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Persistent loss of hippocampal neurogenesis and increased cell death following adolescent, but not adult, chronic ethanol exposure

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Abstract

Although adolescence is a common age to initiate alcohol consumption, long-lasting consequences of exposure to alcohol at this time of considerable brain maturation are largely unknown. In studies utilizing rodents, behavioral evidence is beginning to emerge suggesting that the hippocampus may be persistently affected by repeated ethanol exposure during adolescence, but not by comparable alcohol exposure in adulthood. The purpose of this series of experiments was to explore a potential mechanism of hippocampal dysfunction in adults exposed to ethanol during adolescence. Given that disruption in adult neurogenesis has been reported to impair performance on tasks thought to be hippocampally-related, we used immunohistochemistry to assess levels of doublecortin (DCX), an endogenous marker of immature neurons, in the dentate gyrus (DG) of the hippocampus 3–4 weeks after adolescent (P28–48) or adult (P70–90) intermittent ethanol exposure to 4 g/kg ethanol administered intragastrically. We also investigated another neurogenic niche, the subventricular zone (SVZ), to determine if effects of ethanol exposure were region-specific. Levels of cell proliferation and cell death were also examined in the DG via assessing Ki67 and cleaved caspase-3 immunoreactivity, respectively. Significantly less DCX was observed in the DG of adolescent (but not adult) ethanol exposed animals ~4 weeks post-exposure when these animals were compared to control age-mates. Effects of adolescent ethanol on DCX immunoreactivity were specific to the hippocampus, with no significant exposure effects emerging in the SVZ. In both DG and SVZ there was a significant age-related decline in neurogenesis as indexed by DCX. The persistent effect of adolescent ethanol exposure on reduced DCX in the DG appears to be related to significant increases in cell death, with significantly more cleaved caspase-3 positive immunoreactivity observed in the adolescent ethanol group compared to controls, but no alterations in cell proliferation when indexed by Ki67. These results suggest that a history of adolescent ethanol exposure results in lowered levels of differentiating neurons, likely due at least in part to increased cell death of immature neurons. These effects were evident in

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adulthood, weeks following termination of the chronic exposure, and may contribute to previously reported behavioral deficits on hippocampal-related tasks after the chronic exposure.

Introduction

Evidence is mounting to suggest that the hippocampus may be particularly susceptible to negative consequences of adolescent alcohol (ethanol) exposure. Previous research assessing age-related differences in sensitivity to ethanol has shown that adolescent rats are more affected by ethanol-induced impairments on a hippocampal-dependent spatial task, the Morris water maze MWM), than adults [1, 2]. In line with these behavioral effects, in vitro studies have shown more potent ethanol-induced enhancement of tonic inhibition [3] and antagonism of NMDA receptor synaptic function [4] in the hippocampus of adolescents relative to adults. Research in human adolescents has also suggested that the hippocampus may be susceptible to the effects of adolescent alcohol use, with decreased hippocampal volumes reported in youth who met criteria for alcohol use disorder (AUD) compared to controls [5]; however, it is unclear if these brain differences were present prior to alcohol use. Together these data suggest that the hippocampus may be particularly vulnerable to alcohol exposure during adolescence, which has led to the hypothesis that adolescent alcohol use may lead to long-lasting hippocampal alterations that persist into adulthood.

Recently our lab investigated long-term effects of adolescent ethanol exposure on learning and memory using a Pavlovian fear conditioning paradigm in male Sprague-Dawley rats [6]. Our results were consistent with the suggestion that the hippocampus is persistently affected by adolescent ethanol exposure, with animals that were exposed to ethanol during adolescence (P28–48) showing less retention of context fear, a task thought to be hippocampally-related [7, 8, 9] when tested in adulthood after an ~4 week ethanol free period. No effect of adolescent ethanol exposure emerged during tone fear conditioning, and comparable exposure to ethanol during adulthood (P70–90) did not produce a deficit in context fear memory [6]. These findings are consistent with other studies that reported poor performance on other hippocampally-related tasks, such as Morris water maze [2] and trace fear conditioning [10] in adult animals with a history of ethanol exposure during adolescence. Together these studies suggest that adolescent alcohol use may lead to persistent disruptions in hippocampal functioning.

The mechanisms underlying this apparent adolescent ethanol-induced hippocampal dysfunction have yet to be elucidated. One potential contributor to performance deficits on hippocampally-related tasks may be a long-lasting ethanol-induced disruption of neurogenesis. Indeed, previous reports suggest that irradiation-induced decreases in adult neurogenesis disrupted retention of context fear in rats [11, 12, 13, 14, 15], but not tone fear retention [12, 14], as well as impairing performance on other tasks thought to be hippocampal-dependent (see [16, 17] for review). Furthermore, adolescents given large doses of ethanol showed decreased neurogenesis [18], and chronic exposure to repeated ethanol vapor during adolescence was reported to significantly decrease neurogenesis in adulthood [19]. The latter study, however, did not explore whether a similar decrease would have been evident after comparable adult exposure.

The purpose of the present experiment was to determine whether doublecortin (DCX), an endogenous marker of immature neurons [20], would be decreased in adulthood after adolescent and adult ethanol exposure regimens used in our previous fear conditioning studies. Also explored was whether changes in neurogenesis would be specific to the hippocampus, or were more widespread, affecting another neurogenic niche, the subventricular zone (SVZ). Levels of Ki67, a marker of cell proliferation (see [21] for review), and cleaved caspase-3, a marker of apoptosis [22], were determined to assess the potential influence of neural progenitor cell proliferation and/or cell death, respectively, on DCX expression in the DG of adolescent ethanol exposed adults.

Materials & Methods

Subjects & Design

A total of 40 male Sprague-Dawley rats bred and reared in house at Binghamton University were used in this 2 (exposure: water [H20]; ethanol [EtOH])×2 (exposure age: adolescent, adult) factorial study, with an n=10/group. On the day after birth, postnatal day (P) 1, litters were culled to 8–10 pups, with a sex ratio of 6 males and 4 females retained whenever possible. Pups were housed with their mother in a standard clear plastic tub with shavings until being pair-housed with a same-sexed littermate at the time of weaning (P21). Pair-housed littermates used for this study were randomly assigned to the same age and exposure conditions. All animals were maintained in a temperature-controlled vivarium on a 12:12-h light: dark cycle (lights on 0700), with ad libitum access to food (Purina Rat Chow, Lowell, MA) and water. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health (8th Ed), using protocols approved by the Binghamton University Institutional Animal Care and Use Committee.

Procedure

Exposure—Animals were given 4 g/kg ethanol (EtOH) or an equivalent volume of water (H20) intragastrically every 48 hours during adolescence (P28–48) or adulthood (P70–90) for a total of 11 exposures. After the last exposure day, animals were left undisturbed except for routine animal care until the adolescent ethanol group reached adulthood (P70 and P112 for adolescent and adult exposure groups, respectively). The brain samples and weight data obtained for this study were from a subset of animals that underwent Pavlovian tone fear conditioning for a separate experiment [6]. Perfusions took place 48 hours after the last behavioral testing day for that experiment (i.e., tone fear retention test/extinction session. See Fig 1).

Perfusion and Histology—For perfusion, rats were deeply anesthetized with SleepAway (0.5 mg/kg, i.p.; 26% sodium pentobarbital in 7.8% isopropyl alcohol and 20.7% propylene glycol solution; Fort Dodge Animal Health) and perfused with a 4% solution of paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M phosphate buffer (Sigma-Aldrich, St. Louis, MO). Brains were excised and placed in 4% paraformaldehyde solution for 1 d, followed by transfer to 0.1 M phosphate buffered saline until sectioning. Brains were sliced coronally on a microtome into 40 μm thick sections in a series of 1:12 and stored in cryoprotectant.

For immunohistochemical analyses, tissues were incubated with anti-rabbit DCX (1:1000, Abcam, Cambridge, MA, ab18723), Ki67 (1:400 Abcam, Cambridge, MA, ab66155) or cleaved caspase-3 (1:1200, Cell Signaling, Danvers, MA, #9661) overnight at 4°C. The next day, sections were incubated with biotinylated goat anti-rabbit secondary antibody (1:200, Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Subsequently, avidin-biotin complex (Vector ABC kit, Vector Laboratories) was applied for 1 hour at room temperature. DCX positive cells were visualized using the nickel-enhanced diaminobenzidine (DAB) reaction.

The number of positive neurons was quantified by image analysis software as previously described [23]. Briefly, Bioquant Nova Advanced Image Analysis (R&M Biometric, Nashville, TN) was used for image capture and analysis, using an Olympus BX50 Microscope and Sony DXC-390 video camera linked to the computer. For DCX+ immunoreactivity (IR), the granule cell layer of the dentate gyrus (DG) was outlined and staining density was measured in pixels for the outlined area (pixels/mm2). In a separate set of tissue stained for DCX, the subventricular zone was assessed for staining density in the same fashion as the DG. Positive Ki67 and caspace-3 neurons were counted within the GCL of the DG and expressed as cells per square millimeter. Three to five slices were examined from each brain for each of the four immunohistochemical analyses, with the average value used for statistical analysis.

Statistics—One sample from the adolescent H20 group was excluded as an outlier (> 2 standard deviations from the mean) prior to statistical analysis of DCX in the DG. Final n's were 9–10/ group. ANOVAs were used for all statistical analyses, with Fisher's LSD tests conducted to determine the locus of significant effects.

Results

Body Weight

Effects of ethanol exposure on body weight (g) was examined across the 11 exposure days and at the time of euthanasia ~4 weeks after the exposure period – i.e., on P74 and P116 in the adolescent and adult exposure groups, respectively. Given initial weight difference between the age groups, adolescent and adult weight data were analyzed separately with 2 (exposure: H20, EtOH)×12 (day) repeated measures ANOVAs. The adolescent analysis revealed only a main effect of day [$F(11,198)= 2178.51$, $p<.05$], with adolescents gaining weight similarly across exposure group during the exposure period, and no weight differences evident at P74 (see Fig 1, top panel). The body weight analysis of the adult exposed animals likewise revealed a significant main effect of day [$F(11,198)= 496.30$, $p<.05$], moderated in this case by a significant exposure×day interaction [$F(11, 198)= 10.92$, $p<.05$]. Ethanol-exposed adults weighed significantly less than H20 controls on exposure days 6–11, an effect that was still significant on P116 after a period of abstinence (see Fig 1, bottom panel).

DCX

Hippocampus—DCX+IR in controls decreased by 65±5% between P74 and P116 [F(1, 33)= 6.26, p< .05]. Ethanol treatment in adolescence followed by ~4 weeks of abstinence reduced adult P74 neurogenesis [F(1, 33)= 101.30, p< .05], an effect not evident after ethanol treatment during adulthood (See Fig. 2).

SVZ—DCX+IR did not differ between exposure groups at either exposure age, suggesting that the effect of ethanol on DCX+IR may be specific to the hippocampus. Also, similar to what was seen in the DG, a significant age-related decline in DCX+IR in the SVZ [F (1,34)= 11.38, p<.05] was evident (see Fig. 3).

Ki67 and cleaved caspase-3

Given that adolescent ethanol exposure resulted in persistent decreased DCX+IR in hippocampal DG, we next wanted to determine whether these decreases in adult neurogenesis after adolescent ethanol exposure were a result of decreased neuroprogenitor cell proliferation, increased cell death or a combination of the two. Therefore, we examined Ki67 (proliferation marker) and cleaved caspase-3 (marker of cell death) in the DG of adolescent exposure groups.

No effect of adolescent exposure history was evident in the analysis of Ki67+IR in the DG, suggesting that decreased DCX+IR in adults that were exposed to ethanol as adolescents is not a result of decreased neural progenitor cell proliferation (see Fig. 4a).

Adolescent ethanol-exposed adults had significantly more cleaved caspase-3 +IR in the DG than H20-exposed controls [F(1,18)= 16.63, p< .05] (see Fig. 4b), suggesting that increased cell death may have contributed to the reduction in DG DCX after adolescent ethanol exposure.

Discussion

This is the first study to show that identical ethanol treatments of adolescent and adult rats results in a persistent loss of neurogenesis only in adolescents and not in adults. Despite adolescents showing decreased sensitivity to ethanol during the exposure period in terms of disruption in weight gain, repeated ethanol exposure during adolescence, but not in adulthood, significantly reduced DCX+IR in the DG when assessed ~4 weeks after the final ethanol exposure. DCX+IR did not differ as a function of prior exposure at either age in the SVZ, suggesting that the effect of adolescent ethanol exposure on DCX expression was specific to the hippocampus. Previous studies have found adolescent hippocampal neurogenesis was more sensitive to acute ethanol inhibition than adult neurogenesis [18], and that high levels of neurogenesis in adolescence decline progressively with aging [24, 25]. Consistent with previous studies, we found a significant age3 related decline in both the hippocampal DG and forebrain SVZ, with significantly less DCX+IR observed in P116 (adult exposure groups) than P74 (adolescent exposure groups) animals, regardless of exposure history.

Ethanol inhibition of neurogenesis has been primarily studied in adult rats, with previous studies showing that acute and chronic ethanol reduces hippocampal progenitor proliferation and neurogenesis [26, 27,28,29]. However, ethanol inhibition of neurogenesis in adults returns to age appropriate control levels after about a month of abstinence due to bursts of proliferating progenitors that after a few days mature to microglia [27] and then after about a week of abstinence begin to mature to new dentate gyrus neurons that over time return to a control level of neurogenesis [28]. Although adult neurogenesis rebounds during abstinence following chronic ethanol to age-appropriate levels, treatment of rats with an 8 week ethanol chronic relapsing model of alcoholism did lead to a persistent loss of SVZ neurogenesis [29]. Adolescent intermittent ethanol treatment by oral gavage in this study or by vapor ethanol treatment in a previous study [13] resulted in decreases in neurogenesis that last into adulthood. These intermittent treatments model adolescent weekend drinking and are different from continuous alcoholic-like models that induce a marked physical dependence to alcohol. Studies using a 4 day multiple dose binge treatment in P35 Sprague-Dawley rats that induces physical dependence, find alcohol inhibits neurogenesis, but during withdrawal a rapid rebound increase in proliferation of progenitors restores DG neurogenesis and induces ectopic DCX expression [30] McClain et.al. [30] found abstinence induced neurogenesis correlated with blood alcohol levels over 300 mg/dl. Thus, our finding of a persistent loss of adult neurogenesis after AIE may be specific to intermittent adolescent treatments that do not induce withdrawal seizures or other withdrawal symptoms that can increase neurogenesis.

Adult brain neurogenesis is unique to specific areas that maintain a unique “neurogenic niche”, i.e. a local cellular environment that contains and supports stem cell-like progenitors that can differentiate into multiple brain cell types. DCX has been used as an endogenous marker of neurogenesis, since it is a protein that is transiently expressed in immature neurons from the time neuroblasts are formed until expression of the mature neuronal marker, NeuN [20, 31]. The functional role of DCX is unknown, but may be associated with migration [32, 33] and neurite development [33]. Using DCX as an index of neurogenesis, these data suggest that adolescents may be more susceptible to long-lasting disruptions of hippocampal neurogenesis than adults under the circumstances of the current study. Increased cell death, as indexed by cleaved caspase-3 within the DG, likely contributed to the reduction in DCX+IR in the DG of adults exposed to ethanol during adolescence. These results are consistent with a previous report using an intermittent ethanol vapor exposure regimen during adolescence and also found that decreased survival of progenitors (indexed via cleaved caspase-3) contributed to the loss of neurogenesis [19]. Importantly, the present study extends those findings by providing evidence that persistent attenuations of DCX in adulthood after adolescent ethanol exposure are evident with both oral and vapor routes of ethanol administration (ie., after i.g. administration used in the present study and vapor used in [19]) and not evident when comparable exposure is conducted in adulthood. Therefore, reminiscent of research with prenatal alcohol exposure (see [35] for review), these data suggest that adolescence is another vulnerable period of brain development during which alcohol exposure can result in persistent disruptions of hippocampal neurogenesis.

No effect of adolescent exposure history was evident when assessing Ki67, a marker for neural progenitor cell (NPC) proliferation in the current study. Often a decrease in

neurogenesis (DCX+IR) is associated with a decrease in progenitor proliferation, e.g. a decrease inKi67+IR [20, 31]. Previous studies of intermittent adolescent ethanol vapor treatment found a loss of Ki67+IR two weeks after ethanol treatment, but not eight weeks after treatment [19]. Other studies using a 4 day multiple dose binge ethanol dependence model in adolescence find decreased neurogenesis, without a change in Ki67+IR [36, 37], although there were changes in progenitor cell cycle kinetics [37]. We assessed Ki67 about 4 weeks after adolescent treatment and did not find any differences in Ki67+IR consistent with the majority of studies in adolescents. Thus, adolescent ethanol exposure appears to reduce immature neurons not via alterations in proliferation per se, as indexed via Ki67, but via increasing their demise, as indexed using cleaved capase-3 as a marker of cell death. Together, these findings suggest hippocampal progenitors continue to proliferate following adolescent ethanol exposure, but cell death is increased such that progenitors die before maturing to dentate granule cell neurons. Therefore, the persistent decrease in adult hippocampal neurogenesis following adolescent intermittent ethanol treatment suggests long term changes that result in increased cell death in the neurogenic niche [25].

Adult brain neurogenesis occurs due to unique properties of glia and other cells within the hippocampal dentate gyrus and forebrain subventricular zone that maintain proliferating stem cells which can form new neurons. The mechanisms for the persistent increase in dentate gyrus caspase-3+IR after adolescent ethanol exposure in the current study is likely related to alterations in the neurogenic niche. The age-related decline in neurogenesis is thought to result from multiple factors, including loss of proliferating progenitors, loss of trophic support for progenitors and increased microglial activation and innate immune gene induction [25]. Adolescent intermittent ethanol treatment has been found to increase innate immune gene expression in frontal cortex [38] as well as to reduce trophic factor support of the neurogenic niche [39]. These findings are consistent with a preserved stem cell population in dentate gyrus following adolescent alcohol exposure, as suggested by continuous progenitor proliferation (Ki-67+IR), but an altered neurogenic niche that increases cell death as indicated by the loss of DCX+IR e.g. neurogenesis and increase in cell death (caspase-3+IR). Loss of trophic factors that support neurogenesis (see [39] for review and [40]), and/or increase immune gene induction [25] may both contribute to the persistent loss of neurogenesis following adolescent ethanol exposure.

Given that neurogenesis is implicated in context fear retention [11, 12, 13, 14, 15], it is possible that alterations in neurogenesis in the DG as a result of adolescent ethanol exposure may have contributed to deficits in context fear retention previously observed in a separate group of animals [6]. Alternatively, since increased survival of newly generated neurons has been reported after trace conditioning [41, 42], a possibility arises that tone conditioning and extinction sessions experienced by animals in the current study may have somehow influenced the subsequent levels of neurogenesis. This interpretation is not likely, however, given lack of evidence that tone conditioning or extinction influences subsequent levels of neurogenesis in the hippocampus, nor were there detectable differences in acquisition of conditioning or extinction observed between adolescent ethanol- and water-treated groups (see [6] for behavioral data).

One caveat for the interpretation that reduced neurogenesis after adolescent intermittent ethanol exposure plays a role in deficits context fear retention is that disruptions in context fear conditioning were not evident in P116 control animals relative to their young adult (P74) counterparts [6], despite the marked age-related declines in DCX levels in DG seen between P74 to P116. These data suggest that levels of neurogenesis (indexed via DCX) may be more important for context fear memory in younger adults (P74) than older adult animals (P116). It is also possible that levels of neurogenesis need to be significantly lower than levels seen in the current study to affect context fear memory in older adults, such as the notable declines reached in studies utilizing irradiation-induced decreases in adult neurogenesis (e.g., [11, 12, 13, 14, 15]). Alternatively, these data may suggest that the disruption of context fear memory seen in Broadwater & Spear [6] may not be dependent on production of new neurons, at least as indexed by number of DCX+ cells.

To the extent that these data extend to humans, alcohol consumption during adolescence may lead to long-lasting negative consequences that involve deficits in hippocampal-dependent memory and alterations in survival of neurons in the DG, even after a period of abstinence. Chronic alcoholism is associated with decreases in hippocampal volumes that might be related to a persistent loss of neurogenesis beginning in adolescence [43]. Therefore, these data provide more evidence that adolescence is a particular sensitive period of development to persisting consequences of alcohol exposure, with the hippocampus being a region of vulnerability to long lasting effects.

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Figure 1.

Overview and timeline of manipulations experienced by animals in the current study (n=10/grp). Note that the behavioral data is published elsewhere (see Broadwater & Spear, 2013).

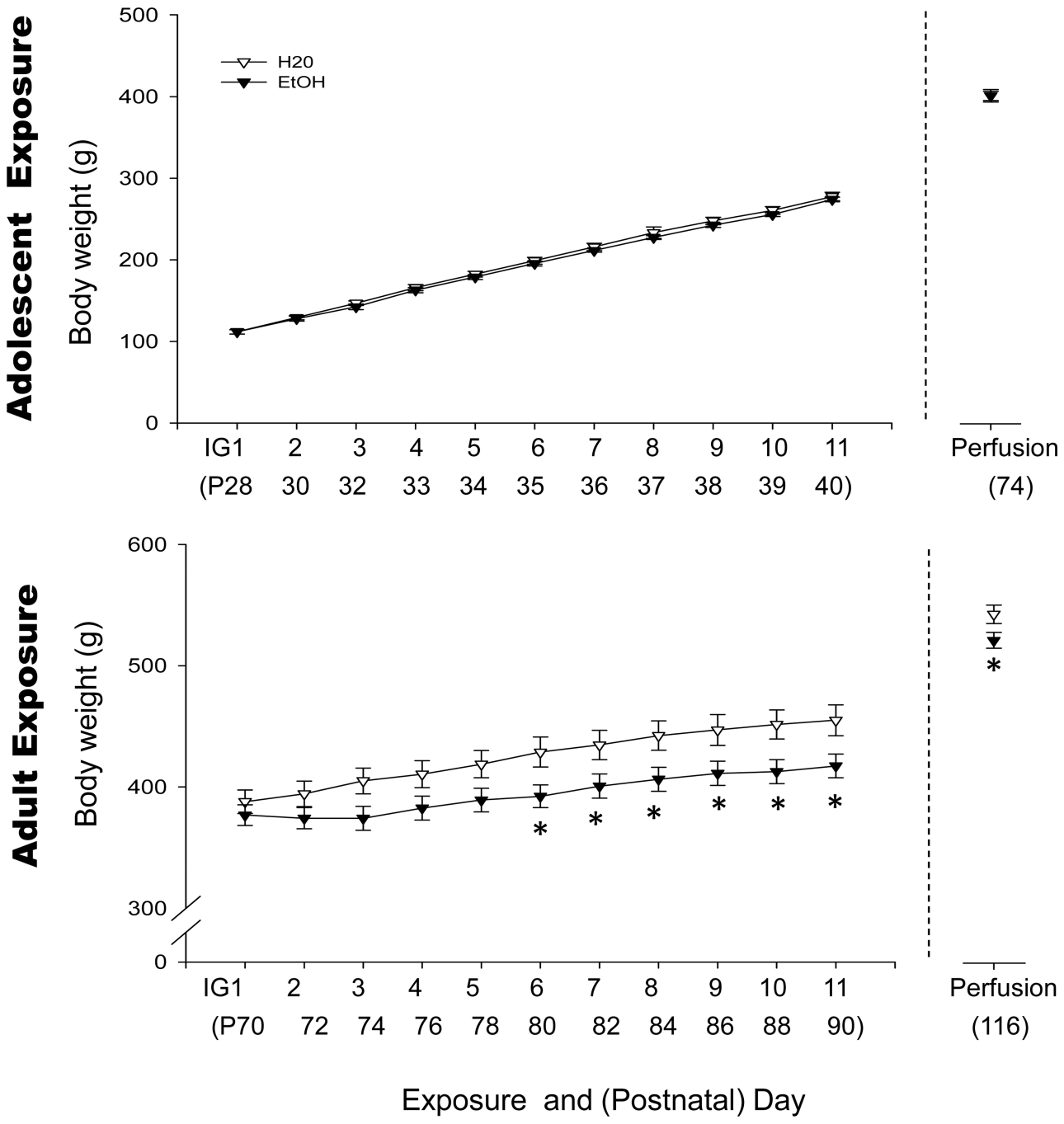

Figure 2.

Weight did not differ between adolescent exposure groups during the exposure period or in adulthood. Adult ethanol exposure resulted in weight deficits starting on the 6th i.g. exposure day (P80), an effect that persisted ~4 weeks after the exposure period [$F(11, 198)= 10.92$, $p<.05$]. Asterisks (*) indicate significant difference from H20 controls.

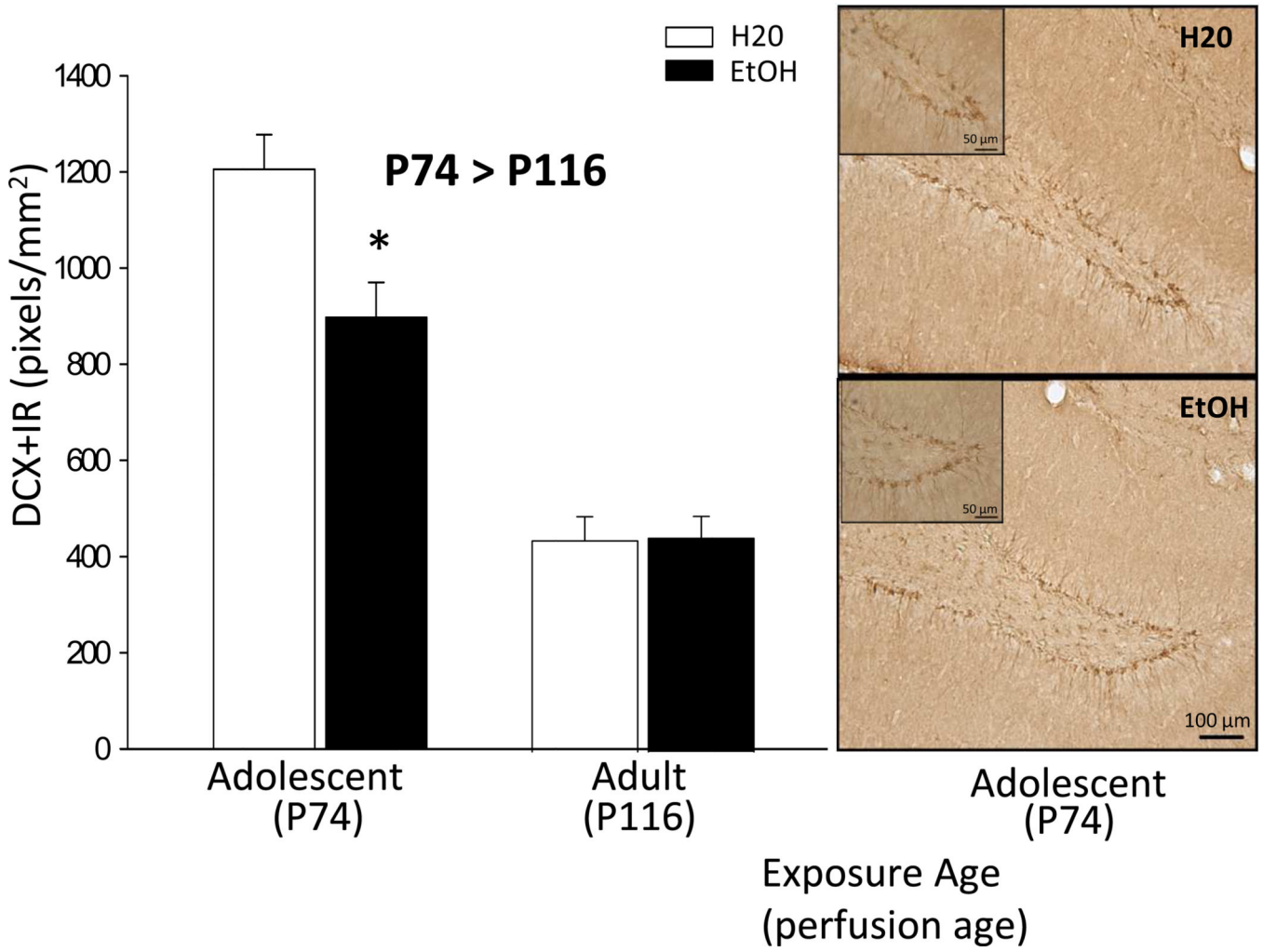

Figure 3.

DCX+IR in the dentate gyrus of the hippocampus in P74 and P116 brains after adolescent or adult exposure, respectively. Adolescent (but not adult) ethanol-exposed animals showed reduced DCX+IR compared to water controls [$F(1, 33)= 101.30$, $p< .05$]. An age-related decline in DCX+IR was also evident [$F(1, 33)= 6.26$, $p< .05$]. Asterisk (*) indicates significant difference from H20 controls.

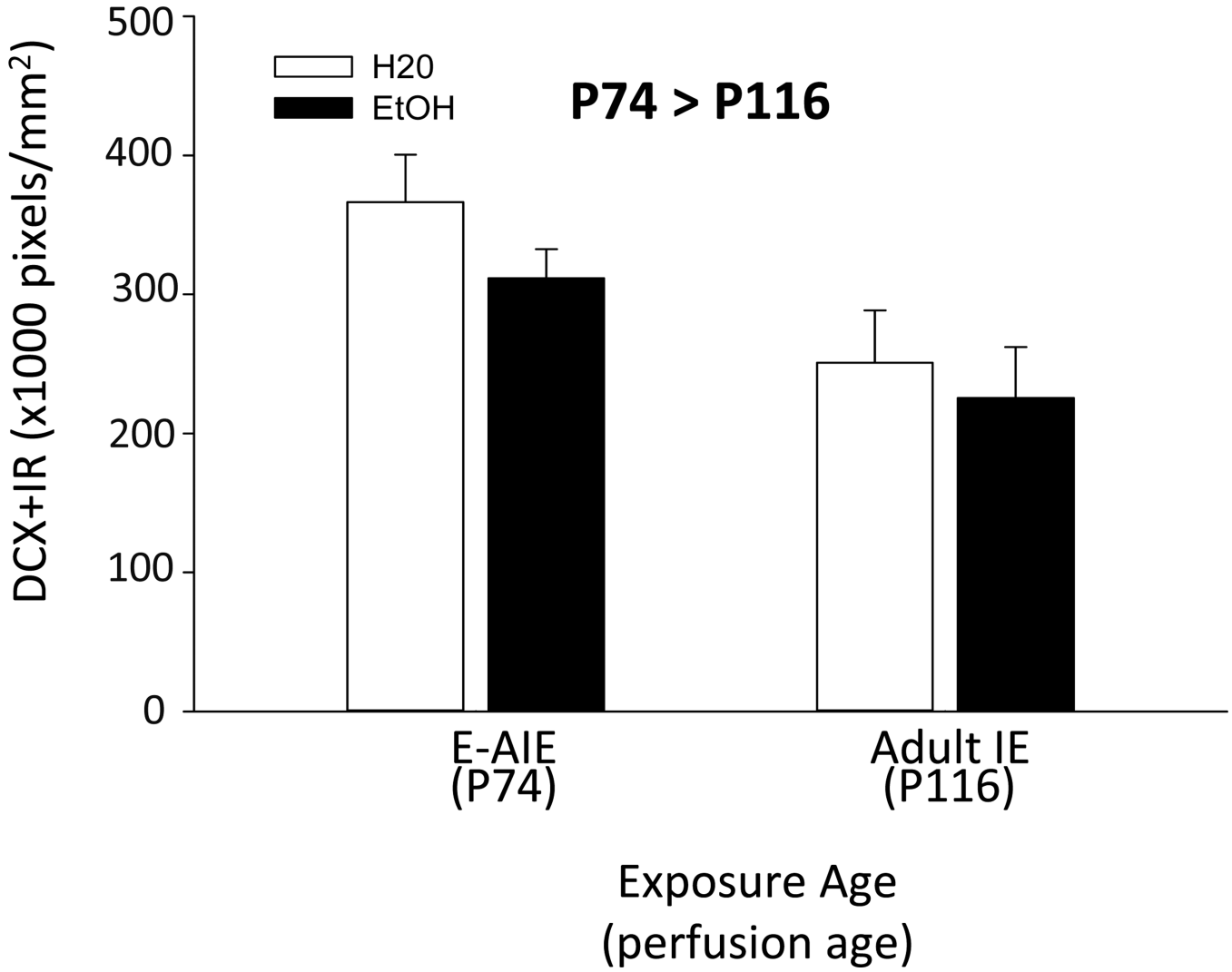

Figure 4.

DCX+IR in the subventricular zone (SVZ) after adolescent or adult exposure, respectively. DCX+IR was not influenced by prior exposure at either age in the SVZ, although an age-related decline in DCX+IR emerged [$F(1,34) = 11.38$, $p < .05$].

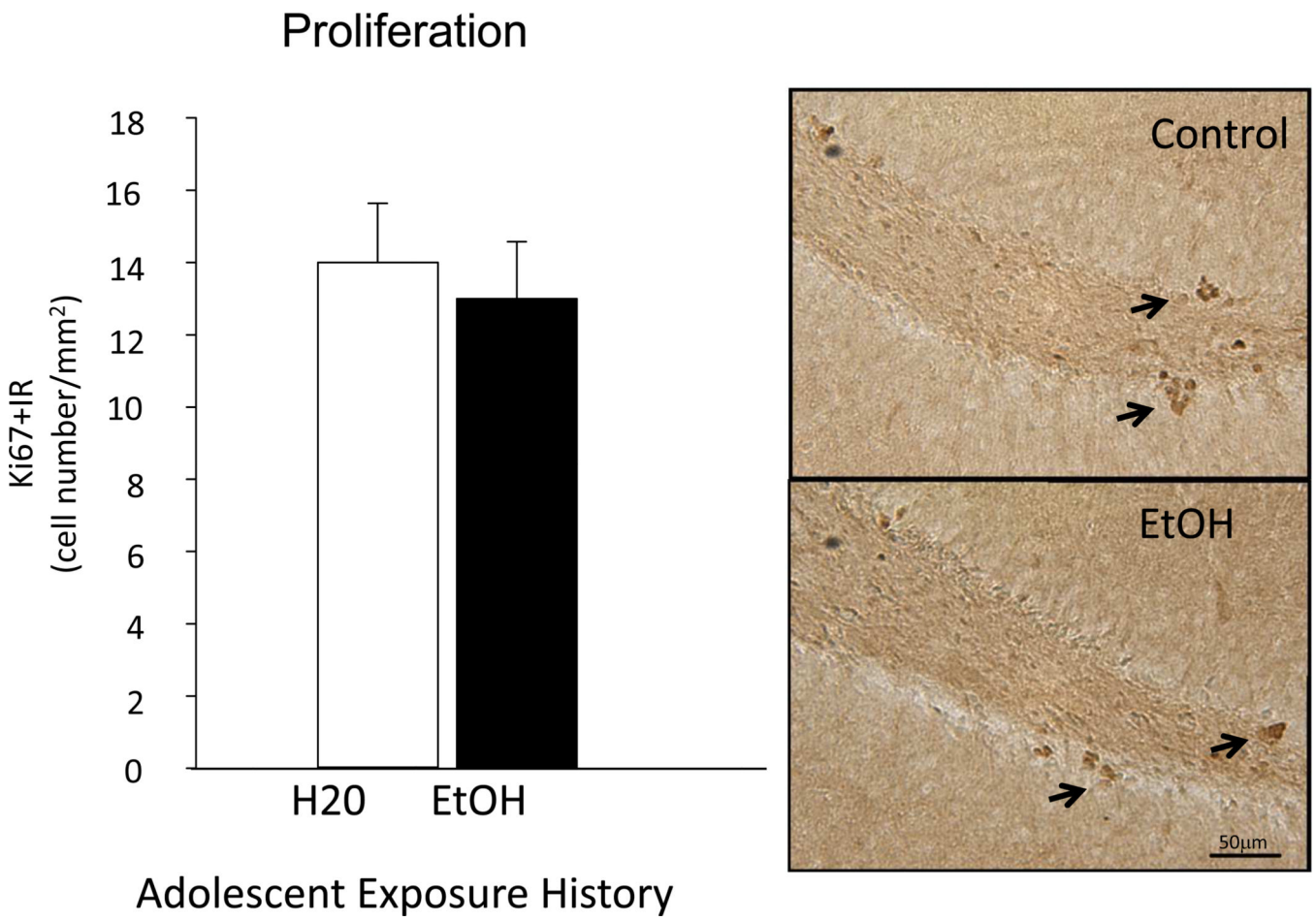

Figure 5.

Ki67+IR in the DG of adolescent exposed adults (P74) did not significantly differ between adolescent exposure groups, suggesting that proliferation of NPC was not influenced by prior adolescent ethanol exposure. See arrows for examplex of ki67+ cells in the pictures on the right panel.

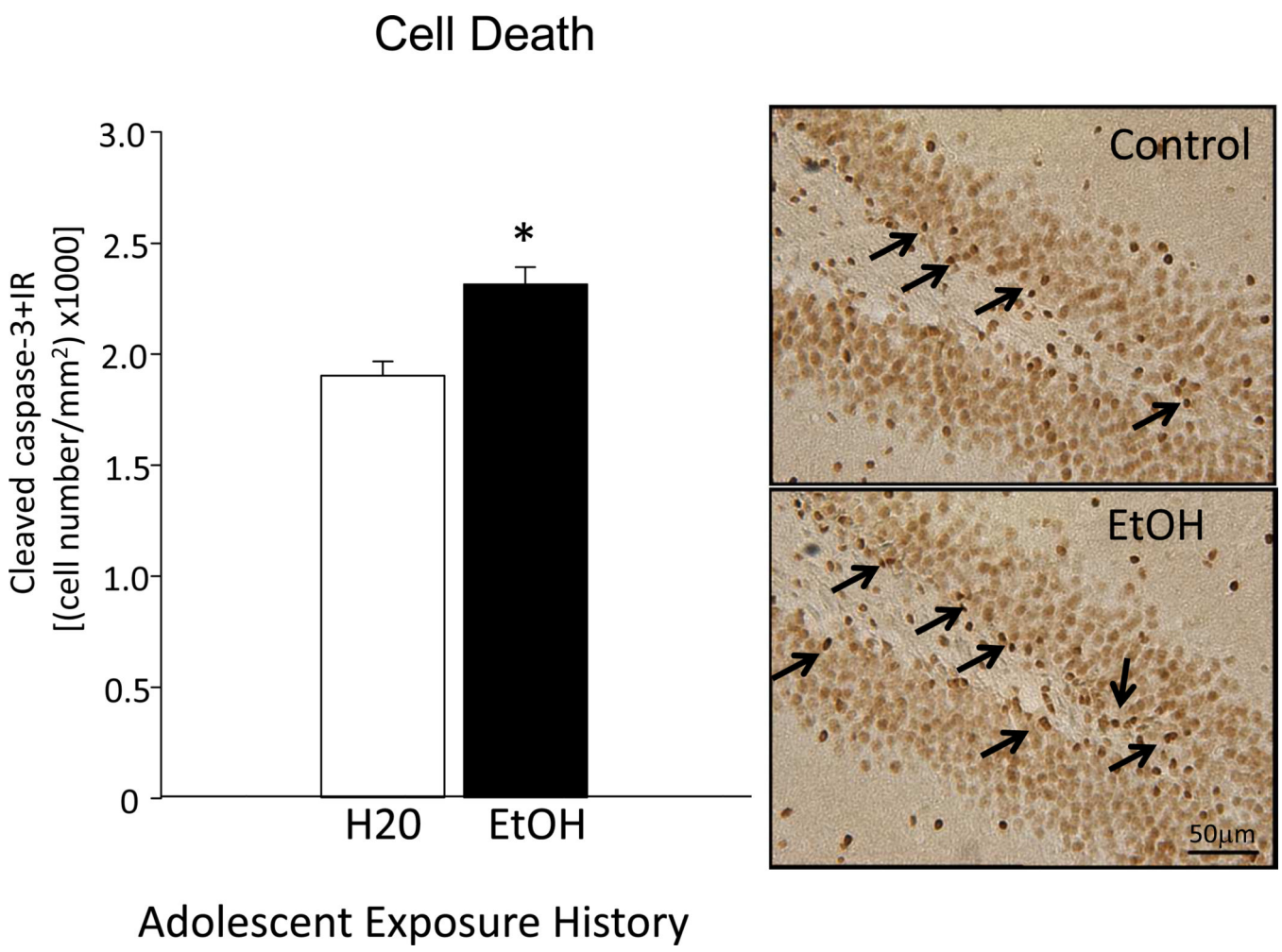

Figure 6.

Cleaved caspase-3+IR, an index of cell death, in the DG of adolescent exposed adults (P74) was significantly increased in the ethanol group compared to H20 controls [$F(1,18)= 16.63$, $p< .05$] (see *). See arrows for examples of caspase-3+ cells (dark stained cells) in the pictures on the right panel.