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Activation of neural stem cells from quiescence drives reactive hippocampal neurogenesis after alcohol dependence

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Abstract

Neural stem cell-driven adult neurogenesis contributes to the integrity of the hippocampus. Excessive alcohol consumption in alcoholism results in hippocampal degeneration that may recover with abstinence. Reactive, increased adult neurogenesis during abstinence following alcohol dependence may contribute to recovery, but the mechanism driving reactive neurogenesis is not known. Therefore, adult, male rats were exposed to alcohol for four days and various markers were used to examine cell cycle dynamics, the percentage and number of neural progenitor cell subtypes, and the percentage of quiescent versus activated progenitors. Using a screen for cell cycle perturbation, we showed that the cell cycle is not likely altered at 7 days in abstinence. As the vast majority of Bromodeoxyuridine-positive (+) cells were co-labeled with progenitor cell marker, Sox2, we then developed a quadruple fluorescent labeling scheme to examine Type-1, -2a, -2b and -3 progenitor cells simultaneously. Prior alcohol dependence indiscriminately increased all subtypes at 7 days, the peak of the reactive proliferation. An evaluation of the time course of reactive cell proliferation revealed that cells begin proliferating at 5 days post alcohol, where only actively dividing Type 2 progenitors were increased by alcohol. Furthermore, prior alcohol increased the percentage of actively dividing Sox2+ progenitors, which supported that reactive neurogenesis is likely due to the activation of progenitors out of quiescence. These observations were associated with granule cell number returning to normal at 28 days. Therefore, activating stem and progenitor cells out of quiescence may be the mechanism underlying hippocampal recovery in abstinence following alcohol dependence.

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alcoholism; ethanol; adult neurogenesis; hippocampus; progenitor cell; neurodegeneration

1. INTRODUCTION

Excessive consumption of alcohol, the defining characteristic of an alcohol use disorder (AUD), results in hippocampal neurodegeneration that may recover in abstinence (Bartels et al., 2007; Beresford et al., 2006; Carlen et al., 1978; Ozsoy et al., 2013; Riley and Walker, 1978; Sullivan et al., 1995). Besides the hippocampus' canonical role in context-dependent memory (Hyman et al., 2006), hippocampal degeneration impacts a variety of neural circuits involved in the development and progression of AUDs through its projections to: a) brain stress systems, including the amygdala (Belujon and Grace, 2011; Mandyam, 2013), b) behavioral control and decision-making centers such as the prefrontal cortices (Godsil et al., 2013) and c) drug seeking and self-administration control regions such as the nucleus accumbens (Belujon and Grace, 2008; Noonan et al., 2010; Vorel et al., 2001). Indeed, hippocampal structural integrity correlates with likelihood of relapse, further supporting its role in AUDs (Chanraud et al., 2007; Mandyam and Koob, 2012; Prendergast and Mulholland, 2012). Therefore, elucidating the mechanisms underlying the maintenance of hippocampal integrity are critical for understanding the neurobiology of the development of AUDs.

In the hippocampus, neurogenesis continues throughout the lifespan (Altman, 1969), maintaining hippocampal integrity and therefore hippocampal function (Clelland et al., 2009; Goncalves et al., 2016; Imayoshi et al., 2008). Newborn granule cells derive from a population of neural stem cells (NSCs) that asymmetrically divide into progenitor cells that then differentiate and mature into granule cells (Bonaguidi et al., 2011; Palmer et al., 1997). These stem and progenitor cells differ in rates of proliferation and their proliferative potential such that different subtypes of progenitors have been described (Kempermann et al., 2004). Thus, the precursors that drive adult neurogenesis are a heterogeneous population of cells with a similarly heterogeneous response to drugs, environment, and insult (Bonaguidi et al., 2011; Kronenberg et al., 2003; Kunze et al., 2006; Lugert et al., 2010).

Dysregulation of adult hippocampal neurogenesis plays roles in psychiatric disorders such as alcohol and drug abuse (Deschaux et al., 2014; Galinato et al., 2017; Mandyam, 2013; Mandyam and Koob, 2012; Nixon, 2006; Nixon and Crews, 2002, 2004; Noonan et al., 2010; Yun et al., 2016). Intoxicating doses of alcohol as one would experience in an AUD reduce adult neurogenesis by inhibiting neural stem cell proliferation (Contet et al., 2013; Crews et al., 2006; Ehlers et al., 2013; Gomez et al., 2015; Sakharkar et al., 2016; see also Olsufka et al., 2017 for review), whereas multiple days of exposure appear necessary to also impact new cell survival (Broadwater et al., 2014; Golub et al., 2015; He et al., 2005; Herrera et al., 2003; Nixon and Crews, 2002; Richardson et al., 2009). Specifically, more chronic exposures or chronic intermittent exposures that mimic an AUD may have a long-term impact on the number of proliferating progenitors and therefore permanently reduce adult neurogenesis (Ehlers et al., 2013; Hansson et al., 2010; Richardson et al., 2009;

Sakharkar et al., 2016; Taffe et al., 2010). The effect of alcohol on adult neural stem cells and adult neurogenesis is suspected to contribute to hippocampal pathology observed in humans with AUDs (Wilson et al., 2017) and animal models of AUDs (Morris et al., 2010a; Sakharkar et al., 2016; Taffe et al., 2010).

The hippocampus is one region susceptible to alcohol neurotoxicity that also recovers with abstinence (Bartels et al., 2007; Beresford et al., 2006; Crews and Nixon, 2009; Sullivan et al., 1995; Wilson et al., 2017). This neurotoxicity and recovery corresponds to similar patterns in alcohol's effects on adult hippocampal neurogenesis (Golub et al., 2015; Hansson et al., 2010; Herrera et al., 2003; Nixon and Crews, 2002; Richardson et al., 2009; Taffe et al., 2010), including a distinct burst in progenitor cell proliferation and subsequent increased neurogenesis during abstinence following alcohol dependence (Geil et al., 2014; Hansson et al., 2010; Mandyam and Koob, 2012; Nixon and Crews, 2004; Somkuwar et al., 2016). This increase in neurogenesis after an insult, termed *reactive neurogenesis*, is common to many forms of brain injury such as ischemia (Jin et al., 2006; Liu et al., 1998), trauma (Dash et al., 2001; Yu et al., 2008) and seizure (Cho et al., 2015; Parent et al., 1997) and may be necessary for recovery after damage (Blaiss et al., 2011; Sun et al., 2013; Sun et al., 2015). However, the mechanisms of reactive- or injury-induced neurogenesis are completely unclear. Therefore, to begin to dissect the mechanism of reactive neurogenesis in a model of a severe AUD, we examined whether alcohol altered cell cycle dynamics or differentially affected subtypes of progenitor cells in the subgranular zone (SGZ) of the hippocampal dentate gyrus.

2. METHODS AND MATERIALS

2.1 Animal model of an AUD

2.1.1 Subjects—Eighty-eight adult, male Sprague-Dawley rats (275–300g; Charles River Laboratories; Raleigh, NC) were used. Rats were ~70 days old, were housed individually and allowed five days of acclimation, including three days of handling, at the University of Kentucky AAALAC-accredited vivarium before initiation of experiments. Rats were maintained on a 12-hour light/dark cycle with *ad libitum* access to food and water except where noted. All protocols were approved by the University of Kentucky Institutional Animal Care and Use Committee prior to experimentation and strictly adhered to the NIH Guide for the Care and Use of Laboratory Animals (NRC, 2011).

2.1.2. Ethanol exposure—A rat model modified from Majchrowicz (Majchrowicz, 1975) was chosen that best mimics human AUDs including high blood ethanol concentrations, overt withdrawal, well-described neurological effects such as alcohol-induced neurodegeneration and cognitive impairments (Collins et al., 1996; Obernier et al., 2002b), and binge-pattern exposure, the pattern most associated with alcohol-induced brain damage (Hunt, 1993). Animals were randomly assigned to either the ethanol or control group and exposed to a four-day alcohol binge identical to that previously described (Morris et al., 2010b; Nixon and Crews, 2004). Briefly, food was removed from the cages and animals were administered a nutritionally complete liquid diet of Vanilla Ensure Plus® (Abbott Laboratories, Columbus, OH) containing either ethanol (25% w/v) or isocaloric amounts of

dextrose via intragastric gavage every eight hours for four days. Ethanol administration began with an initial dose of 5 g/kg with subsequent doses (range = 0 – 5g/kg) titrated based on overt behavioral intoxication scores as described elsewhere (Morris et al., 2010b; Nixon and Crews, 2004). For controls, the average volume of diet administered to the ethanol rats was calculated and dosed at each feeding. Peak blood ethanol concentrations (BECs) were assessed from tail blood samples taken 90 minutes following the seventh dose of ethanol. Blood samples were collected in microcentrifuge tubes containing heparin sodium (10000 units/ml) then centrifuged at $1800 \times g$ for 5 minutes to obtain serum. Samples were stored at -20°C until analyzed in triplicate using an AM1 Alcohol Analyzer (Analox, Lunenburg, MA) calibrated with an external 300 mg/dl ethanol standard.

At 8 hours following the last dose, food was returned and beginning 2 hours later withdrawal behaviors were observed and scored using the scale described (Penland et al., 2001 as reported in Morris et al., 2010b; Nixon and Crews, 2004). Rats were observed in their home cages for 30 minutes of each hour for 16 hours. Mean withdrawal behavior was calculated as the average of the highest score achieved during each hour across the 16 hours.

2.1.3. Tissue preparation—The animal model was conducted over different cohorts (batches of ethanol and control groups) plus animals were sacrificed at various time points following the last dose of diet. In all but one experiment, a factorial design of ethanol and control rats were treated in the binge paradigm, observed for withdrawal behaviors, and sacrificed at 7 (two cohorts, one for cell cycle, one for progenitor analysis), 14 or 28 days (Table 1). One experiment was designed for a one-way time course analysis against a common control group: 2–4 control rats and 4–6 ethanol exposed rats were subjected to the binge paradigm, observed for withdrawal behavior, and sacrificed at 4, 5, 6 and 7 days post binge (Table 2).

All rats were injected with the thymidine analog, bromodeoxyuridine (BrdU; 300 mg/kg i.p.; 1st 7 day group: Sigma-Aldrich, St. Louis, MO; all remaining rats: Roche, Mannheim, Germany), and were euthanized 2 hours later via sodium pentobarbital overdose (Nembutal®, MWI Veterinary Supply, Nampa, IN or Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI). Several rats were removed from the studies due to failure to take up BrdU (Eisch et al., 2000). Rats were then perfused transcardially with 0.1M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA). Brains were extracted, postfixed for 24 hours in 4% PFA, and stored in 0.1M PBS at 4°C until sectioning. Brains were sliced coronally into 40 μm sections using a vibrating microtome (Leica Microsystems, Wetzlar, Germany) beginning at a random start point near Bregma 1.6 and continuing through Bregma -6.3 (Paxinos and Watson, 2009), which covered the entire rostral to caudal extent of the hippocampus. Twelve series of sections were collected and stored in a cryoprotectant in 24-well plates at -20°C until processing. Throughout the analyses, brains were coded such that the observer was blind to the treatment condition.

2.2. Immunohistochemistry (IHC) and histology

2.2.1. 3,3'-Diaminobenzidine Tetrahydrochloride (DAB) IHC—Standard IHC procedures were used as described in detail elsewhere (McClain et al., 2011; Nixon and

Crews, 2004). Briefly, Every 6th (BrdU) or 12th (all others) free-floating tissue section was washed in Tris-Buffered Saline (TBS) then incubated in 0.6–1% H₂O₂ (depending on the antibody) to quench endogenous peroxidases. Following washes in TBS, nonspecific labeling was blocked in blocking buffer (0.1% Triton-X100 and 3% normal serum as appropriate in TBS). Sections were then incubated in primary antibodies for 16–48 hours at 4°C (see Table 3 for antibody specifications). After additional washes in blocking buffer, sections were incubated in species-specific biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) plus 1.5% normal serum. With TBS washes between steps, an avidin-biotin horseradish peroxidase macromolecular complex (Vector Laboratories, Burlingame, CA) was applied and detected with nickel-enhanced DAB (Polysciences, Waltham, MA). Sections were mounted on glass slides, counterstained if needed, and coverslipped using Cytoseal® (Richard Allen Scientific, Kalamazoo, MI). For anti-NeuroD1, anti-Ki67, and anti-phosphoHistoneH3 an additional antigen retrieval step in either 1× standard sodium citrate or Citra buffer (Biogenex, Fremont, CA) for 1 h at 65°C was required. For BrdU, additional DNA denaturing steps were included (Nixon and Crews, 2004). Antibody concentrations were determined from dilution curves and specificity verified by omission of primary antibody.

2.2.2. Fluorescent IHC—Combinations of two or four antibodies were chosen to examine the number of progenitor cells and proliferating progenitor cells as well as to differentiate Type 1, 2a, 2b and 3 progenitor cells in the rat hippocampal subgranular zone (see Table 3 for details). A quadruple labeling approach was utilized to detect the four types of progenitor cells simultaneously (described in Figure 6A). Due to the striking reactive astrocytosis response in this model (Kelso et al., 2011), a progenitor cell marker with nuclear pattern of expression that does not label intermediate filaments was chosen, Sex-determining region Y-related HMG box 2 (Sox2). Briefly, every 12th section was washed in TBS, antigens retrieved in standard sodium citrate at 65°C, blocked in blocking buffer, then incubated in primary antibody in blocking buffer with 10% donkey serum for 48–96 hours at 4°C. After three washes in blocking buffer, sections were incubated in appropriate fluorescent-coupled secondary antibodies, rinsed, and coverslipped with ProLong Gold® anti-fade mounting medium (Invitrogen, Carlsbad, CA).

2.2.3. Cresyl violet staining—Every 12th sections was rinsed in TBS, mounted onto glass slides, dried, and stained with cresyl violet (Acros Organics, Morris Plains, New Jersey): tissue was rinsed in decreasing concentrations of ethanol followed by distilled water (dH₂O) and cresyl violet (0.1% in Walpole Buffer) for 3 minutes. After a quick rinse in dH₂O, tissue was processed through increasing concentrations of ethanol followed by clearing in xylenes (Leasure and Nixon, 2010).

2.3. Quantification

2.3.1. Stereology (NeuroD1, granule cell number)—Unbiased stereology utilizing the optical fractionator method was used to estimate the number of NeuroD1-positive (+) cells in the subgranular zone of the dorsal hippocampal dentate gyrus and the number of granule cells in the granule cell layer using procedures identical to that described in McClain et al. (McClain et al., 2011). Briefly, for each hippocampus section, the subgranular zone or

granule cell layer was traced at 100× magnification using newCAST® Stereology System software (Visiopharm, Hoersholm, Denmark) coupled to a BX-51 Olympus microscope (Olympus, Center Valley, PA), and an average thickness was obtained from three measurements at different locations at 600× (60× oil objective) magnification. Tissue processing yielded a mean section thickness of ~18 µm for NeuroD1 and ~16 µm for cresyl violet and consequently, a dissector height of 14 µm or 12 µm with 2 µm guard zones was used for NeuroD1 or cresyl violet respectively. A 20 µm × 20 µm counting frame and an 80 µm x,y step length were used for NeuroD1. For cresyl violet, a 12.7 µm × 12.7 µm counting frame and a 180 µm x,y step length were used. Cells were counted at 1200× magnification (60× oil objective, plus ×2 magnification changer) and the total number of NeuroD1+ cells in the subgranular zone and the total number of granule cells in the granule cell layer were estimated using the following formula: $N = \Sigma Q \times asf^{-1} \times tsf^{-1} \times ssf^{-1}$ where N is the estimated total number of cells, Q is the summed total number of immunopositive (+) cell profiles that were counted, asf is the area sampling fraction, tsf is the thickness sampling fraction, and ssf is the section sampling fraction (West, 1991). Small darkly colored cells resembling glia were excluded from cell counts. One brain was removed from analysis for poor integrity. Data were presented as mean total cells ± SEM.

2.3.2 Profile Counts—To quantify BrdU+, Ki67+, pHisH₃+, and Sox2+ profiles, a profile counting approach was utilized. This approach was chosen due to the low number and/or heterogeneous distribution of BrdU+, Ki67+, pHisH₃+, and Sox2+ profiles (Crews et al., 2004; Noori and Fornal, 2011), as well as a lack of difference in dentate gyrus (DG) volume after four-day binge ethanol exposure (Leasure and Nixon, 2010). Immunopositive cells in the hippocampal DG were quantified via an Olympus BX-41 microscope (Olympus, Center Valley, PA) at 1000× magnification. Cells located along the subgranular zone, defined as an ~50µm band between the granule cell layer and hilus of the hippocampal dentate gyrus were counted and are presented as mean cells/section ± SEM.

2.3.3. Double label fluorescent IHC—Identification of colocalization was accomplished using an Olympus BX-51 fluorescent-capable microscope equipped with a Proscan II motorized stage and DP70 digital camera. For the BrdU and Sox2 colabel analysis, all BrdU+ cells in the SGZ were counted and analyzed as either positive or negative for Sox2. Similarly, for the Sox2 and Ki67 experiment, all Sox2 cells were counted and analyzed for the presence or absence of Ki67 colocalization. BrdU-Sox2 and BrdU-Ki67 colabeling was verified in a subset of sections via assessment of Z-plane optical stacks at <1.0µm on a confocal microscope (Leica TCS SP5; Wetzlar, Germany).

2.3.4. Quadruple label fluorescent IHC—A Leica TCS SP5 inverted laser scanning confocal microscope (Wetzlar, Germany) was used to obtain Z-plane optical stacks at 0.8 µm thickness using a 63× oil immersion lens (Morris et al., 2010a). Forty cells were sampled per animal, with both individual cells and clusters pseudo-randomly selected from multiple positions along both the superior blade and inferior blade from at least five separate sections per brain. Image-Pro Plus (Version 6.3, Media Cybernetics, Silver Spring, MD, USA) was used to render a 3D model from each section of tissue but only included three channels per stack at a time due to technological limitations of the software. Initially, models using Ki67,

Sox2, and NeuroD1 were uploaded in order to investigate presumed Type 2a, 2b, and 3 progenitor cells (as defined in Figure 4A). A subsequent model using Ki67, GFAP, and Sox2 combined with the data from the first model allowed for the differentiation of Type 1 cells from the Type 2a pool. Surface values for each channel were optimized to reduce background signal without loss of positively stained cells. To confirm the accuracy of the 3D reconstruction, the models were compared to the raw images to ensure inclusion of all positively stained cells.

2.4. Statistical Analysis

Statistical analyses were performed using SPSS statistics (version 20, IBM, Armonk, NY). All data are represented as mean $\pm$ standard error of the mean (SEM). Blood ethanol concentrations and daily ethanol dose were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Intoxication and withdrawal scores were analyzed using the Kruskal-Wallis nonparametric analysis of variance. Histological data were analyzed by appropriate analysis of variance (ANOVA) followed by Bonferroni post hoc tests or Dunnett's multiple comparison test (BrdU time course with collapsed control). Significance in all cases was accepted at $p < 0.05$.

3. RESULTS

3.1. AUD model data

A summary of ethanol intoxication parameters including BEC, intoxication score, daily ethanol dose, and peak withdrawal for each set of experiments is presented in Table 1 and 2. In the first set of experiments, one-way ANOVAs conducted on BEC and ethanol dose revealed a significant effect of cohort on BEC [$F_{(3,31)} = 4.59$; $p < 0.05$] but not on ethanol dose. Post hoc analysis with Bonferroni correction for multiple comparisons revealed that animals in the T28 cohort had significantly higher BECs than the T7 cohort 2 but neither group differed from the remaining cohorts. Intoxication and withdrawal scores were analyzed by Kruskal-Wallis nonparametric tests. Although behavioral intoxication did not differ between cohorts, one of the T7 cohorts differed for withdrawal severity from the other groups ($p < 0.01$). As BEC is only a snapshot of one time point during the binge, BEC was above 300 for all groups and withdrawal severity is expected to be variable (see Nixon and Crews, 2004), all cohorts are considered similar. Furthermore, the range and means for all measures were similar to Experiment 2 (below), supporting that these subject data are similar to that typically expected in this model (Morris et al., 2010b).

For the BrdU time course experiment, all subject data parameters were statistically similar between time points (Table 2). For BEC and ethanol dose, one-way ANOVA failed to show any significant differences between the different time points. For intoxication behavior and withdrawal severity, the Kruskal-Wallis test showed no significant differences across different time points. Therefore, all animals treated were similar in their ethanol exposure and related parameters, as values are similar to those previously reported with the expected variability in withdrawal severity (Morris et al., 2010b; Nixon and Crews, 2004). In summary, differences in various histological or NPC data cannot be attributed to any slight variations in the animal model.

3.2. Time course of NeuroD1 expression confirms reactive neurogenesis

Reactive neurogenesis following binge ethanol exposure was confirmed using NeuroD1 immunoreactivity, as prior work was based on doublecortin immunoreactivity, which may also label oligodendrocytes (Diaz et al., 2013). NeuroD1 is expressed in late stage progenitors committed to a neuronal fate (Gao et al., 2009b). The number of NeuroD1+ cells was estimated at 7 and 14 days post ethanol administration. As shown in Figure 1A–D, NeuroD1+ cells were visible along the subgranular zone (SGZ) of the dentate gyrus of all groups (Gao et al., 2009b). Two-way ANOVA (diet $\times$ time point) indicated significant main effects of diet [$F_{(1, 27)} = 6.48, p < 0.05$] and time point [$F_{(1, 27)} = 22.31, p < 0.001$], as well as a significant interaction [$F_{(1, 27)} = 17.68, p < 0.001$]. Bonferroni post hoc tests revealed a significant increase in the total number of NeuroD1+ cells in the SGZ at 14 days ($p < 0.001$). Thus, the increase in NeuroD1+ cells at day 14 reflects an increased number of new neurons consistent with our previous report that utilized doublecortin (Nixon and Crews, 2004).

3.3 Reactive NPC proliferation starts earlier than previously reported

As our prior report quantified the number of proliferating cells in the entire dentate gyrus (Nixon and Crews, 2004) and subsequent work observed proliferating microglia throughout the hippocampus (Nixon et al., 2008), we quantified the number of BrdU+ cells in the subgranular zone only 4–7 days post-ethanol exposure in order to reveal the extent of reactive cell proliferation. BrdU+ cells were observed along the SGZ in ethanol and control animals for each time point (Figure 2). In controls, one-way ANOVA revealed that BrdU+ cell counts at each time point were not statistically different [$F_{(3,7)} = 3.1; p > 0.05; \eta^2 = 0.568$] and therefore controls were combined into one common control group (as designed). Next, one-way ANOVA comparing BrdU+ cell counts at each time point versus controls revealed a main effect of ethanol exposure [$F_{(4,26)} = 29.69; p < 0.0001$]. Dunnett's multiple comparisons test revealed a significant increase in the number of BrdU+ cells in ethanol groups at 5 ($p < 0.005$), 6 ($p < 0.0005$), and 7 days post binge ($p < 0.0005$) compared to the combined control group (Figure 2). The over three-fold increase at 7 days is similar to that previously reported, while the significant increases in BrdU+ cells at days 5 and 6 suggest that reactive proliferation occurs earlier than previously reported (Nixon and Crews, 2004).

3.4. Cell cycle not altered by ethanol after 7 days of abstinence

We reported previously, and confirmed above, that reactive neurogenesis in abstinence originates with striking cell proliferation within the SGZ that appears to peak at 7 days of abstinence (Nixon and Crews, 2004). Increased cell proliferation is due to either acceleration of the cell cycle and/or an increase in the number of actively proliferating progenitors. In order to probe the mechanism of increased cell proliferation at the peak of this effect, cell cycle effects were screened in this experiment. Using a screen that is sensitive to effects on cell cycle without requiring the large number of animals necessary for cell cycle kinetics studies (Mandyam et al., 2007; McClain et al., 2011), various cell cycle phase-specific markers were examined (Mandyam et al., 2007; McClain et al., 2011): Ki67 for cells in active division, i.e. not G₀ phase (Scholzen and Gerdes, 2000), BrdU for recent S-phase (Nowakowski et al., 1989), and histone H3 serine 10 phosphorylation (pHis-H₃) for the G₂ and M phases (Prigent and Dimitrov, 2003). Finally, the number of cells in the G₁ phase was

estimated by subtracting the number of BrdU+ plus pHisH₃+ cells from the total number of Ki67+ cells (Figure 3A) as the typical G₁ phase marker, Minichromosome maintenance 2 was not specific for the G₁ phase in our hands (data not shown). Three ethanol-exposed animals were omitted from the analysis, for insufficient BrdU uptake (Eisch et al., 2000).

Representative photomicrographs of Ki67+, BrdU+, and pHisH₃+ IHC show expected clusters of cells along the SGZ (Figure 3B–G). Separate one-way ANOVAs revealed significant 2.5–3.6-fold increases in immunopositive cell number at each phase of the cell cycle in ethanol-exposed compared to control rats [Ki67: $F_{(1,11)} = 35.25$, $p < 0.001$; BrdU: $F_{(1,11)} = 30.39$, $p < 0.001$; pHisH₃: $F_{(1,11)} = 21.58$, $p < 0.01$; Figure 3H]. Importantly, Ki67 and BrdU data replicated past work (Nixon and Crews, 2004). Additionally, one-way ANOVA revealed a significant 2-fold increase in the number of cells estimated to be in G₁ phase in ethanol-exposed rats compared to controls [$F_{(1,11)} = 10.47$, $p < 0.01$]. Then, to determine the proportion of cells in each phase of the cell cycle, the number of cells in each phase was divided by the total number of actively-dividing, i.e. Ki67+, cells for both groups. One-way ANOVA for each defined phase, G₁, S, G₂/M, indicated that there were no significant differences between ethanol-exposed and control rats on the proportion of cells in any phase of the cell cycle (Figure 3I). Therefore, altered cell cycle dynamics was ruled out for contributing to reactive neurogenesis at the T7 time point.

3.5. Ethanol exposure increases the proliferation of progenitor cells in the SGZ

Prior alcohol dependence could alter the neural progenitor cell (NPC) pool. Specifically, an increase in the number of proliferating NPCs may be responsible for reactive proliferation at 7 days of abstinence. To first test this hypothesis, Sox2 was used as a marker of NPCs and examined at 7 days of abstinence. Representative photomicrographs of Sox2+ IHC in the dentate gyrus from control (Figure 4A) and ethanol-exposed (Figure 4B) rats show a distinct line of darkly Sox2+ cells along the SGZ, as expected (Suh et al., 2007). Therefore, Sox2+ cells were estimated along the SGZ only as this region is where NPCs are located. One-way ANOVA revealed a significant, 20% increase in Sox2+ cells in the ethanol-exposed SGZ [$F_{(1,14)} = 5.55$, $p < 0.05$] compared to control (Figure 4C). Next, to determine whether these Sox2+ cells were proliferating, dual fluorescent IHC of Sox2 and BrdU was examined (Figure 4D–F). Increased BrdU+ cells (Figure 4G, total BrdU) at 7 days of abstinence was replicated with fluorescent IHC [$F_{(1,13)} = 7.73$, $p < 0.05$] and analysis of Sox2-BrdU colabeling confirmed that the majority of proliferating cells were Sox2+ cells, a value that was slightly higher in the ethanol exposed rats [$91.6 \pm 1.0\%$ and $84.6 \pm 2.8\%$ for ethanol and control rats respectively; $F_{(1,13)} = 6.81$, $p < 0.05$]. This effect translates into a significant two-fold increase in the number of proliferating progenitor cells (Figure 4G, BrdU+Sox2) according to one-way ANOVA [$F_{(1,13)} = 9.03$, $p < 0.05$]. One control animal was removed for insufficient BrdU uptake.

3.6. Prior ethanol exposure activates more Sox2+ progenitor cells out of quiescence

While an increased number of BrdU+ cells was observed and most of those proliferating cells were Sox2+ (Figure 4), BrdU only labels cells that have recently passed through or are in the S-phase of the cell cycle. Thus, we took the opposite approach to examine Sox2+ progenitor cells that were in active cycle (Ki67+). Specifically, to determine if prior four-day

binge ethanol exposure and abstinence shifted the number of Sox2+ progenitor cells from quiescence into active division, we quantified the number and percentage of cells that co-label Ki67 and Sox2 exhaustively throughout the dentate gyrus. In both ethanol and control animals, a vast majority of neural progenitor cells (Sox2+/Ki67-) are maintained in the quiescent state (376.9 ± 23.6 and 452.4 ± 14.6 for control and ethanol rats, respectively) consistent with data that adult stem cells in the adult mammalian forebrain are relatively quiescent (Kronenberg et al., 2003; Morshead et al., 1994). However, one-way ANOVA on the proportion of dividing NPCs (Sox2+/Ki67+; Figure 5) revealed that ethanol-exposed rats at T7 of abstinence show a significant increase in the percentage of actively dividing neural progenitor cells [$F_{(1,13)} = 9.81$, $p < 0.01$]. These data suggest that more progenitors are recruited or “activated” out of quiescence following 4-day binge ethanol exposure.

3.7. All subtypes of neural progenitor cells are increased 7 days following ethanol exposure

Sox2 expression encompasses a variety of progenitor cell subtypes including the Type 2a or transit-amplifying progenitor that is thought to be highly proliferative in response to neurogenic conditions (Kronenberg et al., 2003). Thus, to examine which progenitor subtypes respond at T7 after binge alcohol exposure or its sequelae, a quadruple labeling scheme was undertaken to differentiate Type-1, -2a, -2b, and -3 proliferating NPCs (Figure 6A, based on Kempermann et al., 2004). As proliferating cell subtype is the question, only Ki67+ cells were examined (Figure 6B, 1st column). For each group, 40 cells/rat dentate gyrus were analyzed and the percent of each phenotype is presented in Figure 7A. Individual one-way ANOVAs assessing the effect of ethanol on the percentage of cells in each phenotype were not significant (Figure 7A). However, this analysis fails to consider that a significantly higher number of cells are proliferating at T7 during abstinence following ethanol exposure [$F_{(1,13)} = 13.48$, $p < 0.01$; Figure 7B; left side]. Therefore, the proportion of cells in each phenotype (Figure 7A) was multiplied by the total number of Ki67+ cells (Figure 7B, left side), which revealed a significant increase in the number of cells of each phenotype at T7 (Figure 7B; right side). Individual one-way ANOVAs confirm the increases across Type-1 through Type 3 proliferating NPCs: Type 1 [$F_{(1,13)} = 17.40$, $p < 0.01$]; Type 2a [$F_{(1,13)} = 13.55$, $p < 0.01$]; Type 2b [$F_{(1,13)} = 11.75$, $p < 0.01$]; and Type 3 [$F_{(1,13)} = 55.23$, $p < 0.001$].

3.8 Type 2a progenitor cells respond first at T5 post ethanol

Type 2a cells are typically the most responsive to changes in the niche (Kronenberg et al., 2003). Thus, the increase in proliferation of all progenitor cell types observed at T7 of abstinence, and especially the increase in proliferating Type 1 cells (Figure 7B) coupled with our observation that reactive proliferation starts around T5 (Figure 2), led us to question whether Type 2a cells do respond first at T5 as would be predicted. Therefore, quadruple fluorescent immunohistochemistry identical to experiments presented in Figures 6 and 7 was conducted in the T5 days post binge tissue: 40 Ki67+ cells/rat SGZ were examined and the percent and estimated number of each phenotype is presented in Figure 8. Individual, one-way ANOVAs showed no significant difference in the percentage of cells in each phenotype at T5 days post alcohol (Figure 8A). Again, as this analysis does not incorporate that more cells are proliferating after ethanol exposure [$F_{(1,13)} = 13.48$, $p < 0.01$; Figure 8B; left side],

the proportion of cells in each phenotype (Figure 8A) was multiplied by the total number of Ki67+ cells (Figure 8B, left side). As shown in Figure 8B (right side), one-way ANOVAs confirmed significant increases in the number of Type 2a [$F_{(1,10)} = 15.916$, $p < 0.005$] and Type 2b progenitors [$F_{(1,10)} = 7.215$, $p < 0.05$], but a decrease in Type 3 neuroblasts [$F_{(1,10)} = 31.881$, $p < 0.001$]. This decrease in neuroblasts is consistent with prior alcohol-intoxication inhibited neurogenesis (He et al., 2005; Nixon and Crews, 2002). Critically, there was no effect of prior alcohol exposure on the estimated number of proliferating Type-1 NSCs. Thus, Type-2 progenitors react first, and 2a robustly at T5 days post four-day binge alcohol exposure as predicted by other models (Kronenberg et al., 2003).

3.9 Number of granule cells normalizes in abstinence

We have shown previously that this same four-day binge ethanol exposure reduces the number of dentate gyrus granule cells by 15% when measured prior to withdrawal (Leasure and Nixon, 2010). To determine if granule cell number recovers after reactive neurogenesis, stereology was performed on cresyl violet-stained sections collected from rats 28 days following their last dose of alcohol. One-way ANOVA revealed no difference in the number of granule cells in ethanol rats ($610,738 \pm 27,490$ cells; $n=7$) versus controls ($640,651 \pm 13,264$ cells; $n=4$; $p=0.46$). These data suggest that granule cell numbers recover to control levels following reactive neurogenesis.

4. DISCUSSION

Reactive neurogenesis after CNS insult or neurodegenerative disease has been observed in humans (Gomez-Nicola et al., 2014; Jin et al., 2006; Mattiesen et al., 2009; Sgubin et al., 2007) and animal models (Cho et al., 2015; Liu et al., 1998; Parent et al., 1997; Yu et al., 2008), including in animal models of AUDs (McClain et al., 2014; Nixon and Crews, 2004; Somkuwar et al., 2016). However, the mechanism underlying reactive neurogenesis after alcohol dependence is not understood. Therefore, after ruling out the contribution of cell cycle alterations to reactive neurogenesis, experiments that exhaustively counted the number and percentage of Sox2+ progenitors in active cell cycle (i.e. Ki67+) coupled with a detailed analysis of quadruple fluorescent immunohistochemistry to simultaneously determine Type-1, Type-2a, Type-2b and Type-3 NPC subtypes indicate that reactive neurogenesis during abstinence following four-day binge alcohol exposure is due to NPCs being activated out of quiescence. The activation of NPCs was followed by granule cell numbers returning to normal after one month of abstinence. This first report elucidating the mechanism of alcohol-induced reactive neurogenesis - neural stem and progenitor cell activation - is critical for understanding how the brain recovers during abstinence.

First, as doublecortin may not be specific for neuroblasts (Diaz et al., 2013), we examined NeuroD1, a transcription factor expressed by progenitors committed to a neuronal fate (Lee et al., 1995). NeuroD1, like doublecortin (Nixon and Crews, 2004), was increased at 14 days, but not 7 days following the last dose of ethanol, consistent with concomitant increases in immature neurons one week after increased (reactive) cell proliferation (Brown et al., 2003; Nixon and Crews, 2004). Increased proliferation is driven by either changes in cell cycle or to increases in the number of proliferating NPCs. Thus, using a screen for cell cycle

perturbation (Mandyam et al., 2007; McClain et al., 2011), similar significant increases were observed across all portions of the cell cycle including the entire actively dividing phase (Ki67+), S-phase (BrdU+), and G₂/M phases (pHisH3+) and estimated for G₁ phase (Figure 3). Importantly, the percentage of cells in each phase of the cycle was similar between ethanol and control rats. Therefore, four-day binge ethanol exposure or its sequelae does not likely accelerate the cell cycle to increase proliferation at T7.

The estimated number of Sox2+ cells, however, was increased (~20% after 7 days of abstinence) and analysis of co-expression with BrdU revealed that the majority of these cells are proliferating progenitors. Knowing that subpopulations of proliferating cells respond differently to neurogenic stimuli (Kronenberg et al., 2003), we hypothesized that the Type-2a cells were likely responsible for this reactive increase in proliferation. Surprisingly, in our experiment at T7, the percentage and estimated number of all proliferating NPC subtypes, Type-1, -2a, -2b and -3, were increased similarly, without altering the proportion of cells in any phenotype (Figure 7). These quad label data suggest that although the Type 2a progenitors respond robustly, Type-1 progenitors, the true stem cell, are activated out of quiescence. When this finding is coupled with the exhaustive counting of Ki67 and Sox2 co-labeling at 7 days in abstinence to reveal a shift in the proportion of quiescent progenitors into active division (Figure 5), it allowed us to conclude that reactive neurogenesis is likely due to the activation of neural stem and progenitor cells. The activation of Type-1 progenitors, in particular, is similar to that observed in other forms of brain injury and the totality of the data utilizing multiple markers and these similar observations in other injury models strongly support this interpretation that Type 1 NSC activation may underlie reactive neurogenesis in a model of an AUD (Gao et al., 2009a; Jhaveri et al., 2012; Kunze et al., 2006; Segi-Nishida et al., 2008; Yu et al., 2008). One caveat, however, is that no markers of quiescent NSCs were investigated. Thus, determining a measure by the absence of signal (Ki67-) is a potential limitation, especially considering that Ki67 expression can be low during early G₁ phase. More direct studies of quiescent versus activated NSCs in models of AUDs are warranted.

As we were initially surprised that all progenitor cell subtypes react equivalently at T7, a lingering question remained that the Type 2a activation was merely missed. Thus, we next examined T5 tissue, the time point when reactive NPC proliferation emerged (Figure 2). At the 5-day time point, as predicted (Kronenberg et al., 2003), more Type-2 cells were activated into proliferation (Ki67+) in the ethanol-exposed rats (Figure 8) while Type 1 cells were similar to controls. Thus, Type 2 cells do appear to react before Type 1 and the Type 2a cells proliferate robustly in ethanol-exposed rats versus controls at this T5 time point. Additional work should rule out potential effects of alcohol on the cell cycle at this specific time point (T5), however congruent data in Figure 2 (BrdU) and Figure 8B (Ki67) strongly suggested that the increased number of proliferating cells (increased BrdU+ and increased Ki67+ cells) is likely due to an increased number of proliferating progenitors. Namely, when BrdU and Ki67 data are congruent, the likelihood of the cell cycle playing a role in the change in proliferation is slim (McClain et al., 2011).

Of the host of neuroplastic events, toxicities, and behaviors that comprise alcoholism, alcohol-induced neurodegeneration and/or alcohol withdrawal are the most likely aspects of

this model to elicit NPC activation. As mentioned above, neurodegeneration, brain injury, or seizures activate Type-1 NSC (Gao et al., 2009a; Kunze et al., 2006; Segi-Nishida et al., 2008; Yu et al., 2008). Although this alcohol model includes seizures and withdrawal severity correlates with cell proliferation at T7, eliminating seizures with diazepam does not reduce reactive cell proliferation (Nixon and Crews, 2004). It is possible that diazepam does not blunt all excitatory overactivity that occurs in alcohol withdrawal in the four-day binge model (e.g. Mhatre et al., 2001). Therefore, despite the elimination of convulsions with diazepam, perhaps significant excitatory activity continued (Mhatre et al., 2001) sufficient to activate Type-1 progenitors, as has been shown by activating afferents to the dentate gyrus (Stone et al., 2011). It is of note that both alcohol-induced neurodegeneration and alcohol withdrawal involve glutamatergic excitotoxicity (Prendergast and Mulholland, 2012). Thus, either the alcohol-induced neurodegeneration or alcohol withdrawal that occur with alcohol dependence results in cellular overactivity or excitation which may then drive reactive neurogenesis and Type-1 NSC activation (Ma et al., 2009). The timing of stem cell activation in this AUD model mirrors that observed after induced granule cell excitation (Stone et al., 2011) and, indeed, activity-dependent factors activate the Type-1 NSC population (Jhaveri et al., 2012; Walker et al., 2008). Therefore, increased excitatory activity resulting from the alcohol dependence likely drives NSC activation in abstinence. Indeed, more traditional alcohol dependence models where less acute toxicity is reported also show reactive neurogenesis (He et al., 2009; Somkuwar et al., 2016).

Activity-dependent factors that induce quiescent precursors to enter the cell cycle likely originate in the hippocampal niche (Jhaveri et al., 2012). The neurogenic niche, comprised of glia, endothelial cells, and extracellular matrix, provides the permissive environment for neurogenesis and plays a critical role in regulating stem cells and their activation (Ninkovic and Gotz, 2007). Excessive alcohol exposure and its sequelae dramatically alter the cells and tissues of and surrounding the niche (Crews and Nixon, 2009; Geil et al., 2014; Mandyam, 2013). In addition to nearby neurodegeneration (Collins et al., 1996; Obernier et al., 2002a; Obernier et al., 2002b), striking astrogliosis (Kelso et al., 2011) and microgliosis (Marshall et al., 2013; Peng et al., 2017) have been reported in this model, all of which affect adult neurogenesis (Ekdahl et al., 2003; Geil et al., 2014; Yoneyama et al., 2011).

Data from various models of CNS insult suggest that reactive neurogenesis promotes hippocampal recovery (Blaiss et al., 2011; Gage and Temple, 2013; Mandyam and Koob, 2012; Sun et al., 2013; Yun et al., 2016). Increasing neurogenesis is considered beneficial to hippocampal structure and function (e.g. Sahay et al., 2011) and new neurons born during reactive neurogenesis are integrated into the hippocampal circuitry and associated with recovery of hippocampal function in some models (Sun et al., 2007). Indeed, Type-1 progenitors are required for reactive neurogenesis to repopulate the dentate gyrus (Blaiss et al., 2011; Sun et al., 2013; Sun et al., 2015; Yu et al., 2008). Our data suggest, and future work will examine, whether NPC activation is responsible for restoring hippocampal structure and function after four-day binge alcohol exposure. Recovery of granule cell number is consistent with volume recovery observed in a similar rat model (Zahr et al., 2010) as well as in human alcoholics (Carlen et al., 1978; Gazdzinski et al., 2008; Pfefferbaum et al., 1995). Therefore, the association between reactive neurogenesis and recovery of dentate gyrus granule cell number in a model of an AUD lends additional

support that stem cell activation and reactive neurogenesis may be a means of endogenous self-repair in neurogenic regions such as the hippocampus.

Despite reports of these beneficial effects of stem cell activation, reactive neurogenesis in seizure is neuropathological and drives epileptogenesis (Cho et al., 2015; Parent et al., 1997). Further, the long-term consequences of NSC activation are not understood. The relative quiescence of the NSC pool appears necessary for the long-term maintenance of its proliferative capacity, which implies that reactive proliferation would ultimately lead to stem cell exhaustion (Bonaguidi et al., 2011; Kippin et al., 2005; Lugert et al., 2010). The evidence of stem cell exhaustion or long-term effects on adult neurogenesis after a single four day binge exposure remains limited (Nixon and Crews, 2004). However, humans do not merely binge once and repeated cycles of bingeing and reactive neurogenesis could result in depletion or exhaustion of the stem cell pool. Indeed, more chronic exposures have long-term effects on adult neurogenesis (Broadwater et al., 2014; Ehlers et al., 2013; Hansson et al., 2010; Richardson et al., 2009; Taffe et al., 2010). Therefore, although it remains promising that reactive neurogenesis may contribute to recovery (Mandyam and Koob, 2012), much remains to be studied to determine whether neurogenesis continues at normal rates or whether new neurons are incorporated and contribute to function. Harnessing the endogenous response to beneficially promote neurogenesis and recovery offers promise for the reversal of hippocampal pathology in neurodegenerative and psychiatric disorders such as AUDs.

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References

- Altman J. 1969; Autoradiographic and histological studies of postnatal neurogenesis. 3. Dating the time of production and onset of differentiation of cerebellar microneurons in rats. *Journal of Comparative Neurology*. 136:269–293. [PubMed: 5788129]
- Bartels C, Kunert HJ, Stawicki S, Kroner-Herwig B, Ehrenreich H, Krampe H. 2007; Recovery of hippocampus-related functions in chronic alcoholics during monitored long-term abstinence. *Alcohol Alcohol*. 42:92–102. [PubMed: 17179161]
- Belujon P, Grace AA. 2008; Critical role of the prefrontal cortex in the regulation of hippocampus-accumbens information flow. *J Neurosci*. 28:9797–9805. [PubMed: 18815264]
- Belujon P, Grace AA. 2011; Hippocampus, amygdala, and stress: interacting systems that affect susceptibility to addiction. *Ann N Y Acad Sci*. 1216:114–121. [PubMed: 21272015]
- Beresford TP, Arciniegas DB, Alfors J, Clapp L, Martin B, Du Y, Liu D, Shen D, Davatzikos C. 2006; Hippocampus volume loss due to chronic heavy drinking. *Alcohol Clin Exp Res*. 30:1866–1870. [PubMed: 17067350]
- Blaiss CA, Yu TS, Zhang G, Chen J, Dimchev G, Parada LF, Powell CM, Kernie SG. 2011; Temporally specified genetic ablation of neurogenesis impairs cognitive recovery after traumatic brain injury. *J Neurosci*. 31:4906–4916. [PubMed: 21451029]
- Bonaguidi MA, Wheeler MA, Shapiro JS, Stadel RP, Sun GJ, Ming GL, Song H. 2011; In vivo clonal analysis reveals self-renewing and multipotent adult neural stem cell characteristics. *Cell*. 145:1142–1155. [PubMed: 21664664]

- Broadwater MA, Liu W, Crews FT, Spear LP. 2014; Persistent loss of hippocampal neurogenesis and increased cell death following adolescent, but not adult, chronic ethanol exposure. *Dev Neurosci*. 36:297–305. [PubMed: 24993092]
- Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG. 2003; Transient expression of doublecortin during adult neurogenesis. *J Comp Neurol*. 467:1–10. [PubMed: 14574675]
- Carlen PL, Wortzman G, Holgate RC, Wilkinson DA, Rankin JC. 1978; Reversible cerebral atrophy in recently abstinent chronic alcoholics measured by computed tomography scans. *Science*. 200:1076–1078. [PubMed: 653357]
- Chanraud S, Martelli C, Delain F, Kostogianni N, Douaud G, Aubin HJ, Reynaud M, Martinot JL. 2007; Brain morphometry and cognitive performance in detoxified alcohol-dependents with preserved psychosocial functioning. *Neuropsychopharmacology*. 32:429–438. [PubMed: 17047671]
- Cho KO, Lybrand ZR, Ito N, Brulet R, Tafacory F, Zhang L, Good L, Ure K, Kernie SG, Birnbaum SG, Scharfman HE, Eisch AJ, Hsieh J. 2015; Aberrant hippocampal neurogenesis contributes to epilepsy and associated cognitive decline. *Nat Commun*. 6:6606. [PubMed: 25808087]
- Clelland CD, Choi M, Romberg C, Clemenson GD Jr, Fragniere A, Tyers P, Jessberger S, Saksida LM, Barker RA, Gage FH, Bussey TJ. 2009; A functional role for adult hippocampal neurogenesis in spatial pattern separation. *Science*. 325:210–213. [PubMed: 19590004]
- Collins MA, Corse TD, Neafsey EJ. 1996; Neuronal degeneration in rat cerebrocortical and olfactory regions during subchronic "binge" intoxication with ethanol: possible explanation for olfactory deficits in alcoholics. *Alcohol Clin Exp Res*. 20:284–292. [PubMed: 8730219]
- Contet C, Kim A, Le D, Iyengar SK, Kotzebue RW, Yuan CJ, Kieffer BL, Mandyam CD. 2013; mu-Opioid receptors mediate the effects of chronic ethanol binge drinking on the hippocampal neurogenic niche. *Addict Biol*.
- Crews FT, Mdzinarishvili A, Kim D, He J, Nixon K. 2006; Neurogenesis in adolescent brain is potently inhibited by ethanol. *Neuroscience*. 137:437–445. [PubMed: 16289890]
- Crews FT, Nixon K. 2009; Mechanisms of neurodegeneration and regeneration in alcoholism. *Alcohol Alcohol*. 44:115–127. [PubMed: 18940959]
- Crews FT, Nixon K, Wilkie ME. 2004; Exercise reverses ethanol inhibition of neural stem cell proliferation. *Alcohol*. 33:63–71. [PubMed: 15353174]
- Dash PK, Mach SA, Moore AN. 2001; Enhanced neurogenesis in the rodent hippocampus following traumatic brain injury. *J Neurosci Res*. 63:313–319. [PubMed: 11170181]
- Deschaux O, Vendruscolo LF, Schlosburg JE, Diaz-Aguilar L, Yuan CJ, Sobieraj JC, George O, Koob GF, Mandyam CD. 2014; Hippocampal neurogenesis protects against cocaine-primed relapse. *Addict Biol*. 19:562–574. [PubMed: 23278919]
- Diaz F, McKeehan N, Kang W, Hebert JM. 2013; Apoptosis of glutamatergic neurons fails to trigger a neurogenic response in the adult neocortex. *J Neurosci*. 33:6278–6284. [PubMed: 23575827]
- Ehlers CL, Liu W, Wills DN, Crews FT. 2013; Periadolescent ethanol vapor exposure persistently reduces measures of hippocampal neurogenesis that are associated with behavioral outcomes in adulthood. *Neuroscience*. 244:1–15. [PubMed: 23567812]
- Eisch AJ, Barrot M, Schad CA, Self DW, Nestler EJ. 2000; Opiates inhibit neurogenesis in the adult rat hippocampus. *Proc Natl Acad Sci U S A*. 97:7579–7584. [PubMed: 10840056]
- Ekdahl CT, Claassen JH, Bonde S, Kokaia Z, Lindvall O. 2003; Inflammation is detrimental for neurogenesis in adult brain. *Proc Natl Acad Sci U S A*. 100:13632–13637. [PubMed: 14581618]
- Gage FH, Temple S. 2013; Neural stem cells: generating and regenerating the brain. *Neuron*. 80:588–601. [PubMed: 24183012]
- Galinato MH, Lockner JW, Fannon-Pavlich MJ, Sobieraj JC, Staples MC, Somkuwar SS, Ghofranian A, Chaing S, Navarro AI, Joea A, Luikart BW, Janda KD, Heyser C, Koob GF, Mandyam CD. 2017; A synthetic small-molecule Isoxazole-9 protects against methamphetamine relapse. *Mol Psychiatry*.
- Gao X, Enikolopov G, Chen J. 2009a; Moderate traumatic brain injury promotes proliferation of quiescent neural progenitors in the adult hippocampus. *Exp Neurol*. 219:516–523. [PubMed: 19615997]

- Gao Z, Ure K, Ables JL, Lagace DC, Nave KA, Goebbels S, Eisch AJ, Hsieh J. 2009b; Neurod1 is essential for the survival and maturation of adult-born neurons. *Nat Neurosci.* 12:1090–1092. [PubMed: 19701197]
- Gazdzinski S, Durazzo TC, Weiner MW, Meyerhoff DJ. 2008; Are treated alcoholics representative of the entire population with alcohol use disorders? A magnetic resonance study of brain injury. *Alcohol.* 42:67–76. [PubMed: 18358984]
- Geil CR, Hayes DM, McClain JA, Liput DJ, Marshall SA, Chen KY, Nixon K. 2014; Alcohol and adult hippocampal neurogenesis: Promiscuous drug, wanton effects. *Prog Neuropsychopharmacol Biol Psychiatry.* 54C:103–113.
- Godsil BP, Kiss JP, Spedding M, Jay TM. 2013; The hippocampal-prefrontal pathway: the weak link in psychiatric disorders? *Eur Neuropsychopharmacol.* 23:1165–1181. [PubMed: 23332457]
- Golub HM, Zhou QG, Zucker H, McMullen MR, Kokiko-Cochran ON, Ro EJ, Nagy LE, Suh H. 2015; Chronic Alcohol Exposure is Associated with Decreased Neurogenesis, Aberrant Integration of Newborn Neurons, and Cognitive Dysfunction in Female Mice. *Alcohol Clin Exp Res.* 39:1967–1977. [PubMed: 26365148]
- Gomez R, Schneider R Jr, Quinteros D, Santos CF, Bandiera S, Thiesen FV, Coitinho AS, Fernandes Mda C, Wieczorek MG. 2015; Effect of Alcohol and Tobacco Smoke on Long-Term Memory and Cell Proliferation in the Hippocampus of Rats. *Nicotine Tob Res.* 17:1442–1448. [PubMed: 25744965]
- Gomez-Nicola D, Suzzi S, Vargas-Caballero M, Fransen NL, Al-Malki H, Cebrian-Silla A, Garcia-Verdugo JM, Riecken K, Fehse B, Perry VH. 2014; Temporal dynamics of hippocampal neurogenesis in chronic neurodegeneration. *Brain.*
- Goncalves JT, Schafer ST, Gage FH. 2016; Adult Neurogenesis in the Hippocampus: From Stem Cells to Behavior. *Cell.* 167:897–914. [PubMed: 27814520]
- Hansson AC, Nixon K, Rimondini R, Damadzic R, Sommer WH, Eskay R, Crews FT, Heilig M. 2010; Long-term suppression of forebrain neurogenesis and loss of neuronal progenitor cells following prolonged alcohol dependence in rats. *Int J Neuropsychopharmacol.* 13:583–593. [PubMed: 20334723]
- He J, Nixon K, Shetty AK, Crews FT. 2005; Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. *Eur J Neurosci.* 21:2711–2720. [PubMed: 15926919]
- He J, Overstreet DH, Crews FT. 2009; Abstinence from moderate alcohol self-administration alters progenitor cell proliferation and differentiation in multiple brain regions of male and female P rats. *Alcohol Clin Exp Res.* 33:129–138. [PubMed: 19076732]
- Herrera DG, Yague AG, Johnsen-Soriano S, Bosch-Morell F, Collado-Morente L, Muriach M, Romero FJ, Garcia-Verdugo JM. 2003; Selective impairment of hippocampal neurogenesis by chronic alcoholism: protective effects of an antioxidant. *Proc Natl Acad Sci U S A.* 100:7919–7924. [PubMed: 12792022]
- Hunt WA. 1993; Are binge drinkers more at risk of developing brain damage? *Alcohol.* 10:559–561. [PubMed: 8123218]
- Hyman SE, Malenka RC, Nestler EJ. 2006; Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci.* 29:565–598. [PubMed: 16776597]
- Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R. 2008; Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nat Neurosci.* 11:1153–1161. [PubMed: 18758458]
- Jhaveri DJ, Taylor CJ, Bartlett PF. 2012; Activation of different neural precursor populations in the adult hippocampus: does this lead to new neurons with discrete functions? *Dev Neurobiol.* 72:1044–1058. [PubMed: 22505142]
- Jin K, Wang X, Xie L, Mao XO, Zhu W, Wang Y, Shen J, Mao Y, Banwait S, Greenberg DA. 2006; Evidence for stroke-induced neurogenesis in the human brain. *Proc Natl Acad Sci U S A.* 103:13198–13202. [PubMed: 16924107]
- Kelso ML, Liput DJ, Eaves DW, Nixon K. 2011; Upregulated vimentin suggests new areas of neurodegeneration in a model of an alcohol use disorder. *Neuroscience.* 197:381–393. [PubMed: 21958862]

- Kempermann G, Jessberger S, Steiner B, Kronenberg G. 2004; Milestones of neuronal development in the adult hippocampus. *Trends Neurosci.* 27:447–452. [PubMed: 15271491]
- Kippin TE, Martens DJ, van der Kooy D. 2005; p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes Dev.* 19:756–767. [PubMed: 15769947]
- Kronenberg G, Reuter K, Steiner B, Brandt MD, Jessberger S, Yamaguchi M, Kempermann G. 2003; Subpopulations of proliferating cells of the adult hippocampus respond differently to physiologic neurogenic stimuli. *J Comp Neurol.* 467:455–463. [PubMed: 14624480]
- Kunze A, Grass S, Witte OW, Yamaguchi M, Kempermann G, Redecker C. 2006; Proliferative response of distinct hippocampal progenitor cell populations after cortical infarcts in the adult brain. *Neurobiol Dis.* 21:324–332. [PubMed: 16137890]
- Leasure JL, Nixon K. 2010; Exercise neuroprotection in a rat model of binge alcohol consumption. *Alcohol Clin Exp Res.* 34:404–414. [PubMed: 20028365]
- Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H. 1995; Conversion of *Xenopus* ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science.* 268:836–844. [PubMed: 7754368]
- Liu J, Solway K, Messing RO, Sharp FR. 1998; Increased neurogenesis in the dentate gyrus after transient global ischemia in gerbils. *J Neurosci.* 18:7768–7778. [PubMed: 9742147]
- Lugert S, Basak O, Knuckles P, Haussler U, Fabel K, Gotz M, Haas CA, Kempermann G, Taylor V, Giachino C. 2010; Quiescent and active hippocampal neural stem cells with distinct morphologies respond selectively to physiological and pathological stimuli and aging. *Cell Stem Cell.* 6:445–456. [PubMed: 20452319]
- Ma DK, Kim WR, Ming GL, Song H. 2009; Activity-dependent extrinsic regulation of adult olfactory bulb and hippocampal neurogenesis. *Ann N Y Acad Sci.* 1170:664–673. [PubMed: 19686209]
- Majchrowicz E. 1975; Induction of physical dependence upon ethanol and the associated behavioral changes in rats. *Psychopharmacology.* 43:245–254.
- Mandyam CD. 2013; The Interplay between the Hippocampus and Amygdala in Regulating Aberrant Hippocampal Neurogenesis during Protracted Abstinence from Alcohol Dependence. *Front Psychiatry.* 4:61. [PubMed: 23818882]
- Mandyam CD, Harburg GC, Eisch AJ. 2007; Determination of key aspects of precursor cell proliferation, cell cycle length and kinetics in the adult mouse subgranular zone. *Neuroscience.* 146:108–122. [PubMed: 17307295]
- Mandyam CD, Koob GF. 2012; The addicted brain craves new neurons: putative role for adult-born progenitors in promoting recovery. *Trends Neurosci.* 35:250–260. [PubMed: 22265158]
- Marshall SA, McClain JA, Kelso ML, Hopkins DM, Pauly JR, Nixon K. 2013; Microglial activation is not equivalent to neuroinflammation in alcohol-induced neurodegeneration: The importance of microglia phenotype. *Neurobiol Dis.* 54:239–251. [PubMed: 23313316]
- Mattiesen WR, Tauber SC, Gerber J, Bunkowski S, Bruck W, Nau R. 2009; Increased neurogenesis after hypoxic-ischemic encephalopathy in humans is age related. *Acta Neuropathol.* 117:525–534. [PubMed: 19277687]
- McClain JA, Hayes DM, Morris SA, Nixon K. 2011; Adolescent binge alcohol exposure alters hippocampal progenitor cell proliferation in rats: effects on cell cycle kinetics. *J Comp Neurol.* 519:2697–2710. [PubMed: 21484803]
- McClain JA, Morris SA, Marshall SA, Nixon K. 2014; Ectopic hippocampal neurogenesis in adolescent male rats following alcohol dependence. *Addict Biol.* 19:687–699. [PubMed: 23844726]
- Mhatre MC, McKenzie SE, Gonzalez LP. 2001; Diazepam during prior ethanol withdrawals does not alter seizure susceptibility during a subsequent withdrawal. *Pharmacol Biochem Behav.* 68:339–346. [PubMed: 11267639]
- Morris SA, Eaves DW, Smith AR, Nixon K. 2010a; Alcohol inhibition of neurogenesis: a mechanism of hippocampal neurodegeneration in an adolescent alcohol abuse model. *Hippocampus.* 20:596–607. [PubMed: 19554644]

- Morris SA, Kelso ML, Liput DJ, Marshall SA, Nixon K. 2010b; Similar withdrawal severity in adolescents and adults in a rat model of alcohol dependence. *Alcohol*. 44:89–98. [PubMed: 20113877]
- Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. 1994; Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron*. 13:1071–1082. [PubMed: 7946346]
- Ninkovic J, Gotz M. 2007; Signaling in adult neurogenesis: from stem cell niche to neuronal networks. *Curr Opin Neurobiol*. 17:338–344. [PubMed: 17475475]
- Nixon K. 2006; Alcohol and adult neurogenesis: roles in neurodegeneration and recovery in chronic alcoholism. *Hippocampus*. 16:287–295. [PubMed: 16421863]
- Nixon K, Crews FT. 2002; Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. *J Neurochem*. 83:1087–1093. [PubMed: 12437579]
- Nixon K, Crews FT. 2004; Temporally specific burst in cell proliferation increases hippocampal neurogenesis in protracted abstinence from alcohol. *J Neurosci*. 24:9714–9722. [PubMed: 15509760]
- Nixon K, Kim DH, Potts EN, He J, Crews FT. 2008; Distinct cell proliferation events during abstinence after alcohol dependence: microglia proliferation precedes neurogenesis. *Neurobiol Dis*. 31:218–229. [PubMed: 18585922]
- Noonan MA, Bulin SE, Fuller DC, Eisch AJ. 2010; Reduction of Adult Hippocampal Neurogenesis Confers Vulnerability in an Animal Model of Cocaine Addiction. *J. Neurosci*. 30:304–315. [PubMed: 20053911]
- Noori HR, Fornal CA. 2011; The appropriateness of unbiased optical fractionators to assess cell proliferation in the adult hippocampus. *Front Neurosci*. 5:140. [PubMed: 22207833]
- Nowakowski RS, Lewin SB, Miller MW. 1989; Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *J Neurocytol*. 18:311–318. [PubMed: 2746304]
- Obernier JA, Bouldin TW, Crews FT. 2002a; Binge ethanol exposure in adult rats causes necrotic cell death. *Alcohol Clin Exp Res*. 26:547–557. [PubMed: 11981132]
- Obernier JA, White AM, Swartzwelder HS, Crews FT. 2002b; Cognitive deficits and CNS damage after a 4-day binge ethanol exposure in rats. *Pharmacol Biochem Behav*. 72:521–532. [PubMed: 12175448]
- Olufka, R, Peng, H, Newton, J, Nixon, K. Alcohol effects on adult neural stem cells – a novel mechanism of neurotoxicity in alcohol use disorders. In: Rasmussen, T, editor. *Stem Cells in Toxicology and Teratology*. John Wiley and Sons; New York: 2017. in press
- Ozsoy S, Durak AC, Esel E. 2013; Hippocampal volumes and cognitive functions in adult alcoholic patients with adolescent-onset. *Alcohol*. 47:9–14. [PubMed: 23063480]
- Palmer TD, Takahashi J, Gage FH. 1997; The adult rat hippocampus contains primordial neural stem cells. *Mol Cell Neurosci*. 8:389–404. [PubMed: 9143557]
- Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. 1997; Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci*. 17:3727–3738. [PubMed: 9133393]
- Paxinos, G, Watson, C. *The rat brain in stereotaxic coordinates* / George Paxinos, Charles Watson. Elsevier Academic; London: 2009.
- Peng H, Nickell CRG, Chen KY, Nixon K. 2017; Increased expression of M1 and M2 phenotypic markers in isolated microglia after four-day binge alcohol exposure in male rats. *Alcohol*. 62:29–40. [PubMed: 28755749]
- Penland S, Hoplight B, Obernier J, Crews FT. 2001; The effects of nicotine on ethanol dependence and brain damage. *Alcohol*. 24:45–54. [PubMed: 11524181]
- Pfefferbaum A, Sullivan EV, Mathalon DH, Shear PK, Rosenbloom MJ, Lim KO. 1995; Longitudinal changes in magnetic resonance imaging brain volumes in abstinent and relapsed alcoholics. *Alcohol Clin Exp Res*. 19:1177–1191. [PubMed: 8561288]
- Prendergast MA, Mulholland PJ. 2012; Glucocorticoid and polyamine interactions in the plasticity of glutamatergic synapses that contribute to ethanol-associated dependence and neuronal injury. *Addict Biol*. 17:209–223. [PubMed: 21967628]

- Prigent C, Dimitrov S. 2003; Phosphorylation of serine 10 in histone H3, what for? *J Cell Sci.* 116:3677–3685. [PubMed: 12917355]
- Richardson HN, Chan SH, Crawford EF, Lee YK, Funk CK, Koob GF, Mandyam CD. 2009; Permanent impairment of birth and survival of cortical and hippocampal proliferating cells following excessive drinking during alcohol dependence. *Neurobiol Dis.* 36:1–10. [PubMed: 19501165]
- Riley JN, Walker DW. 1978; Morphological alterations in hippocampus after long-term alcohol consumption in mice. *Science.* 201:646–648. [PubMed: 566953]
- Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R. 2011; Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature.* 472:466–470. [PubMed: 21460835]
- Sakharkar AJ, Vetreno RP, Zhang H, Kokare DM, Crews FT, Pandey SC. 2016; A role for histone acetylation mechanisms in adolescent alcohol exposure-induced deficits in hippocampal brain-derived neurotrophic factor expression and neurogenesis markers in adulthood. *Brain Struct Funct.* 221:4691–4703. [PubMed: 26941165]
- Scholzen T, Gerdes J. 2000; The Ki-67 protein: from the known and the unknown. *J Cell Physiol.* 182:311–322. [PubMed: 10653597]
- Segi-Nishida E, Warner-Schmidt JL, Duman RS. 2008; Electroconvulsive seizure and VEGF increase the proliferation of neural stem-like cells in rat hippocampus. *Proc Natl Acad Sci U S A.* 105:11352–11357. [PubMed: 18682560]
- Sgubin D, Aztiria E, Perin A, Longatti P, Leanza G. 2007; Activation of endogenous neural stem cells in the adult human brain following subarachnoid hemorrhage. *J Neurosci Res.* 85:1647–1655. [PubMed: 17455304]
- Somkuwar SS, Fannon MJ, Staples MC, Zamora-Martinez ER, Navarro AI, Kim A, Quigley JA, Edwards S, Mandyam CD. 2016; Alcohol dependence-induced regulation of the proliferation and survival of adult brain progenitors is associated with altered BDNF-TrkB signaling. *Brain Struct Funct.* 221:4319–4335. [PubMed: 26659122]
- Stone SS, Teixeira CM, Devito LM, Zaslavsky K, Josselyn SA, Lozano AM, Frankland PW. 2011; Stimulation of entorhinal cortex promotes adult neurogenesis and facilitates spatial memory. *J Neurosci.* 31:13469–13484. [PubMed: 21940440]
- Suh H, Consiglio A, Ray J, Sawai T, D'Amour KA, Gage FH. 2007; In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. *Cell Stem Cell.* 1:515–528. [PubMed: 18371391]
- Sullivan EV, Marsh L, Mathalon DH, Lim KO, Pfefferbaum A. 1995; Anterior hippocampal volume deficits in nonamnesic, aging chronic alcoholics. *Alcohol Clin Exp Res.* 19:110–122. [PubMed: 7771636]
- Sun C, Sun H, Wu S, Lee CC, Akamatsu Y, Wang RK, Kernie SG, Liu J. 2013; Conditional ablation of neuroprogenitor cells in adult mice impedes recovery of poststroke cognitive function and reduces synaptic connectivity in the perforant pathway. *J Neurosci.* 33:17314–17325. [PubMed: 24174664]
- Sun D, Daniels TE, Rolfe A, Waters M, Hamm R. 2015; Inhibition of injury-induced cell proliferation in the dentate gyrus of the hippocampus impairs spontaneous cognitive recovery after traumatic brain injury. *J Neurotrauma.* 32:495–505. [PubMed: 25242459]
- Sun D, McGinn MJ, Zhou Z, Harvey HB, Bullock MR, Colello RJ. 2007; Anatomical integration of newly generated dentate granule neurons following traumatic brain injury in adult rats and its association to cognitive recovery. *Exp Neurol.* 204:264–272. [PubMed: 17198703]
- Taffe MA, Kotzebue RW, Crean RD, Crawford EF, Edwards S, Mandyam CD. 2010; Long-lasting reduction in hippocampal neurogenesis by alcohol consumption in adolescent nonhuman primates. *Proc Natl Acad Sci U S A.* 107:11104–11109. [PubMed: 20534463]
- Vorel SR, Liu X, Hayes RJ, Spector JA, Gardner EL. 2001; Relapse to cocaine-seeking after hippocampal theta burst stimulation. *Science.* 292:1175–1178. [PubMed: 11349151]
- Walker TL, White A, Black DM, Wallace RH, Sah P, Bartlett PF. 2008; Latent stem and progenitor cells in the hippocampus are activated by neural excitation. *J Neurosci.* 28:5240–5247. [PubMed: 18480280]

- West MJ. 1991; Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. *Anat Rec.* 231:482–497. [PubMed: 1793176]
- Wilson S, Bair JL, Thomas KM, Iacono WG. 2017 Problematic alcohol use and reduced hippocampal volume: a meta-analytic review. *Psychol Med.* :1–14.
- Yoneyama M, Shiba T, Hasebe S, Ogita K. 2011; Adult neurogenesis is regulated by endogenous factors produced during neurodegeneration. *J Pharmacol Sci.* 115:425–432. [PubMed: 21422724]
- Yu TS, Zhang G, Liebl DJ, Kernie SG. 2008; Traumatic brain injury-induced hippocampal neurogenesis requires activation of early nestin-expressing progenitors. *J Neurosci.* 28:12901–12912. [PubMed: 19036984]
- Yun S, Reynolds RP, Masiulis I, Eisch AJ. 2016; Re-evaluating the link between neuropsychiatric disorders and dysregulated adult neurogenesis. *Nat Med.* 22:1239–1247. [PubMed: 27783068]
- Zahr NM, Mayer D, Rohlfing T, Hasak MP, Hsu O, Vinco S, Orduna J, Luong R, Sullivan EV, Pfefferbaum A. 2010; Brain injury and recovery following binge ethanol: evidence from in vivo magnetic resonance spectroscopy. *Biol Psychiatry.* 67:846–854. [PubMed: 20044076]

Highlights

- Reactive neurogenesis after alcohol dependence is confirmed with NeuroD1
- Recruitment of progenitors out of quiescence likely drives reactive neurogenesis
- Reactive neurogenesis begins earlier than original reports via Type-2a progenitors
- Reactive neurogenesis is not due to alterations in cell cycle
- Type-1 neural stem cell activation has implications for alcoholic neuropathology

Figure 1.

NeuroD1+ immunoreactivity is increased following 14, but not 7, days of abstinence in ethanol-treated rats. Representative photomicrographs of control (A,C) and ethanol-exposed animals (B,D) at 7 days (A,B) and 14 days (C,D) post ethanol exposure. The number of NeuroD1+ cells in the subgranular zone (SGZ) of the hippocampal dentate gyrus was determined using unbiased stereology (E) for ethanol (n=8–9) and controls (n=6–8) and shown as total estimated NeuroD1+ cells $\pm$ SEM. GCL, granule cell layer; scale bar = 100 μ m; inset scale bar = 20 μ m; * p <0.001.

Figure 2.

Alcohol-induced reactive proliferation begins five days post-binge, which is two days earlier than originally reported (Nixon and Crews, 2004). The number of BrdU+ profiles (A) were counted only in the SGZ in order to avoid proliferating microglia (Nixon et al., 2008) and are presented as mean BrdU+ cells/section $\pm$ SEM. Two to four controls were counted at each time point, and upon one-way ANOVA to confirm a lack of difference between time points, were collapsed into a common control group. Representative images are shown for BrdU immunoreactivity in control (B) and ethanol-exposed rats at 4 (C), 5 (D), and 7 (E) days after alcohol exposure. Scale bar = 100 μ m; 20 μ m inset; * p <0.05 versus control.

Figure 3.

Seven days of abstinence following binge ethanol exposure increased the number of actively proliferating cells (Ki67+) without altering cell cycle dynamics. (A) A diagram indicating each stage of the cell cycle and the antibody used for identification: BrdU for cells recently in S-phase (within 2 h), pHisH₃ for cells in the G₂ and M phases, and Ki67 for labelling cells in all phases of active division (not G₀). The G₁ population was estimated by subtracting the sum of BrdU+ and pHisH₃+ cells from number of Ki67+ cells number. Representative photomicrographs from control (B-D, n=7) and ethanol exposed (E-G, n=5) rats are shown for Ki67 (B, E), BrdU (C, F) and pHisH₃ (D, G). (H) Profile counts were used to quantify the number of cells/section due to nonhomogeneous distribution of these markers (Crews et al., 2004; Noori and Fornal, 2011). Abstinence following ethanol exposure yielded a significant increase in the number of proliferating cells in all phases of the cell cycle (H), but did not alter the proportion of cells in any phase of the cell cycle (I). Data are mean ± SEM. BrdU, bromo-deoxy-uridine; pHisH₃, serine 10 phosphorylated Histone H₃; GCL, granule cell layer; scale bar = 100 μm; inset scale bar = 20 μm; **p*<0.05.

Figure 4.

Reactive hippocampal neurogenesis following ethanol exposure and abstinence is due to an increase in the number of proliferating neural progenitor cells. (A,B) Representative photomicrographs of Sox2+ cells in the hippocampus of control (A, n=6) and ethanol-exposed (B, n=8) rats after 7 days of abstinence, quantification of which is shown in C. (D, E) Representative confocal images are shown for Sox2 (D), BrdU (E), and merged (F). Every BrdU+ cell was counted (G, left) and evaluated for Sox2 colabeling (G, right). The original finding of an increase in BrdU was replicated and extended to show that the majority of proliferating cells are Sox2+ NPCs. Data are mean $\pm$ SEM. BrdU, Bromodeoxy-Uridine; GCL, granule cell layer; scale bar = 100 μ m (A); scale bar = 20 μ m (A inset and D). * p <0.05

Figure 5.

Four-day binge ethanol exposure results in recruitment of hippocampal NPCs out of quiescence and into active proliferation at T7 of abstinence. Representative confocal images of Sox2+ (A), Ki67+ (B), and dual-labeled (C) cells were obtained from rats in abstinence at 7 days after binge ethanol exposure (n=8) or control diet (n=6). All Sox2+ cells were evaluated for the presence or absence of Ki67 and presented as the mean percentage of NPCs in active portion of the cell cycle $\pm$ SEM. (D) Progenitor cell activation is evidenced by a shift in the proportion of Sox2+/Ki67– (quiescent) to Sox2+/Ki67+ (proliferating) cells in rats with prior ethanol exposure at this time point. Scale bar = 20 μ m. * p <0.01.

Figure 6.

Adult neurogenesis is a multi-step process that begins with Type 1 stem-like radial glial cells that proliferate and divide asymmetrically to generate a progenitor cell (Type 2a) which can then proliferate and/or differentiate into a Type 2b NPC (circular arrows indicate that the cell is able to proliferate (Kempermann et al., 2004)). Type 2b NPCs can proliferate and/or differentiate into a Type 3 NPC, which can then proliferate and differentiate into an immature neuron. Fluorescent quadruple immunohistochemistry was designed to identify each of the various progenitor cell subtypes as described in A. For example, NeuroD1 is expressed in Type 2b and Type 3 NPCs and is considered to be a Type 2b cell when it co-

expresses with Sox2, but a Type 3 cell when Sox2 is no longer expressed. (B) Representative confocal images are shown: type 1 cell is positive for Ki67, GFAP, and Sox2 but is negative for NeuroD1 (row 1). Type 2a proliferating cells are positive for Ki67 and Sox2 but negative for GFAP and NeuroD1 (row 2). A combination of Ki67+, Sox2+, and NeuroD1+ yet GFAP- signifies a type 2b cell (row 3). Finally, a cell that is only Ki67+ and NeuroD1+ is a type 3 cell (row 4). ML, molecular layer; GCL, granule cell layer; SGZ, subgranular zone; scale bar = 20 μ m.

Figure 7.

Characterization of the proliferating neural progenitor cell pool in the hippocampus of adult rats 7 days after 4 days of binge alcohol exposure. Each Ki67+ cell was investigated for the presence or absence of the other markers. A) NPC subtypes are shown as a percent of total Ki67+ cells counted $\pm$ SEM. (B) In order to approximate the number of cells in each progenitor phenotype, the percentage was multiplied by the total number of Ki67+ cells and these data are presented as mean cells $\pm$ SEM. Control $n=6$, ethanol $n=8$. * $p<0.05$.

Figure 8.

The proliferating pool of NPCs in the hippocampus of adult rats was characterized at 5 days after 4 days of binge alcohol exposure, the point at which reactive proliferation appears to emerge. Each Ki67+ cell was investigated for the presence or absence of the other markers. (A) Progenitor subtypes are shown as a percent of total Ki67+ cells counted $\pm$ SEM. (B) In order to approximate the number of cells in each progenitor phenotype, the percentage in A was multiplied by the total number of Ki67+ cells and these data are presented as mean cells $\pm$ SEM. Control $n=6$, ethanol $n=8$. * $p<0.05$.

Table 1

Animal Model Data

Days post binge	BEC (mg/dl)	INTOX (0–5 scale)	DOSE (g/kg/day)	WD (0–4 scale)
7 days (n=8)	360.8 ± 14.8	1.8 ± 0.1	9.7 ± 0.4	1.9 ± 0.2
7 days (n=8)	307.3 ± 25.3 *	1.8 ± 0.1	9.7 ± 0.4	1.0 ± 0.1 *
14 days (n=9)	377.3 ± 12.9	1.9 ± 0.1	9.3 ± 0.3	1.8 ± 0.2
28 days (n=7)	400.2 ± 17.9	1.9 ± 0.2	9.4 ± 0.5	1.5 ± 0.3

BEC, Blood Ethanol Concentration; INTOX, Intoxication score; WD, Mean Withdrawal

*
p<0.05 indicates significant difference from T28 cohort;

**
p<0.01 compared to all other cohorts

Table 2

BrdU Time course Animals

Days post binge	BEC (mg/dl)	INTOX (0–5 scale)	DOSE (g/kg/day)	WD (0–4 scale)
4 days (n=4)	324 ± 5	1.8 ± 0.1	9.7 ± 0.3	0.6 ± 0.1
5 days (n=6)	330 ± 44	1.6 ± 0.1	10.1 ± 0.4	0.9 ± 0.2
6 days (n=6)	412 ± 11	1.9 ± 0.1	9.2 ± 0.3	0.7 ± 0.2
7 days (n=4)	356 ± 42	1.5 ± 0.4	10.6 ± 1.1	0.8 ± 0.1

BEC, Blood Ethanol Concentration; INTOX, Intoxication score; WD, Mean Withdrawal

Table 3

Antibody Specifications

Primary Antibody	Company; Product no.	Concentration (% serum)	Time in Primary	Secondary antibody (source)
DAB				
Goat anti-NeuroD	Santa Cruz; sc1084	1:1000 (3%)	48 h	Rabbit anti-goat (Vector)
Mouse anti-Ki67	Vector; VP-K452	1:200 (3%)	48 h	Horse anti-mouse (Vector)
Mouse anti-BrdU	Millipore; MAB3424	1:5000 (3%)	16 h	Horse anti-mouse (Vector)
Rabbit anti-pHisH3	Millipore; 06-570	1:1000 (3%)	16 h	Goat anti-rabbit (Vector)
Rabbit anti-Sox2	Millipore; ab5603	1:1000 (3%)	16 h	Goat anti-rabbit (Vector)
Fluorescent				
<i>BrdU+Sox Colabel</i>				
Rabbit anti-Sox2	Millipore; ab5603	1:1000	48 h	AlexaFluor goat anti-rabbit 488 (Invitrogen)
Rat anti-BrdU	Accurate; OBT0030	1:400 (3%)	48 h	AlexaFluor goat anti-rat 546 (Invitrogen)
<i>Quad Colabel</i>				
Mouse anti-Ki67	Vector; VP-K452	1:100	96 h	Donkey anti-mouse 405 (Jackson)
Chicken anti-GFAP	Abcam; ab 4674m	1:1000	96 h	Donkey anti-chicken 488 (Jackson)
Rabbit anti-Sox2	Millipore; ab5603	1:250 (10%)	96 h	AlexaFluor donkey anti-rabbit 546 (Invitrogen)
Goat anti-NeuroD	Santa Cruz; sc1084	1:500	96 h	AlexaFluor donkey anti-goat 633 (Invitrogen)
<i>Sox2+Ki67 Colabel</i>				
Rabbit anti-Sox2	Millipore; ab5603	1:250	96 h	AlexaFluor donkey anti-rabbit 488 (Invitrogen)
Mouse anti-Ki67	Vector; VP-K452	1:100 (10%)	96 h	AlexaFluor donkey anti-rat 546 (Invitrogen)