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Dendritic remodeling of hippocampal neurons is associated with altered NMDA receptor expression in alcohol dependent rats

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Abstract

Prolonged alcohol exposure has been previously shown to impair the structure and function of the hippocampus, although the underlying structural and biochemical alterations contributing to these deleterious effects are unclear. Also unclear is whether these changes persist into prolonged periods of abstinence. Previous work from our lab utilizing a clinically relevant rodent model of alcohol consumption demonstrated that alcohol dependence (induced by chronic intermittent ethanol vapor exposure or CIE) decreases proliferation and survival of neural stem cells in the hippocampal subgranular zone and hippocampal neurogenesis in the dentate gyrus, implicating this region of the cortex as particularly sensitive to the toxic effects of prolonged ethanol exposure. For this study, we investigated seven weeks of CIE-induced morphological changes (dendritic complexity and dendritic spine density) of dentate gyrus (DG) granule cell neurons, CA3, and CA1 pyramidal neurons and the associated alterations in biochemical markers of synaptic plasticity and toxicity (NMDA receptors and PSD-95) in the hippocampus in ethanol-experienced Wistar rats 3h (CIE) and 21 days (protracted abstinence) after the last ethanol vapor exposure. CIE reduced dendritic arborization of DG neurons and this effect persisted into protracted abstinence. CIE enhanced dendritic arborization of pyramidal neurons and this effect did not persist into protracted abstinence. The architectural changes in dendrites did not correlate with alterations in dendritic spine density, however, they were associated with increases in the expression of pNR2B, total NR2B, and total NR2A immediately following CIE with expression levels returning to control levels in prolonged abstinence. Overall, these data provide the evidence that CIE produces profound changes in hippocampal structural plasticity and in molecular tools that maintain hippocampal structural plasticity, and these alterations may underlie cognitive dysfunction associated with alcohol dependence. In addition, the compensatory state concurrent

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with reduced plasticity during protracted abstinence could leave the hippocampus vulnerable to subsequent insult following chronic ethanol exposure.

Keywords

Alcoholism; prolonged abstinence; structural plasticity; NMDA receptor; hippocampus

Introduction

Alcoholism, or the cyclical use, abuse, abstinence, and subsequent relapse of alcohol, is a disorder which has been reported to result in neurobiological deficits in humans. Clinical studies have identified multiple regions of the cortex which are subject to the deleterious effects of chronic alcohol exposure, including the hippocampus, which has been shown to be particularly sensitive to alcohol-induced damage. However, what is less well known is what structural abnormalities, neuronal morphology, and biochemical deviations persist into periods of prolonged abstinence.

Both clinical and pre-clinical studies have clearly demonstrated links between chronic ethanol consumption and impaired hippocampal-sensitive cognitive performance (Brandt et al., 1983; Glenn and Parsons, 1991; Sullivan et al., 2000b; Sullivan et al., 2000a; Sullivan et al., 2002), hippocampal morphology (Bengochea and Gonzalo, 1990; Durazzo et al., 2011), and volume (Sullivan et al., 1995; Agartz et al., 1999; Bleich et al., 2003b; Bleich et al., 2003a; Beresford et al., 2006). Additionally, the hippocampus has been implicated in multiple aspects of ethanol dependence, including reward and seeking of ethanol (Koob and Volkow, 2010), providing evidence that the hippocampus is not only structurally and functionally damaged by chronic ethanol use, but also demonstrates compromised typical hippocampal plasticity mechanisms including structural and biochemical reorganization.

The hippocampus is a substructure of the mammalian limbic system critically important for numerous aspects of cognitive function, stress, and emotional regulation (reviewed in (Bannerman et al., 2014)), as well as having been identified to be sensitive to chronic exposures to alcohol. This region is comprised primarily of three sub-regions: the Cornu Amonis 1 (CA1), the Cornu Amonis 3 (CA3), and the dentate gyrus (DG). Frontal-cortical structures project to the hippocampus via the entorhinal cortex which send perforant path projections (from layers II and III) directly to the dendritic fields of both the granule cells of the DG and the CA3 pyramidal cells. The DG also projects to the CA3 pyramidal cells via the mossy fiber pathway in addition to the CA3 pyramidal neurons being highly self-stimulating. The CA3 pyramidal neurons also send Schaffer Collateral projections to the CA1 pyramidal neurons, which subsequently send projections primarily back to the entorhinal cortex layer V. While the hippocampal structure and function with regards to chronic alcohol use and exposure has been the focus of numerous studies, as yet, no single study has measured the biochemical and structural changes induced by chronic alcohol and subsequent prolonged abstinence in each subregion independently.

While prevailing evidence suggests a direct relationship between chronic ethanol exposure and gross morphological alterations of the hippocampus, as yet, the potential underlying

structural abnormalities of hippocampal neurons following chronic ethanol use have not been well described with only one study noting dendritic retraction in CA1 pyramidal neurons of ethanol exposed animals (McMullen et al., 1984). Functional electrophysiological studies of the hippocampus following chronic ethanol exposure have reported impaired long-term potentiation (LTP; (Fujii et al., 2008)), and altered NMDAR function following ethanol challenge (Sanna and Harris, 1993; Wu et al., 1993; Nelson et al., 2005).

A great emphasis has been placed on understanding the direct impact of chronic ethanol on hippocampal structure and function, however, there has been less work aimed at understanding how long these alterations persist into periods of abstinence. While it has been suggested that some impairments in cognitive function are ameliorated over an extended period in human alcoholics, hippocampal-sensitive functions, such as spatial processing, remain perturbed long into the abstinent period (Fein et al., 2006). To date, there has been no work to describe in a detailed manner how neurons in the three distinct regions of the mammalian hippocampus react to both chronic ethanol and prolonged abstinence. Therefore, we hypothesize that CIE exposure will result in hippocampal pyramidal and granule cell structural abnormalities and aberrant glutamatergic receptor expression, changes which will persist into a period of prolonged abstinence from ethanol exposure.

The goal of the present study was to elaborate on the current knowledge of ethanol toxicity in the hippocampus and expand our understanding of the significant and persistent morphological and biochemical deviations resulting from chronic exposures to ethanol and persisting into a period of abstinence. To the best of our knowledge, there has been no study in rats assessing both the effects of chronic ethanol exposures and prolonged abstinence from ethanol on hippocampal cellular morphology nor biochemical correlates of molecular plasticity. We aim to show that the deviations in DG granule cell and CA3 and CA1 pyramidal cell structure we predict to occur following chronic ethanol exposures will persist into a period of extended abstinence from ethanol exposure, and that these deviations will correspond to aberrant protein expression in the hippocampus.

Materials and Methods

Experimental procedures were conducted 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.

Animals

Adult male Wistar rats (Charles River), weighing 225–275 g and 8 weeks old at the beginning of the experiments, were housed in groups of 2–3 per cage in a temperature-controlled (22°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 8:00 P.M.) with ad libitum access to food and water. All procedures were performed during the dark phase of the light/dark cycle. Thirty-three rats began and completed the study (Control: n=12, Acute Withdrawal: n=12, Prolonged Abstinence: n=9). For each animal, the cortex was dissected into left and right hemispheres upon sacrifice. For all animals, the right hemisphere was

processed for western blot analysis. For 6 of the control animals, 6 of the acute withdrawal animals, and all 9 of the prolonged abstinence animals, the left hemisphere was Golgi stained for the structural analysis portion of this study.

Chronic Intermittent Ethanol (CIE)

Upon arrival to the animal facility, rats were housed in ethanol vapor chambers for 7 weeks, with ethanol on for 14 hours and air only for 10 hours, in a reverse light-dark cycle (lights off at 8am), a cycle of ethanol exposure which has previously been reported as resulting in physical dependence to ethanol (O'Dell et al., 2004; Gilpin et al., 2008; Richardson et al., 2008).

In the chambers, 95% alcohol flows from a large reservoir to a peristaltic pump (model QG-6, FMI Laboratory, Fluid Metering). Alcohol is delivered from the pump to a sidearm flask at a flow rate that can be regulated. The flask is placed on a heater in which the drops of alcohol hitting the bottom of the flask are vaporized. Air flow controlled by a pressure gauge is delivered to the flask and carries the alcohol vapors to the vapor chamber that contains the animal cages. The flow rate was set to deliver vapors that result in blood alcohol levels between 125 and 250 mg% (Figure 1) or 27.2 and 54.4 mM (Gilpin et al., 2008). In this model, rats exhibit somatic withdrawal signs and negative emotional symptoms reflected by anxiety-like responses, hyperalgesia, and elevated brain reward thresholds (Schulteis et al., 1995; Roberts et al., 2000; Valdez et al., 2002; Rimondini et al., 2003; O'Dell et al., 2004; Zhao et al., 2007; Sommer et al., 2008; Edwards et al., 2012; Vendruscolo et al., 2012). Control rats were not exposed to alcohol vapor. Alcohol naïve animals in this study were housed nearly identically to the alcohol-exposed animals (plastic tub containers of identical construction, color, and size, identical bedding, pair housed, ad lib food [same diet as vapor animals] and water, identical animal maintenance schedule [handling, cage changes, weight measures, etc], and light schedule) but outside of the exterior vapor chamber. Blood sampling (tail bleedings) was performed immediately after daily bouts of alcohol vapor exposure in dependent animals twice during the first week of vapor exposure and once during each subsequent week of vapor exposure. Plasma (5 µL) was used for measurement of blood alcohol levels using an Analox AM 1 analyzer (Analox Instruments). The reaction is based on the oxidation of alcohol by alcohol oxidase in the presence of molecular oxygen ($\text{alcohol} + \text{O}_2 \rightarrow \text{acetaldehyde} + \text{H}_2\text{O}_2$). The rate of oxygen consumption is directly proportional to the alcohol concentration. Single-point calibrations were done for each set of samples with reagents provided by Analox Instruments (25–400 mg% or 5.4–87.0 mM). When blood samples were outside the target range (125–250 mg%), vapor levels were adjusted accordingly.

Tissue Collection

Approximately 2 hours (2 h, CIE) or 21 days (21 d, prolonged abstinent, PA) following cessation of ethanol vapor exposure (or age-matched for Controls), rats were anesthetized with isoflourane, then rapidly decapitated and the cortex was immediately removed. The right hemisphere was quickly frozen in dry ice-cooled isopentane and stored at -80°C until further processing. The left hemisphere was immediately submerged in the initial solution of the Golgi-Cox stain per the manufacturer's directions.

Golgi-Cox Staining and Analysis

For determination of the effect of alcohol dependence on hippocampal neuronal architecture, animals were killed by rapid decapitation under light isofluorane anesthesia 2 h, 21 d, or age-matched after the last vapor exposure and whole brain was quickly removed and cut mid sagittally. One half of the brain (the left hemisphere) was processed for Golgi-Cox staining. The other half (right hemisphere) was used for Western blotting analysis. For Golgi-Cox staining, the brain was submerged in Golgi-Cox solution A+B (FD Neurotechnologies Inc.) for 8 d at room temperature, followed by solution C for 4 d at room temperature and stored at -80°C until processed for staining. Frozen brain tissue was coronally cut on a cryostat at 100 μm -thick sections and stained with solution D+E and dehydrated according to manufacturer's instructions. Brains were coded before sectioning to ensure that experimenters were blind to treatments.

To evaluate hippocampal neuron morphology, a Zeiss Axiophot microscope and a computer-based system (Neurolucida; Micro-BrightField) was used to generate three-dimensional neuron tracings that were subsequently visualized and analyzed using NeuroExplorer (MicroBrightField). In order for a neuron to be selected the following four criteria were met: (1) the neuron was in the region of interest (DG [outer granule cell layer of the superior or inferior blade], CA3 or CA1 of the hippocampus, -2.56 to -3.8 mm to bregma), (2) the neuron was distinct from other pyramidal and interneurons to allow for identification of dendrites, (3) the neuron was not truncated or broken, and (4) the neuron exhibited dark, well filled staining throughout including spines.

For each animal, 2 CA3 and CA1 pyramidal neurons in each layer and 4 granule cell neurons in the DG were traced at 40x magnification with an oil immersion lens (equipped with a 10x eye piece). No more than one neuron per region per section was used in the structural analysis. Both the apical and basal trees were traced (representative tracings are provided in Figure 2), and morphological measurements were analyzed separately. For each reconstructed neuron, an estimate of dendritic complexity was obtained using the Sholl ring method. A 3D Sholl analysis was performed in which concentric spheres of increasing radius (starting sphere 10 μm and increasing in 20 μm increments) were layered around the cell body until dendrites were completely encompassed. The number of dendritic intersections at each increment was counted, and results were expressed as total intersections and the number of intersections per radial distance from the soma. Additionally, total dendritic length and longest soma-to-tip length were calculated for each tree of each reconstructed neuron.

For all neurons, spine density was measured at 63x-100x magnification with an oil immersion lens (equipped with a 10x eye piece) on the same dendritic branches used for Sholl analysis. Dendritic spine density was measured on four dendritic segments from both the basal and apical tree. For the basal tree, spines were counted along 10–12 μm segments of 2–5° order branches located at least 50 μm away from the cell body. For the apical tree, spines were counted along 10 μm segments of primary apical branches located at least 80 μm away from the cell body. All measurements of spine density were taken from 1–2- μm -thick dendritic segments to minimize the number of spines hidden by the dendritic shaft and ensure that the number of hidden spines was proportional across all segments counted and

among treatment groups. The total number of dendritic spines visible along both sides of the segment were counted and expressed as number of spines per 10 μm of dendrite. Further, no more than one neuron of each type (CA1, CA3, or DG) from a single section was analyzed.

Western Blot Analysis

Procedures optimized for measuring neuronal levels of both phosphoproteins and total proteins were employed (Edwards et al., 2013). Animals were killed via rapid decapitation under light isoflurane anesthesia 2 h or 21 d after the last vapor exposure (or age-matched for controls). Brains were quickly removed and flash-frozen. Brain tissue was cut at the mid-sagittal axis and the right hemisphere was processed for Western blotting. Tissue punches from dorsal hippocampal formation from 500 μm thick sections were homogenized on ice by sonication in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma), heated at 100 degrees C for five minutes, and stored at -80 degrees 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 (30 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% (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 $^{\circ}\text{C}$ and were incubated with the primary antibody for 16–20 h at 4 $^{\circ}\text{C}$: antibody to NR2B (1:200, Santa Cruz cat. no. sc-9057, predicted molecular weight 178 kDa, observed band $\sim$ 180 kDa), antibody to pNR2B Tyr-1472 (1:200, Cell Signaling cat. no. 4208S, predicted molecular weight 190 kDa, observed band $\sim$ 180 kDa), antibody to NR2A (1:200, Santa Cruz cat. no. sc-9056, predicted molecular weight 178 kDa, observed band $\sim$ 180 kDa), and antibody to PSD-95 (1:500, Millipore, cat. no. 04–1066, predicted band size 95 kDa, observed band $\sim$ 95 kDa). Blots were then washed three times for 15 min in TBST, and then incubated for 1 h at room temperature (24 $^{\circ}\text{C}$), appropriately with horseradish peroxide–conjugated goat antibody to rabbit (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. Blots were then stained Coomassie Blue to normalize to the quantity of protein loaded in each lane (Welinder and Ekblad, 2011). Densitometry was performed using ImageStudio software (Li-Cor Biosciences). X-ray films were digitally scanned at 600dpi 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 the coomassie stained lane corresponding to the band of interest, 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 alcohol naïve animals included on the same blot.

Statistical analysis

The effects of group (control vs. CIE and PA) and distance from the soma on dendritic length were analyzed using two-factor ANOVA, with repeated measures for distance from the soma, followed by Fisher's LSD post hoc tests. One-way ANOVA or unpaired Student's *t* tests were used to compare differences between groups. Data are expressed as mean $\pm$ SEM. Values of $p < 0.05$ were considered statistically significant. Graphs were generated using GraphPad Prism 5.0 software. Images presented here were collected on a Zeiss AxioImagerA2 and imported into Photoshop (version CS6).

Results

BAL (Figure 1)

Blood alcohol concentrations were assessed once per week for the duration of vaporized ethanol exposure. As depicted in Figure 1, animals were gradually exposed to increasing concentrations of vaporized alcohol during the first week, reaching stable BALs between 175–250 mg% for the duration of the CIE exposure.

Sholl Analysis (Figure 2, 3)

Golgi-Cox stained neurons in the DG, CA3, and CA1 were analyzed by Sholl analysis for dendritic complexity and arborization. Representative Golgi-Cox stained tissue is presented in Figure 2A and representative neurons from each region and exposure group are illustrated in Figure 2B. Representative Sholl analysis of an individual neuron is presented in Figure 3A.

Two-way ANOVA (distance from soma x EtOH) analysis of DG granule cell apical dendrites (Figure 3B) showed main effects of distance ($F[15, 1168]=160.0, p<0.0001$) and ethanol (EtOH) ($F[2, 1168]=5.350, p<0.005$). Post-hoc analysis identified significant differences between Control and CIE at 170 μm ($p<0.05$) and Control and Prolonged Abstinence (PA) at 130 μm from Soma. There were no significant differences between CIE and PA at any distance.

Sholl Analysis in the basal dendrites of the CA3 pyramidal cells (Figure 3C), two-way ANOVA analysis (distance x EtOH) resulted in a main effect of distance ($F[15, 576]=76.58, p<0.0001$), EtOH ($F[2, 576]=5.281, p<0.01$), and a main interaction between the two factors ($F[30, 576]=1.665, p<0.05$). Post-hoc analysis found significant differences in dendritic complexity between Controls and CIE at 30 μm ($p<0.05$), 110 μm ($p<0.005$), 130 μm ($p<0.005$), and 150 μm ($p<0.05$); CIE and PA differed significantly at 110 μm ($p<0.05$) and 130 μm ($p<0.05$). PA was not significantly different from Controls at any distance. However, in the apical dendrites of CA3 pyramidal cells (Figure 3D) showed a significant main effect of distance (two-way ANOVA [distance x EtOH], $F[19, 720]=42.44, p<0.001$), with no effect of EtOH.

In the basal dendrites of the CA1 pyramidal neurons (Figure 3E), two-way ANOVA (distance x EtOH) of the data derived from a Scholl analysis revealed a main effect of distance ($F[11, 456]=89.46, p<0.0001$) and EtOH ($F[2, 456]=9.908, p<0.0001$), but no

interaction of the two measures. Post-hoc analysis revealed a significant difference between CIE and PA at 110 μm ($p<0.05$), but CIE and PA individually did not differ from Controls at any point. Two-way ANOVA (distance x EtOH) analysis of CA1 pyramidal apical dendritic complexity (Figure 3F) resulted in a significant main effect of distance ($F[19,680]=47.96$, $p<0.0001$), EtOH ($F[2, 680]=12.21$, $p<0.0001$), and a main interaction ($F[38, 680]=1.510$, $p<0.05$). Post-hoc analysis showed significant differences between Control and CIE at 90 μm ($p<0.01$), 110 μm ($p<0.001$), 130 μm ($p<0.01$), 150 μm ($p<0.01$), and 190 μm ($p<0.05$); CIE and PA were significantly different at 110 μm ($p<0.05$) and 190 μm ($p<0.05$). PA was not significantly different from Controls at any distance.

Dendritic Length

In addition to the dendritic complexity data collected from the Golgi-Cox stained neurons, we assessed soma to tip length of the longest dendrite and total length of all dendrites for each neuron included in the Scholl analysis previously described. There was no effect of CIE or PA on the total dendritic length or the longest soma to tip distance for either the apical or basal dendrites for any hippocampal region (Data not shown).

Spine Density (Figure 4)

We quantified spine density in two distal regions of each Golgi-Cox stained neuron in order to further elucidate the effects of CIE and PA on morphological plasticity. A representative, 10 μm region of dendrite magnified at 100x is presented in Figure 4A to illustrate spine protrusions identified for analysis. One-way ANOVA analysis of spine density in DG granule cells revealed a significant main effect of EtOH ($F[2, 68]=38.87$, $p<0.0001$, Figure 4B), and post-hoc analysis showed a significant increase in spine density in CIE neurons as compared to Controls ($p<0.01$) and PA ($p<0.0001$). Additionally, PA animals had significantly fewer spines than Controls ($p<0.0001$). In CA3 there was no significant effect of treatment in the apical dendrites (Figure 4D), but in the basal dendrites, there was a main effect of EtOH exposure ($F[2, 36]=4.683$, $p<0.05$, Figure 4C) with PA animals having significantly fewer spines than Controls ($p<0.05$) and CIE ($p<0.05$); Control and CIE animals were not significantly different. In the basal dendrites of the CA1 neurons, there was a significant main effect of treatment ($F[2, 33]=27.70$, $p<0.0001$, Figure 4E) and post-hoc analysis indicated that PA neurons had significantly fewer spines than Controls ($p<0.0001$) and CIE ($p<0.0001$) while CIE did not significantly differ from Controls. This was mirrored in the apical dendrites of CA1 neurons, where there was a significant effect of treatment on spine density ($F[2, 33]=20.75$, $p<0.0001$, Figure 4F) with PA neurons having significantly fewer spines than Controls ($p<0.0001$) and CIE ($p<0.001$).

Protein Expression (Figure 5)

In order to quantify a functional change in neuronal function following CIE and PA, we measured the relative expression of proteins of interest in hippocampal tissue. Of particular interest was the glutamatergic receptor NMDA and PSD-95. Respective Western Blots are presented in Figure 5A.

We were particularly interested in the NMDA receptor due to its role in potential glutamatergic excitotoxicity and sensitivity to ethanol exposure. Western blotting and

subsequent One-Way analysis revealed a significant main effect of treatment on the relative expression of the Tyr 1472 phosphorylated NR2B subunit (pNR2B, $F[2, 29] = 11.54$, $p < 0.0005$, Figure 5B) with post-hoc analysis revealing a significant increase in pNR2B expression in CIE tissue compared with Controls ($p < 0.01$) and PA ($p < 0.001$). For total NR2B (tNR2B), there was again a main effect of treatment ($F[2, 29] = 16.25$, $p < 0.0001$, Figure 5C), and post-hoc analysis confirmed that tNR2B expression was elevated relative to Control ($p < 0.005$) and PA ($p < 0.001$). There was no effect of treatment at any level, however, on the ratio of pNR2B to tNR2B (Figure 5E). Since the ratio of tNR2A:tNR2B can also be informative as to the potential for glutamatergic activity, we assessed both the quantity of tNR2A alone and the ratio of tNR2A:tNR2B in each animal. One-way ANOVA analysis revealed a significant main effect of treatment on the relative expression of tNR2A ($F[2, 28] = 6.281$, $p < 0.01$, Figure 5D) which was driven by an increase in tNR2A expression compared to Controls ($p < 0.05$) and PA ($p < 0.01$). For the ratio of tNR2A:tNR2B, there was a main effect of treatment ($F[2, 29] = 3.212$, $p = 0.05$, Figure 5F) with the ratio in CIE animals being significantly higher than in PA animals ($p < 0.05$).

We also quantified the relative expression level of PSD-95, a protein synaptic protein shown to stabilize dendritic spines and anchor NMDA receptors, among others, at the synaptic membrane. However, we found no significant difference between exposure groups in PSD-95 relative expression (Figure 5G).

Discussion

The primary goal of this work was to assess the degree and persistence of morphological and biochemical changes subsequent to chronic intermittent ethanol (CIE) exposure in hippocampal pyramidal and granule cells. We believe that this study constitutes the first detailed reporting of regionally specific deviations from typical cellular morphology and corresponding biochemistry following chronic ethanol exposure, as well as the first to assess the persistence of these deviations on such an extended time course into abstinence. While we predicted that CIE would be detrimental to both the neuronal structure and protein expression in all regions of the hippocampus, the findings were far more complex and regionally distinct.

In humans, chronic alcohol use resulting in alcoholism leads to reduced gray and white matter volume (Pfefferbaum et al., 1992; Fein et al., 2002) with the frontal cortex (Pfefferbaum et al., 1997; Gansler et al., 2000; Tapert et al., 2001; Ratti et al., 2002; Oscar-Berman et al., 2004; Dirksen et al., 2006) and medial temporal lobe structures (including the hippocampus and amygdala) (Jernigan et al., 1991; Sullivan et al., 1995; Fein et al., 2009) being particularly affected. Intriguingly, studies have found that while some structures of the human cortex recover in volume, mass, and white matter content (Pfefferbaum et al., 1995; van Eijk et al., 2013) following periods of abstinence, others, such as the hippocampus, do not (Fein et al., 2009); this implies differential sensitivity of cortical substructures to the damaging effects of alcohol, regionally specific reduced capacity for recovery, or both. Subsequently, cognitive function is recovered in tasks sensitive to those regions which also recover morphologically, such as executive functioning tasks relying primarily on the frontal-cortical areas (Dresler et al., 2012); in contrast, capacity to perform a task reliant on

the hippocampal formation, where damage from chronic alcohol exposure persists into prolonged abstinence, remains impaired (Fein et al., 2006; Fein et al., 2009).

Animal models of chronic ethanol exposure, such as the clinically relevant CIE exposure, have been extensively used to elucidate the cellular and molecular effects of chronic ethanol on the mammalian cortex. Rodents exposed to CIE have similarly impaired cortical volumes as determined by magnetic resonance imaging (Pfefferbaum et al., 2008) and impaired cognitive functioning (Kroener et al., 2012; Van Skike et al., 2012; Trantham-Davidson et al., 2014), and have therefore served as adequate models of the human condition. Using this model, we report that chronic ethanol induces pathologic plasticity in each region of the hippocampus, both structurally and biochemically. In the DG we found significant reductions in the dendritic complexity following CIE which persisted into a period of extended abstinence from CIE, consistent with reports of dendritic retraction in other regions of the hippocampus following chronic ethanol (McMullen et al., 1984). However, CIE exposure resulted in an increase in the density of dendritic spines on granule cell dendrites, while PA resulted in a significant reduction in the density of spine as compared to both CIE and Control animals. In stark contrast, neurons in the CA3 and CA1 regions of the hippocampal formation demonstrated enhanced arborization following CIE exposure, but this increase in the dendritic tree was ameliorated over the period of protracted abstinence with the complexity of pyramidal neuron dendritic trees in prolonged abstinent animals similar to those measured in non-exposed animals. Similar to the DG granule cells, CA3 and CA1 pyramidal cells demonstrated decreased spine density in the abstinent period as compared to CIE and controls, with CIE treatment alone having no significant impact on the density of spines for either region. Therefore, we can conclude that while there is some measure of repair in the hippocampus during periods of abstinence from ethanol, these morphologic changes are structurally and regionally distinct. However, it should be noted that, as described in the methodological approach, a range of dendritic arbors were utilized for the spine density analysis presented here; we are therefore unable to determine if the changes identified here due to chronic ethanol exposure are global effects present in all orders of the dendritic arbor (a global effect) or a specific effect restricted to one select branch. Future studies directed at delineating the details of this abnormality will be of critical importance.

Work from preclinical models of ethanol exposure have also been of critical importance for understanding the molecular mechanisms by which ethanol may be having the observed deleterious effects on neurons and cortical structures. From this work, researchers have established that the glutamatergic transmission system is perturbed following chronic ethanol treatment with the N-methyl-D-aspartate (NMDA) type glutamate receptor being particularly sensitive to ethanol exposures (reviewed in (Ron and Wang, 2009)). As NMDA receptors (NMDARs) are the primary source of glutamatergic transmission in the hippocampus, their regulation, expression, and subunit composition following CIE is critical to understanding the pathological plasticity subsequent to the ethanol exposure. Previous studies of the NMDARs have found expression of the 2B subunit to be up-regulated following ethanol exposure in vitro (Hu and Ticku, 1995; Hu et al., 1996) as well as in vivo in the hippocampus (Nelson et al., 2005; Pian et al., 2010). Additionally, this subunit can be post-transnationally modified via phosphorylation which, depending on the location, can

alter synaptic expression, insertion into the membrane, and channel properties (Ali and Salter, 2001; Nakazawa et al., 2001; Nakazawa et al., 2006). Phosphorylation at tyrosine 1472 have been previously shown to be involved in the maintenance of NR2B-containing receptors at the synapse (Yaka et al., 2002; Lavezzari et al., 2003; Prybylowski et al., 2005; Nakazawa et al., 2006). Therefore, dysregulation of NR2B expression or its phosphorylation state could have significant implications on glutamatergic transmission in ethanol exposed neurons.

Of additional interest is the expression of the 2A subunit and the ratio of 2A to 2B. While increased expression of 2A is linked to improvements in short-term memory in hippocampal-sensitive tasks, increases in 2B expression are observed in association with improvements in long-term memory (Jung and Suh, 2010), and over expression of 2A in genetically modified animals has been linked to impaired hippocampal-sensitive cognitive and electrophysiological function (Cui et al., 2013), findings which were attributed to the increases in the ratio of 2A:2B. The skewed ratio of 2A:2B in these animals resulted in impairments in hippocampal long-term depression (LTD), a process critical for memory consolidation. The 2A subunit, similar to the 2B subunit, has been found to be sensitive to ethanol with respect to expression (Follesa and Ticku, 1996; Kalluri et al., 1998) but establishing comparative sensitivities to ethanol between the two subunits has thus far proved to be challenging for the field at large.

We found significant increases in pNR2B, total NR2B, total NR2A; and the ratio of total NR2A:total NR2B with no difference in PSD-95 expression following CIE treatment with PA animals expressing NMDA subunits in densities similar to those observed in controls. These findings are consistent with other reports of increased NR2A and NR2B expression following chronic ethanol (Nelson et al., 2005; Pian et al., 2010), as well as replicating the finding in previous work that these alterations are ameliorated by some period of abstinence (Nelson et al., 2005).

We believe, that taken together, these findings demonstrate morphological and biochemical pathologic plasticity induced by chronic ethanol in the entirety of the hippocampus, some of which persists into abstinence. There are multiple mechanisms which could be at work in the hippocampal trisynaptic circuit following chronic ethanol exposure, although it should be noted that this is speculation and further investigations into the exact cellular and molecular events occurring in the hippocampus during the development of alcohol dependence and withdrawal will be required in the future. The reduction in the DG morphology could be a protective compensation mechanism for excitotoxic inputs from the perforant path during the intermittent withdrawal periods inherent to CIE exposure, a hypothesis which is supported by the elevated levels of pNR2B, NR2B, and NR2A expression in this study as well as similar phenomena in CA3 pyramidal cells in a study of chronic stress (Christian et al., 2011), but deserves further investigation to confirm.

In contrast to the dendritic retraction observed in the granule cells, the basal dendrites of the CA3 pyramidal neurons along with both the basal and apical dendrites of the CA1 pyramidal neurons demonstrated increased arborization in animals following CIE exposure. We believe that the reduction in granule cell structure results in impaired signaling from the mossy

fibers to the CA3 dendrites, which therefore results in CA3 neurons increasing their morphology to compensate for the loss of signaling and maintain homeostasis. This theory is supported by work Lukoyanov et al. (2000) which demonstrated maintenance of connectivity amongst subregions in spite of a reduction in hippocampal cells in each subregion following chronic alcohol. This increase in morphologic complexity is replicated in CA1 pyramidal neurons. Future electrophysiological studies of the intricate circuitry will further clarify the precise source of the problematic circuitry.

Outside of the intrinsic hippocampal circuitry previously described, it will be of great interest to understand how afferent inputs into the independent subregions of the hippocampus may be further mediating the differential morphological changes we've reported. There is sufficient evidence suggesting that the basolateral amygdala, (BLA) a cortical structure well-described to be sensitive to the deleterious effects of chronic ethanol (reviewed in (Roberto et al., 2012)), directly projects (Pikkarainen et al., 1999; Pitkanen et al., 2000), and receives innervation from (Pitkanen et al., 2000), select regions of the hippocampus. Specifically, the BLA has been shown to send glutamatergic projections to the CA3 and CA1 pyramidal neurons directly (Pikkarainen et al., 1999; Pitkanen et al., 2000), with the CA1 neurons issuing projections directly back to the BLA (Pitkanen et al., 2000). Further, the difference between the persistent retraction of the dendritic arbors of the DG granule cells and the reduction in CA3 and CA1 pyramidal cell arborization to those measured in ethanol naive animals highlights a regional distinction of innervation from the BLA. As discussed previously, prior work has provided evidence for the direct innervation of the CA3 and CA1 regions by BLA glutamatergic neurons (Pikkarainen et al., 1999; Pitkanen et al., 2000), whereas DG granule cells receive indirect innervation from the BLA via the entorhinal cortex and other cortical structures. Therefore, it is possible that the inconsistent findings on structural complexity between the DG and CA regions is the result of the direct and indirect innervation from the BLA, which has been previously shown to demonstrate hyperexcitability following CIE treatment and withdrawal (Lack et al., 2007). We would additionally propose that the prolonged effects of CIE exposure in the granule cells of the DG is related to the unique neurogenic properties of the region (see review by (Drew et al., 2013)); the cells of this region are sensitive to the toxic effects of chronic ethanol (reviewed in (Mandyam and Koob, 2012) and enhanced hippocampal excitability (Ikrar et al., 2013), more so than the pyramidal cells of the CA regions, and therefore are persistently perturbed into a period of extended abstinence from ethanol exposure. Future studies will be required to further elucidate these regional differences.

It will also be of critical interest to understand how the altered cellular morphology in the hippocampus would impact the efferent projections to associated regions of the cortex, particularly those implicated in addiction and drug dependence. Specifically, the nucleus accumbens (NAc) as a central component of the "reward system" in the mammalian cortex has been shown to be critical in the development of addiction to drugs of abuse, including alcohol (for review see (Quintero, 2013)). Activation of hippocampal projections to the NAc have been demonstrated to be required for potentiation of NAc neurons (O'Donnell and Grace, 1995). This relationship could be critical in understanding the role the hippocampus plays in the transition from alcohol abuse to dependency. Further, the neurogenic properties of the dentate gyrus region of the hippocampus have been recently described as both subject

to and a contributing factor to the development of addictive disorders (for review see(Chambers, 2013)). As the dentate gyrus is the primary source of external inputs into the hippocampus, hippocampal projections to affiliated areas, including the NAc, could be impaired following damage to granule cells demonstrated in the work presented here to be subsequent to chronic ethanol exposure, a critical component in the development of alcohol dependence.

Of additional note with regard to the structural findings is the unique observation that only the basal dendritic tree of CA3 pyramidal neurons had significantly larger arbors following CIE treatment, while only the apical tree of the CA1 pyramidal neurons was significantly different from control animals following the chronic alcohol exposure. This could be due to the differential network connections each arbor is exposed to. The primary projections to the apical CA3 dendrites are the mossy fibers from dentate granule cells, associational fibers from nearby CA3 neurons, and the previously described BLA connectivity, while the basal tree of the CA3 cells responds primarily to signals from other CA3 neurons (Spruston N., 2007). In CA1 pyramidal cells, the apical dendrites receive innervations from the Schafer collaterals (originating with the CA3 neurons), direct projections from the entorhinal cortex (layer 3), and the previously described BLA while the basal dendrites are synapsed upon by terminals from the medial septum via the septohippocampal pathway (Amaral and Kurz, 1985; Spruston N., 2007).

The up-regulation of NMDA receptors subsequent to chronic ethanol exposure is potentially a mechanism by which the neuron is attempting to compensate for the repression of signaling characteristic of ethanol exposure. This is supported by the findings of one study utilizing a genetic knock-out of PSD-95, a protein typically found at the synaptic membrane of mature, electrophysiologically active spines which serves to anchor many critical receptors to their synaptic location, NR2B contributed more to NMDA-mediated synaptic current, a finding which was believed to be a functional compensatory mechanism at the mature, PSD95 lacking spines (Beique et al., 2006). Follow-up experiments targeted at examining this idea will be of great interest in the future, as will experiments assessing the expression of other glutamatergic and GABAergic receptors in the hippocampus. However, our findings that CIE results in elevated NMDAR quantities is consistent with previous reports of both increases in the NR2B receptor subunits (Pian et al., 2010) and electrophysiological reports of elevated NMDAR mediated activity following chronic ethanol (Durand and Carlen, 1984; Roberto et al., 2002). Additionally, phosphorylation of the NR2B subunit at tyrosine 1472 plays a critical role in activity dependent trafficking and recycling of the receptor (Roche et al., 2001; Grosshans et al., 2002) and Yagi (1999) similarly reported increased expression of p1472-NR2B following ethanol exposure.

Taken together, this work provides compelling details about the comprised hippocampal plasticity following chronic ethanol exposure as well as the morphological and biochemical profiles following a period of prolonged abstinence. Many of our findings parallel those of previous reports which had evaluated the effects of a shorter course of chronic ethanol and a shorter abstinent period. Nelson et al (2005) reported that the perturbations in the CA1 region of the hippocampus following two weeks of CIE exposure were ameliorated by 7 days post vapor exposure. However, our findings, which include detailed cellular and

molecular analyses from all three hippocampal subregions (rather than just one) and go beyond 7 days to 21 days of ethanol abstinence, provide the first evidence that while the biochemical expression of the cells may have returned to levels observed in ethanol naive animals), some structural abnormalities, such as altered dendritic arborization, persist at levels similar to those found in CIE animals, while the density of dendritic spines increases significantly over the quantities observed in either control or CIE neurons.

Our finding that the structural plasticity following CIE is partially ameliorated in periods of prolonged abstinence could be potentially underlying the partial cognitive recovery in abstinent humans. While reports have demonstrated that working memory and executive functioning capacities are restored in long-term abstinent subjects, spatial memory and reasoning remains impaired (Fein et al., 2006). However, it is not clear if other functions of the hippocampus, such as pattern separation or temporal reasoning, are also affected and to what degree the perturbations might persist into a period of abstinence from ethanol. Future studies targeted at mechanistically link the structural and biochemical abnormalities to altered cognitive function are required to support these theories. Additionally, it will be of critical value to evaluate the influence of these biochemical and structural abnormalities in the hippocampus concurrent with the development of ethanol dependence and withdrawal on self-administration behavior.

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References

- Agartz I, Momenan R, Rawlings RR, Kerich MJ, Hommer DW. Hippocampal volume in patients with alcohol dependence. *Archives of general psychiatry*. 1999; 56:356–363. [PubMed: 10197833]
- Ali DW, Salter MW. NMDA receptor regulation by Src kinase signalling in excitatory synaptic transmission and plasticity. *Current opinion in neurobiology*. 2001; 11:336–342. [PubMed: 11399432]
- Amaral DG, Kurz J. An analysis of the origins of the cholinergic and noncholinergic septal projections to the hippocampal formation of the rat. *The Journal of comparative neurology*. 1985; 240:37–59. [PubMed: 4056104]
- Bannerman DM, Sprengel R, Sanderson DJ, McHugh SB, Rawlins JN, Monyer H, Seeburg PH. Hippocampal synaptic plasticity, spatial memory and anxiety. *Nature reviews Neuroscience*. 2014; 15:181–192.
- Beique JC, Lin DT, Kang MG, Aizawa H, Takamiya K, Huganir RL. Synapse-specific regulation of AMPA receptor function by PSD-95. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:19535–19540. [PubMed: 17148601]
- Bengochea O, Gonzalo LM. Effect of chronic alcoholism on the human hippocampus. *Histology and histopathology*. 1990; 5:349–357. [PubMed: 2134390]
- Beresford TP, Arciniegas DB, Alfors J, Clapp L, Martin B, Du Y, Liu D, Shen D, Davatzikos C. Hippocampus volume loss due to chronic heavy drinking. *Alcoholism, clinical and experimental research*. 2006; 30:1866–1870.
- Bleich, S.; Sperling, W.; Degner, D.; Graesel, E.; Bleich, K.; Wilhelm, J.; Havemann-Reinecke, U.; Javaheripour, K.; Kornhuber, J. *Alcohol and alcoholism*. Vol. 38. Oxford: Oxfordshire; 2003a. Lack

of association between hippocampal volume reduction and first-onset alcohol withdrawal seizure. A volumetric MRI study; p. 40-44.

- Bleich S, Bandelow B, Javaheripour K, Muller A, Degner D, Wilhelm J, Havemann-Reinecke U, Sperling W, Ruther E, Kornhuber J. Hyperhomocysteinemia as a new risk factor for brain shrinkage in patients with alcoholism. *Neuroscience letters*. 2003b; 335:179–182. [PubMed: 12531462]
- Brandt J, Butters N, Ryan C, Bayog R. Cognitive loss and recovery in long-term alcohol abusers. *Archives of general psychiatry*. 1983; 40:435–442. [PubMed: 6838323]
- Chambers RA. Adult hippocampal neurogenesis in the pathogenesis of addiction and dual diagnosis disorders. *Drug and alcohol dependence*. 2013; 130:1–12. [PubMed: 23279925]
- Christian KM, Miracle AD, Wellman CL, Nakazawa K. Chronic stress-induced hippocampal dendritic retraction requires CA3 NMDA receptors. *Neuroscience*. 2011; 174:26–36. [PubMed: 21108993]
- Cui Z, Feng R, Jacobs S, Duan Y, Wang H, Cao X, Tsien JZ. Increased NR2A:NR2B ratio compresses long-term depression range and constrains long-term memory. *Scientific reports*. 2013; 3:1036. [PubMed: 23301157]
- Dirksen CL, Howard JA, Cronin-Golomb A, Oscar-Berman M. Patterns of prefrontal dysfunction in alcoholics with and without Korsakoff's syndrome, patients with Parkinson's disease, and patients with rupture and repair of the anterior communicating artery. *Neuropsychiatric disease and treatment*. 2006; 2:327–339. [PubMed: 19412479]
- Dresler T, Schecklmann M, Ernst LH, Pohla C, Warrings B, Fischer M, Polak T, Fallgatter AJ. Recovery of cortical functioning in abstinent alcohol-dependent patients: prefrontal brain oxygenation during verbal fluency at different phases during withdrawal. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry*. 2012; 13:135–145.
- Drew LJ, Fusi S, Hen R. Adult neurogenesis in the mammalian hippocampus: why the dentate gyrus? *Learning & memory*. 2013; 20:710–729. [PubMed: 24255101]
- Durand D, Carlen PL. Impairment of long-term potentiation in rat hippocampus following chronic ethanol treatment. *Brain research*. 1984; 308:325–332. [PubMed: 6541071]
- Durazzo TC, Tosun D, Buckley S, Gazdzinski S, Mon A, Fryer SL, Meyerhoff DJ. Cortical thickness, surface area, and volume of the brain reward system in alcohol dependence: relationships to relapse and extended abstinence. *Alcoholism, clinical and experimental research*. 2011; 35:1187–1200.
- Edwards S, Guerrero M, Ghoneim OM, Roberts E, Koob GF. Evidence that vasopressin V1b receptors mediate the transition to excessive drinking in ethanol-dependent rats. *Addiction biology*. 2012; 17:76–85. [PubMed: 21309953]
- Edwards S, Baynes BB, Carmichael CY, Zamora-Martinez ER, Barrus M, Koob GF, Gilpin NW. Traumatic stress reactivity promotes excessive alcohol drinking and alters the balance of prefrontal cortex-amygdala activity. *Translational psychiatry*. 2013; 3:e296. [PubMed: 23982628]
- Fein G, Torres J, Price LJ, Di Sclafani V. Cognitive performance in long-term abstinent alcoholic individuals. *Alcoholism, clinical and experimental research*. 2006; 30:1538–1544.
- Fein G, Shimotsu R, Chu R, Barakos J. Parietal gray matter volume loss is related to spatial processing deficits in long-term abstinent alcoholic men. *Alcoholism, clinical and experimental research*. 2009; 33:1806–1814.
- Fein G, Di Sclafani V, Cardenas VA, Goldmann H, Tolou-Shams M, Meyerhoff DJ. Cortical gray matter loss in treatment-naïve alcohol dependent individuals. *Alcoholism, clinical and experimental research*. 2002; 26:558–564.
- Follesa P, Ticku MK. Chronic ethanol-mediated up-regulation of the N-methyl-D-aspartate receptor polypeptide subunits in mouse cortical neurons in culture. *The Journal of biological chemistry*. 1996; 271:13297–13299. [PubMed: 8663153]
- Fujii S, Yamazaki Y, Sugihara T, Wakabayashi I. Acute and chronic ethanol exposure differentially affect induction of hippocampal LTP. *Brain research*. 2008; 1211:13–21. [PubMed: 18423576]
- Gansler DA, Harris GJ, Oscar-Berman M, Streeter C, Lewis RF, Ahmed I, Achong D. Hypoperfusion of inferior frontal brain regions in abstinent alcoholics: a pilot SPECT study. *J Stud Alcohol*. 2000; 61:32–37. [PubMed: 10627094]

- Gilpin, NW.; Richardson, HN.; Cole, M.; Koob, GF. Vapor inhalation of alcohol in rats. In: Crawley, Jacqueline N., et al., editors. *Current protocols in neuroscience / editorial board*. 2008. Chapter 9: Unit 9 29
- Glenn SW, Parsons OA. Impaired efficiency in female alcoholics' neuropsychological performance. *Journal of clinical and experimental neuropsychology*. 1991; 13:895–908. [PubMed: 1779029]
- Grosshans DR, Clayton DA, Coultrap SJ, Browning MD. LTP leads to rapid surface expression of NMDA but not AMPA receptors in adult rat CA1. *Nature neuroscience*. 2002; 5:27–33.
- Hu XJ, Ticku MK. Chronic ethanol treatment upregulates the NMDA receptor function and binding in mammalian cortical neurons. *Brain research Molecular brain research*. 1995; 30:347–356. [PubMed: 7637584]
- Hu XJ, Follesa P, Ticku MK. Chronic ethanol treatment produces a selective upregulation of the NMDA receptor subunit gene expression in mammalian cultured cortical neurons. *Brain research Molecular brain research*. 1996; 36:211–218. [PubMed: 8965641]
- Ikrar T, Guo N, He K, Besnard A, Levinson S, Hill A, Lee HK, Hen R, Xu X, Sahay A. Adult neurogenesis modifies excitability of the dentate gyrus. *Frontiers in neural circuits*. 2013; 7:204. [PubMed: 24421758]
- Jernigan TL, Schafer K, Butters N, Cermak LS. Magnetic resonance imaging of alcoholic Korsakoff patients. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 1991; 4:175–186. [PubMed: 2064717]
- Jung YH, Suh YH. Differential functions of NR2A and NR2B in short-term and long-term memory in rats. *Neuroreport*. 2010; 21:808–811. [PubMed: 20581725]
- Kalluri HS, Mehta AK, Ticku MK. Up-regulation of NMDA receptor subunits in rat brain following chronic ethanol treatment. *Brain research Molecular brain research*. 1998; 58:221–224. [PubMed: 9685652]
- Koob GF, Volkow ND. Neurocircuitry of addiction. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2010; 35:217–238. [PubMed: 19710631]
- Kroener S, Mulholland PJ, New NN, Gass JT, Becker HC, Chandler LJ. Chronic alcohol exposure alters behavioral and synaptic plasticity of the rodent prefrontal cortex. *PloS one*. 2012; 7:e37541. [PubMed: 22666364]
- Lack AK, Diaz MR, Chappell A, DuBois DW, McCool BA. Chronic ethanol and withdrawal differentially modulate pre- and postsynaptic function at glutamatergic synapses in rat basolateral amygdala. *Journal of neurophysiology*. 2007; 98:3185–3196. [PubMed: 17898152]
- Lavezzari G, McCallum J, Lee R, Roche KW. Differential binding of the AP-2 adaptor complex and PSD-95 to the C-terminus of the NMDA receptor subunit NR2B regulates surface expression. *Neuropharmacology*. 2003; 45:729–737. [PubMed: 14529712]
- Lukoyanov NV, Brandao F, Cadete-Leite A, Madeira MD, Paula-Barbosa MM. Synaptic reorganization in the hippocampal formation of alcohol-fed rats may compensate for functional deficits related to neuronal loss. *Alcohol*. 2000; 20:139–148. [PubMed: 10719793]
- Mandyam CD, Koob GF. The addicted brain craves new neurons: putative role for adult-born progenitors in promoting recovery. *Trends in neurosciences*. 2012; 35:250–260. [PubMed: 22265158]
- McMullen PA, Saint-Cyr JA, Carlen PL. Morphological alterations in rat CA1 hippocampal pyramidal cell dendrites resulting from chronic ethanol consumption and withdrawal. *The Journal of comparative neurology*. 1984; 225:111–118. [PubMed: 6539344]
- Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K, Mishina M, Manabe T, Yamamoto T. Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR epsilon 2 (NR2B) subunit of the N-methyl-D-aspartate receptor. *The Journal of biological chemistry*. 2001; 276:693–699. [PubMed: 11024032]
- Nakazawa T, Komai S, Watabe AM, Kiyama Y, Fukaya M, Arima-Yoshida F, Horai R, Sudo K, Ebine K, Delawary M, Goto J, Umemori H, Tezuka T, Iwakura Y, Watanabe M, Yamamoto T, Manabe T. NR2B tyrosine phosphorylation modulates fear learning as well as amygdaloid synaptic plasticity. *The EMBO journal*. 2006; 25:2867–2877. [PubMed: 16710293]

- Nelson TE, Ur CL, Gruol DL. Chronic intermittent ethanol exposure enhances NMDA-receptor-mediated synaptic responses and NMDA receptor expression in hippocampal CA1 region. *Brain research*. 2005; 1048:69–79. [PubMed: 15919065]
- O'Dell LE, Roberts AJ, Smith RT, Koob GF. Enhanced Alcohol Self-Administration after Intermittent Versus Continuous Alcohol Vapor Exposure. *Alcoholism: Clinical & Experimental Research*. 2004; 28:1676–1682.
- O'Donnell P, Grace AA. Synaptic interactions among excitatory afferents to nucleus accumbens neurons: hippocampal gating of prefrontal cortical input. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1995; 15:3622–3639. [PubMed: 7751934]
- Oscar-Berman M, Kirkley SM, Gansler DA, Couture A. Comparisons of Korsakoff and non-Korsakoff alcoholics on neuropsychological tests of prefrontal brain functioning. *Alcoholism, clinical and experimental research*. 2004; 28:667–675.
- Pfefferbaum A, Sullivan EV, Mathalon DH, Lim KO. Frontal lobe volume loss observed with magnetic resonance imaging in older chronic alcoholics. *Alcoholism, clinical and experimental research*. 1997; 21:521–529.
- Pfefferbaum A, Sullivan EV, Mathalon DH, Shear PK, Rosenbloom MJ, Lim KO. Longitudinal changes in magnetic resonance imaging brain volumes in abstinent and relapsed alcoholics. *Alcoholism, clinical and experimental research*. 1995; 19:1177–1191.
- Pfefferbaum A, Zahr NM, Mayer D, Vinco S, Orduna J, Rohlfing T, Sullivan EV. Ventricular expansion in wild-type Wistar rats after alcohol exposure by vapor chamber. *Alcoholism, clinical and experimental research*. 2008; 32:1459–1467.
- Pfefferbaum A, Lim KO, Zipursky RB, Mathalon DH, Rosenbloom MJ, Lane B, Ha CN, Sullivan EV. Brain gray and white matter volume loss accelerates with aging in chronic alcoholics: a quantitative MRI study. *Alcoholism, clinical and experimental research*. 1992; 16:1078–1089.
- Pian JP, Criado JR, Milner R, Ehlers CL. N-methyl-D-aspartate receptor subunit expression in adult and adolescent brain following chronic ethanol exposure. *Neuroscience*. 2010; 170:645–654. [PubMed: 20603193]
- Pikkarainen M, Ronkko S, Savander V, Insausti R, Pitkanen A. Projections from the lateral, basal, and accessory basal nuclei of the amygdala to the hippocampal formation in rat. *The Journal of comparative neurology*. 1999; 403:229–260. [PubMed: 9886046]
- Pitkanen A, Pikkarainen M, Nurminen N, Ylinen A. Reciprocal connections between the amygdala and the hippocampal formation, perirhinal cortex, and postrhinal cortex in rat. A review. *Annals of the New York Academy of Sciences*. 2000; 911:369–391. [PubMed: 10911886]
- Prybylowski K, Chang K, Sans N, Kan L, Vicini S, Wenthold RJ. The synaptic localization of NR2B-containing NMDA receptors is controlled by interactions with PDZ proteins and AP-2. *Neuron*. 2005; 47:845–857. [PubMed: 16157279]
- Quintero GC. Role of nucleus accumbens glutamatergic plasticity in drug addiction. *Neuropsychiatric disease and treatment*. 2013; 9:1499–1512. [PubMed: 24109187]
- Ratti MT, Bo P, Giardini A, Soragna D. Chronic alcoholism and the frontal lobe: which executive functions are impaired? *Acta neurologica Scandinavica*. 2002; 105:276–281. [PubMed: 11939939]
- Richardson HN, Lee SY, O'Dell LE, Koob GF, Rivier CL. Alcohol self-administration acutely stimulates the hypothalamic-pituitary-adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state. *European Journal of Neuroscience*. 2008; 28:1641–1653. [PubMed: 18979677]
- Rimondini R, Sommer W, Heilig M. A temporal threshold for induction of persistent alcohol preference: behavioral evidence in a rat model of intermittent intoxication. *J Stud Alcohol*. 2003; 64:445–449. [PubMed: 12921185]
- Roberto M, Gilpin NW, Siggins GR. The central amygdala and alcohol: role of gamma-aminobutyric acid, glutamate, and neuropeptides. *Cold Spring Harbor perspectives in medicine*. 2012; 2:a012195. [PubMed: 23085848]
- Roberto M, Nelson TE, Ur CL, Gruol DL. Long-term potentiation in the rat hippocampus is reversibly depressed by chronic intermittent ethanol exposure. *Journal of neurophysiology*. 2002; 87:2385–2397. [PubMed: 11976376]

- Roberts AJ, Heyser CJ, Cole M, Griffin P, Koob GF. Excessive ethanol drinking following a history of dependence: animal model of allostasis. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. 2000; 22:581–594. [PubMed: 10788758]
- Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ. Molecular determinants of NMDA receptor internalization. *Nature neuroscience*. 2001; 4:794–802.
- Ron D, Wang J. *Frontiers in Neuroscience*. 2009
- Van Dongen, AM., editor. *Biology of the NMDA Receptor*. Boca Raton (FL): CRC Press; The NMDA Receptor and Alcohol Addiction. Taylor & Francis Group, LLC.
- Sanna E, Harris RA. Recent developments in alcoholism:neuronal ion channels. Recent developments in alcoholism : an official publication of the American Medical Society on Alcoholism, the Research Society on Alcoholism, and the National Council on Alcoholism. 1993; 11:169–186.
- Schulteis G, Markou A, Cole M, Koob GF. Decreased brain reward produced by ethanol withdrawal. *Proceedings of the National Academy of Sciences of the United States of America*. 1995; 92:5880–5884. [PubMed: 7597046]
- Sommer WH, Rimondini R, Hansson AC, Hipskind PA, Gehlert DR, Barr CS, Heilig MA. Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. *Biological psychiatry*. 2008; 63:139–145. [PubMed: 17585886]
- Spruston, NMC. Structural and functional properties of hippocampal neurons. In: Anderson P, MR.; Amaral, D.; Bliss, T.; O'Keefe, J., editors. *The Hippocampus Book*. Oxford: Oxford University Press; 2007. p. 133-202.
- Sullivan EV, Rosenbloom MJ, Pfefferbaum A. Pattern of motor and cognitive deficits in detoxified alcoholic men. *Alcoholism, clinical and experimental research*. 2000a; 24:611–621.
- Sullivan EV, Rosenbloom MJ, Lim KO, Pfefferbaum A. Longitudinal changes in cognition, gait, and balance in abstinent and relapsed alcoholic men: relationships to changes in brain structure. *Neuropsychology*. 2000b; 14:178–188. [PubMed: 10791858]
- Sullivan EV, Fama R, Rosenbloom MJ, Pfefferbaum A. A profile of neuropsychological deficits in alcoholic women. *Neuropsychology*. 2002; 16:74–83. [PubMed: 11853359]
- Sullivan EV, Marsh L, Mathalon DH, Lim KO, Pfefferbaum A. Anterior hippocampal volume deficits in nonamnesic, aging chronic alcoholics. *Alcoholism, clinical and experimental research*. 1995; 19:110–122.
- Tapert SF, Brown GG, Kindermann SS, Cheung EH, Frank LR, Brown SA. fMRI measurement of brain dysfunction in alcohol-dependent young women. *Alcoholism, clinical and experimental research*. 2001; 25:236–245.
- Trantham-Davidson H, Burnett EJ, Gass JT, Lopez MF, Mulholland PJ, Centanni SW, Floresco SB, Chandler LJ. Chronic alcohol disrupts dopamine receptor activity and the cognitive function of the medial prefrontal cortex. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2014; 34:3706–3718. [PubMed: 24599469]
- Valdez GR, Roberts AJ, Chan K, Davis H, Brennan M, Zorrilla EP, Koob GF. Increased ethanol self-administration and anxiety-like behavior during acute ethanol withdrawal and protracted abstinence: regulation by corticotropin-releasing factor. *Alcoholism, clinical and experimental research*. 2002; 26:1494–1501.
- van Eijk J, Demirakca T, Frischknecht U, Hermann D, Mann K, Ende G. Rapid partial regeneration of brain volume during the first 14 days of abstinence from alcohol. *Alcoholism, clinical and experimental research*. 2013; 37:67–74.
- Van Skike CE, Novier A, Diaz-Granados JL, Matthews DB. The effect of chronic intermittent ethanol exposure on spatial memory in adolescent rats: the dissociation of metabolic and cognitive tolerances. *Brain research*. 2012; 1453:34–39. [PubMed: 22464184]
- Vendruscolo LF, Barbier E, Schlosburg JE, Misra KK, Whitfield TW Jr. Logrip ML, Rivier C, Repunte-Canonigo V, Zorrilla EP, Sanna PP, Heilig M, Koob GF. Corticosteroid-dependent plasticity mediates compulsive alcohol drinking in rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2012; 32:7563–7571. [PubMed: 22649234]

- Welinder C, Ekblad L. Coomassie staining as loading control in Western blot analysis. *Journal of proteome research*. 2011; 10:1416–1419. [PubMed: 21186791]
- Wu PH, Mihic SJ, Liu JF, Le AD, Kalant H. Blockade of chronic tolerance to ethanol by the NMDA antagonist, (+)-MK-801. *European journal of pharmacology*. 1993; 231:157–164. [PubMed: 8453972]
- Yagi T. Molecular mechanisms of Fyn-tyrosine kinase for regulating mammalian behaviors and ethanol sensitivity. *Biochemical pharmacology*. 1999; 57:845–850. [PubMed: 10086316]
- Yaka R, Thornton C, Vagts AJ, Phamluong K, Bonci A, Ron D. NMDA receptor function is regulated by the inhibitory scaffolding protein, RACK1. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:5710–5715. [PubMed: 11943848]
- Zhao Y, Weiss F, Zorrilla EP. Remission and resurgence of anxiety-like behavior across protracted withdrawal stages in ethanol-dependent rats. *Alcoholism, clinical and experimental research*. 2007; 31:1505–1515.

Highlights

CIE-induced alterations in structural plasticity persist into protracted abstinence

CIE-induced alterations in NMDA receptor expression is transient

Alterations in hippocampal plasticity may contribute to impaired hippocampal function

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Blood Alcohol Levels

Figure 1. Blood alcohol levels of rats subjected to seven weeks of chronic intermittent ethanol (CIE)

Animals were maintained at blood alcohol levels between 175–250 mg% for the duration of the CIE exposure.

Figure 2. Representative Golgi-Cox stained tissue (A) and neuron tracings (B)
Granule cells, CA3 pyramidal cells, and CA1 pyramidal cells from Control, CIE, and prolonged abstinent (PA) animals were Golgi-Cox stained for morphological assessment including dendritic complexity and length, and spine density. Each image is oriented with the apical dendrites facing the top of the page.

Figure 3. Sholl analysis of hippocampal dendrites

Representative image of a Sholl analysis of a hippocampal pyramidal cell is presented in (A). Granule cells (B), CA3 pyramidal cells (C-D), and CA1 pyramidal cells (E-F) were assessed for dendritic complexity of apical (B, D, and F) and basal (C and E) dendrites in Control, CIE, and PA animals. Red asterisks denote significant differences between Control and CIE animals, blue asterisk denote significant differences between Control and PA groups, and hatch denotes a significant difference between CIE and PA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$.

Figure 4. Spine density of hippocampal dendrite segments

Representative image of a region of dendrite selected for spine density analysis is presented in (A) to illustrate the protrusions identified as spines (arrows). Image generated at 100x, scale bar: 5 μm . Granule cells (B), CA3 pyramidal cells (C-D), and CA1 pyramidal cells (E-F) were assessed for spine density of 10 μm segments of apical (B, D, and F) and basal (C and E) dendrites in Control, CIE, and PA animals. *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001.

Figure 5. Expression of selected proteins in hippocampal homogenate following CIE and PA
 Sample western blots for pNR2B, total NR2B, total NR2A, and PSD95 from all three exposure groups are provided in panel (A). Bar graphs represent the relative expression of pNR2B (B), total NR2B (C), total NR2A (D), the ratio of pNR2B:total NR2B (E), the ratio of total NR2A:total NR2B (F) and PSD95 (G). Values were normalized to controls included on each blot (n=11–12). Asterisks denote significant differences between Control and CIE or PA groups, and hatches denote a significant difference between CIE and PA. *p 0.05, **p 0.01, ***p 0.001, #p 0.05, ##p 0.01, ###p 0.001.