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ORIGINAL ARTICLE

Effects of Chronic Voluntary Alcohol Drinking on Thiamine Concentrations, Endoplasmic Reticulum Stress, and Oxidative Stress in the Brain of Crossed High Alcohol Preferring Mice

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Abstract

Chronic alcohol drinking can damage the central nervous system via many mechanisms. One of these may involve a deficiency of an essential nutrient, thiamine, as a result of chronic alcohol exposure. Although thiamine deficiency (TD) has often been linked to the neuropathology of alcohol-related brain damage, the underlying mechanisms remain to be investigated. The crossed high alcohol preferring (cHAP) mice prefer alcohol to water when they have free access. In this study, we used cHAP mice to determine the effect of chronic voluntary alcohol exposure on thiamine levels and neuropathological changes in the brain. The male cHAP mice were given free-choice access to 10% ethanol (EtOH) and water for 7 months, sacrificed, and thiamine concentrations in the blood plasma and brain were determined by liquid chromatography–mass spectrometry (LC-MS). The expression of thiamine transporters was examined by immunoblotting. In addition, oxidative stress, endoplasmic reticulum (ER) stress, active caspase-3–dependent apoptosis, and neurogenesis in the brain were evaluated. The results indicated that chronic alcohol exposure decreased thiamine levels and thiamine transporters, and increased oxidative stress, ER stress, and neuronal apoptosis in the brains. Interestingly, alcohol exposure also stimulated neurogenesis in the hippocampus which may serve as a compensatory mechanism in response to alcohol-induced brain damage. Our data have demonstrated that cHAP mice are a useful model to study the interaction between chronic alcohol consumption and TD, as well as TD's contributions to the neuropathological processes resulting in alcohol-related brain damage.

Keywords Alcohol use disorders · Nutrition · Neurodegeneration · Thiamine deficiency

Introduction

Chronic alcohol exposure causes serious mental and physical health problems worldwide (Grant et al. 2004; Bouchery et al. 2011). The central nervous system (CNS) is remarkably vulnerable to alcohol; chronic alcohol abuse has serious neuropathological and neurobehavioral consequences (Herrera et al. 2003; Harper and Matsumoto 2005; Johansson et al. 2009; Mukherjee 2013; Oliveira et al. 2015). Chronic alcoholism is commonly associated with decreased absorption/utilization of thiamine (Abdou and Hazell 2015; Galvin et al. 2010).

Thiamine, also known as vitamin B1, is a water-soluble nutrient present in all cells of the human body and is essential for the normal development and function of cells (Kumar 2010). It must be provided in the diet, through food supplements, or medication, because it cannot be synthesized in the body. Once taken up into cells, thiamine is converted into thiamine monophosphate (TMP) or thiamine diphosphate (TDP) by the addition of one or two phosphate groups. TDP is the active form of thiamine that functions as the cofactor involved in the metabolism of sugars and amino acids. In the human body, thiamine is enriched in many tissues including the skeletal muscles, heart, liver, kidney, and

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brain (Martin et al. 2003). The CNS is susceptible to thiamine deficiency (TD) which leads to impaired oxidative metabolism, alterations in neurotransmitters, oxidative stress, endoplasmic reticulum (ER) stress, excitotoxicity, inflammation, and neurodegeneration (Harper 1998; Liu et al. 2017; Wang et al. 2017). TD is the major cause of the Wernicke encephalopathy (Galvin et al. 2010). In western countries, TD is associated with alcoholism, occurring in at least 25–31% and up to 80% of alcoholics (Abdou and Hazell 2015). Some have proposed that TD may underlie alcohol-induced damage to the brain (Martin et al. 2003; Zahr et al. 2011). However, the interaction between alcohol and TD is complex and it is difficult to evaluate their respective contributions to alcohol-induced brain damage in clinical studies. Therefore, relevant animal models are necessary to investigate the interaction between alcohol exposure and TD in the context of alcohol-induced brain damage.

The impact of alcohol on thiamine levels/utilization is usually the result of long-term exposure. Mice that are willing to voluntarily drink alcohol over an extended period of time, achieving pharmacologically relevant blood ethanol concentrations (BECs), may model chronic alcohol abuse in humans. The selectively bred crossed high alcohol preferring (cHAP) mice have been shown to voluntarily consume high amounts of alcohol and demonstrate relatively high BECs that are comparable with those observed in alcohol-dependent humans (Matson and Grahame 2013; Matson et al. 2014). These animals were initially selectively bred from an 8-way inbred strain cross called Heterogeneous Stock (Institute for Behavioral Genetics) for high two-bottle choice alcohol consumption during a 4-week phenotyping period. Subsequently, crossed HAPs were selectively bred from a cross of two HAP lines and show higher intake than either parent line (Oberlin et al. 2010). In this study, we used the cHAP mice to investigate the effects of chronic voluntary alcohol drinking on thiamine levels/transporters in the brain as well as other neuropathological changes. To our knowledge, this is the first study to evaluate whether voluntary alcohol consumption elicits TD, as previous studies used potentially more stressful forced exposure methods such as alcohol vapor exposure or alcohol solutions as the only source of fluid. We show that 7 months of alcohol drinking reduced thiamine levels and the expression of thiamine transporters in the brain of cHAP mice. It also caused oxidative stress, ER stress, expression of active caspase-3, and neurogenesis in some brain regions.

Materials and Methods

Materials

Bromodeoxyuridine (BrdU) was purchased from Thermo Fisher Scientific (Rockford, IL). Anti-cleaved caspase-3 and anti-Ki-67antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-BrdU antibody was purchased from Thermo Fisher Scientific (Waltham, MA). Anti-doublecortin and anti-OCT1 antibodies were purchased from Abcam (Cambridge, MA). Biotinylated goat anti-rabbit IgG was purchased from Vector Laboratories Inc. (Burlingame, CA). Alexa 488-conjugated goat anti-rabbit IgG was purchased from Invitrogen (Carlsbad, CA). Antibodies directed against GRP78, CHOP, eIF2α, ATF6, DNP, and HNE were purchased from Santa Cruz Biotech (Santa Cruz, CA). Anti-SLC19A2 antibody and 3,3′-diaminobenzidine (DAB) were purchased from Sigma-Aldrich (St. Louis, MO). The anti-slc19a3 antibody was purchased from Proteintech (Rosemont, IL).

Animals and Alcohol Exposure

cHAP mice were obtained from Dr. Nicholas J. Grahame at Indiana University-Purdue University Indianapolis (Indianapolis, IN, USA) and housed in the University of Kentucky Medical Center Animal Care Facilities. Animals were maintained in a reverse 12:12 light-dark cycle colony room with lights off at 9:00 a.m. All mice were given ad libitum access to food and water throughout the experiment. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky. Male cHAP mice were given access to alcohol at 60 days of age. The mice had 24-h, free-choice access to 10% ethanol (*v*/*v*) solution and water. Ethanol and drinking water were provided with 50-ml test tube water bottle (#20872, Arcata Pet Supplies, Arcata, CA) fit with a stainless-steel sipper tube (Model OCT-100, Ancare, Belmont, NY). The water bottles were changed every 2 days with freshly prepared ethanol in drinking water or drinking water only. After 7 months of alcohol consumption, the mice were sacrificed, and blood plasma and brain tissues were collected for LC-MS and neurochemical analyses. For immunohistochemical and immunofluorescent studies, mice were anesthetized with intraperitoneal injection of ketamine/xylazine (100 mg/kg/10 mg/kg), and then intracardially perfused with PBS followed by 4% paraformaldehyde in PBS (pH 7.4).

Determination of Blood Ethanol Concentrations

Blood samples were collected at weeks 1 and 25 of free-choice access to ethanol and water. The blood was drawn from the tail veins 4 h after the onset of the dark cycle. The time point was selected because the cHAP mice drink more in the dark, and peak BECs were observed in the dark cycle (Matson and Grahame 2013; Matson et al. 2014). The plasma supernatant was extracted and blood ethanol concentrations (BECs) were measured using an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Quantification of Thiamine Concentrations in the Blood Plasma and Brain

The determination of thiamine concentration in the blood and brain was performed based on a previously described method with some modifications (Kato et al. 2015). Briefly, blood samples were collected in heparin-coated tubes and immediately stored at − 20 °C. A total of 100 μL of 1.2 M ice-cold perchloric acid was added to 100 μL blood sample and stored at 0 °C for 15 min. Plasma was separated in the supernatant after the mixture was centrifuged for at 10,000*g* for 10 min. A total of 100 μL of 0.6 M KOH/1.8 M potassium acetate was then added to 100 μL of supernatant and the mixture was vortexed and centrifuged at 10,000*g* for 10 min. A total of 120 μL supernatant was separated and mixed with 30 μL of acetonitrile/methanol (9:1; *v*/*v*) containing an internal standard. The mixture was then centrifuged at 3639*g* for 10 min. The supernatant was transferred to a new vial, dried with SpeedVac, and reconstituted with H_2O. An aliquot of the samples was injected into a LC-MS/MS system for thiamine analysis. The measurement of total thiamine (TMP and TDP) concentration in the brain was achieved by a modified enzymatic hydrolysis method (Kato et al. 2015; Wielders et al. 2015). Briefly, the brain tissues were dissected, weighed, homogenized in four volumes of water and kept on ice. A total of 30 μL of homogenate was mixed with the equal volume of1.2 M ice-cold perchloric acid and kept at 0 °C for 15 min. After the mixture was centrifuged at 10,000*g* for 10 min, 30 μL of supernatant was separated and mixed with 15 μL of an internal standard solution (1 μM in saline) and 20 μL of 0.6 M KOH/1.8 M potassium acetate. After the mixture was centrifuged at 10,000*g* for 10 min, 25 μL of 4 mg/mL acid phosphatase in saline (pH 5.0) was added to 25 μL supernatant and the mixture was incubated overnight at room temperature. After the enzymatic hydrolysis, 200 μL of acetonitrile/methanol (9:1; *v*/*v*) was added to the mixture, vortexed, and centrifuged at 3639*g* for 10 min. The supernatant was transferred to a new vial, dried with SpeedVac, and reconstituted with H_2O. An aliquot of the samples was injected into a LC-MS/MS system for analyses.

BrdU Labeling, Immunohistochemistry and Immunofluorescent Staining

BrdU is a thymidine analog that is incorporated into the cells during DNA replication and thus BrdU labeling is used to monitor cell proliferation in the brain. After alcohol exposure, five control and five alcohol-exposed mice received an intraperitoneal injection of BrdU (50 mg/kg) for two consecutive days. Twenty-four hours after the last BrdU injection, the mice were anesthetized intraperitoneally with ketamine/xylazine (100 mg/kg/10 mg/kg), and then perfused with 0.1 M potassium phosphate buffer (pH 7.2), followed by 4% paraformaldehyde in PBS (pH 7.4). The brain tissues were dissected and postfixed in 4% paraformaldehyde for 48 h followed by cryoprotection in 30% sucrose-containing PBS at 4 °C. The brains were sectioned (sagittal section or coronal section) on a sliding microtome (Leica Microsystems, Wetzlar, Germany) at a thickness of 10 μm and an interval of 20 μm. The sections were then mounted and dried thoroughly on Superfrost Plus (Fisher) slides.

The immunohistochemical (IHC) staining was performed as previously described (Wang et al. 2007). Briefly, mounted sections were pre-treated and incubated with anti-cleaved caspase-3 (1:200), anti-BrdU antibody (1:50), anti-DCX (1:1000), or anti-Ki-67 antibodies (1:400) overnight at 4 °C followed by incubation with a biotinylated goat anti-rabbit IgG (1:200) for 1 h at room temperature. After rinsing in PBS, the sections were then treated with an avidin-biotinylated-peroxidase kit developed in 3,3′-diaminobenzidine (DAB) containing PBS. The specificity of each antibody on the brain sections was confirmed by comparing with the negative control without using the primary antibody.

Immunofluorescent (IF) staining was performed as previously reported (Ayoub et al. 2005). In brief, pre-treated sections were incubated with anti-BrdU antibody (1:50), anti-DCX (1:200), or anti-Ki67 antibodies (1:400) at 4 °C overnight followed by incubation with Alexa488-conjugated goat anti-rabbit IgG (1:200) for 1 h. The images were recorded with a DP70 digital camera using an Olympus BX61 microscope. Negative controls without using primary antibody were also performed to assure the specificity of each antibody. To quantify BrdU-, DCX-, or Ki67-positive cells, a 20× objective was used to count stained cells from five microscopic fields in the SVZ or DG area from each brain. The average of the positive cells from 4 to 5 consecutive sections in each brain was analyzed using the Image lab 5.2 software. For each treatment group, five animals were used and analyzed.

Immunoblotting

The immunoblotting was performed as previously described (Ke et al. 2011). Briefly, the mice were anesthetized with ketamine/xylazine. The brain tissues were dissected and lysed with RIPA buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM PMSF, 0.5% NP-40, 0.25% SDS, 5 μg/ml leupeptin, and 5 μg/ml aprotinin] on ice for 30 min. The cell lysates were centrifuged at 15,000*g* for 15 min at 4 °C. The supernatant was collected and the protein concentration was measured using a protein assay kit. Aliquots of 30 μg of the protein were loaded and separated on an SDS-polyacrylamide gel by electrophoresis. The proteins were then transferred to nitrocellulose membranes followed by blocking with 5% bovine serum albumins (BSA), 0.01 MTBS (pH 7.4), and 0.1% Tween-20 (TBST) at room temperature for 1 h. The membranes were probed with primary antibodies at 4 °C

overnight. After three washes in TPST, the membranes were incubated with a horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody. The immune complexes were detected by the enhanced chemiluminescence method. The experiments were replicated three times and the density of immunoblotting was quantified by densitometry.

Data Analysis

All of the statistical analyses were performed using unpaired *t* test in the software GraphPad Prism 6 (GraphPad Software; La Jolla, CA). All values were reported as mean ± SEM. Differences were considered significant if the *p* value was less than 0.05.

Results

Chronic Alcohol Exposure Decreases Thiamine Levels and Thiamine Transporters in the Brain

The BECs were measured 1 week and 25 weeks after alcohol exposure. The BECs were 97.15 ± 15.12 mg/dL and 92.02 ± 12.05 mg/dL for mice exposed to alcohol for 1 week and 25 weeks, respectively. Total thiamine concentration was measured after the enzymatic hydrolysis. The concentrations of endogenous total thiamine and exogenous total thiamine-d3 were also measured. Each sample was measured three times to assure intra-assay precision. Furthermore, we measured the intra-assay precision of samples spiked with low, intermediate, and high additions of TDP. The thiamine concentrations in the blood and brain determined in the current study were consistent with that in a previous study (Kato et al. 2015). As shown in Table 1, although there was no significant difference in the plasma thiamine levels in control and alcohol-exposed mice, the thiamine concentration in the brain was significantly lower in mice exposed to alcohol [$t(11) = 2.684$, $p = 0.0213$]; the thiamine concentrations in the brain of control mice and alcohol-exposed mice were 4.88 nmol/g and 2.85 nmol/g, respectively. Thus, alcohol exposure reduced the brain thiamine levels by 42%.

We next determined the effect of alcohol exposure on the expression of thiamine transporters. SLC19A2 and SLC19A3 are important thiamine transporters that are widely expressed (Zhao and Goldman 2013). Organic cation transporter 1 (OCT1), another thiamine transporter, plays an important role in thiamine uptake in the liver (Liang et al. 2018). As shown in Fig. 1, chronic alcohol exposure reduced the expression of SLC19A3 [$t(6) = 2.56$, $p = 0.043$] without affecting SLC19A2 and OCT1 in the brain. Interestingly, alcohol exposure decreased the levels of SLC19A2 [$t(7) = 3.74$, $p = 0.007$] and OCT1 [$t(7) = 4.80$, $p = 0.002$] without altering SLC19A3 in the liver.

Table 1 Thiamine concentrations in the plasma and the brain

Average thiamine concentration	Plasma (nmol/ml)		Brain (nmol/g)	
	Control	EtOH	Control	EtOH
Mean	0.12	0.16	4.88	2.85*
SEM	0.02	0.05	0.93	0.18

The effect of chronic alcohol exposure on thiamine concentrations in the plasma and brain of male cHAP mice. Male cHAP mice voluntarily drank alcohol for 7 months as described in the "Materials and Methods" section. The plasma and brain samples were assayed for total thiamine contents by LC-MS/MS as described in the "Materials and Methods" section. For the plasma samples, $n = 6$ for both control and EtOH group. For the brain samples, $n = 5$ and 8 for control and EtOH groups, respectively. Asterisk denotes a statistical difference, $p < 0.05$

Chronic Alcohol Exposure Induces Oxidative Stress, ER Stress, and Activation of Caspase-3 in the Brain

Since thiamine deficiency (TD) is associated with oxidative stress and ER stress, both of which play a critical role in alcohol-induced brain damage (Yang and Luo 2015; Liu et al. 2017), we investigated the effect of chronic alcohol exposure on oxidative stress and ER stress in the brain. 4-hydroxynonenal (4-HNE) and 2,4-dinitrophenol (DNP) have been used as sensitive and reliable biomarkers for lipid peroxidation and protein oxidation, respectively (Perluigi et al. 2014). Chronic alcohol drinking increased the expression of 4-HNE [$t(6) = 2.83$, $p = 0.014$] and DNP [$t(6) = 4.78$, $p = 0.003$] in the brain, indicating the induction of oxidative stress (Fig. 2a). Furthermore, chronic alcohol drinking upregulated the expression of a number of protein markers for ER stress, such as GRP78 [$t(8) = 2.38$, $p = 0.045$], CHOP [$t(6) = 2.91$, $p = 0.013$], p-eIF2α [$t(6) = 2.09$, $p = 0.04$], and ATF6 [$t(8) = 2.82$, $p = 0.023$], indicative of ER stress (Fig. 2b).

We next sought to determine whether alcohol-induced neuronal apoptosis in the brain. As shown in Fig. 3, the immunoblotting analysis indicated that chronic alcohol exposure caused an increase in the expression of cleaved caspase-3 [$t(8) = 2.50$, $p = 0.037$], the active form of caspase-3 (Fig. 3a), suggesting apoptosis in the brain. The IHC study confirmed that cleaved-caspase-3-positive cells were observed in the prefrontal cortex (PFC) and the dentate gyrus (DG) of the hippocampus (Fig. 3b).

Chronic Alcohol Exposure Stimulates Neurogenesis in the Brain

We investigated the effect of chronic alcohol exposure on the neurogenesis in the subventricular zone (SVZ) and DG of the hippocampus. As shown in Fig. 4a and c, alcohol exposure increased the number of BrdU-positive cells [$t(6) = 2.84$, $p = 0.029$] and Ki-67-positive cells [$t(8) = 2.38$, $p = 0.044$] in the SVZ, indicating enhanced proliferation of neural progenitors.

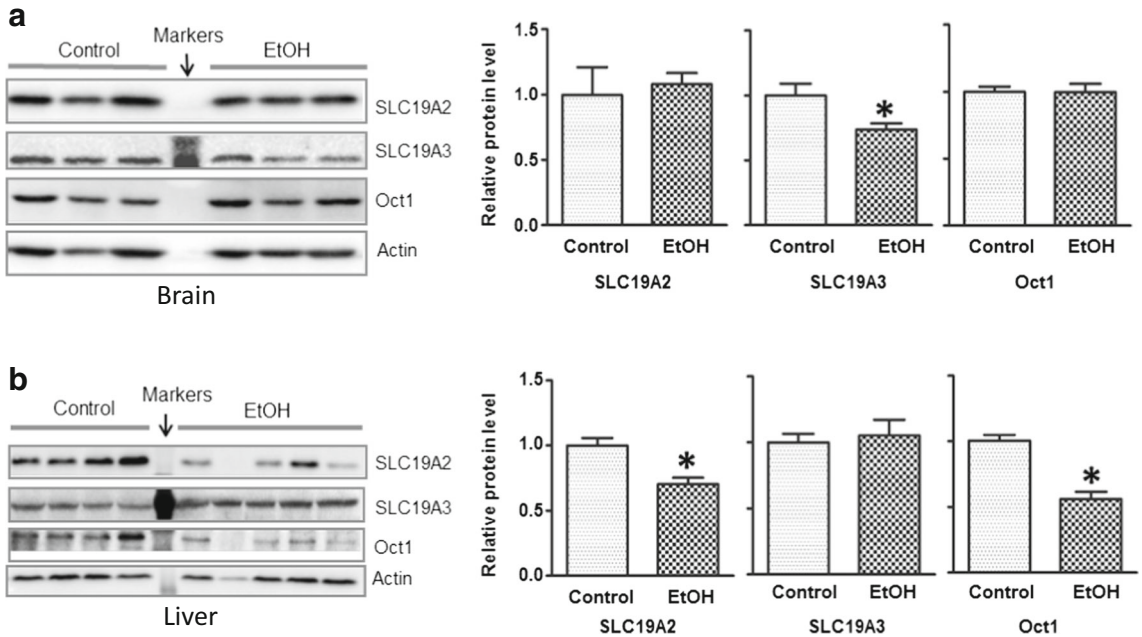

Fig. 1 Effect of chronic alcohol exposure on the expression of thiamine transporters in the brain and liver of cHAP mice. Male cHAP mice voluntarily drank alcohol for 7 months as described in the "Materials and Methods" section. **a** The expression of SLC19A2, SLC19A3, and OCT1 in the cerebrum was examined by immunoblotting. The expression of actin served as a loading control. The relative amounts of SLC19A2, SLC19A3, and OCT1 were quantified and normalized to the expression of actin. The experiment was replicated three times, and the results were expressed as the mean ± SEM, $n = 4$ or 5 for each group. $^*p < 0.05$ denote statistically significant difference from the control group. **b** The relative expression of SLC19A2, SLC19A3, and OCT1 in the liver was examined by immunoblotting, and quantified and normalized to the expression of actin. Each data point was the mean ± SEM of three independent experiments, $n = 4$ or 5 for each group. $^*p < 0.05$ denotes a statistically significant difference from the control group

Alcohol exposure also increased the number of BrdU-positive cells ($t(8) = 2.44$, $p = 0.040$] and DCX-positive cells [$t(6) = 2.47$, $p = 0.049$] in the DG of the hippocampus (Fig. 4b and c).

Discussion

We used cHAP mice to study the effect of chronic voluntary alcohol drinking on thiamine levels in the blood and brain. Seven months of alcohol exposure in mice is equivalent to 20 years of drinking in humans extrapolated from the proportion of lifespan. In our study, the BECs were consistently at ~ 90 mg/dl as measured at 1 and 25 weeks after alcohol exposure, which are consistent with the standard of binge drinking defined by the National Institute on Alcohol Abuse and Alcoholism. However, the BECs were lower than previous reports using this mouse line, which showed that the BECs averaged as high as 250 mg/dl, in the dark cycle following 3 weeks of alcohol access (Matson and Grahame 2013; Matson et al. 2014). The reason for this discrepancy is currently unclear, although we took blood somewhat earlier in the dark cycle, at 4 h after onset of dark as opposed to 7 h in these earlier papers.

We showed here that chronic voluntary alcohol consumption for 7 months caused a 42% decrease in the concentration of total thiamine in the brain, while there was little change in the blood samples (Table 1). A previous study investigated the effect of involuntary exposure to alcohol on blood thiamine levels in rats (Vedder et al. 2015). In that study, male Sprague Dawley rats had access to EtOH (20% *v*/*v*) in drinking water as their sole source of fluid for 6 months. Similar to our results, BEC averaged 98.8 mg/dl at month 6, which when the blood samples were measured 4 h into the dark cycle. In this study, the active form of thiamine (TDP) in the blood was shown to be decreased by alcohol exposure (Vedder et al. 2015). However, the thiamine contents in the brain were not measured in their study. This discrepancy may result from the difference in animal species and the forms of thiamine that were assayed, or from the fact that forced alcohol drinking placed additional stress on the animals in that study. Forced alcohol drinking studies such as Vedder et al. (2015) require pair-fed controls, and even rats maintained on 6% ethanol (as opposed to the 20% used in the Vedder et al. 2015) show a variety of changes related to nutrition and energy balance (Pravdova et al. 2009). In the present study, we did not need nutritional or stress controls because animals drank alcohol voluntarily. Additionally, we determined the levels of total thiamine, while they measured TDP in the blood. In a future study, we may need to examine the TDP in both brain and blood. A previous study showed that short-term (4 days) binge alcohol exposure fails to change the thiamine concentration (TMP and TDP) in the blood of Wistar rats (Zahr et al. 2010).

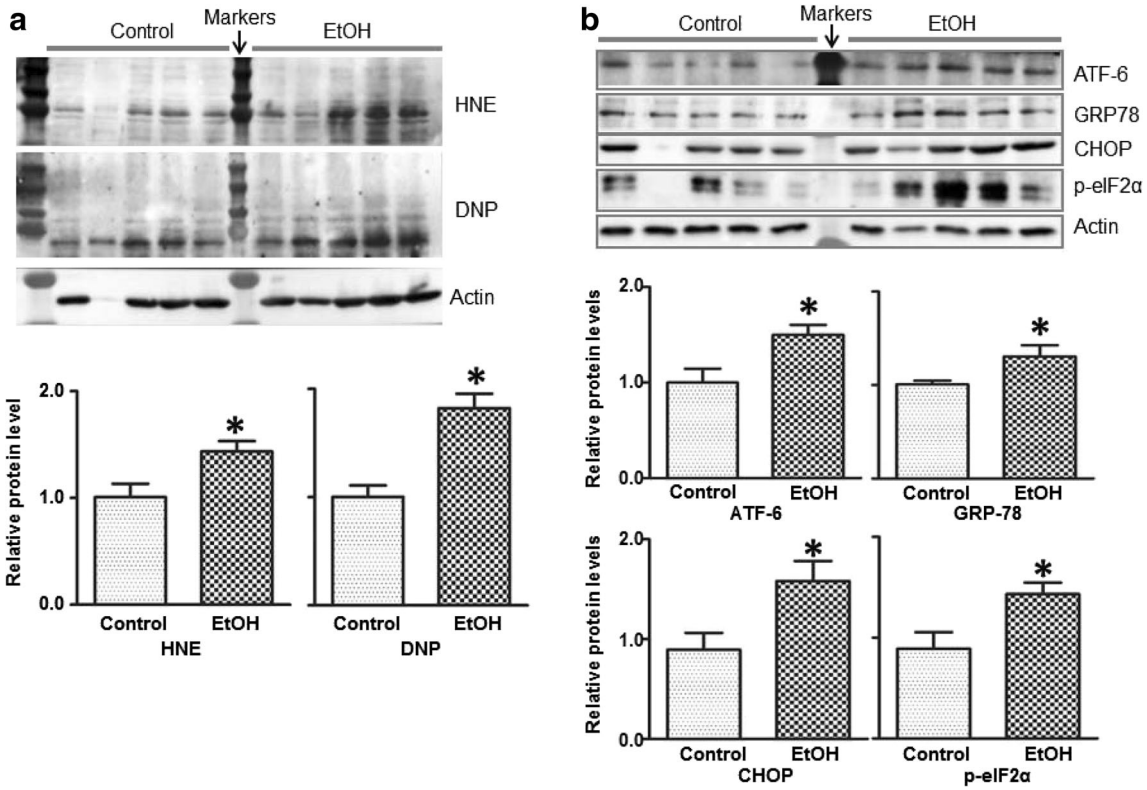

Fig. 2 Effect of chronic alcohol exposure on markers of oxidative stress and ER stress in the brain. Male cHAP mice voluntarily drank alcohol for 7 months as described in the "Materials and Methods" section. **a** The expression of 4-HNE and DNP in the cerebrum was determined by immunoblotting. The relative amounts of 4-HNE and DNP were quantified and normalized to the expression of actin. **b** The expression of ER stress markers, GRP78, CHOP, p-eIF2α, and ATF6 in the cerebrum was determined by immunoblotting. The relative amounts of expression were quantified and normalized to the expression of actin. The experiment was replicated three times, and the results were expressed as the mean ± SEM, $n = 4$ or 5 for each group. $^*p < 0.05$ denotes a statistically significant difference from the control group

This is consistent with clinical results indicating that the impact of alcohol drinking on thiamine generally results from chronic exposure.

It is possible that alcohol-drinking animals may consume less food because alcohol provides a significant source of calories, which may thereby reduce dietary thiamine intake. Ideally, a caloric control (drinking isocaloric sucrose or other carbohydrates) should be included. However, including sucrose or other carbohydrates may cause its own problems. These additives are much more palatable than alcohol and without pharmacological effects, so mice finish consuming their allotted amount of fluid in a short time, and then go without, creating binge-like behavior and large day-to-day changes in caloric intake. We would also note that the use of pair-fed, isocaloric controls is common in studies in which mice are forced to consume alcohol, which typically reduces their caloric intake and causes stress secondary to that forced exposure. Neither is true of the current study, which is highly innovative in the use of voluntary drinking, the only kind of drinking linked to thiamine deficiency in humans. In our alcohol-drinking paradigm, approximately 21% of calories are from alcohol. If the animals compensate completely for these empty calories, they would reduce their chow (and corresponding thiamine intake) by about 21%. Vitamins including thiamine are oversupplied by at least 30–40% more than the requirement in animal diets, so that this relatively small change in food intake would not alter thiamine levels in the system. In addition, if a 21% reduction in food intake causes a global reduction of thiamine in the system, the first thing we would expect is a reduction of thiamine in the blood. However, our data indicate that chronic alcohol drinking actually increased thiamine levels in the blood by 33% (but it is not statistically different). In contrast, chronic alcohol drinking caused a 42% reduction of thiamine in the brain, which was a statistically significant reduction. This is consistent with our data showing chronic alcohol drinking downregulated thiamine transporters in the brain. This suggests that lower thiamine levels in the brain resulted from reduced thiamine transporters after chronic alcohol drinking, not from reduced thiamine in the diet or blood. Our conclusion is further supported by a recent study showing it is alcohol-induced disruption of thiamine transporters in the brain but not thiamine in the diet that caused reduction thiamine levels in the brain (Abdul-Muneer et al. 2018).

That said, due to its short half-life and lower storage, a continuous dietary supply of thiamine is critical. As a water-

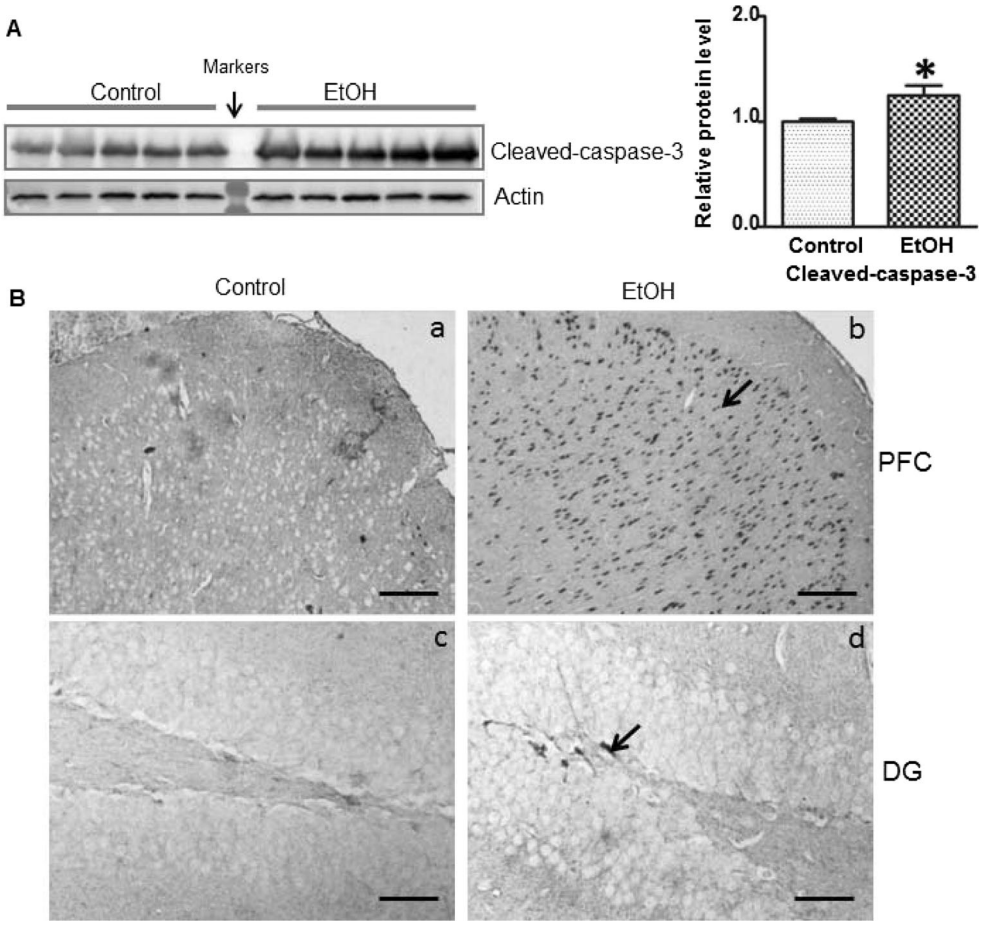

Fig. 3 Effect of chronic alcohol exposure on the expression of activated caspase-3. Male cHAP mice voluntarily drank alcohol for 7 months as described in the "Materials and Methods" section. **a** The expression of cleaved caspase-3 in the cerebrum was determined by immunoblotting. The relative amounts of expression were quantified and normalized to the expression of actin. The result was expressed as the mean ± SEM, $n = 5$ for each group. *$p < 0.05$ denotes a statistically significant difference from the control group. **b** The expression of cleaved caspase-3 in the prefrontal cortex (PFC) and the dentate gyrus (DG) of the hippocampus was examined by immunohistochemistry (IHC); bar = 50 μm. Arrows indicate cleaved caspase-3-positive cells

soluble vitamin, thiamine can enter cells or tissues or cross the blood-brain barrier through both active transport and passive diffusion, depending on the concentration of thiamine (Kumar 2010). Thiamine serum concentration is not an accurate marker for tissue samples and is not a reliable indicator of thiamine status (Kumar 2010). For example, Kato et al. (2015) showed that the thiamine levels in the plasma but not brain were significantly altered by knocking out organic cation transporter 1 (OCT1) and OCT2. Consistent with this idea, our results provide direct evidence indicating that chronic alcohol exposure can reduce thiamine levels in the brain without affecting blood thiamine levels. The mechanisms underlying alcohol-induced loss of thiamine in the brain are currently unclear. The loss may result from impaired transportation through the BBB or defective function of thiamine transporters. Indeed, our results demonstrate that alcohol drinking reduced the expression of SLC19A3 in the brain (Fig. 1).

SLC19A2 and SLC19A3 are two main thiamine transporters expressed in many tissues including the intestine, placenta, kidneys, and brain (Guerrini et al. 2009). SLC19A2 and SLC19A3 were implicated in the pathophysiology of alcohol-associated thiamine deficiency (Guerrini et al. 2009). Genetic defects in SLC19A2 mainly present extra-neurological features, such as diabetes mellitus, megaloblastic anemia, and sensorineural hearing loss, whereas SLC19A3 defects have prominent neurological involvement (Ortigoza-Escobar et al. 2016). Therefore, alcohol-induced downregulation of SLC19A3 in the brain may account for lower thiamine contents in the brain and possibly other neurological consequences. OCT1 plays an essential role in thiamine uptake in the liver and is involved in modulating hepatic glucose and lipid metabolism (Liang et al. 2018). It is also a key drug transporter (Lozano et al. 2013; Liang et al. 2018). We showed that alcohol drinking reduced OCT1 and SLC19A2 in the liver. OCT1 defects may affect the response of healthy hepatocytes or liver cancer cells to cationic drugs, such as metformin and sorafenib, respectively (Lozano et al. 2013). Decreased OCT1 and SLC19A2 in the liver may alter liver functions and drug metabolism. It would be interesting to study the effect of alcohol on thiamine contents in the liver as well as liver functions. However, further investigation of alcohol on these endpoints is beyond the scope of the current study.

There are a number of potential mechanisms that could account for TD-induced damage to the CNS. These include apoptotic cell death, decreased synaptic transmission, mitochondrial dysfunction, excessive cytokine production, oxidative stress, and ER stress (Kumar 2010; Liu et al. 2017).

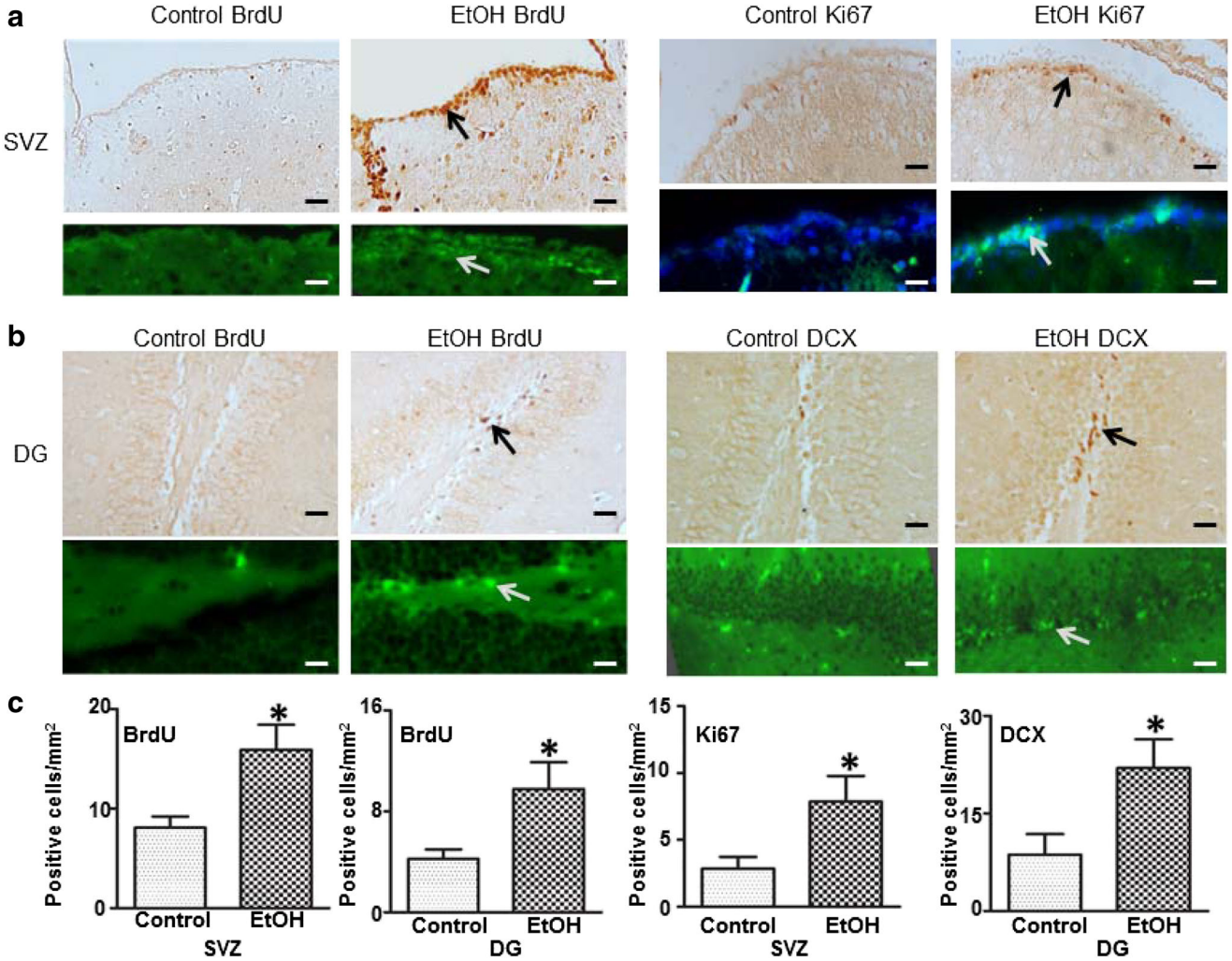

Fig. 4 Effect of chronic alcohol exposure on neurogenesis in the subventricular zone (SVZ) and DG of the hippocampus. Male cHAP mice voluntarily drank alcohol for 7 months as described in the "Materials and Methods" section. Both control and alcohol-exposed mice received BrdU injection. BrdU-positive cells in the SVZ (**a**) and DG (**b**) were determined by IHC and immunofluorescent staining. Arrows indicate BrdU-positive cells; bar = 50 μm. The expression of Ki67-positive cells in the SVZ (**a**) and doublecortin (DCX) in the DG of the hippocampus (**b**) was also examined by IHC and immunofluorescent staining. Arrows indicate Ki67- and DCX-positive cells; bar = 20 μm. **c** The number of BrdU-, Ki67-, and DCX-positive cells in the SVZ and DG was quantified as described in the "Materials and Methods" section. The results are expressed as mean ± SEM, $n = 4$ or 5 for each group. *$p < 0.05$ denotes a statistically significant difference from the control group

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) production and the capability of the cell to detoxify oxidants (Fischer and Maier 2015). The brain is a vulnerable organ affected by oxidative stress due to its high oxygen consumption and high production of ROS. Oxidative stress has been proposed as a key mechanism for alcohol-induced brain damage (Hernandez-Vazquez et al. 2016). ER regulates posttranslational protein modification, protein folding, and transport (Bravo et al. 2013). Accumulation of unfolded or misfolded proteins in the lumen of ER will cause disturbance in ER homeostasis and ER stress (Ozcan and Tabas 2012). Sustained ER stress results in apoptotic cell death (Yang and Luo 2015; Perri et al. 2016). We have previously demonstrated that TD induced ER stress in the CNS (Wang et al. 2007). Our current study indicated an alcohol-induced reduction of thiamine levels/transporters in the brain was accompanied by oxidative stress and ER stress. Therefore, it is likely that alcohol-induced oxidative stress and ER stress via TD. However, alcohol may also induce oxidative stress and ER stress independent of TD (Ke et al. 2011; Wang et al. 2012). Regardless of the underlying mechanisms, alcohol-induced oxidative stress and ER stress may profoundly affect the structures and functions of the brain.

Chronic alcohol exposure caused caspase-3 activation in the prefrontal cortex and the DG of the hippocampus, indicative of apoptosis (Fig. 3). This is consistent with the previous findings that these regions are vulnerable to chronic alcohol exposure (Bhupanapadu Sunkesula et al. 2008; Johansson et al. 2009; Fowler et al. 2014; Wang et al. 2018). A number of studies demonstrate that chronic alcohol exposure induces

caspase-3 activation in these regions. For example, chronic alcohol exposure using 20% ethanol as the sole source of fluid for 6 months activated caspase-3 in the hippocampus of male C57BL/6 mice (Wang et al. 2018). Another study showed that alcohol exposure by 10% ethanol in the drinking water for 3 months activated caspase-3 in the hippocampus and prefrontal cortex of male Wistar rats (Bhupanapadu Sunkesula et al. 2008).

Neurogenesis in adult brains from the neural stem cells (NSCs) takes place mainly in the subgranular zone (SGZ) in the DG of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles. Many animal studies have shown that alcohol intoxication (typically through forced exposure) results in an overall decrease in neurogenesis, while increased neurogenesis has been observed during abstinence after alcohol dependence (Geil et al. 2014). For example, excessive drinking during alcohol dependence inhibited hippocampal neurogenesis in adult rats (Richardson et al. 2009). Short-term binge alcohol exposure inhibited neurogenesis in the hippocampus of adolescent rats (Morris et al. 2010). However, in human alcoholics, hippocampal neurogenesis increases after cessation of alcohol consumption (Kühn et al. 2014). Similarly, in adult rats, 7 days of abstinence following binge alcohol exposure increased the neurogenesis in the hippocampus (Hayes et al. 2018). Voluntary alcohol consumption in a two-bottle choice paradigm for 2 weeks inhibited neurogenesis in adult SVZ but not DG of the hippocampus of adult C57Bl/6 mice (Campbell et al. 2014). Alcohol exposure through liquid diet (10% ethanol) for 2 weeks inhibited neurogenesis in the SVZ and SGZ of adult male Wistar rats (Rivera et al. 2015). However, some studies indicate that alcohol exposure may stimulate neurogenesis in adult mice. For example, two-bottle free-choice drinking of 10% ethanol and water for 2 months increased the neurogenesis in the DG of the hippocampus of C57Bl/6 mice (Aberg et al. 2005). Two weeks of alcohol exposure using a 7% (*v*/*v*) ethanol liquid diet caused a twofold increase in the number of proliferating cells in the SGZ of DG of Swiss-Webster mice (Pawlak et al. 2002). We showed that chronic alcohol exposure increased the neurogenesis in the SGZ and SVZ. The discrepancy in alcohol-induced alterations in neurogenesis may involve one or more of several factors: forced exposure versus voluntary drinking; binge versus chronic exposure; type of animal model and population used, animal's BEC at the time of sacrifice/analysis; the time in abstinence prior to analysis; the age of the animals; and the approach used to label proliferating cells. The selectively bred mice we used in the present study had the advantage of allowing us to model the effects of pharmacologically relevant BECs without the potential stress of forced alcohol exposure. The increased neurogenesis in mice after alcohol consumption observed in this study could be a compensatory mechanism in response to alcohol-induced neuronal damage in the brain.

In summary, we demonstrated that chronic voluntary alcohol drinking decreased thiamine levels/transporters in the brain of cHAP mice, which was accompanied by oxidative stress, ER stress, and apoptotic cells death in some brain regions. Alcohol also increased neurogenesis in the SVZ and DG of the hippocampus, which may be a compensatory response to alcohol-induced damage. Therefore, cHAP mice comprise a good animal model to study the interaction among chronic alcohol exposure, thiamine status, and oxidative stress/ER stress.

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Compliance with Ethical Standards All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Kentucky.

Conflict of Interest The authors declare that they have no competing interests.

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