sitting or rearing up on the object was not counted as exploration. A discrimination index was then calculated (exploration of novel object – exploration of familiar object / total exploration time); positive index values indicate the subject explored the novel object more frequently. As with other tests, behavior was scored by two individuals blinded to the treatment conditions.

Fear conditioning

All subjects were placed in a Plexiglas shock box (Colbourne Instruments) for 2 min; then a 30-s white noise (90 dB) burst was delivered, co-terminating in a 2-s foot shock (1.5 mA). This procedure is repeated at an inter-stimulus interval of 1 min for a total of two shocks over a 5-min trial. Freezing behavior (motionless except for respirations) was monitored at 5-s intervals by an observer blinded to the condition.

Cued fear conditioning was tested in subsequent trials for each subject 24 h after the initial ones. In these trials there was a 3-min baseline followed by 3 min of the same white noise from the training sessions (no shocks were given). Cue-dependant fear conditioning was determined by subtracting freezing during the baseline condition from that during the white noise.

Protein quantification

Samples for the blots were homogenized tissue from either the cerebellar or cerebral regions of experimental animals (PND 34 group). These tissues were harvested approximately 48 h after the last behavioral test was conducted (PND 94), following rapid decapitation of the animals, and were flash frozen using liquid nitrogen.

Tissues collected were homogenized with ice-cold homogenizing buffer (50 mM Tris, 150 mM sodium chloride, 3.5 mM sodium dodecyl sulfate, 12 mM sodium deoxycholate, and 15 mM Triton X-100, pH 8.0) and protease inhibitor cocktail tablets (Roche, Grenzach-Wylen, Germany). Protein concentrations were measured with the Pierce™ BCA protein assay kit (Thermo Scientific, USA). Cerebellar and cerebral protein lysates (40 µg/well) were separated by SDS-PAGE (12.5%) under reducing conditions and transferred to a nitrocellulose membrane (Thermo Scientific, USA). Briefly, blots were blocked with blocking buffer for 1 h at 4 °C (5% not-fat dried milk in TBST). After blocking, blots were incubated with anti-caspase-3 polyclonal antibody (1/1000, v/v; catalogue # 9662S, lot # 0018), anti-cleaved caspase-3 monoclonal antibody (1/1000, v/v; catalogue # 9664S, lot # 21), anti-NF-κB monoclonal antibody (1/1000, v/v; catalogue # 8242S, lot # 0009), anti-phosphor-NF-κB p65 monoclonal antibody (1/1000, v/v; catalogue # 3033S, lot # 16), anti-PKCγ polyclonal antibody (1/1000, v/v; catalogue # 43806S, lot # 0001), or anti-phosphor-PKC (pan) polyclonal antibody (1/1000, v/v; catalogue # 9379S, lot # 0002) (all from Cell Signaling, USA) for 20 h at 4 °C. All blots were also incubated with anti-α/β tubulin polyclonal antibody (1/1000, v/v; catalogue # 2148S, lot # 0007), as a loading control. Blots were washed 3 times with TBST and incubated with HRP conjugated-secondary antibody (1/5000, v/v; catalogue # 7074S, lot # 0026, Cell Signaling, USA) for 1 h at room temperature. Protein bands were visualized with the commercial Western Lightening© chemiluminescence kit (HRP-catalyzed system; Perkin Elmer, USA).

Statistical analysis

Results between groups for motor testing were initially analyzed using mixed-design ANOVAs to determine significance of a main effect. Subsequent analyses for motor tests, all other behavioral tests, and western blotting were conducted using the independent-samples *t* test. The threshold for significance in all experiments was set at $\alpha < .05$. All data was analyzed using SPSS (IBM, Armonk, USA).

Results

Motor testing

Periadolescent (PND 34) groups tested during our initial study had significant differences between control and treated groups at the 15 RPM speed, but not at 5 or 10 RPM. A mixed-design ANOVA with treatment condition (control, ethanol) as a between-subjects factor and testing day (day 1, 2, 3, 4, 5, 11, 15, 20, or 35) as a within-subjects factor revealed a main effect of the condition ($F(1, 10) = 8.89, p = .014$). Simple main effect analysis showed that whether the subjects were treated with ethanol had a significant effect on latency to fall from the rotarod ($p = .018$). Further analyses with a series of independent-samples t tests revealed significance for testing days 2 ($t(10) = 2.92, p = .015$), 3 ($t(10) = 2.53, p = .030$), 4 ($t(10) = 2.97, p = .014$), 5 ($t(10) = 2.39, p = .038$), 11 ($t(10) = 2.98, p = .014$), 15 ($t(10) = 3.25, p = .009$), and 20 ($t(10) = 3.09, p = .011$); with a return to no between-group difference on day 35 ($p = .060$; Fig. 2). A pre-adolescent group (PND 26) was also tested on this same paradigm and showed significant differences between groups at 15 RPM with a main effect of condition ($F(1, 10) = 7.98, p = .018$) on testing days 3 ($t(10) = 2.50, p = .031$), 4 ($t(10) = 4.07, p = .002$), 5 ($t(10) = 4.90, p = .001$), 11 ($t(10) = 2.67, p = .023$), 15 ($t(10) = 3.50, p = .006$), and 20 ($t(10) = 4.30, p = .002$); with a return to no between-group difference on day 35 ($t(10) = 1.03, p = .326$; Fig. 2).

The results above were performed as an initial study to ascertain whether our ethanol administration procedure would affect behavior. A new periadolescent group (PND 34) was subsequently treated before a series of behavioral tests. This new group was tested on an accelerating rotarod paradigm

and showed no significant differences in performance (data not shown, all $p > .05$). No significant difference was found in this group with a cage hang test on PND 46 and 50 (data not shown, $t(22) = .110, p = .913$).

As noted in the methods, a separate group of periadolescent animals underwent an identical ethanol exposure paradigm as the first and were tested for withdrawal effects shortly after treatment (1 day after the last ethanol exposure). There was no withdrawal effect on the subject's propensity for locomotion ($p = .11$) or movement speed ($p = .14$) when this was measured using a simple open arena (Supp. Fig. 1).

Anxiety-like behavior

Interestingly, the amount of time spent in the open arm of the EPM was significantly higher in the ethanol-treated group when compared to controls ($t(22) = -2.50, p = .020$; Fig. 3); however, there was no difference between groups with respect to the frequency of crossing between the open/closed arms. Subjects previously treated with ethanol also spent significantly more time in the light side ($t(22) = -2.78, p = .011$; Fig. 3) of the light-dark box. There were no significant differences between groups with respect to the total number of crossings between the light to dark sides. However, there was also an increased preference for the light side of the box noted in the groups being tested for withdrawal effects. Previously seen on PND 79 in the first ethanol-treated group, this significant increase appears shortly after treatment as well ($t(22) = -2.65, p = .015$; Fig. 4). Finally, there was no statistically significant difference seen between groups on the open field, acoustic startle, or fear conditioning tests (all $p > .05$; Supp. Fig. 2).

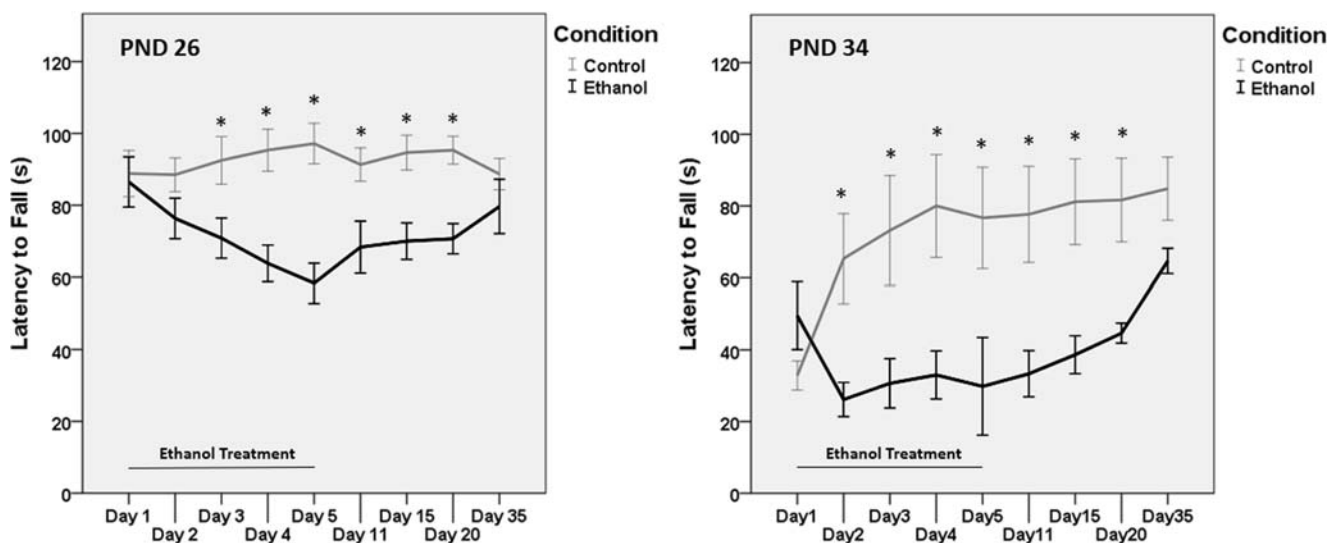


Fig. 2 Fixed-speed rotarod testing revealed a significant difference between ethanol-treated and control subjects in both the early adolescent (PND 26; $n = 12$ per treatment group; $*p < .05$ days 3–20) and

periadolescent (PND 34; $n = 12$ per treatment group; $*p < .05$ days 2–20) age groups. In both graphs “Day 1” corresponds to the first experimental PND for each group (PND 26 or PND 34)

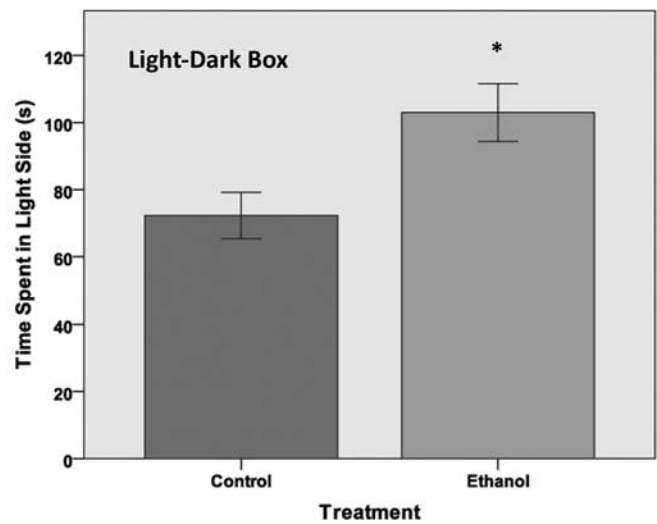
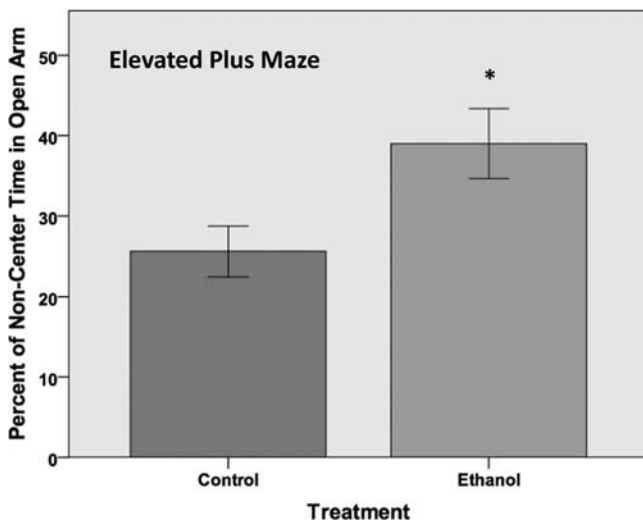


Fig. 3 Anxiety-like behavior testing revealed significant differences between control and ethanol-treated groups on the elevated plus maze ($n = 12$ per group; $*p < .05$) and light-dark box ($n = 12$ per group;

$*p < .05$) tasks. Significant results on these tests within our model imply a lack of behavioral inhibition on the part of ethanol-treated animals

Novel object recognition

There were no significant differences between the two groups in total exploration time of the objects, either during the training or testing phases of the experiment. Analysis of the discrimination index value showed a significant difference between groups ($t(22) = 2.40, p = .025$). A higher value for the control group indicated that they preferred the novel object during the testing trials of NOR, while the ethanol-treated group had no strong preference for either object (Fig. 5), suggesting a deficit in the ethanol-treated group.

Western blotting

All western blot results were determined to be within the combined linear dynamic ranges of both the target and loading

control. Caspase-3 levels were significantly elevated in the cerebella of ethanol-treated animals compared to control subjects ($t(22) = -2.32, p = .030$). NF-kB protein levels were also elevated in ethanol-treated groups ($t(22) = -2.20, p = .038$; Fig. 6). Activated forms of the proteins were also elevated with exposure to ethanol as can be seen in the cleaved form of caspase-3 ($t(14) = -2.73, p = .016$) and phosphorylated NF-kB ($t(14) = -2.57, p = .022$; Fig. 7). These results indicate that neurodegeneration has occurred in the cerebella of animals exposed to ethanol and this is likely related to the behavioral differences observed between groups. PKC was also elevated in both its non-active ($t(14) = -3.18, p = .007$) and phosphorylated ($t(14) = -4.38, p = .001$) states. There

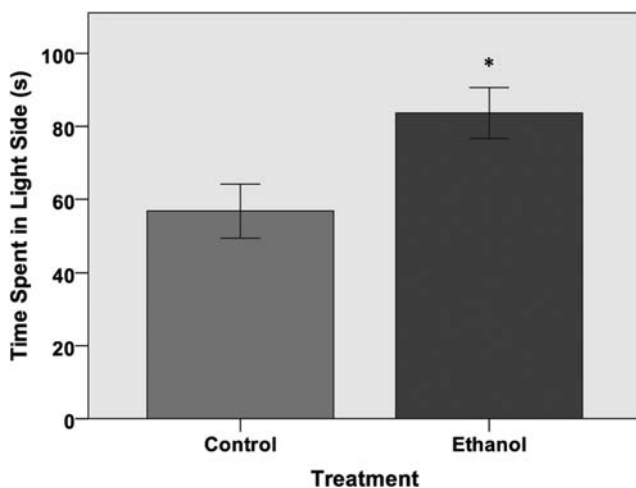


Fig. 4 Withdraw experiments showed that subjects’ performance on the light-dark box test was impaired similarly the day after treatment ($n = 12$ per group; $*p < .05$) as it was when tested on PND 79

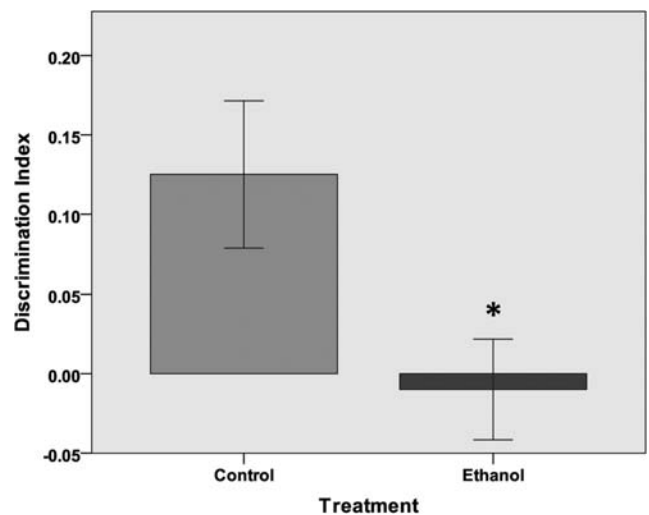


Fig. 5 Calculated discrimination index values were significantly different between control and ethanol-treated groups ($n = 12$ per group; $*p < .05$), indicating that the ethanol-treated group was unable to distinguish between a familiar and novel object after delay on the novel object recognition task

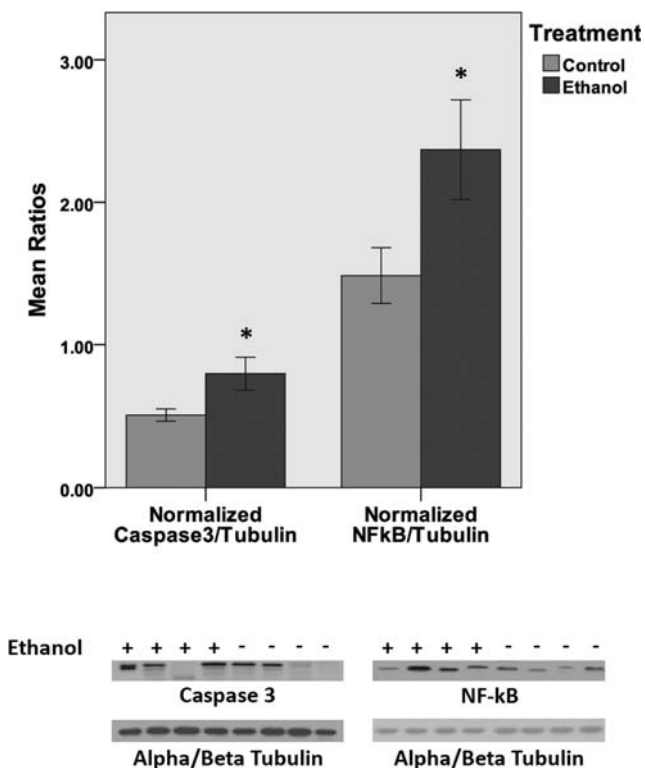


Fig. 6 Significant differences were seen between the control and ethanol groups in the expression levels of the caspase-3 ($n = 12$ per group; $*p < .05$) and NF-kB ($n = 12$ per group; $*p < .05$) proteins in the cerebellum

was no significant difference in the ratio of the two forms to one another between groups since both forms of PKC were increased in ethanol-treated subjects (Fig. 8). All significant blot results in this project were found only in cerebellar tissue. Finally, an independent-samples t test was conducted to compare tubulin densitometry levels between control ($M = 28,325.00$, $SD = 2891.68$) and ethanol-treated ($M = 28,566.67$, $SD = 2573.84$) groups indicating no significant difference ($t(22) = -.062$, $p = .95$).

When homogenized lysates of cerebral cortex were examined, there were no significant difference between groups detected (Supp. Fig. 3). Additionally, there were no significant differences detected when the ratios of activated proteins to base protein level (PKC, caspase-3, and NF-kB) was compared between groups (Supp. Fig. 4).

Discussion

We have previously found long-term adverse effects of ethanol administration in adolescent rats on motor function, which were evident weeks after exposure (Forbes et al. 2013). In this previous study, we used a binge drinking paradigm in which animals received intraperitoneal injections of ethanol (total of eight injections over a 2-week period), resulting in very high

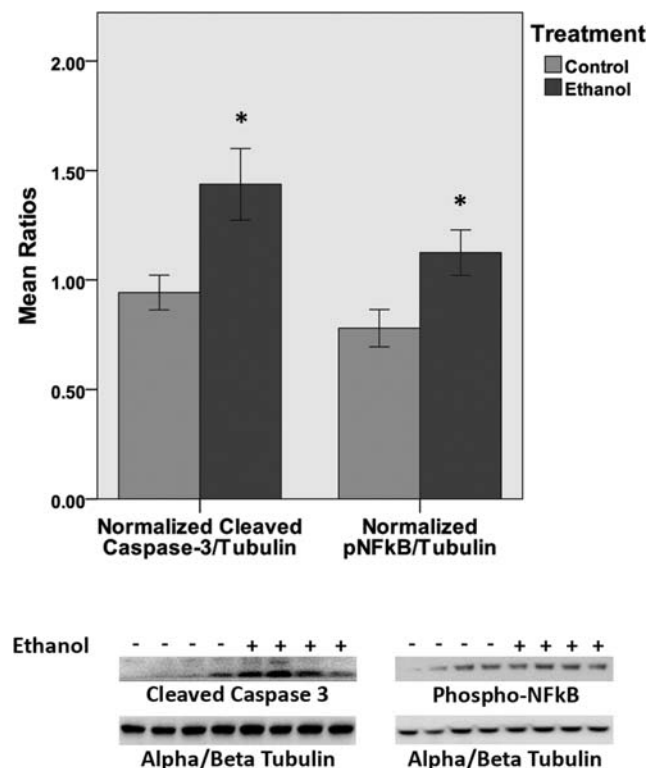


Fig. 7 Activated versions of the proteins of interested were also upregulated, as evidenced by increased levels of cleaved caspase-3 ($n = 12$ per group; $*p < .05$) and phosphorylated NF-kB ($n = 12$ per group; $*p < .05$) proteins between groups in the cerebellum

BACs of 303.2 ± 24.5 mg/dl in males. In the current study, exposure to ethanol vapors for 2 h resulted in BACs of 172 ± 18 mg/dl, which we feel is a more realistic BAC that would be achieved by adolescent individuals who are participating in binge drinking. We chose our exposure paradigm to mimic binge drinking episodes at an age when the brain is still undergoing rapid development. Most studies using vapor administration of ethanol have monitored the effects of chronic exposure to alcohol (Becker and Hale 1993; McCool and Chappell 2015; Nentwig et al. 2019; Topper and Valenzuela 2014).

Although a direct translation of age in rats to that in humans is difficult, some estimates are that PND 25–42 in rats is roughly equivalent to 10–18 years in humans, which is considered the early-mid adolescent period (Spear 2015). Our early adolescent exposure (PND 26–30) would then be equated to years 10–12 in humans, while our periadolescent exposure (PND 34–38) is closer to 15–17 years in humans, and therefore represents binge drinking over about 2 years in the adolescent period. We administered what could then be considered a low to moderate amount of ethanol over the course of 5 days, and yet still saw deficits that persisted into adulthood, several weeks after the last alcohol exposure. Unlike chronic studies, we did not observe any obvious signs of withdrawal in this model. We observed no effects on overall

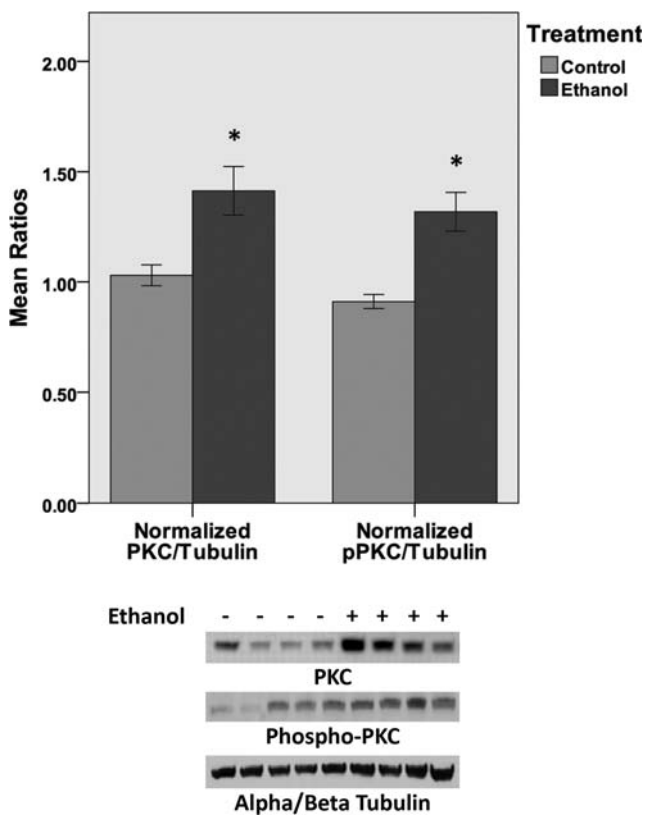


Fig. 8 Levels of both PKC ($n = 8$ per group; $*p < .01$) and pPKC ($n = 8$ per group; $*p < .001$) proteins were also elevated in cerebellar tissue when ethanol-treated groups are compared to controls

movement when tested on the day after last ethanol exposure. We also tested rats in the light-dark box which has been used in withdrawal animals (McCool and Chappell 2015) and we did observe a significant increase in time spent in the light side of the box 1 day after the last alcohol exposure. However, it is difficult to determine if this was a true withdrawal effect, especially as this effect was observed when tested in other animals on PND 79.

Overall the results indicate that binge drinking during adolescence produces persistent behavioral and biological alterations that can last well into adulthood even if alcohol exposure has ceased. Motor testing showed significant deficits caused by ethanol administration on the rota-rod task (Fig. 2). It is interesting that this was one of the deficits which recovered over time after the cessation of ethanol administration. Experiments with the periadolescent group on the accelerating rota-rod did not show any significant differences, which was surprising since it is believed to be a more difficult task than the fixed-speed rota-rod.

It has been established that high levels of behavioral inhibition in childhood predict an increased chance of developing anxiety disorders later in life (Biederman et al. 2001; Campbell-Sills et al. 2004). Behavioral inhibition relates to the tendency to experience distress and to withdraw from unfamiliar situations, people, or environments. Where a decrease

of behavioral inhibition is a well-known consequence of acute ethanol consumption in humans (de Wit Crean and Richards 2000), it is plausible the altered behavior seen in this study is a long-term extension of this effect. Therefore, the ethanol-treated groups' responses in the anxiety-like behavior testing (i.e., EPM and DLB) may reflect a chronic decrease of behavioral inhibition or increase in risk taking behavior, but further testing would need to be done in order to more definitively confirm this.

Previous studies have found that pre-natal exposure to ethanol can affect object recognition and spatial learning (Kim et al. 1997), it can decrease performance on a NOR task when given acutely (Ryabinin et al. 2002), and that chronic ethanol intake can produce object memory deficits in adult rats before going through alcohol withdrawal (Garcia-Moreno et al. 2002). Our results are the first, to our knowledge, which demonstrate that a relatively short exposure to ethanol during adolescence can not only produce these deficits, but that they persist long after the acute effects of ethanol consumption are over. This highlights the high susceptibility of the brain to alcohol-induced damage during a critical stage of neurodevelopment.

Increases to both proinflammatory (NF- κ B) and proapoptotic (caspase-3) protein expression in the cerebellum, even at 60 days post-exposure, indicates a biological basis for the accompanying aberrant behavioral features. Studies in hippocampal brain slice cultures found that ethanol exposure increases NF- κ B binding to DNA which in turn induced the production of proinflammatory cytokines TNF α , MCP-1, and IL-1 β (Zou and Crews 2010). It was interesting that there was a broad-based increase of cellular protein production in all western blots run from the ethanol-treated group. Previous studies have shown that the phosphorylation state of NF- κ B can be affected by other drugs of abuse (Zhang et al. 2011). Also similar to our findings, Pascual et al. (2007) found long-term motor dysfunction and increased levels of inflammatory mediators (cyclooxygenase-2 and inducible nitric oxide synthase) in brain tissue using a model of intermittent ethanol exposure in adolescent rats. A lack of statistically significant differences seen in cerebral tissue western blotting was likely due to the large variety of brain regions which were homogenized together. In future experiments, the brain needs to be dissected into smaller regions (i.e., hippocampus, prefrontal cortex, etc.) prior to flash freezing the tissue in order to facilitate a more detailed analysis.

Although acute ethanol consumption is correlated with decreased proinflammatory protein production, either chronic consumption or consumption with extenuating factors will lead to increased production of these proteins in adults (Szabo et al. 2007). Although the fully developed adult physiology can tolerate acute ethanol intake, the fact that adolescent brains are still undergoing development seems to make them more susceptible to long-term behavioral alterations especially in the domain of learning and memory (Crews et al. 2000; Guerri and Pascual 2010).

Adolescent binge ethanol exposure had effects on the cerebellum up to 60 days after treatment, which is considered early adulthood in rats (Sengupta 2013; Spear 2015) and thus extending the findings of existing studies focused on acute outcomes. In addition to the persistent behavioral deficits/changes, protein quantification indicated increased expression of the NF- κ B/pNF- κ B, caspase-3/cleaved caspase-3, and PKC/pPKC proteins. The complex interaction of these proteins with behavioral output is highlighted by these findings. Although there are some limitations to the external validity of the model with respect to the timing of alcohol consumption, we believe it is a reasonable approximation of human behavior seen in adolescents today (Johnston et al. 2018). Future studies should focus on determining the specific sensitive period during which the brain is most susceptible to these alterations and how these alterations might be inhibited by blocking the effects of the proteins whose expression has been increased as a result of treatment.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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