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Spatial and Sex-Dependent Responses of Adult Endogenous Neural Stem Cells to Alcohol Consumption

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SUMMARY

Chronic alcohol abuse results in alcohol-related neurodegeneration, and critical gaps in our knowledge hinder therapeutic development. Neural stem cells (NSCs) are a subpopulation of cells within the adult brain that contribute to brain maintenance and recovery. While it is known that alcohol alters NSCs, little is known about how NSC response to alcohol is related to sex, brain region, and stage of differentiation. Understanding these relationships will aid in therapeutic development. Here, we used an inducible transgenic mouse model to track the stages of differentiation of adult endogenous NSCs and observed distinct NSC behaviors in three brain regions (subventricular zone, subgranular zone, and tanycyte layer) after long-term alcohol consumption. Particularly, chronic alcohol consumption profoundly affected the survival of NSCs in the subventricular zone and altered NSC differentiation in all three regions. Significant differences between male and female mice were further discovered.

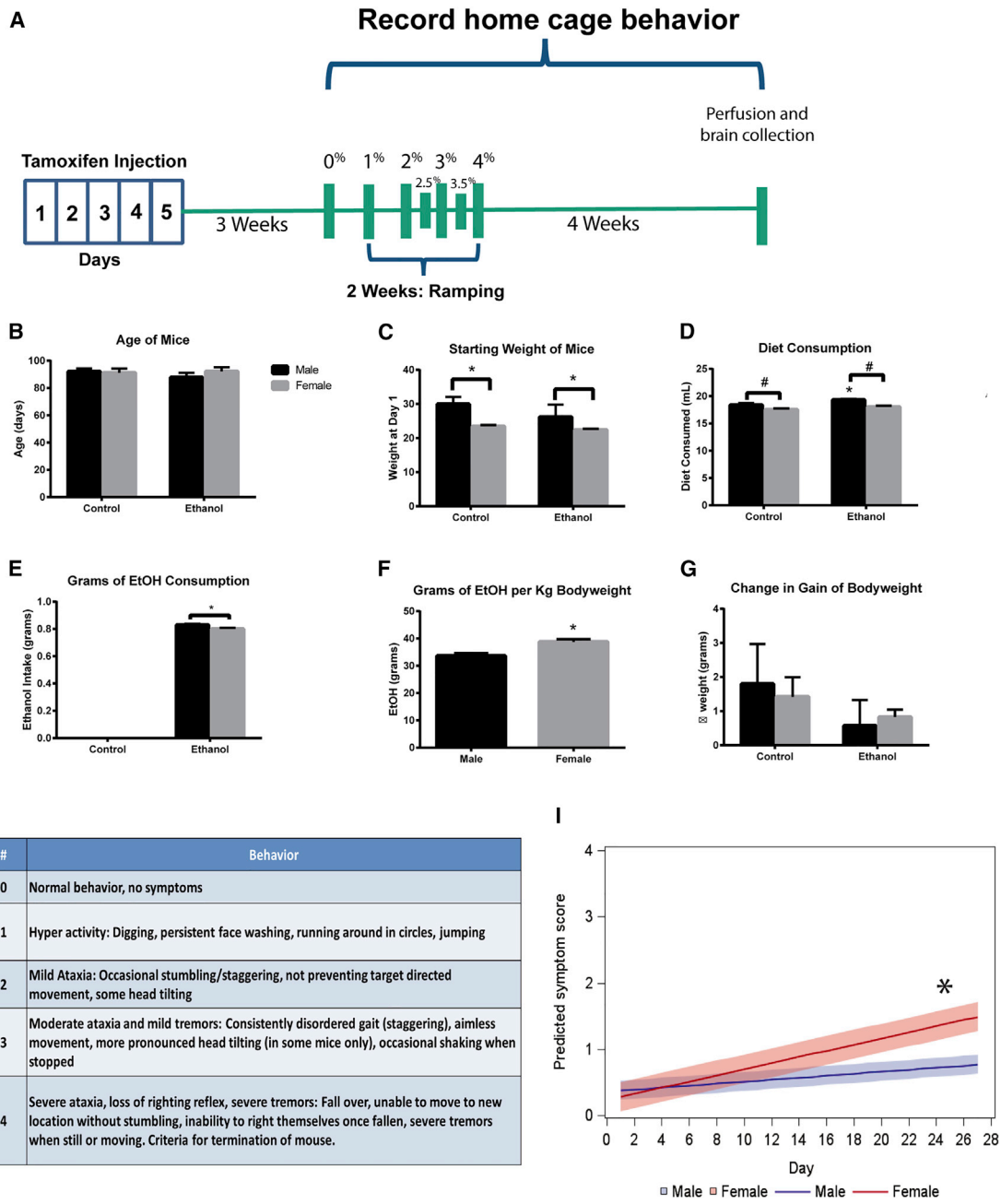
INTRODUCTION

Alcohol abuse is the third leading cause of preventable death in the United States, with almost 30% of the population meeting the criteria for alcohol use disorder at some point in life (Grant et al., 2015). It is established that alcohol misuse has detrimental effects on the brain and leads to deficits in cognitive and behavioral function, as well as a condition referred to as alcohol-related neurodegeneration (Zahr et al., 2011; Vetreno and Crews, 2015; Staples and Mandyam, 2016).

Several factors affect the response of the brain to alcohol, such as age, sex, duration, and quantity of alcohol consumed (Oscar-Berman and Marinkovic, 2003; Squeglia et al., 2014). There are also regional patterns of degeneration and glial pathology observed in patients with a history of chronic alcohol abuse (Pfefferbaum et al., 1992, 1993; Cullen and Halliday, 1994; Agartz et al., 1999; Zahr et al., 2011). As with neurodegenerative diseases, neural damage observed in chronic alcoholism is speculated to be not only caused by neuronal death, but also by inhibition of neurogenic processes (He et al., 2005; Crews et al., 2006; Morris et al., 2010; Winner et al., 2011). Thus, the effect of alcohol on neural stem cells (NSCs) has become a topic of interest due to the critical role NSCs play in maintaining neurogenesis throughout life, and possibly in mediating neuro-regeneration after insults (Leasure and Nixon, 2010; Maynard and Leasure, 2013).

The two most commonly studied NSC-containing regions in the adult brain are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus (Crews and Nixon, 2003; Kempermann et al., 2015). In these regions, alcohol consumption impairs NSC proliferation and neurogenesis (Rice et al., 2004; Crews et al., 2006; Broadwater et al., 2014; McClain et al., 2011; Campbell et al., 2014). Recently, the tanycyte layer (TL) of the third ventricle was discovered to contain cells with NSC properties; however, the impact of alcohol on this cell population has not been investigated (Robins et al., 2013). While studies have investigated the effect of alcohol in the SVZ and SGZ, a comparative examination of how NSCs in all three regions (SVZ, SGZ, and TL) respond to alcohol at different stages of differentiation has not been conducted. Despite the broad knowledge that males and females respond differently to alcohol clinically, the role of sex differences in NSC response to alcohol consumption is not well explored. These two unaddressed issues leave substantial gaps in knowledge of how the brain recovers following chronic alcohol abuse.

To address these gaps in knowledge, we utilized recent advances in genetic inducible fate mapping which provides a tool to study longitudinal changes of endogenous NSC



#	Behavior
0	Normal behavior, no symptoms
1	Hyper activity: Digging, persistent face washing, running around in circles, jumping
2	Mild Ataxia: Occasional stumbling/staggering, not preventing target directed movement, some head tilting
3	Moderate ataxia and mild tremors: Consistently disordered gait (staggering), aimless movement, more pronounced head tilting (in some mice only), occasional shaking when stopped
4	Severe ataxia, loss of righting reflex, severe tremors: Fall over, unable to move to new location without stumbling, inability to right themselves once fallen, severe tremors when still or moving. Criteria for termination of mouse.

Figure 1. Experimental Model

(A) Schematic of experimental paradigm.
(B) Average age of mice at start of experiment.
(C) Average bodyweight of mice at the start of experiment.
(D) Average daily diet consumption.
(E) Average grams of ethanol consumed daily.
(F) Average grams of ethanol consumed per kilogram of mouse bodyweight.
(G) Average changes in gain of bodyweight.
(H) Chart showing the scoring system evaluation criteria.

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populations in the adult brain (Lagace et al., 2007; Taupin, 2007). This technology allows us to evaluate differentiation and population changes of endogenous NSCs in response to alcohol. Specifically, we analyzed the effect of chronic alcohol consumption on NSC populations in adult mice and concentrated on the three regions in the mouse brain that are known to contain NSCs: SVZ, SGZ, and TL. In addition, we compared NSC responses in males and females to determine the role of sex in response to alcohol.

RESULTS

Differences in Tolerance to Alcohol Consumption

Adult *Nestin*-CreER^{T2}:R26R-YFP bitransgenic mice were used to trace the fate of endogenous NSCs following tamoxifen induction, and chronic ethanol feeding with the Lieber-DeCarli ethanol liquid diet (Figure 1A). Both pair-fed control and ethanol-fed mice were given *ad libitum* access to their respective diets and water. To ensure that both ethanol and control mice received diets with the same caloric value, maltose-dextrin was used to calorie balance the diets. At the start of the study, males weighed more than females; however, there was no significant difference between the body weights of control or ethanol groups (Figures 1B and 1C). Male mice in both groups had a higher average daily diet consumption compared with their female counterparts. Control males consumed 5% more diet than control females, and ethanol males consumed 7% more than ethanol females (Figure 1D). Due to the greater consumption, male mice consumed more grams of ethanol compared with females; however, females consumed 13% more grams of ethanol per kilogram of body weight (Figures 1E and 1F). There were no significant changes in body weight gains, but mice in the ethanol group tended to have less weight gain (Figure 1G).

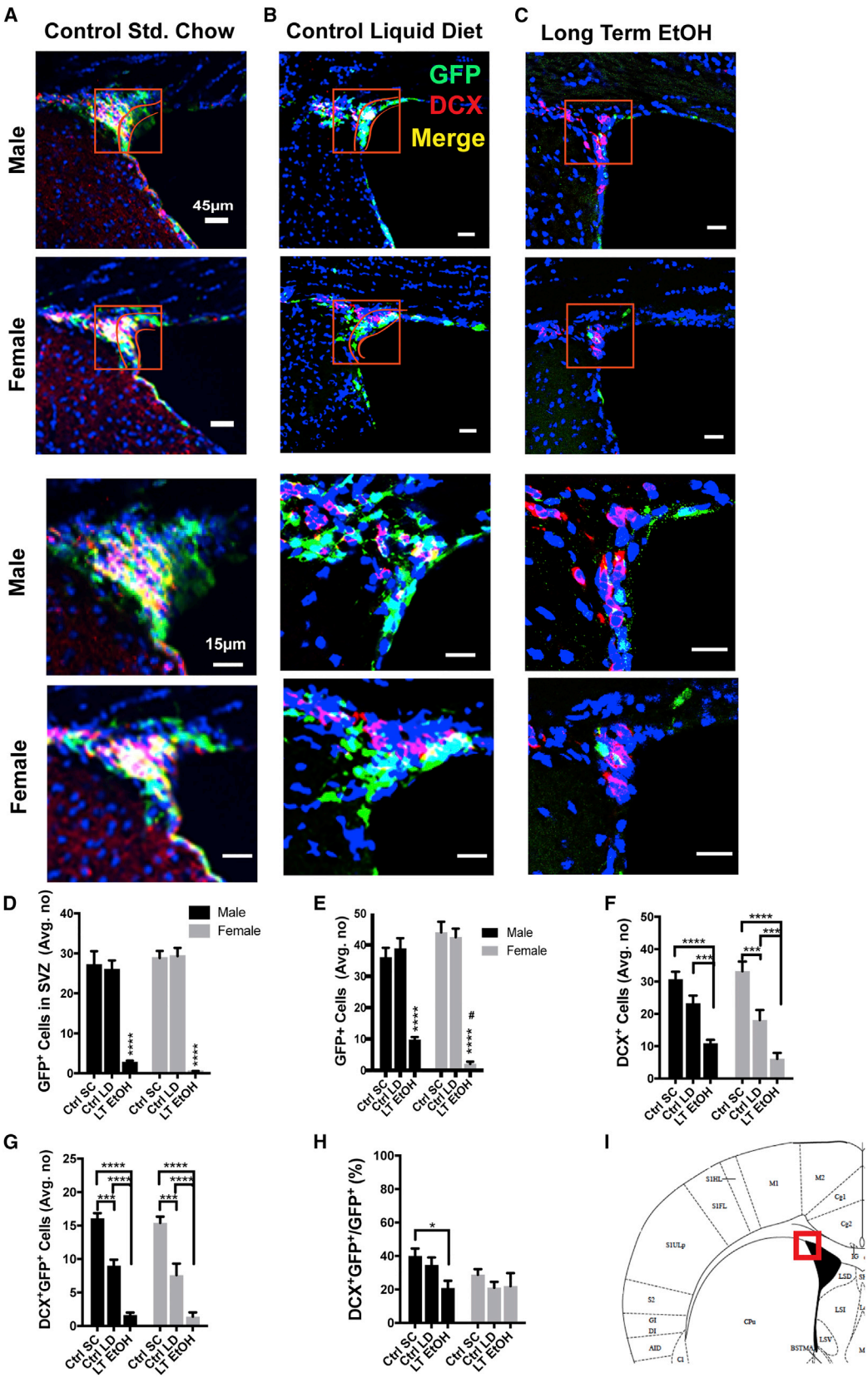
Similar to humans, mice showed individual variations in response to alcohol intake, ranging from ataxia to loss of righting reflex. To evaluate ethanol intoxication, an ordinal scale was created based upon home cage behaviors ranging from asymptomatic to severe on a scale of 0–4, respectively (Figure 1H). These behaviors have previously been associated with intoxication in mice (Crabbe et al., 2008). All animals in the ethanol group tolerated the 2-week ramping stage without abnormal behaviors. Among 25 mice fed with 4% ethanol, 4 males (30.8%) and 4 females (33.3%) were kept on a sustained 4% ethanol diet for 28 days with mild symptoms and thus were included in the "long-term" study. On the other hand, 9 males (69.2%) and 8 females (66.7%) showed severe symptoms of intoxication reaching a score of 4 between 6 and 24 days with 4% ethanol. Five males and 4 females died between 6 and 24 days of 4% ethanol, and were not included in this study. Other mice with severe symptoms were euthanized between 6 and 11 days and not included for further histopathological analyses in this study. Ethanol females displayed more severe symptoms of intoxication and escalated in severity at a higher rate than males ($p = 0.009$) (Figure 1I). Blood alcohol analysis showed a wide range of blood alcohol concentrations (BACs) and blood acetaldehyde concentrations, which did not correlate with behavioral severity for either sex (Figure S1). One source of variability may be due to blood samples collected in the morning, while mice typically feed in the first few hours of the dark cycle. In addition, since mice have unrestricted access to the diet it is possible that mice with higher BACs consumed the diet more recently than mice with lower BACs.

Effect of Alcohol Consumption on SVZ NSCs in the Lateral Ventricle

We focused on the rostral lateral ventricle, which harbors endogenous NSCs and is an area of active neurogenesis in adult mammalian brains (Lim and Alvarez-Buylla, 2014). The *Nestin*-CreER^{T2};R26R-YFP transgenic mice allowed us to trace the NSCs that were present at the time of tamoxifen injection by their expression of yellow fluorescent protein (YFP) (Lagace et al., 2007). Tamoxifen, administered at 2 months of age, enabled expression of the YFP reporter gene in *Nestin*-expressing NSCs in adult mice. The YFP signal was further enhanced by immunohistochemistry using a GFP antibody. Since the YFP reporter gene was activated by tamoxifen, only cells expressing *Nestin* at the time of tamoxifen injection would be labeled with YFP. Since YFP is constitutively active, all progeny cells from that induced population will also express YFP, regardless of differentiation or division. This provides the capacity to study a population of NSCs and their progeny in response to alcohol consumption. This genetic tracing technique allowed us to examine three phases of NSCs: NSCs (GFP$^+$), newly differentiated NSCs (DCX$^+$GFP$^+$ or GFAP$^+$GFP$^+$), and neurons or astrocytes that were present before tamoxifen induction (DCX$^+$ or GFAP$^+$). Cells that were double-labeled with GFP and a marker of differentiation (DCX or GFAP) represent newly differentiated cells after tamoxifen induction of the YFP expression in *Nestin*$^+$ cells. Cells that

(I) Graph comparing the severity of alcohol-induced symptoms over time. Red line, female; blue line, male; shadow, error bars. Values are medians in each day evaluated by a random slope model.

Values are shown as mean ± SEM, *$p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with other sex in the same group, male mice n = 13, female mice n = 12; two-way ANOVA with Tukey's test. See also Figure S1.



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differentiated prior to tamoxifen induction and no longer expressed *Nestin* would not be co-labeled with GFP. In standard chow controls, there were more DCX^+ and DCX^+GFP^+, and fewer $GFAP^+GFP^+$ cells, indicating that the liquid diet itself may influence cell proliferation (Patten et al., 2013) and probably neurogenesis (Figures 2 and 3), likely due to the high fat in the liquid diet (Park et al., 2010). Thus, ethanol treatment was compared with liquid diet controls. Male and female mice on control diets had comparable numbers of GFP^+ NSCs in the SVZ region (Figures 2A and 2B). Mice receiving the ethanol diet exhibited significant alterations of SVZ NSCs in a temporal- and sex-dependent manner. In the SVZ region (indicated by orange lines), long-term treatment decreased GFP^+ cells by 98% in females and 89% in males (Figures 2A–2D). The total number of GFP^+ cells was reduced by 94% in females and 74% in males in the origin of the rostral migratory stream (oRMS) (indicated by the red box) (Figures 2A–2C and 2E).

Next we examined the neurogenic capacity of SVZ NSCs after ethanol consumption by using the doublecortin (DCX) antibody, a marker of newly differentiated neuroblasts and immature neurons (Klempin et al., 2011). Males and females receiving the liquid diet had fewer DCX^+ and DCX^+GFP^+ cells in the oRMS compared with standard chow controls (Figures 2A–2C, 2F, and 2G). In the liquid diet controls, DCX^+GFP^+ accounted for 35% and 22% of the total GFP cells in control males and females, respectively (Figures 2B and 2G). Long-term ethanol consumption significantly decreased DCX^+ expression by 54% in males and 66% in females (Figures 2C and 2F). In addition, DCX^+GFP^+ cells were reduced by 82% in males and 87% in females (Figures 2C and 2G). Males but not females demonstrated a reduction in the percent of GFP^+ cells becoming DCX^+ following long-term ethanol consumption (Figure 2H). The greater loss of DCX^+GFP^+ cells (Figure 2G) than that of the total DCX^+ cells indicates that recently differentiated NSCs are more susceptible to ethanol-mediated toxicity than older differentiated cells. To further confirm the effect of chronic ethanol consumption on neurogenesis of NSCs, dual immunolabeling with GFP and NeuN (a mature neuronal marker) was conducted in the olfactory bulb. As shown in Figure 3, chronic ethanol treatment significantly reduced the numbers of newly generated mature neurons ($NeuN^+GFP^+$) and GFP^+ cells in the olfactory bulb, exhibiting a trend similar to DCX^+GFP^+ in the oRMS (Figure 3).

We further assessed the effect of ethanol consumption on NSC astroglial differentiation by using glial fibrillary acidic protein (GFAP) as a glial marker. In the SVZ region of the control mice, the majority of GFP^+ cells were co-labeled with GFAP (Figures 4A–4C). To determine astrogliogenesis from the SVZ NSCs expressing GFAP, we quantified those GFP and GFAP co-labeled cells in the oRMS (indicated by the red box) (Liu et al., 2010). No significant differences were found in the number of $GFAP^+$ cells between the standard chow and control liquid diets; however, females in the liquid diet control group had significantly less $GFAP^+$ cells compared with their male counterparts (Figures 4A–4D). In ethanol-treated mice, males showed a trend of reduction in $GFAP^+$ cells, albeit statistically insignificant, compared with both standard chow and control liquid diets (Figures 4C and 4D). $GFAP^+GFP^+$ cells decreased by 90% and 97% in males and females, respectively (Figures 4C and 4E). Furthermore, the percent of GFP^+ cells expressing $GFAP^+$ was significantly reduced in females (92%) and had a trend of reduction in males (Figure 4F). Ethanol-treated females had a significantly lower percentage of GFP^+ cells expressing $GFAP^+$ compared with their male counterparts. Individual immunohistochemical stain images can be found in the Supplemental Experimental Procedures (Figure S2).

In summary, these data show the NSCs in the SVZ are sensitive to long-term ethanol consumption, more so in females. Chronic ethanol consumption altered neurogenesis by inducing a drastic loss of DCX^+GFP^+ neuroblasts/immature neurons in the oRMS, and a subsequent loss of $NeuN^+GFP^+$ in the olfactory bulb. Also, alcohol consumption decreased the survival of newly differentiated astrocytes ($GFAP^+GFP^+$).

Effect of Alcohol Consumption on SGZ NSCs in the Hippocampus

Next, we examined the SGZ in the dorsal hippocampus, given its role in cognitive function and active adult neurogenesis (Kempermann et al., 2015). The standard chow controls had similar numbers of GFP^+ cells; however, the

Figure 2. Neurogenesis in the SVZ following Ethanol Consumption

(A–C) Representative images of standard chow (Ctrl SC), liquid diet control (Ctrl LD), and long-term ethanol (LT EtOH) male and female mouse brains stained with stem cell marker (GFP green), neuronal marker (DCX red), and merged with nuclear maker DAPI (blue). The regions in the red boxes are shown enlarged at the bottom.

(D–H) Quantification of average total GFP^+ cells in SVZ and origin of the rostral migratory stream (D), GFP^+ within the SVZ (E), DCX^+ (F), and DCX^+GFP^+ (G), and the percentage of DCX^+GFP^+ over total GFP^+ cells (H).

(I) Schematic image of representative region quantified (bregma from 0.5 to 1.08) and outlined in the red box.

Values are shown as mean ± SEM, n = 3 mice per sex per group. $^*p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared with controls, two-way ANOVA with Tukey's test. $^{\#}p < 0.05$ compared with male counterpart, two-way ANOVA with Sidak's test. Scale bars, 45 μm, and 15 μm in enlarged images. See also Figure S2.

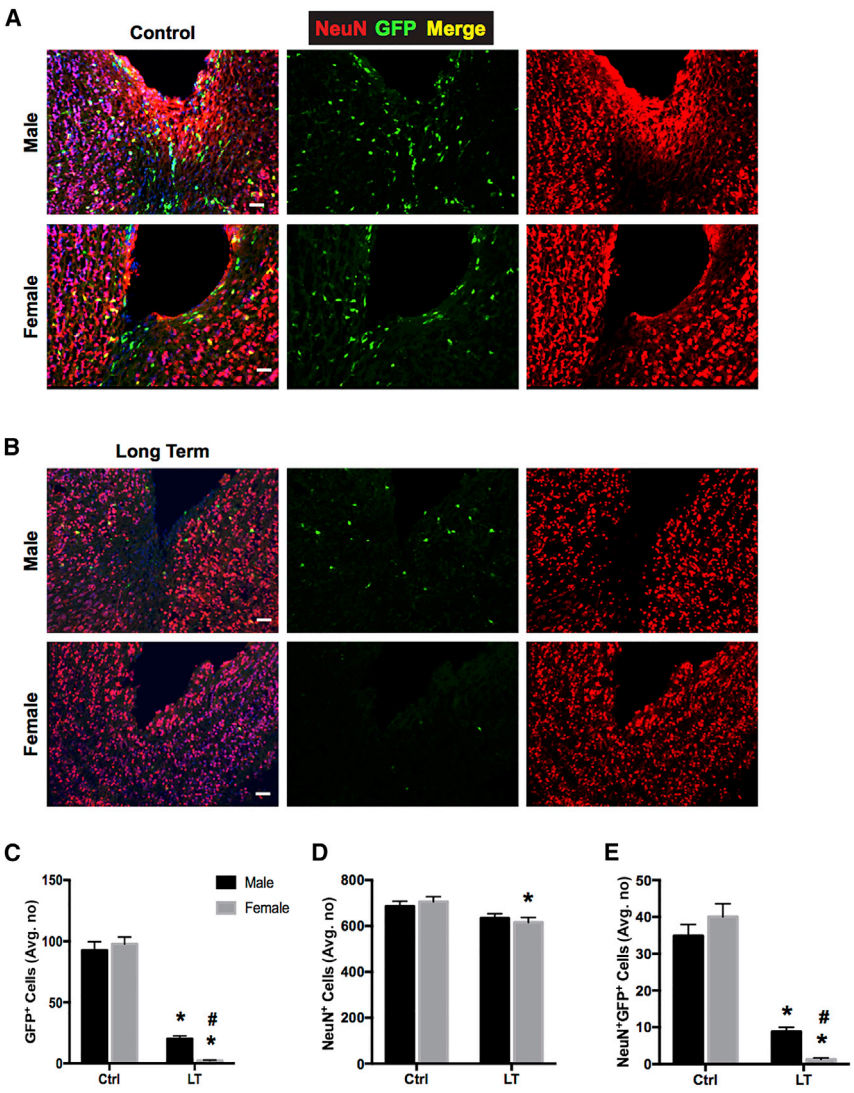

Figure 3. Neurogenesis in the Olfactory Bulb following Ethanol Consumption

(A and B) Representative images of control and long-term ethanol male and female mouse brains stained with stem cell marker (GFP green), mature neuronal marker (NeuN red), and merged with nuclear maker DAPI (blue). Scale bars, 32 μm. n = 3 mice per sex per group.

(C–E) Quantification of GFP^+, $NeuN^+$, and GFP^+NeuN^+ cells in the olfactory bulb.

Values are shown as mean ± SEM, $^*p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with male counterpart, two-way ANOVA with Tukey's test, n = 3 mice per sex per group.

liquid diet controls displayed sex differences, with female mice in the control group having approximately twice the number of GFP^+ cells as their male counterparts (Figures 5A–5D). The SGZ GFP^+ cells also showed a sex-dependent response to ethanol consumption. Alcohol treatment significantly reduced the numbers of GFP^+ cells in both males and females (Figures 5C and 5D).

When evaluating SGZ neurogenesis, liquid diet control males and females had similar levels of DCX^+ and DCX^+GFP^+ cells. Standard chow controls had significantly more DCX^+ cells compared with liquid diet controls (Figures 5A, 5B, and 5E). In the long-term ethanol cohort, DCX^+ cells were significantly decreased in males and females when compared with liquid diet controls (Figures 5A–5C, and 5E). There was a 49% and 65% decrease in males and females, respectively (Figure 5E). There was also a trend of reduction in both males and females in the DCX^+GFP^+ cell population, albeit statistically insignificant (Figures 5C and 5F). Males exhibited a significant reduction (63%) in the percentage of GFP^+ cells labeled with DCX when compared with both standard chow and liquid diet controls, whereas females only had a trending decrease compared with standard chow controls (Figure 5G).

When assessing SGZ astrogliogenesis, we found no significant differences in $GFAP^+$, $GFAP^+GFP^+$, or percentage of GFP^+ cells expressing GFAP between males and females within the control diets (Figures 6A, 6B, 6D, and 6E). In addition, all comparisons between standard chow and control liquid diets were similar (Figures 6A, 6B, and 6D–6F). Female mice in the ethanol group had a significant reduction in $GFAP^+$ cells compared with both standard chow and liquid diet controls; however, males only had a significant reduction relative to the standard chow group (Figures 6C and 6D). Long-term ethanol consumption did

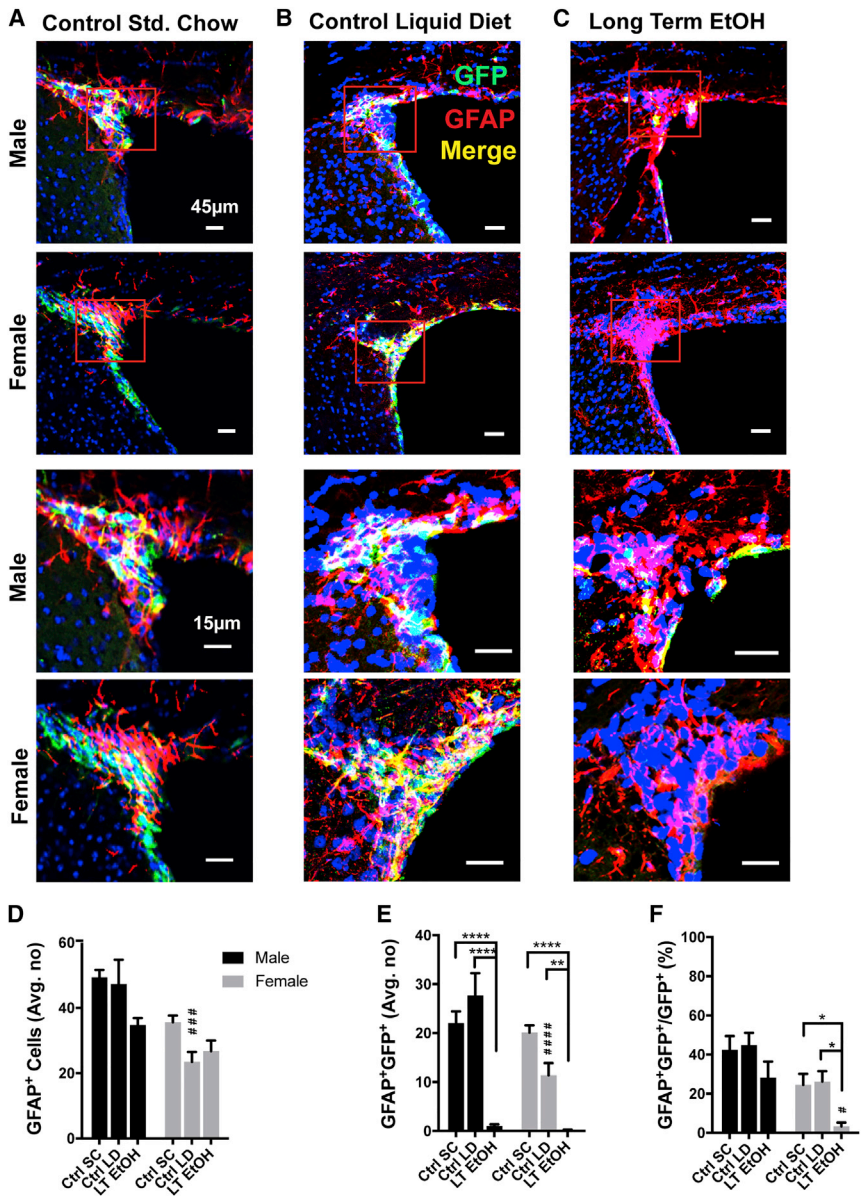

Figure 4. Astrogliogenesis in the SVZ following Ethanol Consumption

(A–C) Representative images of standard chow, liquid diet control, and long-term ethanol male and female mouse brains stained with stem cell marker (GFP green), astrocyte marker (GFAP red), and merged with nuclear maker DAPI (blue). The region in the red box is shown enlarged at the bottom.

(D–F) Quantification of average $GFAP^+$ (D) and $GFAP^+GFP^+$ (E), and the percentage of $GFAP^+GFP^+$ over total GFP^+ cells (F).

Scale bars, 45 μm, and 15 μm in enlarged images. Values are shown as mean ± SEM, n = 3 mice per sex per group. *p < 0.05, **p < 0.01, ****p < 0.0001 compared with controls, two-way ANOVA with Tukey's test. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ compared with other sex counterparts, two-way ANOVA with Sidak's test. See also Figure S2.

not have an impact on $GFAP^+GFP^+$ populations, or the percentage of GFP^+ cells expressing GFAP in males or females (Figures 6C, 6E, and 6F). These data suggest that $GFAP^+$ populations in the female SGZ are more sensitive to changes following ethanol consumption compared with males, and that $GFAP^+$ populations respond differently to alcohol in the SGZ compared with the SVZ. Individual immunohistochemical stain images can be found in Figure S3.

In summary, long-term alcohol consumption reduced neurogenesis in both males and females. In addition, females, but not males, had a significant reduction in $GFAP^+$ cells.

Effect of Chronic Alcohol Consumption on TL NSCs in the Third Ventricle

The third region we evaluated was the TL of the third ventricle. This region was selected because it has recently been shown to contain cells with NSC potential (Robins et al., 2013; Haan et al., 2013). However, in our study we found little evidence of newly differentiating neurons (Figure S4), therefore our primary focus was on GFP^+ and $GFAP^+$ cell populations. In this region $GFAP^+$ cells can label astrocytes as well as tanycytes, which are specialized ependymal cells with elongated morphology that share characteristics of astrocytes and radial glia (Rodriguez et al., 2005; Robins et al., 2013). The primary role of tanycytes is to

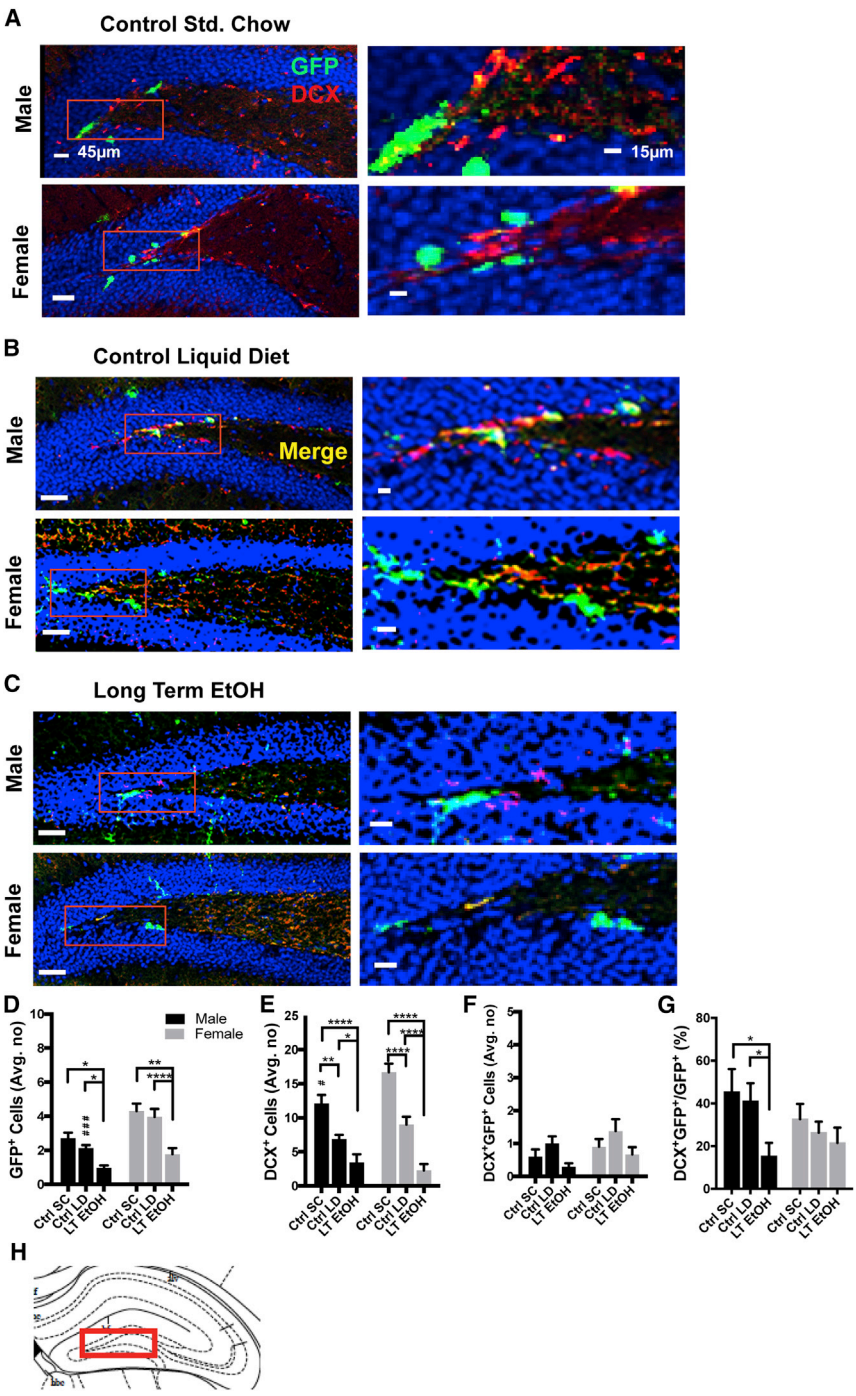

Figure 5. Neurogenesis in the SGZ following Ethanol Consumption

(A–C) Representative images of standard chow, liquid diet control, and long-term ethanol male and female mouse brains stained with stem cell marker (GFP green), neuronal marker (DCX red), and merged with nuclear maker DAPI (blue). The regions in the red boxes are shown enlarged on the right.

(D–G) Quantification in SGZ of average GFP^+ (D), DCX^+ (E), and DCX^+GFP^+ (F), and the percentage of DCX^+GFP^+ over total GFP^+ cells (G).

(H) Schematic image of representative region that was quantified (bregma from −1.58 to −2.16) and outlined in the red box. Scale bars, 45 μm, and 15 μm in enlarged images. Values are shown as mean ± SEM, n = 3 mice per sex per group. *p < 0.05, **p < 0.01, ****p < 0.0001 compared with controls, two-way ANOVA with Tukey's test. #p < 0.05, ###p < 0.001, compared with other sex counterparts, two-way ANOVA with Sidak's test. See also Figure S3.

communicate chemical signals from the ventricles to the hypothalamus (Rodriguez et al., 2005). Control mice in both standard chow and liquid diet groups had similar numbers of GFP^+, $GFAP^+$, and $GFAP^+GFP^+$ cells between both sexes (Figures 7A, 7B, and 7D–7F).

Following long-term ethanol consumption, both males and females exhibited significant decreases of GFP^+ in the TL region by 56% and 40%, respectively (Figures 7C and 7D). Interestingly, females had an increase in $GFAP^+$ cells (75%), whereas males experienced a 67% decrease in $GFAP^+$ cells (Figure 7E). Females did not have a significant reduction in the number of $GFAP^+GFP^+$ cells; however, males experienced a 67% decrease in $GFAP^+GFP^+$ cells when compared with liquid diet (Figure 7F). Neither males

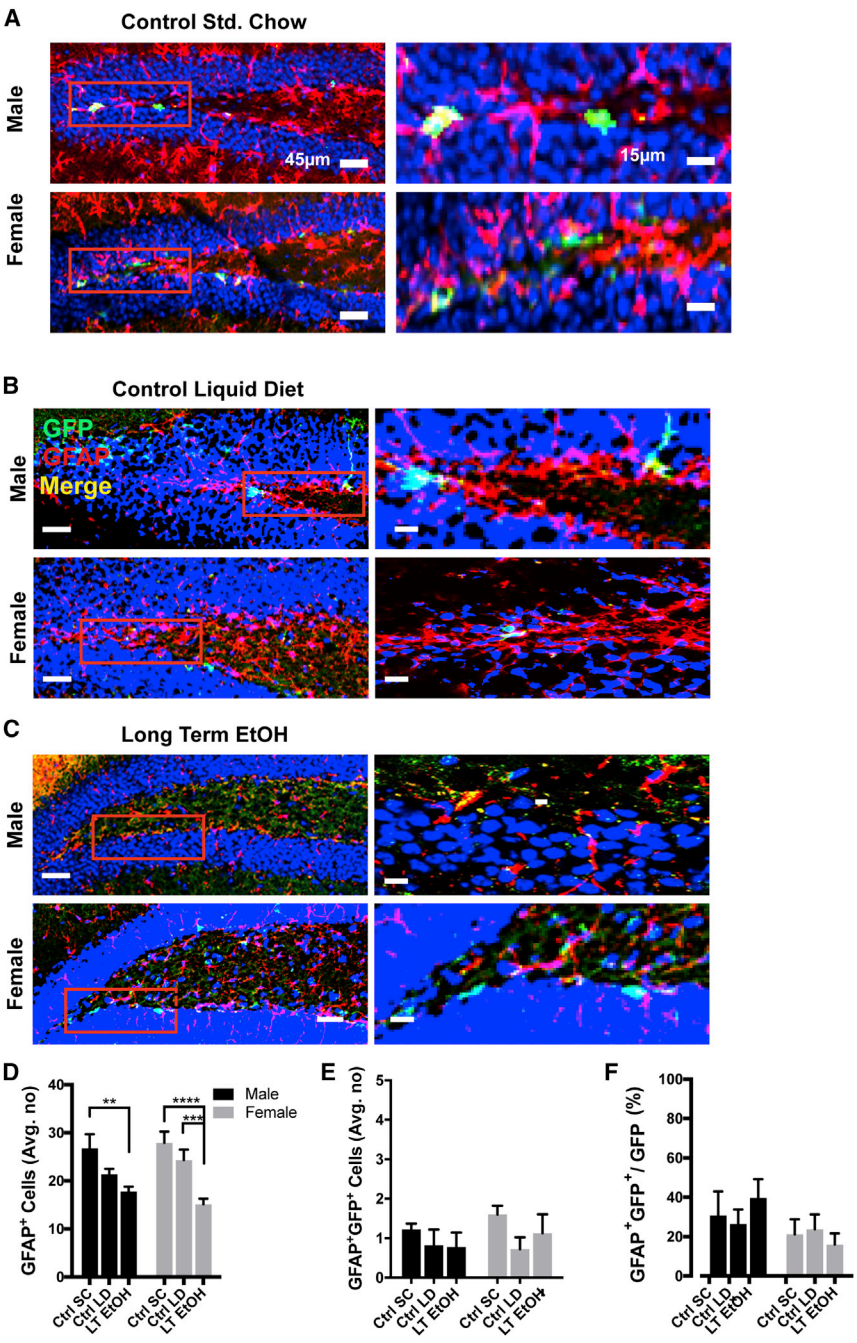

Figure 6. Astrogliogenesis in the SGZ following Ethanol Consumption

(A–C) Representative images of standard chow, liquid diet control, and long-term ethanol male and female mouse brains stained with stem cell marker (GFP green), astrocyte marker (GFAP red), and merged with nuclear maker DAPI (blue). The region in the red box is shown enlarged on the right.

(D–F) Quantification in SGZ of average $GFAP^+$ (D) $GFAP^+GFP^+$ (E), and percentage of $GFAP^+GFP^+$ over total GFP^+ cells (F).

Scale bars, 45 μm, and 15 μm in enlarged images. Values are shown as mean ± SEM, n = 3 mice per sex per group. **p < 0.01, ***p < 0.001, ****p < 0.0001, two-way ANOVA with Tukey's test compared with controls. See also Figure S3.

nor females had a change in the percentage of GFP^+ cells differentiating into $GFAP^+$ following long-term alcohol consumption (Figure 7G). Individual immunohistochemical stain images can be found in the Supplemental Experimental Procedures (Figure S5).

In summary, these data show different behavior of NSCs in the TL compared with both SVZ and SGZ. Long-term alcohol decreased NSCs in males and females, and increased total $GFAP^+$ cells without changes of astrogliogenesis in females.

DISCUSSION

Our study is to employ a genetic inducible fate mapping model to study the effects of chronic alcohol intake on adult brain NSCs and their progeny, comparatively, in three main brain regions in both male and female mice. A particular interesting finding is the observation of changes in the hypothalamic NSCs in response to alcohol consumption. One limitation in this study was the high

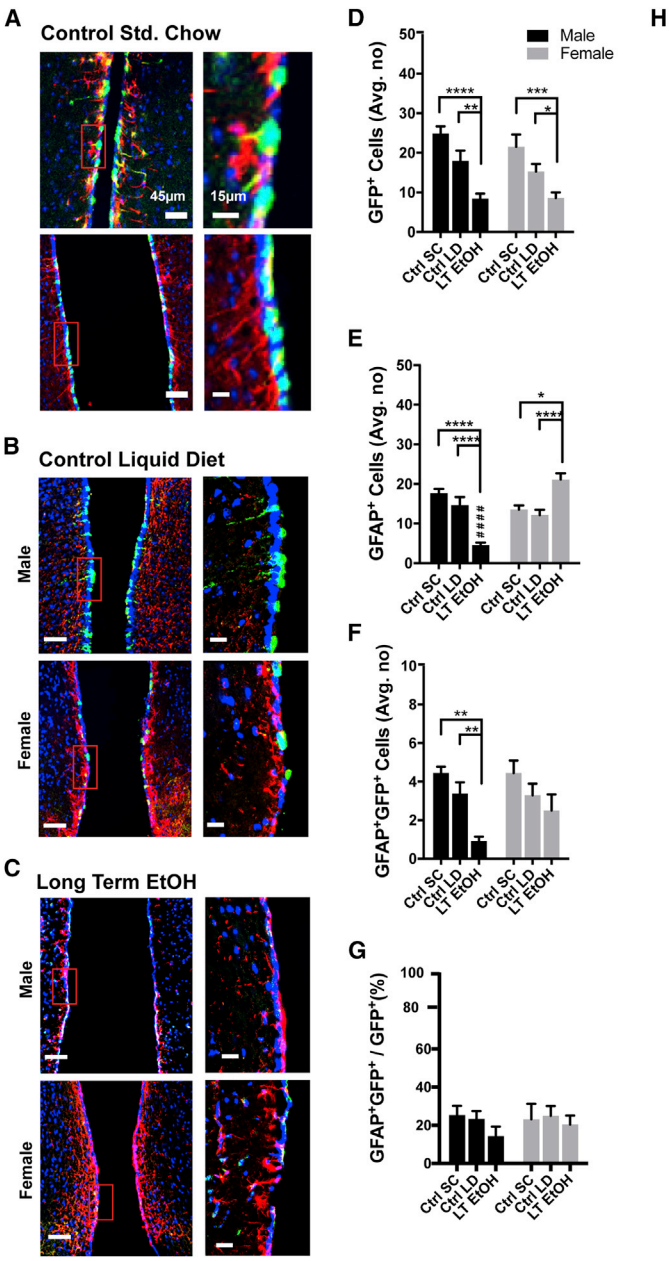

Figure 7. Astrogliogenesis in the TL following Ethanol Consumption

(A–C) Representative images of standard chow, liquid diet control, and long-term ethanol male and female mouse brains stained with stem cell marker (GFP green), astrocyte marker (GFAP red), and merged with nuclear maker DAPI (blue). The region in the red box is shown enlarged on the right.

(D–G) Quantification in the TL of average GFP^+ (D), $GFAP^+$ (E), and $GFAP^+GFP^+$ (F), and the percentage of $GFAP^+GFP^+$ over total GFP^+ (G) cells.

(H) Schematic representative image of region quantified (bregma from −1.58 to −2.16) and outlined in the red box.

Scale bars, 45 μm, and 15 μm in enlarged images. Values are shown as mean ± SEM, n = 3 mice per sex per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with controls, two-way ANOVA with Tukey's test. ####p < 0.0001 compared with female counterpart, two-way ANOVA with Sidak's test. See also Figures S4 and S5.

mortality of mice, likely due to the administration of ethanol since mice in the ethanol group were not given a choice of control diet. However, this model eliminates the variables in other models, such as ethanol dose and caloric intake that also impact NSCs (Geil et al., 2014; Stangl and Thuret, 2009).

In terms of astrocytes, we observed regional and sex differences in astrocytic responses following alcohol consumption. The net changes of $GFAP^+$ cells resulted from a combination of NSC astrogliogenesis and astrocyte reactivation, and varied among brain regions. Previous studies evaluated regional and durational differences in GFAP expression (Franke, 1995; Franke et al., 1997; Dalcik et al., 2009); however, our study further examined both variables together with sex differences in adult astrocyte response to alcohol consumption. In addition, using the genetic induced fate mapping technology, we were able to distinguish the newly NSC-differentiated astrocytes from the reactivated astrocytes. Some studies reported increases in GFAP immuno-labeled cells in the hippocampus following alcohol consumption (Dalcik et al., 2009; Kane et al., 2014). In contrast, we found no alteration of GFAP expression in males, but decreased GFAP in female hippocampal dentate gyrus. Using a similar dosing paradigm, and in

agreement with us, Franke and colleagues also observed similar GFAP responses (Franke, 1995; Franke et al., 1997). These suggest that both ethanol dosing regimen and sex play key roles in astrocytic responses.

Use of the transgenic fate tracing mice also enables more accurate study of NSC population and differentiation. In previous studies, bromodeoxyuridine (BrdU) has been used as a surrogate marker for proliferative NSCs. However, because BrdU labels all proliferative cells, it is challenging to distinguish proliferating NSCs from newly differentiated DCX^+ cells that are actively dividing (Klempin et al., 2011; Kempermann et al., 2004; Taupin, 2007). Nevertheless, we did observe decreased neurogenesis in response to chronic alcohol consumption in SGZ and SVZ that is similar to previous reports (Nixon and Crews, 2002; Herrera et al., 2003; He et al., 2005; Crews et al., 2006; Hansson et al., 2010; Golub et al., 2015).

One distinguishing aspect of our study is that using a genetic inducible fate tracing mouse model enabled us to identify three distinct phases contributing to neurogenesis and evaluate the unique susceptibility of each phase to ethanol. The three phases are NSCs (GFP^+), newly differentiated immature (GFP^+DCX^+) and mature (GFP^+NeuN^+) neurons, and immature neurons (GFP^-NeuN^+) that were present before tamoxifen induction. In the SVZ, we discovered that NSCs lining the ventricle are most susceptible to ethanol, followed by newly differentiated NSCs, and, finally, cells that had differentiated before the onset of the study. This subtle, yet important, distinction between the stages of NSC differentiation is crucial in developing an understanding of how ethanol progressively impacts the adult brain. Furthermore, our data also suggested the potential mechanisms underlying alcohol-induced neurogenesis deficits in SVZ, including a reduced NSC pool, inhibition of neuronal differentiation, and/or reduced migration of newly differentiated neurons. The latter has also been well documented in rodents treated with alcohol during neural development (Miller, 1993).

We found that NSCs lining the SVZ of the rostral lateral ventricles were most susceptible to the effects of ethanol. The significant reduction in the number of GFP^+ NSCs, and particularly the absence of GFP labeling in regions lining the lateral ventricle, suggested cytotoxicity as a mechanism. However, it is possible that inhibition of proliferation also contributed to the reduction of GFP^+ NSCs, which has been reported in previous studies (Golub et al., 2015; Hansson et al., 2010; Nixon and Crews, 2002). One explanation for this heightened effect is the proximity of SVZ NSCs to the cerebrospinal fluid. It has previously been shown that cerebrospinal fluid can directly influence cell behavior and neurogenesis (Zappaterra and Lehtinen, 2012). Direct exposure to ethanol and other toxic metabolites of ethanol, such as acetaldehyde, may contribute to amplified sensitivity of this population. There was a robust increase in $GFAP^+$ cells in the region surrounding the SVZ during a short-term ethanol treatment (our unpublished data). One reason may be that the insult of ethanol and metabolites on the SVZ initiates an injury response leading to local astrocyte reactivation (Anderson et al., 2014; Brahmachari et al., 2006). The mechanism and extent to which astrocyte reactivation contributes to changes in SVZ neurogenesis presents a unique opportunity for future studies. On the other hand, a previous study did not detect significant reduction of SVZ cell proliferation in the postmortem brains of a small cohort of chronic alcoholic patients (Sutherland et al., 2013). Although this needs to be further confirmed with more human brains, it calls for animal models of chronic alcohol abuse that more closely mimic what occurs in humans.

The toxic effect of chronic ethanol intake on the SGZ NSCs and neurogenesis appears to be less severe when compared with the SVZ cells, in agreement with a vapor ethanol administration model (Hansson et al., 2010). Furthermore, we found a significant increase of NSCs in the male SGZ after short-term ethanol treatment (our unpublished data). While this finding seems contradictory to some previously published literature (Nixon and Crews, 2002; Crews et al., 2006; Morris et al., 2010; Campbell et al., 2014), we believe that the discrepancies may be due to different alcohol administration paradigms. On the other hand, our finding is in agreement with those reported by Pawlak et al. (2002), who also observed an increase in NSC proliferation in the dentate gyrus following 14 days of ethanol liquid diet consumption. In addition, moderate ethanol consumption has also been shown to increase adult NSC proliferation in the dentate gyrus (Aberg et al., 2005). Further investigation into mechanisms for this transient increase of NSCs *in vivo* is still needed; but injury-induced NSC proliferation may be a likely mechanism (Deierborg et al., 2010; Petrenko et al., 2015). The less severe effect of long-term ethanol intake on the SGZ NSCs indicates the potential of these cells to regain their neurogenic function. Indeed, several studies demonstrated a compensatory increase of SGZ NSC proliferation after abstinence from binge drinking or chronic ethanol vapor exposure (Nixon and Crews, 2004; Somkuwar et al., 2016). Along this line, it remains to be determined what amount of ethanol intake at what time period of exposure would lead to irreversible damage to NSCs, which will most likely depend on species, brain region, and sex.

The TL is an intriguing region, given its novelty in the NSC field as well as its relationship with the surrounding brain regions such as the dorsomedial hypothalamus, paraventricular nucleus, and arcuate nucleus (Robins et al., 2013; Haan et al., 2013; Rodriguez et al., 2005). Tanycytes are the primary cells found lining the TL of the third

ventricle, and their main function is to relay signals from the CSF to the surrounding regions that are critical for regulating functions such as feeding, sleep, and water balance (Robins et al., 2013; Rodriguez et al., 2005). Robins et al. (2013) showed that dorsal α-2 tanycytes present along the third ventricle in the region of the arcuate nucleus were unique in their possession of NSC-like potential. However, in our study, we observed GFP^+ cells in the α-1 tanycyte region as well. This suggests that α-1 tanycytes may also have some NSC properties. Further studies should be conducted to verify this finding.

While recent evidence has indicated that there may be active adult neurogenesis in the TL (McNay et al., 2012; Haan et al., 2013), we saw very little DCX^+ staining and no changes in response to ethanol consumption. In the previous studies evaluating neurogenesis in this region, neuronal marker NeuN was used, which may be one explanation for the discrepant findings in our study (McNay et al., 2012; Haan et al., 2013). NeuN labels more mature neurons, whereas DCX labels immature migratory neurons. Alternatively, the discrepancy in finding of TL neurogenesis in hypothalamus may be attributed to different animal age, i.e., 18-week-old mice in our study versus 8- to 12-week-old mice in others (Rojczyk-Golebiewska et al., 2014). Further investigation with multiple neuronal markers in animals with a larger age range would be beneficial to elucidate the presence and degree of neurogenesis in this region.

While we did not observe substantial neurogenesis, the changes in NSCs and $GFAP^+$ cells in the TL were quite interesting. Long-term alcohol consumption resulted in a decrease in NSCs, but not as dramatic of a decrease as was observed in the SVZ. Since NSCs in the TL line the third ventricle, we expected that their behavior would be similar to NSCs in the SVZ of the lateral ventricle; however, this was not observed in the present study. Increases of $GFAP^+$ cells were in females following alcohol consumption. Given the role that tanycytes play in communication with the hypothalamus and paraventricular nucleus in regulation of feeding behavior and other endocrine-related functions, further studies are necessary to better understand the effect of alcohol on this cell population (Rodriguez et al., 2005). Since the morphology of the cells in our study look similar to tanycytes, and that GFAP and *Nestin* are expressed in tanycytes, further studies are needed to fully validate the identity of this cell population.

Probably the most interesting finding from this work is that regional location and sex played pivotal roles in determination of NSC response to ethanol consumption. Behaviorally, females showed more severe symptoms of intoxication over a shorter period of time compared with males. Females also consumed more grams of ethanol per kilogram of bodyweight. In the SVZ, females had a higher susceptibility to the detrimental effects of ethanol consumption. Their NSCs, DCX^+ cell, and co-labeled newly differentiated neurons were reduced significantly compared with males. However, similar responses to ethanol were seen in the SGZ in both males and females. In the TL, long-term consumption initiated a greater increase in $GFAP^+$ cells in females compared with males. It is known, clinically, that males and females have differing capacities to metabolize ethanol (Oscar-Berman and Marinkovic, 2003). This may be one possible explanation for the sex differences observed in NSC response. If males and females produce varying levels of ethanol metabolites, these metabolites could be affecting the CNS more than males. In addition, it would be of great value to further interrogate the role of the endocrine system in NSC response to ethanol; especially given the implication of the TL in NSC response to ethanol. Altogether, our findings suggest that NSCs exhibit unique behavior and susceptibility in response to ethanol, depending on the stage of differentiation, regional niche, duration of ethanol consumption, and sex.

EXPERIMENTAL PROCEDURES

Animals

Nestin-$CreERT^2$:R26R-YFP bitransgenic male and female mice were generated by crossing the C57BL/6-Tg(Nes-cre/ERT2)KEisc/J strain with the B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J strain (both obtained from The Jackson Laboratory), and have been described elsewhere (Lagace et al., 2007). Mice were genotyped by PCR using genomic DNA from tail snip to confirm the presence of Cre and YFP *trans*-genes (see Supplemental Experimental Procedures). All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, and conducted in accordance with the *Guide for the Care and Use of Laboratory Animals*, and maintained on a 12-hr light/dark cycle.

Tamoxifen-Induced Recombination

Stock solutions of tamoxifen (Sigma-Aldrich, St. Louis, MO) were prepared at 20 mg/mL in corn oil (Sigma). Mice, 8- to 10-weeks-old, received intraperitoneal injections of 2 mg of tamoxifen once a day for 5 consecutive days. A 3-week interval was left before ethanol treatment to enable maximum recombination and washout of immediate effects of tamoxifen. It has previously been shown that tamoxifen treatment of these mice does not elicit any sex-dependent effect on males or females (Lagace et al., 2007).

Ethanol Treatment

Mice, 12- to 14-weeks-old, were randomly assigned to either the control (male n = 8, female n = 9) or experimental group (male n = 13, female n = 12) using the "randomize" (RAND) function in Microsoft Excel. Mice in each group were age and sex matched to control for variability. Following randomization, mice were given *ad libitum* access to water and a Lieber-DeCarli liquid diet containing complete nutrients for rodents (Dyets, Bethlehem, PA, catalog no. 710260), as described previously (Brandon-Warner

et al., 2012). Pair feeding was done, in which ethanol calories were replaced by equivalent calories of maltose-dextrin in the diet for control mice. The amount of ethanol in the liquid diet was slowly increased from 1% to 4% over the course of 2 weeks. Mice in the ethanol group were maintained on a 4% ethanol in the liquid diet for up to 4 weeks. Both the control and ethanol-containing liquid diets were made fresh daily. Each day, between 9:00 and 11:00 a.m., the feeding bottles were removed from the cage, the remaining liquid diet was measured and subtracted from the original diet administered. All mice were euthanized between 8:00 and 11:00 a.m. See Supplemental Experimental Procedures for blood alcohol and acetaldehyde analysis.

Home Cage Behavior Scoring

Mice were observed daily in home cages during exchange of liquid diet tube. Behavior was recorded and scored on a 0- to 4-point scale based on an ordinal scale (Figure 2A). 0 = control (asymptomatic) behavior and 4 = severely impaired behavior. Mice that reached level 4 or bodyweight reduction over 20% were terminated and not used for the chronic group. Behavioral data were analyzed using a random slope model.

Immunohistochemistry

At the termination of the experiments, mice were anesthetized with ketamine/xylazine (90 and 10 mg/kg), and perfused with cold 5 mL PBS and 30 mL of 4% paraformaldehyde via an intra-cardial injection. Mouse brains were collected, post-fixed in 4% paraformaldehyde for 2 days at 4°C, and then infiltrated with 30% sucrose for 5 days at 4°C before embedding in OCT medium. Tissue was serially sectioned at a thickness of 30 μm using a Leica cryostat machine. Detection of induced YPF was enhanced by immunohistochemical staining with chicken anti-GFP antibodies (Aves Labs, Tigard, OR, catalog no. GFP-1020). In addition, sections were incubated with antibodies against GFAP (Thermo Fisher Scientific, Waltham, MA, catalog no. PA110019), DCX (Abcam, San Francisco, CA, catalog no. ab18723), or NeuN (Thermo Fisher Scientific, catalog no. MAB377). Following primary antibody incubation at 4°C overnight, sections were incubated with Alexa Fluor secondary antibodies (goat-anti-chicken 488, catalog no. A11039; and goat-anti-rabbit 568, catalog no. A11011).

Imaging and Cell Counting

All images were acquired on a Nikon D-ECLIPSE C1 confocal microscope, using the Nikon EZ-C1 3.91 software. Blinded cell counting was performed using NIS Elements software. Positive identification of cells was based on both immunostaining and morphological characteristics. The SVZ of the lateral ventricle was counted from bregma 0.50 to 1.08, and both the SGZ of the hippocampus and the TL of the third ventricle were counted from bregma −1.58 to −2.16. Four sections (both hemispheres) from each region, spanning a total of 580 μm longitudinally, were counted for each antibody. Average total positive cells were performed using triplicate mice for each region and antibody.

Statistics

The number of mice required for analysis was calculated based on our previous results and power analyses using G*Power 3.1.7. A number of 3 was needed to provide statistical significance at $p < 0.05$ with a power of 0.80 for the histological data and an $n = 11$ for behavioral analysis. A two-way ANOVA with various *post-hoc* tests was used to analyze the alcohol intake-related data and the immunohistochemical data, and multiple comparisons were done with Tukey's test, Holm-Sidak's test, or Sidak's test. All statistical analyses were conducted using GraphPad Prism v.6 software. Each group contained three mice per sex, and a p value less than 0.05 was considered statistically significant, and a p value greater than 0.05 but less than 0.1 was considered a trend. Behavioral data were analyzed using a random intercept model by SAS9.4 (Snijders, 2011). Two-sided tests were conducted and a p value less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.10.007.

AUTHOR CONTRIBUTIONS

E.L.M., B.K., K.A.C., K.T.D., and P.W. contributed intellectual knowledge and expertise in experimental design and data analysis. E.L.M. performed the majority of the experiments. E.L.M. and P.W. wrote the manuscript. J.G., Y.F.K., and M.J.R. assisted with data analysis. T.J.D. provided technical assistance in conducting some key experiments. All authors reviewed and approved manuscript.

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