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Hippocampal neural progenitor cells play a distinct role in fear memory retrieval in male and female CIE rats

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Abstract

Adult male and female GFAP-TK transgenic rats experienced six weeks of chronic intermittent ethanol vapor inhalation (CIE). During the last week of CIE, a subset of male and female TK rats were fed with Valcyte to ablate neural progenitor cells (NPCs). Seventy-two hours after CIE cessation, all CIE and age matched ethanol naïve controls experienced auditory trace fear conditioning (TFC). Twenty-four hours later all animals were tested for cue-mediated retrieval in the fear context. Adult male CIE rats showed a significant burst in NPCs paralleled by reduction in fear retrieval compared to naïve controls and Valcyte treated CIE rats. Adult female CIE rats did not show a burst in NPCs and showed similar fear retrieval compared to naïve controls and Valcyte treated CIE rats, indicating that CIE-mediated impairment in fear memory and its regulation by NPCs was sex dependent. Valcyte significantly reduced Ki-67 and NeuroD labeled cells in the dentate gyrus (DG) in both sexes, demonstrating a role for NPCs in reduced fear retrieval in males. Valcyte prevented adaptations in GluN2A receptor expression and synaptoporin density in the DG in males, indicating that NPCs contributed to alterations in plasticity-related proteins and mossy fiber projections that were associated with reduced fear retrieval. These data suggest that DG NPCs born during withdrawal and early abstinence from CIE are aberrant, and could play a role in weakening long-term memory consolidation dependent on the hippocampus.

Keywords

NeuroD; Ki-67; CIE; GluN; Synaptoporin; Trace Fear Conditioning

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Author contributions

MCS and CDM contributed to the study design, MJF, KKM, JW, LWQ, BS, JS, RJM, SSS, MJT, DCP contributed to data collection and analysis, MJF, KKM, BS, JS and CDM performed data analysis, and CDM wrote the manuscript.

Conflict of Interest

The authors declare no competing financial interests in relation to the work described.

Introduction

Moderate to severe alcohol use disorder has been reported to result in cognitive deficits in humans (Brandt *et al.*, 1983; Glenn & Parsons, 1991; Sullivan *et al.*, 2000). Clinical studies have identified the deleterious effects of chronic alcohol exposure in the hippocampus, which has been shown to be particularly sensitive to alcohol-induced damage (Bengochea & Gonzalo, 1990; Sullivan *et al.*, 1995; Durazzo *et al.*, 2011). However, what is less well known is how neuroadaptations in the hippocampus produced by alcohol contribute to alcohol-induced behavioral deficits dependent on the hippocampus.

Pavlovian conditioning is a form of associative learning where a neutral conditional stimulus (CS; e.g. tone) becomes associated with an aversive unconditional stimulus (US; e.g. footshock). Through pairings of the two stimuli, the CS comes to elicit a conditional response (CR). Trace fear conditioning (TFC) is a procedure where termination of the CS precedes onset of the US. That is, a "trace interval" is imposed between the CS and US; thus, the two stimuli are temporally discontiguous. It has been established that TFC with a trace interval greater than 10s depends on the hippocampus (Huerta *et al.*, 2000; McEchron *et al.*, 2000; Quinn *et al.*, 2002; Chowdhury *et al.*, 2005). Notably, when adult rats that experienced ethanol neonatally are tested on a TFC paradigm, they demonstrate hippocampus-specific memory impairments (Goodfellow *et al.*, 2016). These impairments are paralleled by neuroadaptations in hippocampal glutamate receptor function and signaling (Goodfellow *et al.*, 2016), suggesting that ethanol-induced hippocampal glutamatergic neuroadaptations, a form of hippocampal plasticity, could contribute to ethanol-induced impairments in TFC (Bast *et al.*, 2003; Szapiro *et al.*, 2003).

The adult hippocampus also harbors neural stem cells and these cells generate neural progenitor cells (NPCs; Ki-67+ cells) that give rise to transiently amplifying neuroblasts and immature neurons (NeuroD+ cells) that mature into granule cell neurons in the dentate gyrus (DG) through a process called neurogenesis (Enikolopov *et al.*, 2015; Goncalves *et al.*, 2016). Adult neurogenesis in the hippocampus contributes to hippocampal plasticity and certain hippocampal functions (Toni *et al.*, 2008; Ming & Song, 2011; Sahay *et al.*, 2011; Spalding *et al.*, 2013; Frankland & Josselyn, 2016). For example, types of learning that depend on the hippocampus, including TFC, increase the number of immature neurons (doublecortin+ or NeuroD+ cells) without enhancing proliferation of NPCs (Ki-67+ cells or 2-hour-old bromodeoxyuridine+ cells), suggesting that NeuroD+ young neurons are involved in the formation of trace memories (Gould *et al.*, 1999). Furthermore, mechanistic studies show that young neurons in the hippocampus play a role in TFC, with studies in adult rats suggesting neurogenesis is needed for formation of trace memories involving fearful stimuli (Shors *et al.*, 2001; Shors *et al.*, 2002; Achanta *et al.*, 2009).

In the context of ethanol experience, several widely accepted rodent models of moderate to severe alcohol use disorder demonstrate reduction of neurogenesis during ethanol experience (Nixon, 2006; Mandyam & Koob, 2012; Staples & Mandyam, 2016). One such model, namely the chronic intermittent ethanol vapor exposure (CIE) model, implements daily cycles of intoxication via ethanol vapors and withdrawal to induce clinical signs of alcoholism, such as somatic withdrawal symptoms and escalated ethanol drinking in rats

(Valdez *et al.*, 2002; O'Dell *et al.*, 2004). Proliferation (Ki-67+cells), differentiation (doublecortin+ cells) and neurogenesis of newly born neurons is also hindered in adult rats that experience CIE (Richardson *et al.*, 2009; Hansson *et al.*, 2010); however, this suppression is transient, and forced abstinence from CIE produces a rebound or burst effect observed as increases in NPCs (Hansson *et al.*, 2010; Somkuwar *et al.*, 2016). Few studies have investigated the capacity of the NPCs born during the rebound effect to survive and express neuronal and glial markers, and findings indicate reduced stability and neurogenesis of NPCs born during excessive proliferation (Somkuwar *et al.*, 2016). We therefore hypothesize that NPCs born during forced abstinence from CIE are aberrant and contribute to memory impairments dependent on the hippocampus. We used the pharmacogenetic rat model (Snyder *et al.*, 2016) in which NPCs can be selectively and inducibly ablated. Our data demonstrates that NPCs born during the proliferative burst in abstinent CIE male rats contribute to reduced fear retrieval when tested in a TFC paradigm, and inhibiting this process assisted with maintaining intact fear responses. Our data also demonstrates sex specific effects of this phenomena as females did not demonstrate burst of NPCs during withdrawal and abstinence from CIE and had intact fear responses.

Methods

Animals:

Transgenic rats expressing HSV-TK under the human GFAP promoter (GFAP-TK) were generated on a Long–Evans background (Snyder *et al.*, 2016). These rats were bred at the Scripps Research Institute. Rats were weaned at 21–24 d of age, pair-housed, and genotyped by PCR (TransnetYX). The rats were housed two-three/cage in a temperature-controlled (22°C) vivarium on a 12 h/12 h light/dark cycle (lights on 8:00 P.M- 8:00 A.M.). All procedures were performed during the dark phase of the light/dark cycle. Food and water access was available *ad libitum*. All rats weighed approximately 180-250 g and were 8 weeks old at the beginning of the study. All experimental procedures were carried out in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Chronic Intermittent Ethanol vapor exposure (CIE):

During CIE, male (n = 39, housed 2 per cage) and female (n = 39, housed 3 per cage) rat cages were housed in specialized chambers and exposed to a 14-h on / 10-h off schedule for the alcohol vapors. Using a peristaltic pump (model QG-6, FMI Laboratory, Fluid Metering), 95% ethanol from a large reservoir was delivered to a heated flask at a regulated flow rate (95% ethanol vaporized at a drip rate of 2.5 - 4 mls per min for 14h a day followed by 10h of withdrawal). The vaporized ethanol was carried to the vapor chambers containing the rat cages by controlled air flow (regulated by a pressure gauge). The air and ethanol flow rates were optimized to result in blood alcohol levels (BALs) between 125 and 250 mg/dl of or 27.2 and 54.4 mM (Gilpin *et al.*, 2008a); these BALs are 2-3 times the BAL observed in binge drinking, but not high enough to abolish righting reflex (Ernst *et al.*, 1976; Courtney & Polich, 2009). CIE males and females either experienced TFC (n = 25) or not (n =14) and were euthanized 96h after cessation of CIE.

Tail bleeding for BAL measures:

For measuring BALs, tail bleeding was performed on male and female rats, once a week on the same day every week, during hours 13-14 of CIE (total 14h exposure/day) according to (Gilpin *et al.*, 2008b). Rats were gently restrained while the tip of the tail (1 mm) was punched with a 22g needle. Tail blood (0.2 ml) was collected and centrifuged. Plasma (5 μL) was used for measurement of blood alcohol levels (BALs) using an Analox AM1 analyzer (Analox Instruments USA Inc., MA). Single-point calibrations were performed for each set of samples with reagents provided by Analox Instruments (25–400 mg/dl or 5.4–87.0 mM). When blood samples were outside the target range (125–250 mg/dl), vapor levels were adjusted accordingly. Mean BALs during the 6 week CIE exposure is reported in Figure 1.

Estrous Cycle Tracking:

All female rats were vaginally swabbed with a sterile cotton swab soaked in 0.9% saline. Samples were applied to Superfrost® Plus slides and dried overnight and stained with Cresyl Echt Violet Solution (Abcam) to determine stage of estrous based on cell morphology (Cora *et al.*, 2015). Vaginal swabs were performed after the last CIE session and after fear retrieval session.

Suppression of Neurogenesis:

Neurogenesis was suppressed by feeding the animals the orally available prodrug, valganciclovir (Valcyte, Roche), which is enzymatically converted to ganciclovir. Valcyte (7.5 mg) was given in a 0.5 g pellet of a 1:1 mixture of ground chow and peanut butter. To minimize neophobia, rats were exposed to the chow–peanut butter mixture in their home cage for 2-4 days prior to drug treatment. On drug treatment days, each rat was separated into an empty cage without bedding and individual Valcyte pellets were placed on the wall of the cage and monitored for feeding activity to ensure consistent dosing. Once the animal consumed the drug pellet the animal was moved back to the housing chamber. The entire feeding procedure lasted between 4-7 minutes and care was taken to reduce any stressful experience. Valcyte treatment (1x/d) was initiated during week 6 of CIE and was continued until the day of euthanasia. All CIE animals consumed the vehicle or Valcyte chow/peanut butter pellet (+/− Valcyte; males: n = 11 $Valcyte^{-}$, n = 14 $Valcyte^{+}$; females, n = 15 $Valcyte^{-}$, n = 10 $Valcyte^{+}$).

Trace Fear Conditioning:

Apparatus: Fear conditioning was conducted in a set of four identical chambers housed within sound-attenuating boxes (Med Associates chambers connected to AnyMaze interface and Video tracking system). The floor was composed of stainless steel rods through which 0.5mA shocks were delivered. Each chamber was illuminated by an overhead 7.5-W bulb and was connected to its own shock generator-scrambler. Ventilation fans provided constant background noise (~60 dB). Chambers were cleaned with a solution of quatricide disinfectant between animals. All training and testing sessions were conducted in the same chamber for each rat.

Training: 72h after CIE cessation, CIE males (n = 25) and females (n = 25) and CIE naïve males (no CIE, n = 16) and females (no CIE, n = 13) were trained with TFC. We used a conditioning protocol based on previous reports to produce approximately equivalent freezing levels in both sexes. For training sessions of trace conditioning, the animals received 5 series of CS-US presentations that occurred with varied inter-trial intervals. The CS was a 30-sec tone cue (80 dB) and the US was a 1-sec foot shock (0.5 mA). The CS and US were separated by an empty 45-sec trace interval. The first CS presentation occurred following a 3-min baseline period and the final shock was followed by a 1-min post-shock period.

CS retrieval in fear context: Twenty-four hours after training, animals were placed back in their original chambers for 3-min baseline period after which they were presented with 5 CS only presentations with each CS (30-sec tone cue) separated by 45s inter-interval. Immediately after the retrieval test, animals were returned to their home cages.

Assess freezing behavior: Freezing behavior has been reported in a variety of ways: e.g. compute freezing during acquisition and retrieval without considering baseline freezing (Gewirtz & Davis, 1997; Marsicano *et al.*, 2002; Schafe *et al.*, 2005; Han *et al.*, 2007; Gogolla *et al.*, 2009; Monfils *et al.*, 2009), or by subtracting baseline freezing (Reijmers *et al.*, 2007). We report freezing behavior during acquisition and retrieval using the latter method, where baseline freezing was subtracted from each rat to compute freezing during acquisition and retrieval (Jacobs *et al.*, 2010).

Brain tissue processing:

The groups of animals used for behavior and brain tissue processing is indicated in supplementary Figure 1. One hour after the fear retrieval session, TFC rats (no CIE and CIE), age matched experimentally naïve controls (n = 6 males, n = 6 females) and CIE only (TFC naïve rats euthanized 72h or 96 h after CIE) males (n = 14) and females (n = 14) were killed by rapid decapitation and the brains were isolated, and dissected along the midsagittal plane. The experimentally naïve group (control group) did not experience CIE or TFC. They were housed in cages similar to the CIE cages and were handled similarly. They were habituated to the TFC chamber, and did not experience any CS or US. They were not given any vehicle or Valcyte diet and were not tail bled for blood sampling. The left hemisphere was snap frozen and the right hemisphere was postfixed in 4% paraformaldehyde for immunohistochemistry. For tissue fixation, the hemispheres were incubated at room temperature for 36 hours and subsequently at 4°C for 48 hours with fresh paraformaldehyde replacing the old solution every 24 hours. Finally, the hemispheres were transferred to sucrose solution (30% sucrose with 0.1% sodium azide) for cryoprotection and stored until tissue sectioning was conducted (Cohen *et al.*, 2015). Subsequently, the tissue was sliced in 40μm sections along the coronal plane on a freezing microtome. Sections were stored in a 1xPBS solution with 0.1% sodium azide at 4°C for histochemical analysis.

Immunohistochemistry (IHC):

Every eighteenth section through the hippocampus (anterio-posterior −2.5 to −6.3 mm from bregma) was mounted on Superfrost® Plus slides and dried overnight (Somkuwar *et al.*,

2016). Six to eight sections per rat were stained for Ki-67 (1:700, Rabbit polyclonal, Thermo Scientific) and NeuroD (1:500, Goat polyclonal, Santacruz Biotechnology) followed by biotin-tagged secondary antibodies and visualized with DAB. For Ki-67 and NeuroD analyses, all immunoreactive cells in the subgranular zone and granule cell layer were counted per animal. In addition to cell counting, area measures of the granule cell layers were also determined for each section for each animal using StereoInvestigator software (MBF), and the raw cell counts per section per animal were divided by the area of the granule cell layer and are indicated as cells per mm2 of the granule cell layer per animal.

For morphometric analysis of the density of mossy fiber projections, dorsal hippocampal sections (representing −2.56 and −4.8mm from bregma, 4 sections per rat) were separately stained for synaptoporin (1:50, Rabbit polyclonal, SynapticSystems) followed by biotin-tagged secondary antibodies and visualized with DAB (Staples *et al.*, 2017). For mossy fiber density, images were captured at 10x and synaptoporin in the hilus of the dorsal DG was evaluated by quantifying DAB stain (% area stained; (Galinato *et al.*, 2018)) using ImageJ software (NIH). Briefly, the mossy fiber tracts were contoured using the polygonal selection feature. A circular area above the CA3 was used to quantify non-specific/ background staining.

Western Blotting:

Procedures optimized for measuring both phosphoproteins and total proteins were employed (Kim *et al.*, 2015). Tissue punches from dorsal hippocampal formation enriched in the dentate gyrus from 500 μm thick sections were homogenized on ice by sonication in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1mMEDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma), heated at 100 °C for five minutes, and stored at−80 °C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA). Samples were mixed (1:1) with a Laemmli sample buffer containing β-mercaptoethanol. Each sample containing protein from one animal was run (20 μg per lane) on 8–12% SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes (PVDF pore size 0.2 μm). Blots were blocked with 2.5% bovine serum albumin (for phosphoproteins) or 5% milk (w/v) in TBST (25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for 16–20 h at 4 °C and were incubated with the primary antibody for 16–20 h at 4 °C: antibody to tGluN2A (1:200, Santa Cruz Biotechnology cat. no. sc-9056, predicted molecular weight 177 kDa, observed band ~170 kDa), antibody to tCamKII (1:200, Abcam cat. no. ab52476, predicted molecular weight 47 and 60 kDa, observed band ~47 and 60 kDa), antibody to tumor necrosis factor alpha (TNFα; Abcam, ab9635; 1:500, predicted molecular weight 20 kDa, observed band ~17 kDa (Kinsella *et al.*, 2016)). Blots were then washed three times for 15min in TBST, and then incubated for 1 h at room temperature (24 °C), appropriately with horseradish peroxide-conjugated goat antibody to rabbit or horseradish peroxide–conjugated goat antibody to mouse (1:10,000, BioRad) in TBST. After another three washes for 15 min with TBST, immunoreactivity was detected using SuperSignal West Dura chemiluminescence detection reagent (Thermo Scientific) and collected using HyBlot CL Autoradiography film (Denville Scientific) and a Kodak film processor. For normalization purposes, membranes were incubated with 0.125% coomassie stain for 5 minutes and

washed three times for 5-10 minutes in destain solution. Densitometry was performed using ImageStudio software (Li-Cor Biosciences). X-ray films were digitally scanned at 600 dpi resolution, then bands of interest were selected in identically sized selection boxes within the imaging program which included a 3 pixel extended rectangle for assessment of the background signal. The average signal of the pixels in the 'background' region (between the exterior border of the region of interest selection box and the additional 3 pixel border) was then subtracted from the signal value calculated for the band of interest. This was repeated for coomassie, and the signal value of the band of interest following subtraction of the background calculation was then expressed as a ratio of the corresponding coomassie signal (following background subtraction). This ratio of expression for each band was then expressed as a percent of the drug naïve control animals included on the same blot.

Statistical analyses:

Parametric statistical analysis were used to analyze our datasets based on the assumption that our data fit a normal distribution and satisfy the sample size for adequate statistical power. Changes in BALs was assessed as a repeated measures two-way ANOVA (sex × week). For TFC analysis, the main dependent variable was the amount of time rats spent engaged in freezing behavior. Freezing was defined as the absence of all movement except for that required for respiration. The average percent time spent freezing was calculated using the AnyMaze software (StoeltingCo.com). The AMI scoring parameters were chosen and freezing was analyzed as a percentage of each minute during the baseline, training and testing sessions. None of the behaviors were hand scored. Baseline freezing was evaluated as percent freezing, and freezing during training and testing sessions are indicated as change in percent freezing from baseline freezing. Changes in freezing behavior during TFC acquisition was assessed as repeated measures two-way ANOVA (TFC session × treatment) in each sex. Changes in freezing behavior during CS retrieval was analyzed by one-way ANOVA in each sex. Cell counts for each marker (expressed as positive cells per mm^2) and raw values of protein expression from Western blotting were analyzed by three-way ANOVA (sex × CIE × TFC). Significant interaction or ANOVA was followed by post-hoc analysis using Newman-Keuls multiple comparisons test. All graphs and statistical analysis were generated using Graph Pad version 7 for PC and $p<0.05$ was considered statistically significant.

Results

CIE produces higher BALs in females compared to male rats

Adult male and female rats experienced CIE for 6 weeks (Figure 1a-b). 72h after the last CIE session, CIE rats and age matched controls were trained on TFC (Figure 1c-d) and 24h later were tested for CS retrieval (Figure 1e-f). BALs were monitored during CIE in males and females. BALs increased as a function of the alcohol flow rates and remained within an acceptable range over the six week period in male and female rats (Figure 1b). The amount of alcohol experienced by each rat reached the desired range by the second week of CIE exposure as indicated by the increases in BALs. There were sex differences in BALs while vapors were delivered, with females demonstrating greater BALs than males during weeks 3 to 6. Repeated measures two-way ANOVA demonstrated a significant sex × week interaction

(F[5,269]=3.1, p = 0.008), main effect of weeks of CIE (F[5,269]=39.1, p < 0.001) and main effect of sex (F[1,269]=44.1, p < 0.001) over the six weeks of CIE exposure. Post hoc analysis demonstrated higher BALs in females during weeks 3 to 6 compared with males (ps < 0.05; Figure 1b).

CIE and TFC does not alter estrus phase in females

We determined the estrus phase of females in CIE naïve and CIE groups before TFC and after TFC. The number of females in each phase are as follows: (CIE naïve: diestrus (D)- 1, proestrus (P)- 5, estrus (E)- 13; CIE: D-4, P-9, E-12; CIE naïve + TFC: D-3, P-2, E-14; CIE + TFC: D-5, P-4, E-16). Two-way ANOVA did not reveal significant effects of CIE and TFC on estrus cycle stages (effect of treatment F (3, 6) = 0.7619, p = 0.5), however, revealed changes in estrus phase before and after TFC in both groups (estrus phase F (2, 6) = 24.11, p = 0.001). These findings indicate that CIE and TFC did not alter estrus cycle in females, supporting previous findings (Lebron-Milad *et al.*, 2013; Priddy *et al.*, 2017).

Abstinent CIE females show enhanced freezing during acclimation and this is abolished by Valcyte treatment

General locomotor activity was assessed in both sexes in all treatment groups before training period. Two-way ANOVA did not detect a significant treatment × sex interaction F[2,72] = 0.2, p = 0.8, or main effect of treatment F[2,72] = 0.8, p = 0.4, however, detected a main effect of sex F[1,72] = 9.5, p = 0.002; supplementary Figure 2). Baseline freezing behavior was obtained during the 3 minute acclimation period before training (Figure 2a). All the groups within each sex were evaluated separately. Freezing behavior did not differ between control males and females during acclimation (Figure 2b). Baseline freezing was higher in CIE females compared with control females and this effect was abolished with Valcyte treatment (Figure 2b). Valcyte treated males and females did show differences in freezing behavior. Two-way ANOVA demonstrated a significant treatment × sex interaction F[2,72] = 8.888, p = 0.004, main effect of treatment F[2,72] = 5.245, p = 0.007, and main effect of sex F[1,72] = 6.392, p = 0.01. Post hoc analysis indicated higher freezing in CIE^{-} Valcyte females compared to controls and CIE^{+} Valcyte females (ps < 0.05; Figure 2b). Therefore, freezing behavior during training and CS retrieval is presented as change in percent freezing during training or retrieval period from percent freezing during baseline to eliminate the confounding effect of baseline freezing (Jacobs *et al.*, 2010).

Abstinence from CIE does not alter freezing during training in male and female rats

In males, repeated measures two-way ANOVA did not detect a significant training × treatment group interaction (F[6,148] = 1.558, p = 0.16) and CIE treatment (F[2,148] = 0.7563, p = 0.43), however, detected a significant effect of training (F[3,148]=218.4, p < 0.001; Figure 2c). In females, repeated measures two-way ANOVA did not detect a significant training × treatment group interaction (F[6,140]=1.5, p = 0.17) and CIE treatment (F[2,140]=0.7, p = 0.49), however, detected a significant effect of training (F[3,140]=292.6, p < 0.001; Figure 2d). All animals learned the CS-US association and demonstrated significant freezing behavior by trial 2 of the 5 trials. In order to determine whether the rats encoded the timing of CS and US freezing behavior was measured during the inter-trial interval (ITI). In males and females, freezing behavior did not significantly differ between

the first and the last ITI (no interaction, no effect of treatment or training). However, in males and females, repeated measures indicated a significant reduction in freezing behavior during ITI 5 compared with tone 5 and trace 5, indicating that freezing behavior was maximal during CS and US and declined during the ITI (males: $F[3,140]=292.6$, $p < 0.001$; Figure 2c; females ($F[3,140]=292.6$, $p < 0.001$; Figure 2d)).

Abstinence from CIE does not alter context freezing in male and female rats

Twenty-four hours following training, rats were returned to the training context for 3 min with no tones or footshocks presented (Figure 3a). There were no significant differences in context freezing in males $F[2,37] = 0.75$, $p = 0.4$ (Figure 3b) or females $F[2,35] = 2.7$, $p = 0.07$ (Figure 3c) by one-way ANOVA.

Abstinence from CIE reduces CS retrieval in male rats without effecting CS retrieval in female rats

On testing day, after context freezing testing, male and female rats experienced CS-only trials. Freezing was measured during CS. In males, repeated measures two-way ANOVA demonstrated a session × treatment interaction ($F[8,132] = 2.0$, $p = 0.05$), main effect of treatment ($F[2,33] = 6.4$, $p = 0.004$) and main effect of testing ($F[4,132] = 3.3$, $p = 0.012$) during CS. Posthoc analysis revealed that CIE rats with intact NPCs showed reduced freezing during CS during the first three CS trials ($p<0.05$). Valcyte prevented this effect during CS, and reduced latency to freezing behavior during CS periods. By the fifth trial all male rats showed similar freezing during CS (Figure 3d).

In females, repeated measures two-way ANOVA demonstrated a session × treatment interaction ($F[8,140] = 2.3$, $p = 0.02$), main effect of treatment ($F[2,35] = 3.7$, $p = 0.03$) and main effect of testing ($F[4,140] = 3.1$, $p = 0.017$) during CS. Posthoc analysis revealed that CIE rats with Valcyte showed reduced freezing during CS during the fourth CS trials ($p<0.05$). Valcyte enhanced latency to freezing behavior during CS trials (Figure 3e).

Abstinence from CIE and TFC produce distinct effects on NPCs and immature neurons in male and female rats

The effects of abstinence from CIE on NPCs (Ki-67 and NeuroD cells; (Galinato *et al.*, 2018)) in males and females was investigated. We first evaluated whether 72h of abstinence would produce an increase in NPCs in TK rats (Hansson *et al.*, 2010; Somkuwar *et al.*, 2016). Next, in order to determine whether the alterations in Ki-67 cells and NeuroD cells seen at the 72h abstinence time point were transient or long lasting, we examined the number of Ki-67 and NeuroD cells at 72h and 96h after CIE was terminated. This allowed us to evaluate whether the rebound burst in NPCs was specific to the 72h time point. For Ki-67 cells, two-way ANOVA showed a significant sex × CIE interaction ($F[2,37] = 4.2$, $p = 0.02$), and did not show a main effect of CIE or sex. Posthoc analysis revealed lower number of Ki-67 cells in no CIE females compared to males and lower number of cells in 72h males compared to no CIE males ($p<0.05$; Figure 4a). For NeuroD cells, two-way ANOVA showed a significant sex × CIE interaction ($F[2,37] = 4.2$, $p = 0.02$), and did not show a main effect of CIE or sex. Posthoc analysis revealed higher number of NeuroD cells in no CIE females compared to males and higher number of cells in 72h males compared to no CIE males

(p<0.05; Figure 4b). In addition to the immunohistochemical analysis of the proliferation/immature neuron markers, we evaluated changes in a marker of neuroimmune response, TNFα, as previous findings in adult rats indicate that the burst in neuronal progenitors that occurs during abstinence from chronic alcohol experience is followed by an increase in expression of neuroimmune markers (Nixon & Crews, 2004; Nixon *et al.*, 2008; Marshall *et al.*, 2016; Peng *et al.*, 2017). Two-way ANOVA did not detect a significant sex × CIE interaction ($F[2,37] = 2.04$, $p = 0.14$), however, detected a main effect of sex ($F[1,37] = 10.3$, $p = 0.002$). Posthoc analysis revealed higher expression of TNFα at 96h time point compared with no CIE animals and higher expression of TNFα in 96h males compared to 96h females (p<0.05; Figure 4c).

We next determined the effects of TFC on NPCs in male and female rats. Immunohistochemical analysis of Ki-67 cells did not show a sex × CIE × TFC interaction ($F[5,108] = 0.264$, $p = 0.608$), did not show a significant sex × TFC interaction ($F[3,108] = 0.99$, $p = 0.30$), however, showed a significant sex × CIE interaction ($F[3,108] = 11.6$, $p = 0.001$) and a significant CIE × TFC interaction ($F[3,108] = 5.2$, $p = 0.02$). Posthoc analysis revealed lower number of Ki-67 cells in females vs. males (p<0.05) and reduced the number of Ki-67 cells in CIE males compared to naïve controls (p<0.05). CIE did not alter the number of Ki-67 cells in females. Posthoc analysis revealed a lower number of Ki-67 cells in males vs. females that experienced TFC during abstinence (p<0.05) and reduced number of Ki-67 cells in Valcyte treated males and females that experienced TFC during abstinence compared with vehicle treated rats (p<0.05). TFC did not alter the number of Ki-67 cells in males and females that were CIE naïve (Figure 4d).

The effects of abstinence from CIE on immature neurons in males and females were investigated. Immunohistochemical analysis of NeuroD cells did not show a sex × CIE × TFC interaction ($F[5,93] = 1.9$, $p = 0.16$), however, showed a significant sex × TFC interaction ($F[3,93] = 4.04$, $p = 0.04$), a significant sex × CIE interaction ($F[3,93] = 5.7$, $p = 0.019$) and a significant CIE × TFC interaction ($F[3,93] = 5.5$, $p = 0.02$). Posthoc analysis revealed higher number of NeuroD cells in females vs. males (p<0.05) and increased number of NeuroD cells in CIE males compared to naïve controls (p<0.05). CIE did not alter the number of NeuroD cells in females. Posthoc analysis showed that TFC increased the number of NeuroD cells in males that were CIE naïve and CIE experienced (p<0.05). TFC did not alter the number of NeuroD cells in females that were CIE naïve and abstinent from CIE. Valcyte treated males and females that experienced TFC during abstinence showed reduced number of NeuroD cells compared with vehicle treated males and females that experienced TFC during abstinence (p<0.05; Figure 4e).

NPCs generated during abstinence prevent TFC-induced plasticity in glutamatergic receptor expression in male rats

The effects of abstinence from CIE on density of tGluN2A and tCaMKII receptor expression in males and females were investigated. Immunoblotting analysis for tGluN2A did not show a sex × CIE × TFC interaction ($F[5,111] = 0.24$, $p = 0.63$) and a significant sex × CIE interaction ($F[3,111] = 0.7$, $p = 0.40$), however, showed a significant sex × TFC interaction ($F[3,111] = 12.4$, $p = 0.001$) and a significant CIE × TFC interaction ($F[3,111] = 7.8$, $p =$

0.006). Posthoc analysis revealed that in females, TFC in CIE naïve and CIE rats did not alter the density of tGluN2A and Valcyte treatment did not affect the density of tGluN2A. In males, TFC in CIE naïve animals increased the density of tGluN2A ($p<0.05$); this effect was not evident with TFC in CIE males. Importantly, Valcyte treatment in TFC-CIE males effected the density of tGluN2A and increased it to the levels in TFC-CIE naïve animals ($p<0.05$; Figure 5).

Immunoblotting analysis for tCaMKII did not show a sex × CIE × TFC interaction ($F[5,111] = 0.09$, $p = 0.74$), a significant sex × CIE interaction ($F[3,111] = 0.09$, $p = 0.75$), and a significant CIE × TFC interaction ($F[3,111] = 0.01$, $p = 0.92$); however, showed a significant sex × TFC interaction ($F[3,111] = 5.3$, $p = 0.023$). Posthoc analysis revealed that in females, TFC in CIE naïve and CIE rats increased the density of tCaMKII ($p<0.05$), and Valcyte treatment did not have any effects. In males, TFC in CIE naïve and CIE animals did not alter the density of tCaMKII (Figure 5).

NPCs generated during abstinence prevent TFC-induced plasticity in mossy fiber projections in male rats

The effects of abstinence from CIE on density of mossy fiber projections in males and females were investigated. Immunohistochemical analysis of synaptoporin did not show a sex × CIE × TFC interaction ($F[5,93] = 0.3$, $p = 0.84$) and a significant sex × CIE interaction ($F[3,93] = 0.8$, $p = 0.36$), however, showed a significant sex × TFC interaction ($F[3,93] = 16.04$, $p < 0.001$) and a significant CIE × TFC interaction ($F[3,93] = 10.5$, $p = 0.002$). Posthoc analysis revealed lower expression of synaptoporin in CIE males and females compared with controls, and lower expression in CIE females vs. CIE males ($ps<0.05$). In females, TFC in CIE naïve and CIE rats did not alter the density of synaptoporin and Valcyte treatment did not affect the density of synaptoporin. In males, TFC in CIE naïve animals reduced the density of synaptoporin ($p<0.05$); this effect was not evident with TFC in CIE males. Importantly, Valcyte treatment in TFC-CIE males effected the density of synaptoporin and reduced it to the levels in TFC-CIE naïve animals ($p<0.05$; Figure 6).

Discussion

The present results are the first to show that NPCs born during abstinence from chronic ethanol experience play a direct role in reduced expression of tone conditioning (CS retrieval) when trained using a TFC procedure, and that these effects are sex specific. Mechanisms underlying NPC-induced reduction in CS retrieval include reduced expression of GluN2A receptors and enhanced expression of synaptoporin in the DG of male rats. These findings help demonstrate that the rebound proliferation of NPCs in the DG in ethanol withdrawn male rats is aberrant and contributes to deficits in hippocampal dependent behaviors in adult subjects.

Formation and expression of fear memories in a time-limited manner depend on a functional and intact hippocampus (Frankland & Bontempi, 2005; Beeman *et al.*, 2013; Doron & Goshen, 2017; Woods & Kheirbek, 2017). Mechanistic studies undisputedly demonstrate that the dorsal hippocampus and specifically the DG are involved in the acquisition, consolidation, and expression of TFC (McEchron *et al.*, 2000; Quinn *et al.*, 2002; Yoon &

Otto, 2007; Quinn *et al.*, 2008; Beeman *et al.*, 2013; Pierson *et al.*, 2015). However, mechanistic studies show that the role of adult hippocampal neurogenesis in TFC is controversial, with studies in adult rats indicating that neurogenesis is needed for formation of trace memories involving fearful stimuli (Shors *et al.*, 2001; Shors *et al.*, 2002; Achanta *et al.*, 2009) and others in adult mice demonstrating that neurogenesis is not necessary for formation of trace memories, however, buffers against nonassociative, anxiogenic effects of a fearful stimuli (Seo *et al.*, 2015). In addition, it has been demonstrated that increasing neurogenesis by wheel running or systemic injections of a pro-neurogenesis drug memantine in mice after formation of fear memories reduces CS retrieval or produces amnesic effects in response to the CS in a novel context (Ishikawa *et al.*, 2016; Gao *et al.*, 2018). Therefore, it appears that mature and newly born granule cell neurons in the DG play distinct roles in formation of fearful memories, and time-limited retrieval of fear memories (Beeman *et al.*, 2013; Seo *et al.*, 2015; Gao *et al.*, 2018).

In the context of alcohol use disorder (AUD), short-term and prolonged ethanol exposure does not alter acquisition of fear responses in delay fear conditioning and TFC (Melia *et al.*, 1996; Gould, 2003; Weitemier & Ryabinin, 2003; Holmes *et al.*, 2012; Broadwater & Spear, 2013; Hunt & Barnet, 2016; Goodfellow *et al.*, 2018), however, produces deficits in CS retrieval, seen as reduced freezing or amnesic effects in response to the CS in a novel context (Gould, 2003; Weitemier & Ryabinin, 2003; Hunt & Barnet, 2016; Goodfellow *et al.*, 2018). The impaired and amnesic hippocampal functioning in ethanol experienced animals could be resulting from neuroplastic and neuroadaptive changes in the DG (Gilmartin & McEchron, 2005; Czerniawski *et al.*, 2012; Pierson *et al.*, 2015). For example, several neuroplastic and neuroadaptive changes are seen in the hippocampus after chronic ethanol experience (reviewed in (Nixon, 2006; Mandyam, 2013; Zorumski *et al.*, 2014; Kutlu & Gould, 2016; Montesinos *et al.*, 2016)). Such neuroplastic changes could involve the proliferative burst in the NPCs in the DG 72 hours into withdrawal from CIE, a timeframe associated with negative affect (Hansson *et al.*, 2010; Somkuwar *et al.*, 2016). The effect this proliferative burst has on hippocampal functioning, particularly emotional memories dependent on the hippocampus, remained unclear and were determined in the current study.

We used transgenic rats in which neural progenitors can be selectively and inducibly ablated (Snyder *et al.*, 2016; Galinato *et al.*, 2018). Male and female rats experienced CIE for 6 weeks and were withdrawn from CIE. Female rats showed greater BALs compared with males when maintained at similar drip-rate/body weight ratio in the vapor chambers. This is consistent with the previous literature in humans and rodents that demonstrate sex differences in alcohol drinking, pharmacokinetics, peak BAL and alcohol elimination rate (Rivier *et al.*, 1992; Rivier, 1993; Thomasson, 1995; Priddy *et al.*, 2017). Male and female rats that received Valcyte during withdrawal and early abstinence showed a reduction in Ki67+ cells and NeuroD+ cells indicating that Valcyte abolished the rebound burst in NPCs. Valcyte^{-} CIE female rats with intact NPCs showed enhanced freezing responses compared to CIE naïve rats and Valcyte^{+} CIE rats during the baseline session that occurred at 72 hours of abstinence. Enhanced freezing during the baseline session is suggested to indicate anxiety-like behavior (Brandao *et al.*, 2008; Pettersson *et al.*, 2015), a phenotype that is demonstrated in female rats after chronic ethanol experience (Getachew *et al.*, 2008). Valcyte prevented the rebound burst in NPCs in CIE female rats and reduced freezing

responses during baseline session, demonstrating a role for NPCs generated during early abstinence in anxiety-like behavior. Enhanced freezing during baseline session in Valcyte$^-$ CIE females did not affect freezing behavior during acquisition of fear responses in TFC and during CS retrieval, indicating that these behaviors were not predicted by the anxiety-like response or high degree of context fear generalization in Valcyte$^-$ CIE female rats (Seo *et al.*, 2015). Unlike Valcyte$^-$ CIE females, Valcyte$^-$ CIE male rats did not show altered freezing behavior during baseline session compared with no CIE and Valcyte$^+$ CIE rats. Valcyte$^-$ CIE male rats did not differ in acquiring TFC compared to no CIE and Valcyte$^+$ CIE male rats. These results in CIE female and male rats show that CIE did not alter learning of TFC, a finding in line with previous reports conducted with single dose of ethanol experience (Weitemier & Ryabinin, 2003; Hunt & Barnet, 2016; Goodfellow *et al.*, 2018). Valcyte$^-$ CIE males showed normal freezing during context retrieval and reduced freezing during CS retrieval when compared with no CIE and Valcyte$^+$ CIE males. In CIE males, Valcyte treatment recovered the freezing response during CS retrieval without altering context retrieval. This is an important finding, as a previous report shows that Valcyte treatment enhanced context retrieval in CIE naïve mice, suggesting that loss of NPCs in normal mice induced impaired associative conditioning to the trace CS (Seo *et al.*, 2015). These findings also indicate that NPCs in normal rodents supports conditioned fear to the trace CS, and this relationship is abolished in CIE rats. Furthermore, it is tempting to consider that the suppression of freezing in Valcyte$^-$ CIE males may reflect a preferential disruption by CIE in the temporal component of TFC. These findings suggest that chronic prolonged ethanol experience via CIE may have selectively disrupted the hippocampus-dependent ability of timing, but may not have impaired the ability to acquire a conditioned freezing response *per se*. Taken together, these data suggests that exposure to CIE may create a higher vulnerability for hippocampal impairment in male subjects when compared with female subjects. Additionally, the proliferative burst in NPCs that is seen 72 hours into withdrawal from CIE in males plays a mechanistic role in disrupted consolidation of TFC.

Recent studies have demonstrated a mechanistic role of GluNs in acquisition and CS retrieval, when subjects were trained in a TFC paradigm (Huerta *et al.*, 2000; Quinn *et al.*, 2005; Jarome *et al.*, 2012; Holehonnur *et al.*, 2016). For example, blockade of GluN receptors prior to training disrupts the consolidation of fear memories (Stiedl *et al.*, 2000) and similarly, genetic deletion of hippocampal GluNs disrupts TFC (Huerta *et al.*, 2000). The GluN2A and GluN2B subunits mark the principal GluN subtypes, and expression of the GluN2A determines qualitative and functional properties of hippocampal neurons (Monyer *et al.*, 1994). Our findings demonstrate that TFC enhances GluN2A expression in no CIE males and Valcyte$^+$ CIE males and this increase correlated with intact consolidation of fear memories. We also show that reduced GluN2A subunits in Valcyte$^-$ CIE male rats correlated with reduced consolidation or amnesic effects, indicating that rebound effect of NPCs during early abstinence prevented neuroadaptations in GluNs in the DG. Whether newly born NPCs born during the proliferative burst differentially express GluNs to modulate neuroplasticity and behavior is yet to be determined and is an interesting future pursuit (Hagihara *et al.*, 2011). In female rats, TFC enhanced tCaMKII expression and this effect was evident in all TFC groups, supporting previously reported role of tCaMKII in fear memory consolidation (Jarome *et al.*, 2016). Taken together, these findings demonstrate sex-specific alterations in

plasticity-related proteins in the DG, whose expression correlates with consolidation of fear memories in subjects trained on TFC.

In the DG, NPCs and newly born GCNs contribute to the density of mossy fiber tracts (Romer *et al.*, 2011). Given the inverse correlation between mossy fiber density and GluN2 expression in the DG (Ni *et al.*, 2009), we determined the density of mossy fiber projections in male and female rats. Our findings demonstrate that during early abstinence, mossy fiber density in the hilus was significantly reduced in both genders. These findings support the previously indicated reduced mossy fiber density in ethanol experienced subjects (Cadete-Leite *et al.*, 1989; Feller *et al.*, 1991; Brandao *et al.*, 1996). Notably, mossy fiber density was significantly enhanced by TFC in females and this correlated with enhanced expression of CaMKII, indicating a cellular mechanism for enhanced learning and memory behaviors in females. In no CIE and Valcyte^{+} CIE males, TFC reduced mossy fiber density and this correlated with enhanced expression of GluN2A. The inverse relationship between mossy fiber density, GluNs and learning and memory function dependent on the hippocampus has been previously reported (Ni *et al.*, 2009), suggesting distinct neurobiological mechanisms underlying fear consolidation in female versus male subjects (Bouchet *et al.*, 2017; Keiser *et al.*, 2017).

In conclusion, our findings demonstrate a direct role of NPCs that were born during early abstinence from prolonged exposure to CIE in males in reduced CS retrieval after TFC. We show plasticity-related adaptations in the DG that correlate with rebound burst of NPCs. Additional neuroimmune responses in the DG in concert with aberrant NPC proliferation could contribute to the memory deficits in CIE male rats, and exploring these mechanisms could be critical in linking microglial proliferation in the DG in ethanol-induced disruption in fear consolidation (Klaus *et al.*, 2016; Marshall *et al.*, 2016; Peng *et al.*, 2017; Goodfellow *et al.*, 2018).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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- Withdrawal from chronic ethanol increases neural progenitor cells in the dentate gyrus in male rats
- Increase in neural progenitor cells is mechanistically linked to chronic ethanol-induced amnesic effects
- Aberrant burst in neural progenitor cells regulates plasticity related proteins in the dentate gyrus

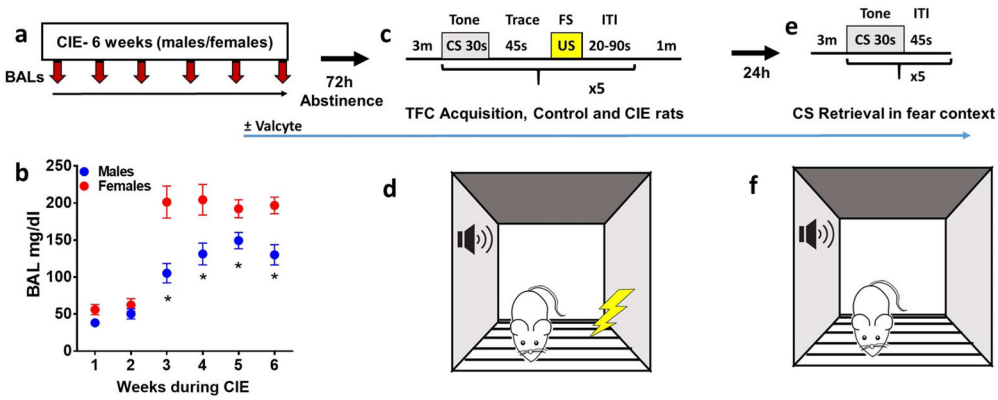

Figure 1:

Schematic of CIE weeks and TFC procedure in TK rats. (a) Timeline of CIE, blood collection for BALs during CIE and Valcyte administration. (b) BALs during each week of CIE in male and female TK rats. *p<0.05 vs. females. n = 38 males, n = 34 females. 72h after cessation of CIE, CIE naïve and CIE male and female TK rats were trained on TFC (c-d) and 24h after training all animals were tested for CS retrieval (e-f). Freezing was monitored during the 3 minute baseline session and during CS and trace intervals.

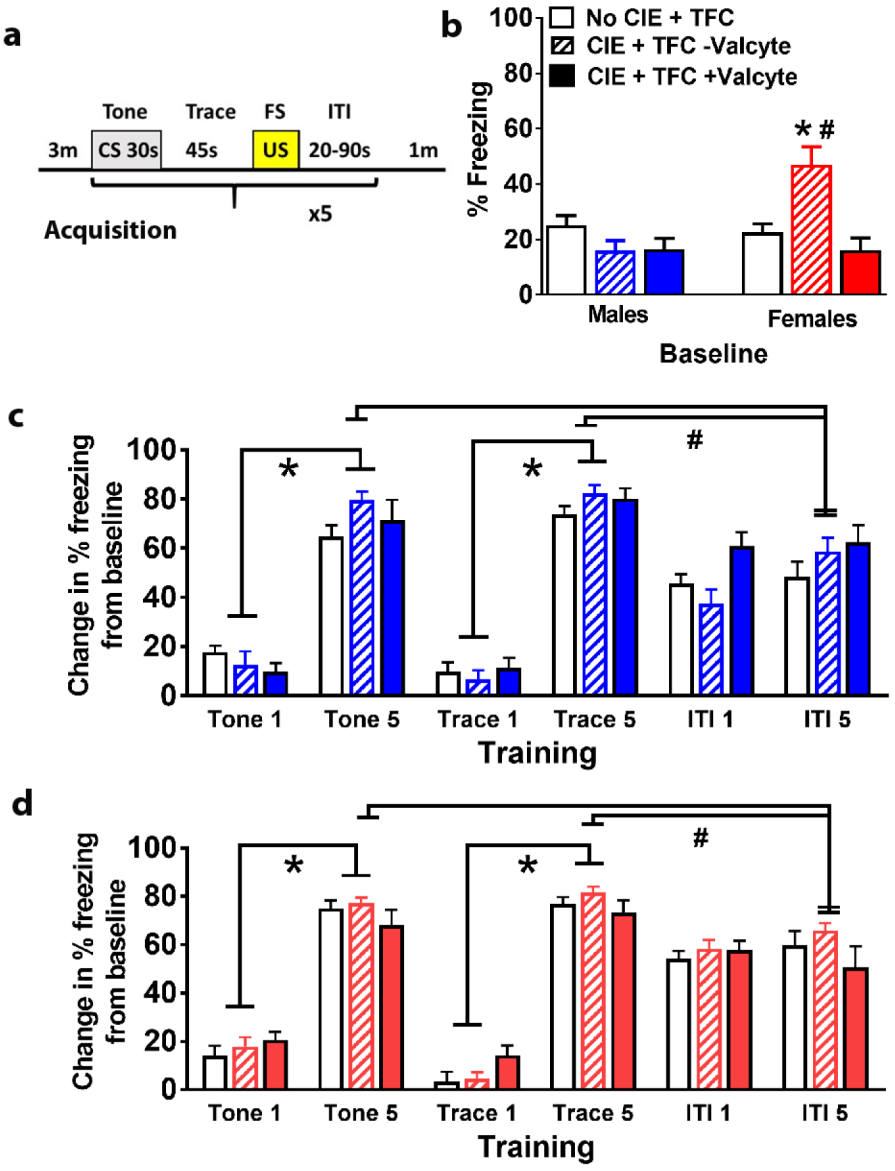

Figure 2:

Male and female TK rats after CIE have distinct responses during TFC. (a) Schematic of TFC protocol. (b) Mean (±SE) freezing did not differ between treatment groups in males during the 3 min baseline. Mean freezing was significantly higher in Valcyte^{-} CIE females compared to other treatment groups during the 3 minute baseline. *p<0.05 vs. CIE naïve rats, #p<0.05 vs. Valcyte^{+} CIE rats. (c) Mean (±SE) freezing (change in freezing from baseline) in males did not differ between groups during the CS 1 and trace 1 period, during the CS 5 and trace 5 period and ITI 1 and ITI 5 periods. Freezing increased significantly from the CS 1 to CS 5 and from trace 1 to trace 5. *p<0.05 vs. tone and trace 1. (d) Mean (±SE) freezing (change in freezing from baseline) in females did not differ between groups during the CS 1 and trace 1 period, during the CS 5 and trace 5 period and ITI 1 and ITI 5 periods. Freezing increased significantly from the CS 1 to CS 5 and from trace 1 to trace 5. *p<0.05 vs. tone and trace 1. n = 16 CIE naïve males, n = 11 Valcyte^{-} CIE males, n = 14

Valcyte^{+} CIE males; n = 13 CIE naïve females, n = 15 Valcyte^{-} CIE females, n = 10 Valcyte^{+} CIE females.

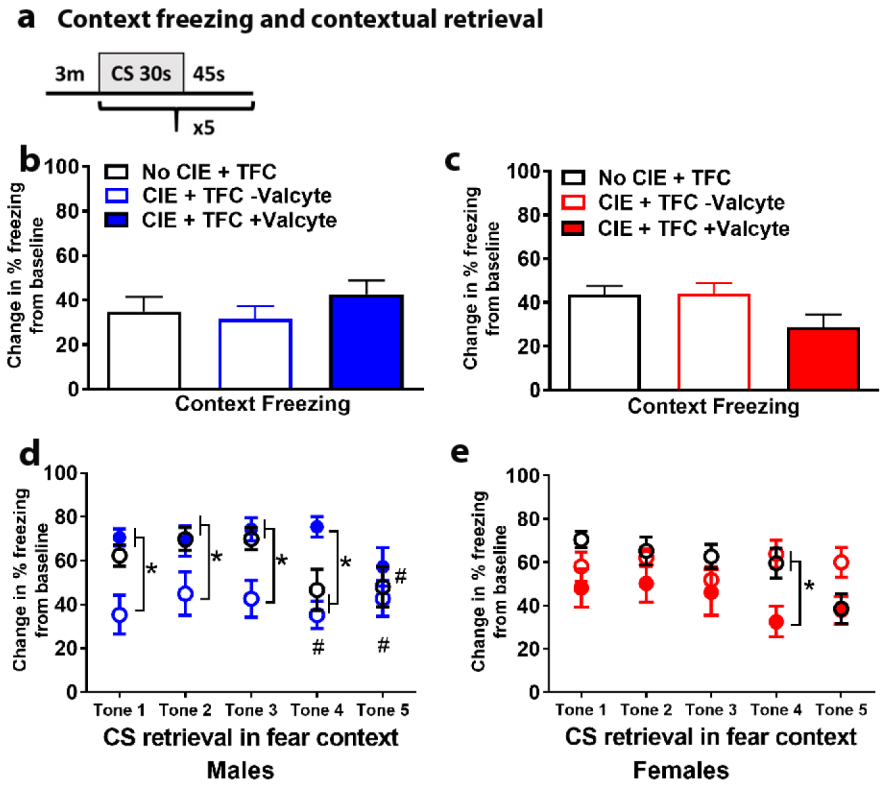

Figure 3:

Male and female TK rats after CIE have distinct responses during CS retrieval. (a) Schematic of CS retrieval protocol. (b-c) Mean (±SE) freezing (change in freezing from baseline) did not differ between treatment groups in males (b) and females (c) during the 3 min context retrieval. (d) Mean (±SE) freezing (change in freezing from baseline) in males show that Valcyte^{-} CIE rats had reduced freezing during CS retrieval during tones 1-4 compared with CIE naïve rats and Valcyte^{+} CIE rats. *p<0.05 vs. Valcyte^{-} CIE rats; #p<0.05 vs. tone 1. (e) Mean (±SE) freezing (change in freezing from baseline) in females show no significant effect of treatment on CS retrieval during tones 1-3. During tone 4, Valcyte^{+} CIE rats show reduced freezing compared with CIE naïve rats and Valcyte^{-} CIE rats. *p<0.05 vs. Valcyte^{+} CIE rats.

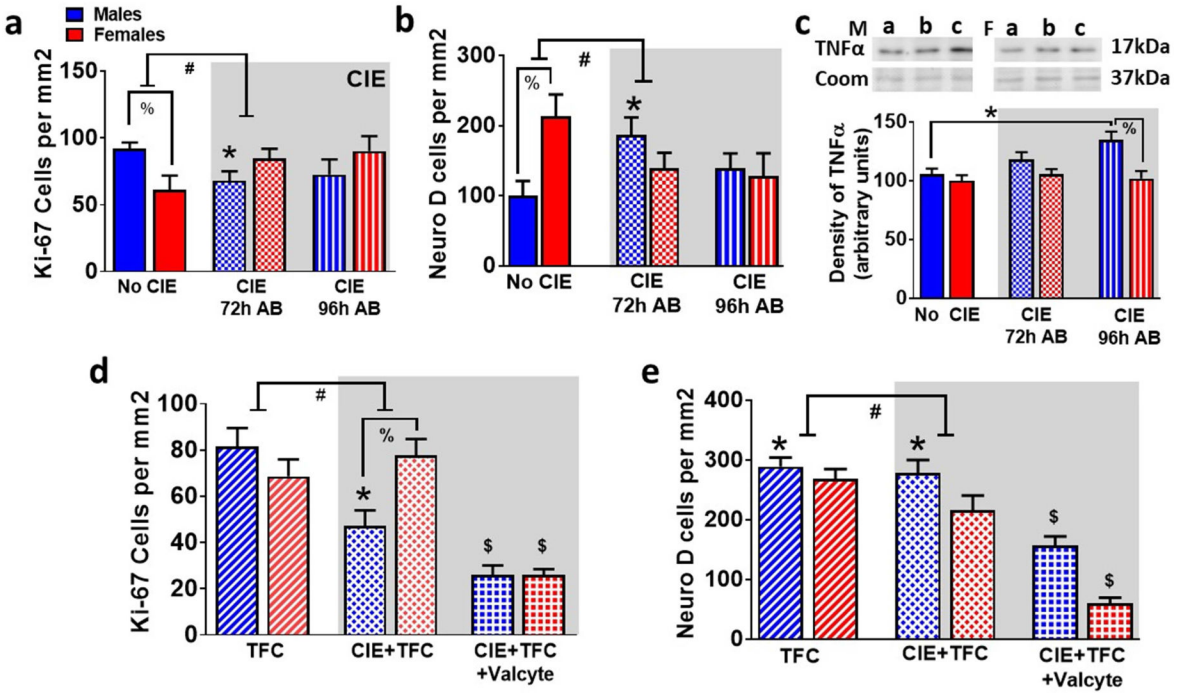

Figure 4:

Valcyte reduces proliferation and differentiation of NPCs in male and female TK rats. (a-b) Quantitative analysis of the total number of Ki-67 (a) and NeuroD (b) labeled cells in no CIE and CIE rats. AB, abstinence. (c) Density of TNFα by Western blotting analysis. Representative immunoblots of TNFα and coomassie from males (M, left panel) and females (F, right panel). a, no CIE; b, 72h AB; c, 96h AB. (d-e) Quantitative analysis of Ki-67 (d) and NeuroD (e) cells from TFC and CIE + TFC groups. Data shown are represented as mean +/− SE. #$p<0.05$ indicating significant interactions; *$p<0.05$ compared with drug/behavior naïve controls; $^{\%}p<0.05$ vs males; $^{\$}p<0.05$ vs. CIE+TFC group by three-way ANOVA followed by posthoc tests. n = 6 behavior naïve males, n = 9 CIE males, n = 16 CIE naïve TFC males, n = 11 Valcyte^{-} CIE TFC males, n = 14 Valcyte^{+} CIE TFC males; n = 6 behavior naïve females, n = 9 CIE females n = 13 CIE naïve TFC females, n = 15 Valcyte^{-} CIE TFC females, n = 10 Valcyte^{+} CIE TFC females.

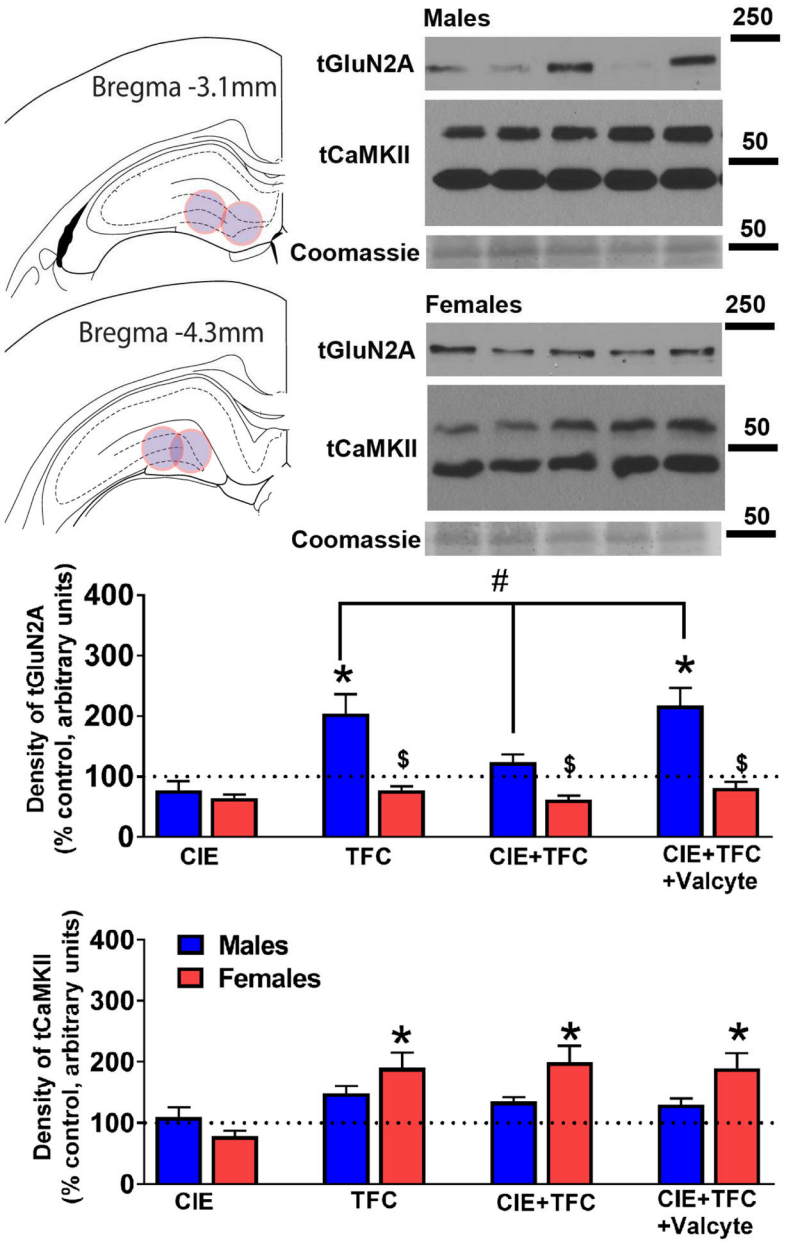

Figure 5:

GluN2A and CaMKII expression is distinctly effected by TFC and CIE in male and female rats. (a) Schematic showing location of tissue punches taken in the dorsal DG of the hippocampus. (b-c) Representative Western blots for protein expression in male (b) and female (c) DG enriched tissue. Lane 1- Naïve control; 2- CIE; 3- TFC; 4- CIE+TFC; 5- CIE +TFC+Valcyte. Molecular weight of ladder in kDa is indicated to the right of the blots. (d-e) Density of protein expression for total GluN2A and CaMKII in dorsal DG from male and female rats. #$p<0.05$ indicating significant interactions; *$p<0.05$ compared to drug and sucrose naïve age matched controls; $^{\&}p<0.05$ vs. CIE+TFC-Valcyte; $p<0.05$ vs. males by three-way ANOVA followed by posthoc tests. Data shown are represented as mean +/− SEM. n = 6 behavior naïve males, n = 9 CIE males, n = 16 CIE naïve TFC males, n = 11

Valcyte^{-} CIE TFC males, n = 14 Valcyte^{+} CIE TFC males; n = 6 behavior naïve females, n = 9 CIE females n = 13 CIE naïve TFC females, n = 15 Valcyte^{-} CIE TFC females, n = 10 Valcyte^{+} CIE TFC females.

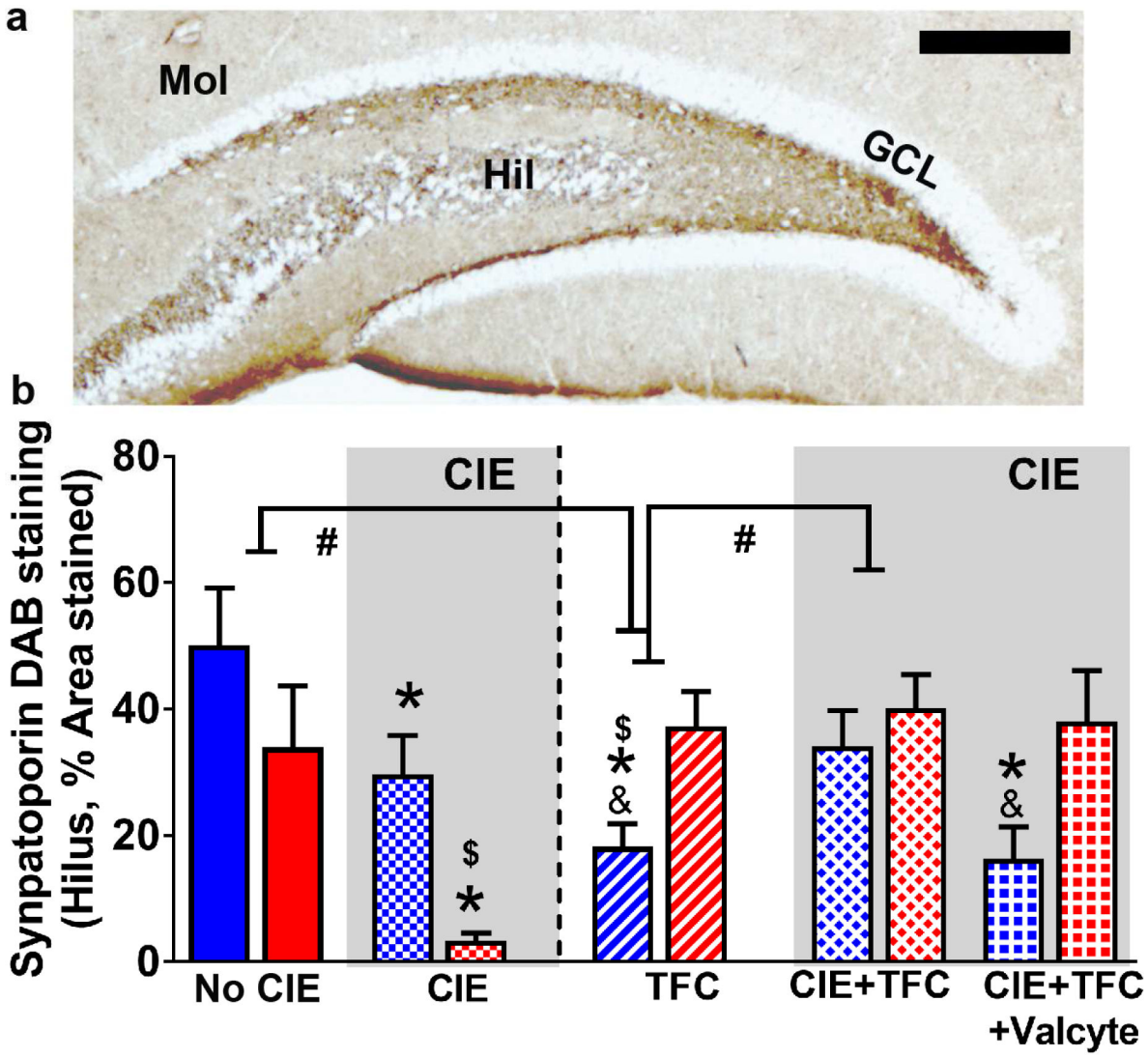

Figure 6:

CIE and TFC differently alter mossy fiber tracts in the DG in male and female rats. (a) Photomicrograph of a section from one control male rat through the anterior dorsal hippocampus stained with synaptoporin. Staining revealed mossy fiber tracts and terminal fields in the hilus (Hil), and the CA3 pyramidal projections. Molecular layer (Mol), granule cell layer (GCL). Scale bar in (a) is 500 um. (b) Quantitative analysis of the density measures in the hilus. Data shown are represented as mean +/− SEM. #$p<0.05$ indicating significant interactions; *$p<0.05$ compared to drug and sucrose naïve age matched controls; $^{\&}p<0.05$ vs. CIE+TFC-Valcyte; $p<0.05$ vs. males by three-way ANOVA followed by posthoc tests. n = 6 behavior naïve males, n = 9 CIE males, n = 16 CIE naïve TFC males, n = 11 Valcyte^{-} CIE TFC males, n = 14 Valcyte^{+} CIE TFC males; n = 6 behavior naïve females, n = 9 CIE females n = 13 CIE naïve TFC females, n = 15 Valcyte^{-} CIE TFC females, n = 10 Valcyte^{+} CIE TFC females.