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Permanent impairment of birth and survival of cortical and hippocampal proliferating cells following excessive drinking during alcohol dependence

Heather N. Richardson^{+, *}, Stephanie H. Chan^{*}, Elena F. Crawford, Youn Kyung Lee, Cindy K. Funk, George F. Koob, and Chitra D. Mandyam

Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, La Jolla, CA, USA.

Abstract

Experimenter-delivered alcohol decreases adult hippocampal neurogenesis, and hippocampal-dependent learning and memory. The present study used clinically relevant rodent models of nondependent limited access alcohol self-administration and excessive drinking during alcohol dependence (alcohol self-administration followed by intermittent exposure to alcohol vapors over several weeks) to compare alcohol-induced effects on cortical gliogenesis and hippocampal neurogenesis. Alcohol dependence, but not nondependent drinking, reduced proliferation and survival in the medial prefrontal cortex (mPFC). Apoptosis was reduced in both alcohol groups within the mPFC, which may reflect an initiation of a reparative environment following alcohol exposure as decreased proliferation was abolished after prolonged dependence. Reduced proliferation, differentiation, and neurogenesis was observed in the hippocampus of both alcohol groups, and prolonged dependence worsened the effects. Increased hippocampal apoptosis and neuronal degeneration following alcohol exposure suggests a loss in neuronal turnover and indicates that the hippocampal neurogenic niche is highly vulnerable to alcohol.

Keywords

prefrontal cortex; subgranular zone; self-administration; alcohol vapor; Ki-67; bromodeoxyuridine; doublecortin; activated caspase-3; Fluoro-Jade C

Introduction

Alcoholism is a chronically relapsing disorder characterized by cycles of repeated high alcohol intake and negative emotional consequences of withdrawal that contribute to excessive drinking and susceptibility to relapse (Breese et al., 2005; Heilig and Egli, 2006; Koob, 2003). Investigating how chronic alcohol affects the nervous system in rodent models can help

Correspondence: Chitra D. Mandyam, Ph.D., Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, 10550 North Torrey Pines Road, SP30-2400, La Jolla, CA 92037 USA. Office: (858) 784-9039, Fax: (858) 784-8851, email: cmandyam@scripps.edu.

⁺*Present address:* Department of Psychology-Neuroscience, Center of Neuroendocrine Studies, University of Massachusetts-Amherst, Tobin Hall, 135 Hicks Way, Amherst, MA 01003, USA.

^{*}H.N.R. and S.H.C. contributed equally to this work.

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elucidate the neurobiological mechanisms contributing to the pathology of alcoholism in humans (Heilig and Egli, 2006). Similar to human alcoholics, alcohol-dependent animals exhibit emotional distress and uncontrolled excessive alcohol consumption following periods of withdrawal (File et al., 1989; Koob, 2003; Overstreet et al., 2002; Roberts et al., 2000; Valdez et al., 2002). Thus, rodent models of alcohol dependence are ideal tools for investigating the neurobiological changes associated with chronic alcohol use and dependence.

Alcohol exposure impairs the structure of, and function dependent on, the hippocampus (Abe et al., 2004; Beresford et al., 2006; Morrisett and Swartzwelder, 1993; Sullivan et al., 1995; Sullivan et al., 2000; Walker et al., 1980) and frontal cortex (Bechara et al., 2001; De Bellis et al., 2005; Langen et al., 2002; Miguel-Hidalgo, 2005), but the underlying cellular mechanisms contributing to these deleterious effects are unclear. Various alcohol administration paradigms, including forced acute and chronic exposure and voluntary chronic exposure, have demonstrated alcohol-induced changes in adult hippocampal neurogenesis (for review, see (Nixon, 2006), a phenomenon implicated in maintaining hippocampal structure, integrity, and function (Brown et al., 2003a; Kempermann, 2002; Markakis and Gage, 1999; Ramirez-Amaya et al., 2006). However, little is known about the impact of alcohol on prefrontal cortical plasticity, a brain region injured by alcohol abuse (Bechara et al., 2001; De Bellis et al., 2005). Clinically relevant animal models of alcohol drinking and dependence are seldom used in investigations of alcohol on neural plasticity but could help clarify the functional significance of the effects of alcohol on the brain. Examining the effects of self-administered alcohol with and without a history of alcohol dependence on plastic, regenerative events in the medial prefrontal cortex (mPFC) and hippocampus holds significant potential for testing the hypothesis that newly born neurons and glia are important for the development and perhaps consequences of alcohol dependence.

The present study investigated the effect of nondependent limited access alcohol drinking and excessive drinking during dependence on adult cortical and hippocampal cell genesis in an established animal model of alcohol dependence. In this model, rats trained to self-administer alcohol and made dependent using chronic alcohol (e.g., several weeks of intermittent exposure to alcohol vapors) exhibit blunted neuroendocrine and enhanced limbic stress sensitivity, and excessive drinking during acute withdrawal (Funk et al., 2006; Funk et al., 2007; O'Dell et al., 2004; Richardson et al., 2008; Walker and Koob, 2007) and prolonged abstinence from alcohol vapors (Gilpin et al., 2008b; Rimondini et al., 2002; Sommer et al., 2008; Zhao et al., 2007) compared with self-administering rats housed under control conditions (nondependent rats). For related models see (Overstreet et al., 2002; Rimondini et al., 2002; Roberts et al., 1996; Valdez et al., 2002)).

Materials and methods

Animals

Thirty-one adult, male Wistar rats (Charles River), weighing 180-200 g at the start of the experiment, were housed two per cage in a temperature-controlled vivarium under a reversed light/dark cycle (lights off 06.00 h to 18.00 h). Animals were divided into six groups: (1) control ($n = 9$), received no training or alcohol exposure; (2) trained ($n = 3$), trained on the self-administration paradigm for 3 weeks (30 minute access to sweetened solution *vs.* water 5 days a week); (3) nondependent alcohol self-administering ($n = 5$), initially trained to self-administer alcohol *vs.* water for 3 weeks (30 minute access to alcohol, 5 days a week) and housed for 6 weeks similarly to the dependent groups described below but without exposure to alcohol vapors, during which they were tested for alcohol intake via alcohol self-administration twice a week (30 minute access); (4) alcohol dependent ($n = 4$), trained to self-administer alcohol *vs.* water for 3 weeks (30 minute access to alcohol, 5 days a week) and exposed to intermittent chronic alcohol vapors for the following 6 weeks to induce dependence during which they were

tested for alcohol intake via alcohol self-administration twice a week; (5) prolonged nondependent ($n = 6$), trained to self-administer alcohol vs. water for 3 weeks and housed under control air conditions (no alcohol vapors) for the following 10 weeks during which they were tested for alcohol self-administration twice a week up to 6 weeks and were discontinued from testing for the additional 4 weeks; (6) prolonged dependent ($n = 4$), trained to self-administer alcohol vs. water for 3 weeks and exposed to intermittent chronic alcohol vapors for the following 10 weeks during which they were tested for alcohol self-administration twice a week up to 6 weeks and were discontinued from testing for the additional 4 weeks. The main difference between the nondependent and dependent conditions is the exposure to intermittent alcohol vapors in the dependent groups and higher intake of alcohol during self-administration sessions. After several weeks of intermittent vapor exposure, dependent animals show mild physical dependence (Richardson et al., 2008) and robust motivational dependence, characterized by increased willingness to work for alcohol (Walker and Koob, 2007) and excessive, binge-like drinking patterns (O'Dell et al., 2004; Richardson et al., 2008). Thus, the total amount of alcohol exposure differed greatly between the dependent and nondependent groups. Dependent rats self-administered alcohol in 30 min sessions and received 14 hours of vapor daily. As such, the dependent groups maintained blood alcohol levels 150 mg% for approximately 14.5 hours per day versus the nondependent groups, which self-administered alcohol to maintain blood alcohol levels not exceeding 50 mg% for 30 min per day (Richardson et al., 2008). Food and water were available *ad libitum*. Rats were subjected to alcohol self-administration and were 21–25 weeks old when intracardially perfused. All procedures were performed during the dark phase of the light/dark cycle. 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.

Operant self-administration apparatus

The self-administration system consisted of test chambers (Coulbourn Instruments, Allentown, PA) contained within wooden sound-attenuated ventilated cubicles. The test chambers were equipped with two retractable levers located 4 cm above the grid floor and 4.5 cm to either side of a small stainless steel receptacle containing two drinking cups. Two infusion pumps (Razel Scientific Instruments, Stamford, CT) were connected to the system in which a lever press resulted in the delivery of 0.1 ml of solution. Tap water was delivered to one dish, and the experimental solution (i.e., sweetened solution or alcohol) was delivered to the other dish. Fluid delivery and recording of operant self-administration were controlled by a computer. Lever presses were not recorded during the 0.5 s inter-response time-out interval when solution was being delivered.

Solutions for oral self-administration

Alcohol (10% w/v) was prepared with 95% ethyl alcohol and tap water. Glucose (3%) and/or saccharin (0–0.125%; Sigma, St. Louis, MO) was added to the water or alcohol solutions to achieve the appropriate concentration. Sweetened alcohol was only used in the early stages of training and was faded out so that animals were lever pressing for 10% w/v alcohol (unsweetened) versus water within the first few weeks of training.

Acquisition of operant alcohol self-administration

Animals were trained to self-administer alcohol or water orally in a concurrent, two-lever, free-choice contingency. A continuous reinforcement (fixed ratio-1, FR1) schedule of reinforcement was used in which each lever press was reinforced. Animals acquired alcohol self-administration using a variation of the previously described saccharin fading free-choice operant conditioning protocol (Samson, 1986). The modified procedure in the present study

utilized a sweetened solution containing 3% glucose and 0.125% saccharin (Sigma, St. Louis, MO) instead of water restriction and 0.2% saccharin to initiate and maintain operant responding (Funk et al., 2006). Animals respond for the sweetened solution within 1-2 training sessions, thereby circumventing the need for water restriction to initiate lever-pressing. Operant sessions during training were conducted 5 days per week between 09.00 h and 15.00 h (lights on at 06.00 h). Operant sessions were 30 min in duration, except during the initial days of training in which sessions lasted up to 2 h to permit acquisition of responding for the sweetened solution. Alcohol (ethanol, 10% w/v) then was added to the sweetened solution, and once mean responding stabilized (around 1 week; Fig. 1a), the glucose was removed from the solution, leaving only 0.125% saccharin and 10% w/v alcohol. Animals were kept at this stage until mean responding again stabilized (around 1 week; Fig. 1a), and saccharin concentrations were gradually reduced in ~50% successive steps over 2-10 days, ultimately leaving an unsweetened 10% w/v alcohol solution. Animals then were maintained on 10% w/v alcohol for at least 3 weeks, and stable responders ($\pm 25\%$ across three consecutive sessions and averaging >10 presses for alcohol) were evenly divided into two groups matched for baseline responding and exposed to intermittent alcohol vapors (alcohol dependent) or air (nondependent alcohol self-administering) as described below.

Dependence induction by alcohol vapor chambers

A recent modification of the alcohol dependence model was made to reflect the natural progression of alcohol dependence in which alcohol exposure occurs in a series of extended exposures followed by periods of withdrawal (O'Dell et al., 2004). Chronic exposure to intermittent alcohol vapor exposure elicits even higher alcohol self-administration than continuous vapor (O'Dell et al., 2004), and the intermittent procedure therefore was used to induce dependence in trained animals in the present study. Vapors were delivered on a 14 h on/10 h off schedule for 4 weeks before post-vapor testing began. This schedule of exposure has been shown to induce physical dependence (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 125-250 mg% (g/dL) or 27.2-54.4 mM (Gilpin et al., 2008a).

After 4 weeks of vapor exposure, post-vapor alcohol self-administration testing was conducted twice per week during acute withdrawal (2-4 h after cessation of daily vapor exposure) to confirm in alcohol dependent animals that alcohol self-administration increased compared with pre-vapor levels. Notably, excessive drinking in dependent animals results in a rapid elevation in blood alcohol levels exceeding 80 mg% ("binge drinking," as defined by NIAAA) within 15 min and peaking around 100 - 150 mg% during the 30-min self-administration session, whereas nondependent drinking produces blood alcohol levels that rarely exceed 50 mg% (Richardson et al., 2008).

Measurement of blood alcohol levels

Blood sampling (tail bleedings) was performed immediately after daily bouts of alcohol vapor exposure in dependent animals. Blood samples were also obtained from nondependent animals (exposed to control air) at the same time to control for handling during blood sampling. Plasma (5 μ L) 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%), vapors levels were adjusted. Blood alcohol levels were always undetectable in nondependent animals (air exposure only).

Bromodeoxyuridine injection

Bromodeoxyuridine (BrdU) was administered to a subset of rats to quantify survival of proliferating cells in nondependent and alcohol dependent self-administering rats. After 6 weeks of exposure to alcohol vapors (dependent) or control air (nondependent), 4 dependent and 6 nondependent rats were selected randomly and administered one injection of 150 mg/kg BrdU, i.p. (Boehringer Mannheim; dissolved in 0.9% saline and 0.007N NaOH at 20 mg/ml), at the same time into acute withdrawal (2–4 h) at which time they were normally tested for operant responding. Four alcohol-naïve rats received BrdU injections also at this time. “Prolonged nondependent” ($n = 6$) and “prolonged dependent” ($n = 4$) rats were not tested for post-air/vapor alcohol self-administration after the BrdU injection, but both groups were returned to their chronic treatment environment (i.e., intermittent alcohol vapor exposure for dependent animals or air control for nondependent animals) for 28 days until perfusion.

Tissue preparation

Naïve rats with ($n = 4$, Set II) or without BrdU ($n = 5$, Set I), naïve trained ($n = 3$, Set I), nondependent ($n = 5$, Set I), dependent ($n = 4$, Set I), prolonged nondependent ($n = 6$, Set II), and prolonged dependent rats ($n = 4$, Set II) were anesthetized with chloral hydrate and perfused transcardially as described previously (Mandyam et al., 2004). Serial coronal 40 μ m sections were obtained on a freezing microtome, and sections from the mPFC (bregma 3.7 to 2.2) and hippocampus (bregma –1.4 to –6.7; (Paxinos and Watson, 1997) were stored in 0.1% Na₂S₂O₃ in 1X PBS at 4°C.

Antibodies and immunohistochemistry

The following primary antibodies were used for immunohistochemistry (IHC): Rabbit monoclonal anti-Ki-67 (1:1000; LabVision), mouse anti-BrdU (1:100; Becton Dickinson), goat polyclonal anti-DCX (1:700; Santa Cruz Biotechnology), and rabbit polyclonal anti-activated caspase-3 (AC-3; 1:500; Cell Signaling Technology). The left and right hemisphere of every ninth section through the mPFC and hippocampus were slide-mounted and dried overnight prior to IHC. Slides were coded prior to IHC, and the code was not broken until after analysis was complete. All incubations were performed at room temperature unless otherwise indicated. Slide-mounted sections were subjected to pretreatment steps as described previously (Mandyam et al., 2004). Slides were incubated with 0.3% H₂O₂ for 30 min to remove any endogenous peroxidase activity. Nonspecific binding was blocked with 5% serum and 0.5% Tween-20 in 0.1M PBS for 60 min and incubated with the primary antibody (in 5% serum and 0.5% Tween-20) for 18–20 h. After washing with 0.1M PBS, the sections were exposed to biotinylated secondary IgG for 1 h (1:200; Vector Laboratories). After secondary antibody incubation, slides were incubated in ABC for 1 h (Vector Laboratories), and staining was visualized with 3,3-diaminobenzidine (DAB; Pierce Laboratories). Sections were counterstained with Fast Red (Vector Laboratories). Omission or dilution of the primary antibody resulted in lack of specific staining, thus serving as a negative control for antibody experiments.

Microscopic analysis and quantification

Cells in the mPFC (i.e., Ki-67-, AC-3-, and BrdU-immunoreactive [IR] cells in the cingulate, infralimbic, and prelimbic cortices) and SGZ (Ki-67-, doublecortin [DCX]-, AC-3-, and BrdU-IR cells touching and within three cell widths inside and outside the hippocampal granule cell-

hilus border) were quantified with a Zeiss Axiophot photomicroscope (400×) using the optical fractionator method. Cells from each bregma region were summed and multiplied by 9 to give the total number of cells.

Detailed quantification was performed for DCX-IR cells. DCX is a marker for young neurons, and the developmental stages of young neurons can be further delineated using morphological analysis. Early-phase DCX-IR cells represent a mixed population of proliferating late progenitors, transiently amplifying neuroblasts, and post-mitotic migrating cells that become immature neurons (Mandyam et al., 2008b). The late-phase DCX-IR cells, however, represent differentiating mature cells that co-label with mature neuronal markers such as NeuN (Kuhn et al., 2005; Mandyam et al., 2008b). Specifically early phase (immature) DCX-IR cells can be differentiated from the late phase (mature) DCX-IR cell types, with early phase cells having short processes and late phase cells having long processes that extend into the molecular layer of the dentate gyrus (Brown et al., 2003a; Brown et al., 2003b; Couillard-Despres et al., 2005; Mandyam et al., 2008a; Plumpe et al., 2006; Rao and Shetty, 2004; Serio et al., 1996). The early phase and late phase DCX-IR cells were quantified separately.

For BrdU phenotype analysis, every 27th section through the hippocampus was triple-labeled with BrdU (CY3), NeuN (CY5), and GFAP (CY2). All BrdU-IR cells in the SGZ, approximately 20 (naïve: 27 ± 3 ; nondependent: 20 ± 0.7 ; dependent: 18 ± 2) BrdU-IR cells from each rat were scanned and analyzed for BrdU/NeuN, BrdU/GFAP or BrdU alone labeling. All labeling was visualized and analyzed using a confocal microscope (LaserSharp 2000, version 5.2, emission wavelengths 488, 568, and 647 nm; Bio-Rad Laboratories). The percent of BrdU-IR cells that were NeuN positive or GFAP positive or GFAP/NeuN negative in relation to the total number of BrdU cells were analyzed from each rat. For calculated phenotype analyses the ratio of BrdU-IR cells that were BrdU/NeuN positive and BrdU/GFAP positive in relation to the total number of BrdU cells were analyzed from each rat. The values for mature neurons, mature glia and other phenotype were computed by multiplying the ratio of double-labeled-IR cells and total number of cells from each rat. All microscopic quantification and analysis were made by an observer blind to the study.

Fluoro-Jade C (FJC) staining

FJC staining was performed as previously described (Schmued et al., 2005). Every 18th section through the hippocampus was slide mounted and air dried prior to FJC treatment. Slides were first incubated with alcohol-sodium hydroxide mixture (solution containing 1% sodium hydroxide in 80% alcohol) for 5 min followed by 2 min washes with 70% alcohol and distilled water. Second, sections were incubated with potassium permanganate (0.06% potassium permanganate) for 10 min and rinsed in distilled water for 2 min. Lastly, sections were incubated with FJC (0.0001% solution of FJC dye, Millipore) combined with 4',6-diamidino-2-phenylindole (DAPI; 0.0001% solution, Roche) dissolved in 0.1% acetic acid for 10 min followed by three distilled water washes. The slides were air dried and coverslipped. FJC-immunoreactive cells in the granule cell layer of the hippocampus were visualized and quantified under confocal microscope. Total number of cells from each rat were multiplied by 18 and are reported as total number of degenerating cells per rat.

Measurement of hippocampal granule cell number

Quantitative analysis to obtain unbiased estimates of the total number of granule cells was performed on a Zeiss Axiophot Microscope equipped with MicroBrightField Stereo Investigator software (MicroBrightField), a three-axis Mac 5000 motorized stage (Ludl Electronics Products), a digital charge-coupled device ZVS video camera (Zeiss), a PCI color frame grabber, and computer workstation. All contours were drawn at low magnification using a Zeiss Plan-Apochromat 10× objective N/A 0.32, and the contours were realigned at high

magnification using a Zeiss Plan Apochromat 63× oil objective N/A 1.4. The total number of granule cells was calculated by an unbiased stereological estimation in which the average density of the granule cells was multiplied by total volume of the granule cell layer of the hippocampal dentate gyrus (Donovan et al., 2006). Every 18th section (eight sections from each rat; cut section thickness 40 μm; measured section thickness 21 μm) through the dentate gyrus of the hippocampus counterstained with Nuclear Fast Red was saved in strict anatomical order for quantitative analysis. Granule cell layer volume was estimated using StereoInvestigator software that employs the Cavalieri method (West and Andersen, 1980; West et al., 1991). The average density of granule cells was found by examinations from all portions of the granule cell layer. Over 400 cells were counted at 630× with an oil objective (N/A 1.4), a 10 μm × 10 μm × 2 μm counting grid, and a 2 μm top and bottom guard zone. The total number of cells was determined by multiplying the average density of cells (cells/μm³) by the total volume of the granule cell layer (West et al., 1991). Granule cell layer volume and cell number estimates were made by an observer blind to the study.

Data analysis

Alcohol self-administration data and Ki-67, DCX, AC-3, BrdU and FJC data comparing nondependent alcohol self-administering and alcohol dependent groups were analyzed by non-matching or one-way analysis of variance (ANOVA), phenotypic analysis were analyzed by two-way ANOVA using GraphPad Prism 5. All analyses were followed by Bonferroni's or Tukey's *post hoc* tests. Values of $p < 0.05$ were considered statistically significant.

Results

Alcohol self-administration behavior: nondependent alcohol self-administration and excessive alcohol self-administration during alcohol dependence (termed henceforth as nondependent drinking and alcohol dependence)

The experimental timeline of operant training, dependence induction, BrdU injection, and perfusion are shown in Fig. 1a. Two sets of two groups of alcohol exposure were used: **Set I**, nondependent alcohol self-administering and alcohol-dependent animals perfused 2–4 h after the last air/alcohol vapor exposure; **Set II**, prolonged nondependent and prolonged dependent rats received one injection of BrdU and were continued on air/alcohol vapor exposure for 28 days beyond Set I perfusion date to measure BrdU survival, and were perfused 2–4 h after air/alcohol vapor exposure (Fig. 1a). The animals from the two nondependent groups did not differ in alcohol intake and self-administration behavior during baseline training (i.e., prior to air control). The animals from the two dependent groups did not differ in alcohol intake and self-administration behavior during baseline training (prior to alcohol vapors). Chronic intermittent exposure to alcohol vapors elicited increased alcohol self-administration in both groups of alcohol-dependent animals compared to baseline alcohol self-administration and compared to nondependent animals (post-vapor behavioral data was averaged from self-administration sessions over 5 days before day 77 Set I or Set II). Specifically, alcohol intake (g/kg) was increased post-vapor in dependent animals compared to alcohol intake (g/kg) post-air control in nondependent animals (Set I: $F_{3,20} = 5.4$, $p = 0.008$; Fig. 1b; Set II: $F_{3,19} = 5.9$, $p = 0.006$; Fig. 1d). Furthermore, dependent animals had increased lever responding for alcohol post-vapor compared to pre-vapor (baseline) and compared to lever responding for alcohol in nondependent animals post-air control (post-vapor lever presses for alcohol, average day 70–75, Set I: $F_{3,15} = 5.6$, $p = 0.01$; Fig. 1c; Set II: $F_{3,19} = 11.4$, $p = 0.0003$; Fig. 1e). Alcohol vapor chambers were set to elicit blood alcohol levels averaging 150–200 mg% in the dependent groups. As expected, nondependent groups (exposed only to air) had no reliable blood alcohol levels: (dependent: (Set I: 193.2 ± 17.7 mg%; Set II: 159.7 ± 9.7 mg%; $p = 0.14$) versus nondependent: Set I and II combined, 10 ± 0.5 mg%, $F = 227.6$, $p < 0.0001$). Previous work shows the temporal profile of elevations in blood alcohol levels *during* self-administration

sessions, in which nondependent animals rarely elevate blood alcohol levels above 50 mg%, where as dependent animals rapidly increase blood alcohol levels 2-3 fold that of nondependent animals (often reaching blood alcohol levels as high as 100-150 mg% during a 30 min session (Richardson et al., 2008).

Alcohol dependence alters apoptosis, proliferation and survival in the mPFC

Sections comprising mPFC from the naïve, naïve-trained, nondependent and alcohol-dependent animals (Set I, brain tissue collected on day 77) were processed and examined for changes in cell death (apoptosis; AC-3-IR cells, Fig 2b), proliferation (Ki-67-IR cells (Bacchi and Gown, 1993), Fig 2a), and survival (Set II, BrdU injected on day 77 and brain tissue collected on day 96; BrdU-IR cells, Fig 2c). Four bilateral sections (bregma 3.7-2.2, Fig 3a) from each rat were used for cell quantification analysis. There were approximately 0-3 AC-3-IR cells, 50-60 Ki-67-IR cells, and 25-30 BrdU-IR cells in each bilateral brain section analyzed from control rats. Training for the sweetened solution fading procedure did not alter apoptosis. Nondependent drinking and alcohol-dependent animals showed a significant decrease in apoptosis compared with drug-naïve and trained controls ($F_{3,17} = 9.26$, $p < 0.01$, Fig 3b). Training for the sweetened solution fading procedure did not alter proliferation. Nondependent drinking did not produce a significant change in proliferation compared with drug-naïve and trained controls ($p > 0.05$), whereas alcohol dependence decreased proliferation compared with drug-naïve and trained controls ($F_{3,17} = 16.71$, $p < 0.001$, Fig 3c) and nondependent animals ($F_{3,17} = 16.71$, $p < 0.05$, Fig 3c). Alcohol dependence also decreased survival in the mPFC ($F_{2,14} = 6.5$, $p < 0.05$, Fig. 3d). We next evaluated the effects of prolonged nondependent and dependent environment on proliferation in the mPFC (Set II, brain tissue collected on day 96). Surprisingly, although decreased proliferation was observed in dependent animals (Fig. 3c) it was not in the prolonged dependent group ($F_{2,14} = 2.6$, $p = 0.11$, Fig. 3e).

Nondependent drinking and alcohol dependence alter cell death, proliferation, immature neurons, survival and neurogenesis in the hippocampal SGZ

Hippocampal sections from naïve, naïve-trained, nondependent and alcohol dependent animals (Set I, brain tissue collected on day 77) were processed and examined for changes in cell death – apoptosis (AC-3-IR; Fig. 2f) and neuronal degeneration (Fluoro-Jade C-IR; Fig. 2g), cell proliferation (Ki-67-IR; Fig. 2d), immature neurons (DCX-IR; Fig. 2e), survival (Set II, injected with BrdU on day 77 and brain tissue collected on day 96; BrdU; Fig. 2h), neurogenesis (BrdU/NeuN Fig. 2i) and gliogenesis (BrdU/GFAP Fig 2p). Training for the sweetened solution fading procedure did not alter cell death (apoptosis and neuronal degeneration), proliferation and immature neurons.

Cell death was analyzed by probing for two markers, AC-3 to measure apoptotic cell death and Fluoro-Jade C to measure neuronal degeneration. Neuronal degeneration was not observed in the naïve and trained controls. Alcohol-dependent animals showed increased neuronal degeneration in the granule cell layer compared to naïve and trained controls, and nondependent animals ($F_{3,17} = 19.9$, $p < 0.001$, Fig. 4a). Nondependent animals showed increased apoptosis compared with controls and alcohol-dependent animals ($F_{3,17} = 8.5$, $p = 0.002$, Fig. 4b), while apoptosis was not changed in alcohol-dependent animals compared with controls ($p > 0.05$, Fig. 4b).

Nondependent drinking and alcohol dependence significantly altered SGZ cell proliferation (effect of alcohol, $F_{3,17} = 58.38$, $p < 0.0001$, Fig. 4c). Nondependent drinking animals showed reduced proliferation to the same extent as alcohol dependent animals ($p < 0.001$, Fig. 4c) compared with drug-naïve and trained controls. DCX-IR cells were quantified to assess changes in young immature neurons. An interaction was observed between alcohol intake and immature neurons (early and late phase DCX cells, $F_{3,17} = 4.3$, $p = 0.017$, Fig. 4d), with

decreases in early-phase DCX-IR cells induced by alcohol. Nondependent drinking and alcohol dependent animals showed a significant decrease in only early-phase DCX-IR cells compared with naïve and trained controls (early phase cells, $p < 0.05$, Fig. 4d). Late-phase DCX-IR cells were unchanged in all groups ($p > 0.05$, Fig. 4d).

Both nondependent and dependent groups show significant reductions in BrdU cell number (survival, $F_{2,14} = 16.9$, $p < 0.01$, Fig. 4e) compared with naïve control. Alcohol dependence decreased percent of cells that were NeuN positive (neurogenesis, $F_{2,27} = 72.17$, $p < 0.01$, Fig. 4f) compared with nondependent drinking and control conditions. Mature neurons were calculated by applying the percentage of BrdU cells that colabeled with NeuN to the actual BrdU cell counts. Nondependent drinking animals showed reduced mature neurons compared to naïve controls, and alcohol dependence showed an even greater decrease, significantly different from nondependent and naïve groups ($F_{2,27} = 72.17$, $p < 0.01$, Fig. 4g).

Prolonged exposure to nondependent and alcohol dependent environment differentially alters proliferation, immature neurons and granule cell neurons in the hippocampal SGZ

Sections from naïve, prolonged nondependent, and prolonged dependent animals (Set II, brain tissue collected on day 96) were processed for Ki-67 and DCX IHC to quantify proliferation and immature neurons. Proliferation was robustly decreased by prolonged nondependent and dependent conditions ($F_{2,14} = 63.8$, $p < 0.001$, Fig 5a) compared with controls. Notably, alcohol-induced reduction in proliferation in Set II animals were nearly identical to those in Set I animals (Fig. 4c vs. 5a) despite 28 days of no further access to alcohol self-administration and continued exposure to air control (prolonged nondependent animals) or alcohol vapors (prolonged dependent animals). Likewise, alcohol-induced reduction in the total number of DCX-IR cells of prolonged nondependent animals of Set II was similar to nondependent animals of Set I compared to naïve controls (Fig. 4d vs. 5b), thus showing no signs of reversal after cessation of alcohol. On the other hand, prolonged dependence showed a greater decrease in total DCX-IR cells compared to nondependent drinking ($F_{2,33} = 27.02$, $p < 0.01$, Fig. 5b), the decrease in total number of DCX-IR cells was due to decreases in late phase cells ($F_{2,33} = 27.02$, $p < 0.01$, Fig. 5b), which indicates that prolonged exposure to alcohol vapors further attenuated the immature neuron population. Prolonged environment of nondependent drinking and alcohol dependence decreased hippocampal granule cell neurons ($F_{2,14} = 4.9$, $p = 0.02$, Fig. 5c).

Discussion

Animal models of nondependent drinking and excessive drinking during alcohol dependence are useful in modeling distinct patterns of alcohol use in humans that range from casual drinking to alcoholism. The present study shows that chronic drinking alters adult neurogenesis in the hippocampus *prior* to dependence— suggesting that alcohol-induced changes in neurogenesis may proceed and possibly cause the neurodegeneration and hippocampal deficits associated with chronic alcoholism. Notably, alcohol dependence reduces proliferative capacity in the prefrontal cortex and hippocampus with distinct underlying mechanisms specific to each brain region. For example, nondependent drinking and alcohol dependence reduced cortical apoptosis, which might serve to compensate for the toxic effects of alcohol on existing neurons, as suggested in a recent report showing down-regulation of apoptotic pathways in alcoholics (Johansson et al., 2009). Alcohol dependence produced no change in hippocampal apoptosis but increased neuronal degeneration, indicating that the later stages of chronic high alcohol use and dependence are characterized by increased cell death via non-apoptotic pathways, decreasing neuronal turnover in the hippocampus. Such regulation by alcohol dependence in the two brain regions suggests that proliferating cells from each region may play critical roles in the consequences of long-term exposure to alcohol associated with dependence.

The effects of alcohol on proliferation of cortical precursors has been studied *in vitro*, in which alcohol decreased proliferation of cultured neocortical neurons (Jacobs and Miller, 2001). The present study demonstrates the dynamic regulation of adult mPFC proliferative capacity and underscores the specific influence that alcohol dependence has on cell birth, survival, and death *in vivo*. Alcohol dependence reduced proliferation and survival in the mPFC, suggesting that the level of alcohol exposure was critical for producing changes in the gliogenic integrity of the mPFC. Decreased survival of mPFC cells may be due to decreases in S phase cells during BrdU incorporation or decreased maturation and survival of S phase cells with chronic alcohol exposure. Ki-67 analysis suggests that the former mechanism is true, albeit further studies incorporating a time course of BrdU labeling are needed to detect the latter mechanism. Additional labeling studies with phenotypic makers for immature neurons, mature neurons, astrocytes and oligodendrocytes in the mPFC will also allow us to delineate specific effects of alcohol dependence on the phenotype of surviving cortical precursors. Nondependent drinking showed a trend towards decrease in proliferation with no effect on survival. It is possible that larger sample sizes would allow for the detection of reduced Ki-67 cells in the mPFC of nondependent animals to suggest that moderate alcohol exposure also impacts proliferation. Both nondependent drinking and alcohol dependence *reduced* apoptosis, perhaps indicating compensation of the mPFC gliogenic niche and predicting the eventual neurotoxic response to chronic alcohol after extended intake. This hypothesis is supported by the opposing effects of prolonged alcohol dependence on proliferation. For example, early dependence (6 weeks of vapors) reduced proliferation and the reduction was normalized after 4 weeks of further exposure to alcohol vapors. The normalization of proliferation after prolonged dependence suggests that compensatory mechanisms (e.g., further decreases in cell death) may be enhanced during the additional 4 weeks of exposure to intermittent alcohol vapors to reverse the negative effects of alcohol on proliferation. An alternative explanation is that the effects of alcohol on proliferation during dependence may be specific to high levels of voluntary oral self-administration of alcohol (rather than passive exposure to alcohol vapors), such that 4 weeks of abstinence from alcohol self-administration rescued proliferation in the prolonged dependent group. Whether the newly generated proliferating cells after prolonged dependence eventually mature to attain a phenotype is questionable and warrants detailed investigation.

Several alcohol models have been employed to study the effect of alcohol exposure on hippocampal proliferation. Notably, there are discrepancies on alcohol-induced effects on proliferation (Nixon, 2006), and they may be attributable to differences in alcohol delivery methods, blood alcohol levels, withdrawal time after the last exposure to alcohol prior to tissue collection, or the use of endogenous vs. exogenous markers for cell quantification. The present study utilized a clinically relevant model of dependence for adult alcoholism producing high blood alcohol levels and an endogenous marker (Ki-67) to label and quantify proliferation. Both nondependent drinking and alcohol dependence equally reduced proliferation. A lack of difference in cell proliferation between the nondependent and dependent groups could be explained by the extended exposure to alcohol self-administration in both groups, and that moderate, albeit nondependent, alcohol intake produces robust effects on hippocampal proliferation (data herein; Ieraci and Herrera, 2007; Nixon and Crews, 2002). An alternate explanation could be that the cell cycle parameters of proliferating progenitors in the dependent group are altered. For example, although Ki-67's expression is tightly regulated and corresponds to cell proliferation (Dayer et al., 2003; Mandyam et al., 2007), some proliferating cells frozen in certain parts of the cell cycle, such as G₁ (Scholzen and Gerdes, 2000) could still express the protein, suggesting that Ki-67 labeling may over-estimate proliferating cells actively progressing through the cell cycle. Because we did not observe quantitative differences in Ki-67-IR cells in the alcohol dependent group versus nondependent group, one might speculate that some Ki-67-IR cells in the dependent group may be representing the 'trapped' population, which inflates the number of 'proliferating' cells observed. Additional markers would be needed to test this hypothesis. Nonetheless, it appears that exposure to even modest

amounts of alcohol may cause long-term changes in the hippocampal proliferative environment, resulting in reduced neurogenic capacity in the hippocampus.

The next two stages of hippocampal neural stem cell development, cell migration and differentiation, were visualized and quantified using the endogenous marker DCX. As previously shown with experimenter-delivered alcohol exposure paradigms (He et al., 2005), nondependent drinking and alcohol dependence decreased total DCX-IR cells. Additional morphological analysis in the present study assessed the specific affects of alcohol on DCX-IR cell types (early phase and late phase). Both nondependent and dependent conditions were associated with reductions in early-phase DCX-IR cells in the hippocampal SGZ. As such, alcohol exposure in the present study did not attenuate the number of late-phase DCX-IR cells, although chronic forced exposure to alcohol has been shown to impair dendritic outgrowth of late-phase DCX-IR cells (He et al., 2005), suggesting morphological marring of late-phase cells. Combining independent results from Ki-67 and DCX analysis, initial chronic alcohol exposure appeared to preferentially hinder the proliferation of hippocampal progenitors, and dependence-induced decreases in cell birth and *not* cell maturation may contribute to the neuronal loss seen in alcoholism.

The last stage of hippocampal neurogenesis, cell survival and maturation was visualized by injecting BrdU to label a pulse of proliferating S phase cells after which, the rats were continued in their alcohol paradigms for 4 weeks (air/vapor exposure) without further self-administration. Nondependent drinking and alcohol dependence decreased survival and neurogenesis of SGZ progenitors, and alcohol dependence reduced neurogenesis to a greater extent compared to nondependent drinking. The altered ratio in the phenotype of surviving cells after dependence suggests greater and perhaps permanent impairment of hippocampal neurogenesis due to the higher amount of alcohol exposure during dependence and long-lasting injury to the hippocampal neurogenic environment. Thus, to evaluate the effects of additional 4 weeks of continued alcohol vapor exposure on SGZ progenitors, proliferation and immature neurons were quantified from the prolonged nondependent and alcohol dependent rats. Notably, proliferation was not further decreased after prolonged dependence, suggesting that minimal levels (threshold) of proliferation is persistent in the hippocampal SGZ, and that continued chronic alcohol exposure does not disrupt this threshold of proliferation. Note that immature neurons were further reduced only in the dependent group suggesting that dependence-induced decreases in neurogenesis were due to more robust effects on differentiation and maturation of hippocampal progenitors. Thus it appears that progression of dependence targets distinct population of progenitors, where proliferation is initially altered, followed by alteration in differentiation and maturation of progenitors, ultimately leading to decreased hippocampal neurogenesis.

Reduced hippocampal neurogenesis with alcohol self-administration and dependence may promote hippocampal neuronal loss through multiple mechanisms. Studies using forced alcohol paradigms to investigate the effect of alcohol on neural plasticity have suggested that alcohol reduces proliferation and neurogenesis in the adult hippocampus through increased cell death (He et al., 2005; Herrera et al., 2003; Obernier et al., 2002). Active programmed cell death (apoptosis) and neuronal degeneration were analyzed in the present study. Nondependent drinking increased hippocampal apoptosis. Apoptotic cell death was not observed in dependent animals but increased neuronal degeneration was evident, suggesting that cells may be dying in dependent animals through other cell death pathways. Long-term exposure to moderate to high alcohol may produce cell death via apoptosis and necrosis (Obernier et al., 2002), which could underlie decreased granule cell number and eventual decreases in hippocampal volume. Perhaps, nondependent drinking first initiates programmed cell death, which is then followed by passive non-programmed degeneration of neurons with increasing alcohol exposure during dependence.

Taken together, the cellular alterations produced by both moderate (nondependent) and excessive (dependent) alcohol use may be extended into other behavioral effects of alcohol addiction. For example, human alcoholics are known to suffer from hippocampal-dependent cognitive deficits, including impulsivity and deficits in spatial learning, short-term memory, and executive function (Uekermann et al., 2003), possibly reflecting chronic alcohol-induced decreased hippocampal neurogenesis. Although we do not provide a direct link to demonstrate that hippocampal neurogenesis is a vulnerability factor for alcohol dependence (Meshi et al., 2006), we demonstrate that altered plasticity in the mPFC and SGZ produces neuroadaptations in the PFC and hippocampus that may contribute to increased alcohol-drinking or may perpetuate excessive alcohol drinking.

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Figure 1. Alcohol self-administration: timeline of experimental procedure and behavior responses
 (a) Time-line of alcohol self-administration by the sweetened solution fading procedure indicated in days (SS, sweetened solution; Alc, alcohol; BrdU, bromodeoxyuridine). Alcohol intake (g/kg, **b**) and operant responses (**c**) from Set I rats before and after chronic exposure to control air (nondependent rats) or 6 weeks of alcohol vapors (dependent rats; bars reflect self-administration averages from the last 4-5 sessions before day 77). Alcohol intake (g/kg, **d**) and operant responses (**e**) from Set II rats before and after chronic exposure to control air (prolonged nondependent animals) or 10 weeks of alcohol vapors (prolonged dependent animals; bars reflect self-administration averages from the last 4-5 sessions before day 77). Data are expressed as mean $\pm$ SEM. Set I, $n = 3-5$; Set II, $n=4-6$ per group. * $p < 0.05$ compared with

nondependent group by one-way ANOVA. $^{\#}p < 0.05$ compared with dependent pre-vapor period by paired t -test.

Figure 2. Qualitative representative images from the mPFC and SGZ

(a-c, mPFC) Representative images of Ki-67-IR cells in the infralimbic cortex (a) and AC-3-IR cell (b) and BrdU-IR cell (c) in the cingulate cortex from one naïve control rat. Arrowhead in (a-c) point to the immunoreactive cells. Ki-67-IR cells were almost always seen in pairs. Scale bar in (b) is 20 μm applies to a-c.

(d-p, SGZ) Representative images of Ki-67-IR cells (d), DCX-IR cells (e), and AC-3-IR cells (f) in the SGZ from one control drug naïve animal, and Fluoro-Jade C-IR cells (g) in the granule cell layer from one dependent animal, (h) BrdU-IR cells, (i-p) fluorescent triple labeled BrdU-IR cells (BrdU/NeuN-IR neuron, i-l; BrdU/GFAP-IR astrocyte, m-p) in the SGZ from one control drug naïve animal. Fluorescent BrdU is labeled with CY3 (red), NeuN is labeled with CY5 (blue) and GFAP is labeled with CY2 (green). Scale bar in (d) is 20 μm applies to (d, f,

g), **(e)** is 50 μm (**h**) is 20 μm and in **(p)** is 10 μm applies **(i-p)**. Arrowhead in **(d)** points to a Ki-67-IR cell, **(e)** late phase DCX-IR cell, **(f)** AC-3-IR cell, **(g)** fluorescently labeled Fluoro-Jade C-IR cell in FITC (green), **(h)** BrdU-IR cell **(i-l)** BrdU/NeuN-IR cell, and **(m-p)** BrdU/GFAP-IR cell. Arrow in **(e)** points to early phase DCX-IR cell. Orientation of the dentate gyrus is indicated by hilus (H), molecular layer (Mol), and granule cell layer (GCL).

Figure 3. Alcohol dependence decreases mPFC cell death, proliferation, and survival

(a) Schematic of bregma regions and the area of prefrontal cortex included for quantification. Area shaded in gray included the cingulate cortex (Cg), prelimbic cortex (PrL), and infralimbic cortex (IL). (b-c) Rats from Set I were used for this analysis. Quantitative analysis of total number of apoptotic cells (activated caspase-3-IR, **b**) and Ki-67-IR proliferating cells (**c**), in the mPFC. (d-e) Rats from Set II were used for this analysis. Quantitative analysis of total number of BrdU-IR mature cells (**d**), and Ki-67-IR proliferating cells (**e**) in the mPFC. Data are expressed as mean $\pm$ SEM. Set I, $n = 3-5$ per group; Set II $n = 4-6$ per group. * $p < 0.05$ compared with naive controls. # $p < 0.05$ compared with trained controls. ^a $p < 0.05$ compared with nondependent group by one-way ANOVA.

Figure 4. Nondependent drinking and alcohol dependence increase cell death and decrease proliferation, immature neurons, survival and neurogenesis in the hippocampal subgranular zone (a-d) Rats from Set I were used for this analysis. Quantitative analysis of cell counts of (a) cell death neuronal degeneration (Fluoro-Jade C-IR cells), (b) apoptosis (AC-3-IR cells), (c) proliferation (Ki-67-IR cells), (d) total immature neurons (early and late phase cells). (e-g) Rats from Set II were used for this analysis. (e) Total number of BrdU-IR cells. (f) Phenotypic ratio of BrdU-IR cells that are NeuN positive neurons, GFAP positive astrocytes or other phenotype in the hippocampal granule cell layer. (g) Calculated number of neurons, astrocytes or other phenotypes in the hippocampal granule cell layer. Data are expressed as mean $\pm$ SEM. $n = 3-6$ per group. * $p < 0.05$ compared with naive controls, $^{\#}p < 0.05$ compared with trained controls, $^ap < 0.05$ compared with nondependent rats, $^bp < 0.05$ compared with dependent rats, one-way ANOVA.

Figure 5. Prolonged nondependent and alcohol-dependent environment decreases proliferation, immature neurons and granule cell neurons in the hippocampus

Rats from Set II were used for this analysis. (a-c) Quantitative analysis of total number of Ki-67-IR (a), DCX-IR (b) cells in the SGZ and granule cell neurons in the GCL (c). Data are expressed as mean ± SEM. $n = 4-6$ per group. * $p < 0.05$ compared with naive control. ^a $p < 0.05$ compared with nondependent rats, one-or two-way ANOVA.