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# Evaluating the Effect of Chronic Alcohol, Cocaine, and Co-administration on Endogenous Adult Neural Stem Cell Survival, Differentiation, and Proliferation

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# Evaluating the Effect of Chronic Alcohol, Cocaine, and Co-Administration on Endogenous Adult Neural Stem Cell Survival, Differentiation, and Proliferation

**by**

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# Dedication

*"Success is not measured by what you accomplish, but by the opposition you have encountered, and the courage with which you have maintained the struggle against overwhelming odds." –Orison Swett Marden*

**To my husband:** You were my rock when the rest of my world was quicksand. I have overcome the biggest obstacles in my life because you were right there by my side. You teach me every day what selfless love is, and give me the strength to keep going, no matter what. Thank you for the sacrifices you made to help me realize my dream.

**To my mother:** You taught me to believe in myself, even when nobody else does. You're the strongest woman I know, and because of that, you taught me how to be strong and let nothing hold me back. But most importantly, you taught me how to love myself by loving me. Thank you for all the late nights, laughs, and tears. You were always there with a smile and a cup of tea to help get me through.

**To my father:** You taught me the value of family. You're the most loving family man I know. Thank you for always being there for me, through all the ups and downs, and showing me that that no matter what happens, you will always be there and I can always come home. Thank you for inspiring me to be a more loving person every day. You always had valuable words of wisdom that challenged me to think and put things in perspective when I felt lost.

**To my brother:** You taught me compassion and the value of enjoying life. You are the strongest and most resilient person I have ever known. No matter what happened, you took it in stride and always managed to bring a smile to my face, even when everything seemed bleak. You were always there to call me out when I was wrong and help me see all sides of a situation. I admire your energy, perseverance, and compassion, and hope each day I can learn a little more of it from you.

**To my grandmothers:** You taught me to seize every opportunity that came my way. You may not have understood everything I was working on, but you were always so excited to hear about it. Your excitement and enthusiasm, even about the smallest things, meant more to me than you will ever know. At times, it was what kept me going when I felt completely worn out. No matter what, you were always there with an eager ear and words of praise.

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Thank you for watching over me, and I want you to know that I made it.

Pop, this  is for you.

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# Evaluating the Effect of Chronic Alcohol, Cocaine, and Co-Administration on Endogenous Adult Neural Stem Cell Survival, Differentiation, and Proliferation

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Cocaine and alcohol are two of the most commonly co-abused substances, and the third most fatal drug combination. Efforts in drug addiction research primarily focus on preventing or stopping abuse; however little work is being done to reverse brain damage incurred by chronic drug abuse. Neural stem cells (NSCs) are a promising target to stimulate brain recovery, yet little is known about the effect of long term co-abuse of alcohol and cocaine on this cell population. Additionally, sex differences in NSC behavior following chronic drug abuse have yet to be evaluated. This is the first study to evaluate regional and sex-dependent responses of endogenous adult NSCs to chronic treatment with alcohol and cocaine. I sought to elucidate the response of adult endogenous NSCs to chronic alcohol and cocaine treatment using an inducible lineage tracing mouse model. This model enables us to trace a given population of NSCs in response to drug treatment. In this dissertation I will briefly discuss the role of adult NSCs in homeostatic and pathological conditions, as well as recent technological advances for studying NSCs. Next I will describe findings which show regional, temporal, and sex dependent responses of endogenous NSCs to alcohol; specifically changes in survival and neurogenesis. Finally, I will summarize data interrogating the impact of chronic cocaine, alcohol, and co-administration on NSC survival and differentiation, and how these changes correlate with cognitive and behavioral changes.

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# List of Abbreviations

| | |
|---|---|
| UTMB | University of Texas Medical Branch |
| GSBS | Graduate School of Biomedical Science |
| TDC | Thesis and Dissertation Coordinator |
| ESCs | Embryonic Stem Cells |
| iPSCs | Induced Pluripotent Stem Cells |
| NSC | Neural Stem Cells |
| SVZ | Subventricular Zone |
| SGZ | Subgranular Zone |
| TL | Tanycyte Layer |
| RMS | Rostral Migratory Stream |
| oRMS | Origin of the Rostral Migratory Stream |
| OB | Olfactory Bulb |
| Ctrl | Control |
| Coc | Cocaine |
| EtOH | Ethanol |
| Combo | Combination of Alcohol and Cocaine |
| CE | Cocaethylene |
| BAC | Blood Alcohol Content |
| IHC | Immunohistochemistry |
| GFAP | Glial Fibrillary Acidic Protein |
| DCX | Doublecortin |

| | |
|---|---|
| GFP | Green Fluorescent Protein |
| ADH | Alcohol dehydrogenase |
| ALDH | Aldehyde dehydrogenase |
| CES1 | Carboxylesterase-1 |
| CYP3A4 | Cytochrome-P450-3A4 |
| ß-Actin | Beta Actin |
| HRP | Horse-radish Peroxidase |
| CD | Context Discrimination |
| EZM | Elevated Zero Maze |
| SP | Sucrose Preference |
| CSF | Cerebrospinal Fluid |

# CHAPTER 1: INTRODUCTION

## STEM CELL OVERVIEW

Stem cells are broadly defined as a cell population capable of self-renewing and giving rise to various cell types. During development embryonic stem cells (ESCs) comprise 100% of the inner cell mass of the blastocyst. ESCs are pluripotent meaning they can give rise to any cell type, and have indefinite potential to self-renew. At the fetal stage of development stem cells are about 1-10% of the cell population and at this point are multipotent, meaning their differentiation potential is restricted to certain lineages. For example, fetal neural stem cells only have the ability to differentiation into neural and glial lineages. In the adult, stem cells are located within specific niches in various tissues.

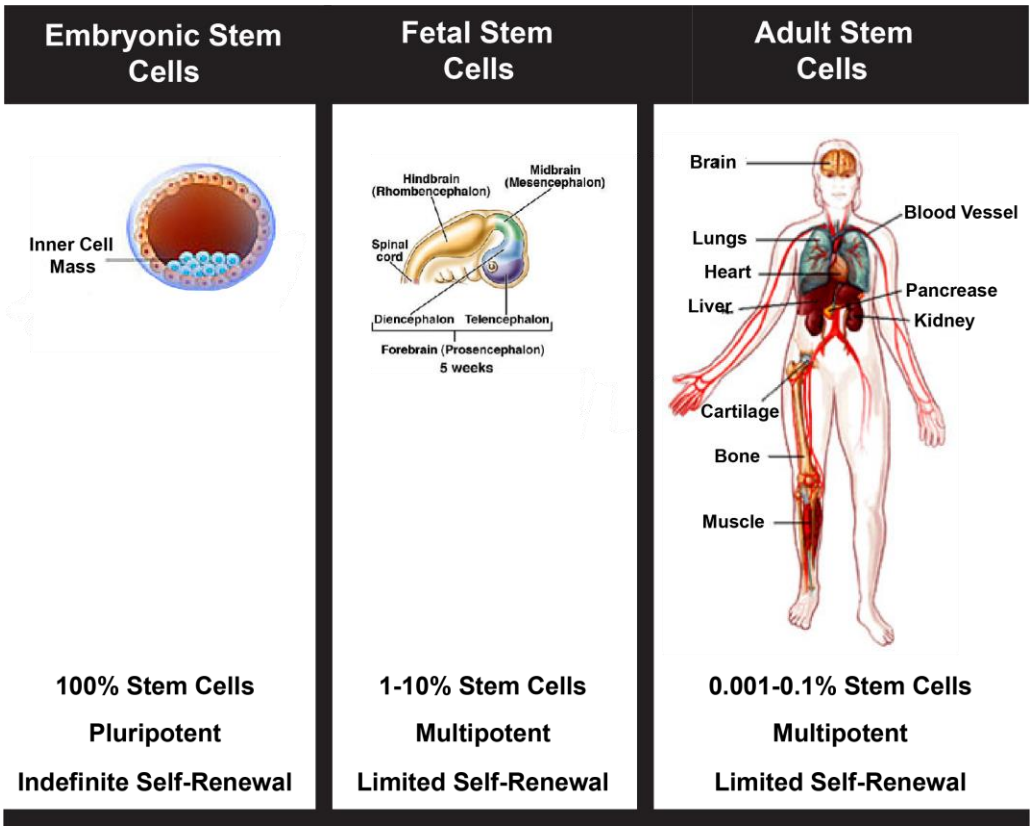


Illustration 1: Stem cell populations. Above diagram shows relative abundance, differentiation potential, and self-renewal properties of stem cells at three stages of development: embryonic, fetal, and adult.

Adult neural stem cells (NSCs) are a multi-potent sub-population of cells broadly defined by their ability to self-renew and give rise to neurons, glia, or oligodendrocytes (Gage, 2000; Taupin and Gage, 2002; Kriegstein and Alvarez-Buylla, 2009; Ming, 2005; Lim and Alvarez-Buylla, 2014). NSCs persist throughout adulthood and contribute to normal brain maintenance and repair (Taupin and Gage, 2002; Imayoshi, 2008; Gage, 2002). Survival, proliferation, and differentiation of NSCs is strictly regulated; however, endogenous and exogenous factors such as genetics, exercise, infection, and drugs can profoundly impact the fate of these cells (Dominguez-Escriba, 2006; Maynard, 2013; Yoneyama, 2011; Deierborg, 2010; Nebel, 2016). Alterations in NSC survival, proliferation, and differentiation have been implicated in a number of mental health and nervous system disorders (Imayoshi, 2008; Kang, 2016; Christian, 2010; McGrath, 2017; Apple, 2017; Nixon, 2010; Yoneyama, 2014). Despite their critical importance, much remains unknown about NSCs since, historically, they have been notoriously difficult to study (Reekmans, 2012; Nam, 2015). In recent years, advances in stem cell culture systems and inducible transgenic animal models have paved the way for more in-depth studies on NSCs and the impact of endogenous and exogenous stimuli on their fate (Jakel, 2004; Bernau, 2016; Decarolis, 2013; Lagace, 2007; Ortega, 2011; Chow, 2015). This review will address the most recent advances in NSC tracing technology and how these technologies contribute to improving neurological and mental health.

## NEURAL STEM CELLS IN THE ADULT BRAIN

NSCs in the adult brain exist as a heterogeneous population, which can be sub-divided based on expression of certain protein markers (Lim and Alvarez-Buylla, 2014;

Zhao, 2008; Codega, 2014; Gebara, 2016). To date, three main subpopulations of NSCs have been identified. One population is Type I or Type B NSCs. Type B cells are radial glia-like NSCs characterized by expression of glial fibrillary acidic protein (GFAP) and lack of *Nestin* expression (Kriegstein and Alvarez-Buylla, 2009; Ming, 2005; Lim and Alvarez-Buylla, 2014; Codega, 2014; Alvarez-Buylla and Lim, 2004). These cells are quiescent and do not actively undergo division or differentiation, though they are believed to be self-renewing (Alvarez-Buylla and Lim, 2004; Sanai, 2004). The second sub-group are referred to as Type II or Type C cells (Kriegstein and Alvarez-Buylla, 2009; Lim and Alvarez-Buylla, 2014; Codega, 2014; Alvarez-Buylla and Lim, 2004). Type C cells give rise to immature neuroblasts and are characterized by *Nestin* expression and active cell division (Alvarez-Buylla and Lim, 2004). Due to their actively dividing nature and differentiation potential, Type C cells are sometimes referred to as transient amplifying cells (TACs) or intermediate progenitor cells (IPCs) (Kriegstein and Alvarez-Buylla, 2009; Ming, 2005; Codega, 2014). Finally, Type III, or Type A, cells are the immature neuroblasts produced from Type C cells. Type A cells are doublecortin (DCX) positive and *Nestin* negative (Ming, 2005; Alvarez-Buylla and Lim, 2004; Doetsch and Alvarez-Buylla, 1996). Classification of NSCs has been extensively studied; however, with the emergence of new technologies the understanding of NSC populations is constantly evolving. There is still further need for studies to elucidate mechanisms governing NSC behavior, particularly their survival, proliferation, and differentiation.

In the adult brain there are two primary regions containing NSCs actively undergoing neurogenesis: the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone of the dentate gyrus of the hippocampus (SGZ) (Ming, 2005; Gage, 2002). The SVZ is a dynamic region surrounding the ventricles, with multiple cell types and direct contact with cerebrospinal fluid (CSF) (Lim and Alvarez, 2014). It was previously

thought only ependymal cells directly contacted the CSF, however recent studies have shown that Type B cells send projections to contact CSF (Lim and Alvarez-Buylla, 2014). Additionally, molecular signals in the CSF have been shown to influence NSC survival, proliferation, and differentiation (Zappaterra and Lehtinen, 2012). In the SVZ, Type A cells begin to migrate away from the SVZ toward the olfactory bulb via the rostral migratory stream (RMS) (Lim and Alvarez-Buylla, 2014; Doetsch and Alvarez-Buylla, 1996; Figueres-Onate and Lopez-Mascaraque, 2016). Migratory cells in the RMS use a chain-like migration mechanism, surrounded by astrocytes. The migrating neurons mature as they reach the olfactory bulb, where they contribute to olfaction processing after becoming local interneurons in the granular, periglomerular, and glomerular layers (Ming, 2005; Lim and Alvarez-Buylla, 2014; Figueres-Onate and Lopez-Mascaraque, 2016).

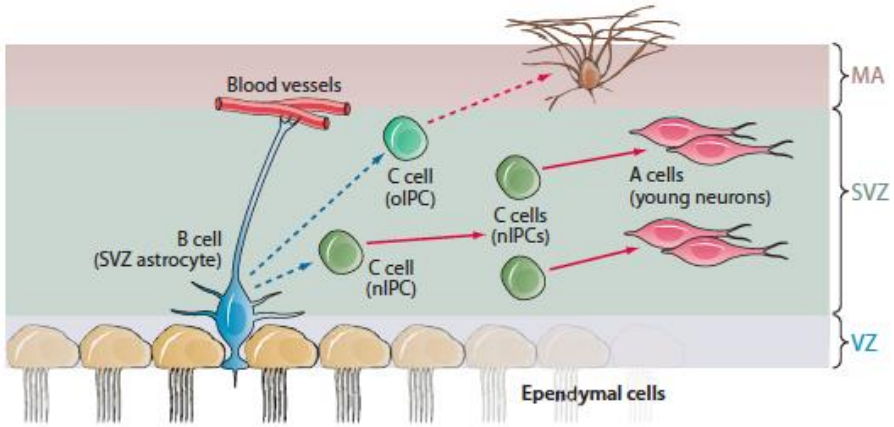



Illustration 2: Stem cells in the SVZ. The above illustration is taken from (Kriegstein and Alvarez-Bullya, 2009). It demonstrates the three main stages of NSCs present in the SVZ (A, B, and C cells).

In the dentate gyrus, NSCs primarily reside in the SGZ. NSCs in the SGZ will produce thousands of cells each day, however only a small subset of those that are able to successfully integrate will survive, the rest will be pruned under homeostatic conditions (Taupin and Gage, 2002). The new neurons that survive will migrate into the granular cell

layer, and send axons into the CA3 region of the hippocampus (Markakis and Gage, 1999). These cells synaptically integrate into the dentate gyrus and receive excitatory signals from the entorhinal cortex (Markakis and Gage, 1999). Adult-born neurons that successfully integrate into the synaptic network of the dentate gyrus contribute to learning and memory, particularly pattern separation (Clelland, 2009).

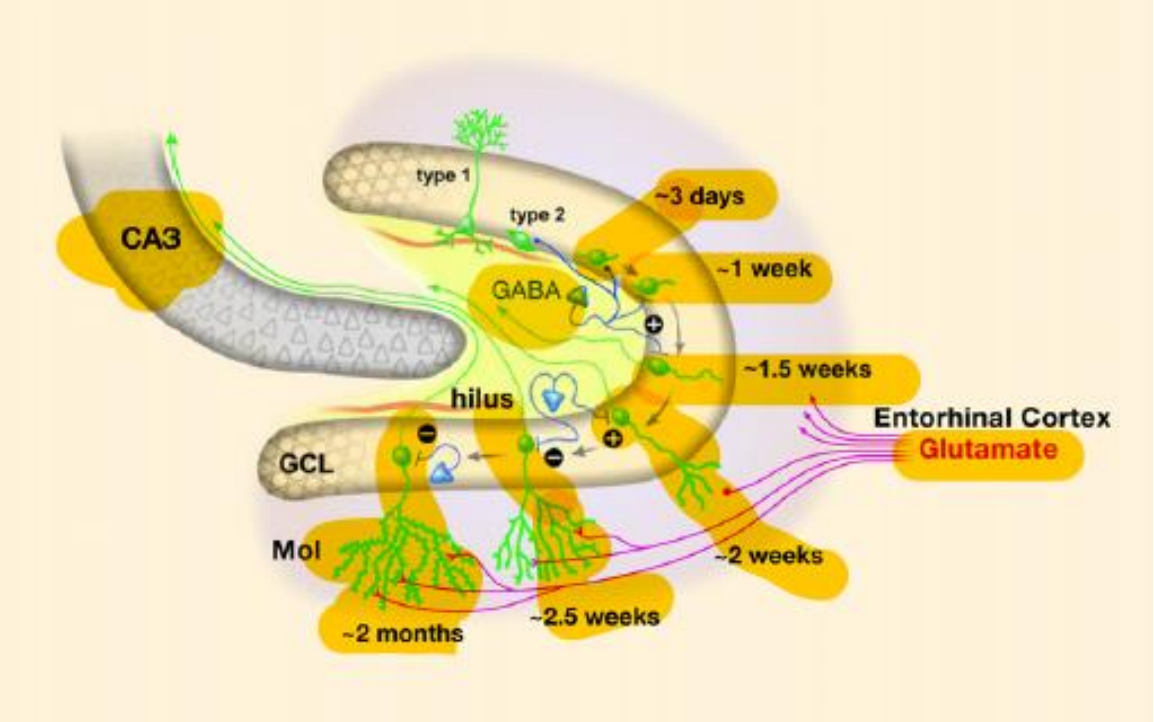



Illustration 3: Stem cells in the SGZ. The above illustration is taken from (Zhao, 2008) and demonstrates the various types of NSCs in the SGZ and their migration patterns over time.

## ADVANCES IN TOOLS FOR STUDYING NSCS

Recent advances in genetic fate mapping and adult neural stem cell isolation have now implicated the tanycyte layer of the third ventricle (TL) as an important region containing cells with neural stem cell like potential (Robins, 2013; Chaker, 2016). The TL is well known for harboring specialized glial cells called tanycytes. The primary role of

tanycytes is to relay signals from the CSF to the hypothalamus. Information relayed from the tanycytes to the hypothalamus is important for signaling key hypothalamic functions such as feeding and endocrine regulation (Bolborea, 2013; Benford, 2017; Goodman, 2015; Rodriguez, 2005). While tanycytes have been identified for some time, recent technological advances such as transgenic lineage tracing mouse models have provided new insight into this cell population and revealed a novel site of potential adult neurogenesis (Chaker, 2016; Kokoeva, 2007; Perez-Martin, 2010; Wang, 2012; Xu, 2005). The recent discovery of NSC-like cells in the tanycyte layer exemplifies the important contributions of technological advances in the NSC field.

The ability to accurately study NSCs *in vitro* is vital in understanding mechanisms that regulate NSC survival, proliferation, and differentiation. In 2006 Takahashi and Yamanaka developed a way to reprogram adult fibroblasts into pluripotent stem cells using four essential transcription factors (Yamanaka and Takahashi, 2006; Takahashi and Yamanaka, 2006). This research greatly contributed to current understanding of stem cell biology and enabled rapid expansion of the field. The advantage of induced pluripotent stem cells (iPSCs) is that skin fibroblasts, or other differentiated cell samples, can be readily obtained from human study participants (Wen, 2014; Juopperi, 2011). After isolating a patient's cells, they can be induced into a stem cell state, and since they are the patient's own cells, they retain the same genetic composition. This is valuable particularly in regards to the study of developmental disorders such as Autism Spectrum Disorder or schizophrenia (Wen, 2014; Juopperi, 2011). It is possible to observe aberrant development in the iPSCs of the patient in order to better understand the pathophysiology of the disorder (Wen, 2014; Juopperi, 2011). Additionally, other genetic or drug screens can be performed *in vitro* with the hopes of improving treatment efficacy.

Advances in culture methods for primary human fetal NSCs also provide a unique system to model human disease, conduct drug screening, and examine stem biology (McGrath, 2017; Jakel, 2004; Svendsen, 1998; Barrows, 2016; Lopez-Garcia, 2016; Mich, 2014). With any stem cell culture, the difficulty is maintaining the appropriate balance of growth factors required to maintain the “stem-ness” of the culture. In 1998 Svendson and colleagues published a method to maintain primary human fetal neural stem cells in culture (Svendson, 1998). Since then, multiple labs have expanded on this protocol and maintained these cells in culture for extended periods of time, and altered growth factor concentrations to prime and differentiate primary NSCs into neuronal and glial lineages (Codega, 2014; Lopez-Garcia,2016; Gao; 2006). An advantage of primary human fetal NSC culture is that no genetic manipulation is required, as opposed to iPSCs, which could be a potential confounding variable in studies (Svendsen, 1998).

*In vitro* culturing of either primary NSCs or iPSCs provides a system in which NSCs can be rapidly and easily manipulated in a controlled environment. Additionally, the ability to expand NSCs in culture enables utilization of NSCs for transplantation studies. NSC transplantation is not only a promising therapeutic intervention, it also provides valuable information on how neurogenic niches influence NSC survival, differentiation, and migration (Gao; 2006; Wu, 2002; Tarasenko, 2007; Lamanna, 2017). For instance, a primary impediment in NSC transplantation was that transplanted stem cells frequently did not generate neurons when grafted into non-neurogenic regions of the adult brain. To overcome this obstacle, Wu and colleagues showed that human fetal NSCs, primed *in vitro*, were able to generate region specific cholinergic neurons when grafted into rats (Wu, 2002). This study demonstrates how utilizing *in vitro* methods can greatly enhance stem cell transplantation efficacy and overcome obstacles associated with neurogenic niches *in vivo*. Additionally, the technique developed by Walker and Kempermann in 2014 has proved immensely valuable to the NSC field (Walker and Kempermann, 2014). Their

technique enables researchers to isolate and culture NSCs from the SGZ and SVZ of the same rodent brain (Walker and Kempermann, 2014). In this way, researchers can study regional differences in adult NSCs and their neurogenic niches within the same brain samples. This eliminates individual differences and cell samples can be used for a variety of assays from epigenetics to differentiation.

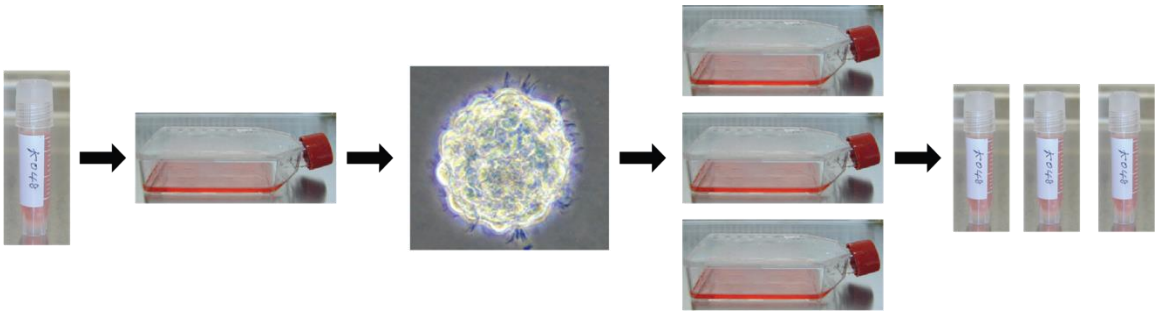


Illustration 4: The above illustration shows the method employed to expand human fetal NSC populations for use in *in vitro* studies.

*In vivo* studies of NSCs present an entirely new set of unique challenges. One of the greatest challenges has been how to label and trace endogenous NSCs. For some time, 5-Bromo-2'-deoxyuridine (BrdU) was the gold standard to study NSCs. BrdU is a thymidine analog that incorporates into cells during the S phase, thereby labeling all proliferative cells. This method has long been used to label transient amplifying cells in the SGZ and SVZ due to the ease of administration and detection (Taupin, 2007). Typically an incubation time of minutes to hours is used to measure currently proliferative cells, whereas days to weeks is used to trace the lineage of dividing cells (Taupin, 2007; Crews, 2004; Leasure and Nixon, 2010). Despite the many advantages of BrdU labeling, there are some distinct pitfalls that must be considered (Taupin, 2007; Caldwell, 2005). Since BrdU labels all proliferative cells it can only be used as a surrogate for transient amplifying NSCs. BrdU can also label other proliferative cells such as immature neurons, and also cells undergoing DNA repair (Taupin, 2007; Klempin, 2011). Additionally, BrdU results

vary greatly depending on dose administered, duration of treatment and time after treatment before cells are analyzed (Taupin, 2007). Furthermore, as a cell divides, the BrdU signal becomes more and more diluted (Taupin, 2007). Therefore, if BrdU is used to trace NSCs over an extended period of time, there is a risk for inaccurate BrdU signal. BrdU can also cause cytotoxic responses which could alter results when examining NSC population, particularly since injury responses can induce proliferation of NSCs (Caldwell, 2005; Zhang, 2014). A new thymidine analogue, 5-Ethynyl-2'-deoxyuridine (EdU), has recently started being utilized (Chehrehasa, 2009). While EdU has proven to be less cytotoxic than BrdU, the other caveats associated with BrdU still apply to EdU (Chehrehasa, 2009). In order to control for some of these variables, other proliferative markers such as Ki-67 and PCNA are commonly used in conjunction with markers for cell death like Fluor-Jade B and activated Caspase3 (Taupin, 2007).

While BrdU and EdU continue to be important tools for studying NSC populations, the emergence of transgenic technology has risen to the challenge of overcoming some key obstacles in the NSC field. One of the most important developments is inducible transgenic fate tracing mouse models. To label endogenous NSCS, *Nestin* or glutamate aspartate transporter (GLAST) serve as conditional drivers of a reporter gene in a Cre-Lox mouse model system. This enables the study of endogenous NSC proliferation, differentiation, and survival at a certain period of time, over extended periods of time, and response to a treatment. In these mouse models, all cells contain a reporter gene, such as yellow fluorescent protein (YFP) or a lacZ gene, flanked by two loxP sites (Decarolis, 2013; Lagace, 2007; Robins, 2013; Mori, 2006; Ninkovic, 2007). These loxP sites prevent expression of the reporter gene. Cells expressing NSC markers, such as *Nestin* or GLAST, will also express a Cre-recombinase enzyme linked to a mutated estrogen receptor (Cre-ER). When the animal is treated with tamoxifen, tamoxifen activates the mutated estrogen receptor which, in turn, translocates to the nucleus. The Cre-

recombinase, once in the nucleus, will cleave the loxP sites flanking the reporter gene. This methods ensures that only cells expressing the NSC marker at the time of tamoxifen treatment are expressing YFP. However, the reporter gene is typically under the control of a housekeeping gene promoter such as Rosa26 (Lagace, 2007; Robins, 2013). Therefore, the reporter gene will remain constitutively expressed in that cell, as well as all progeny cells, even if the cell differentiates and loses the marker protein expression (e.g. *Nestin* or GLAST). This provides the capacity to study a population of neural stem cells and their progeny in over time in response to treatments.

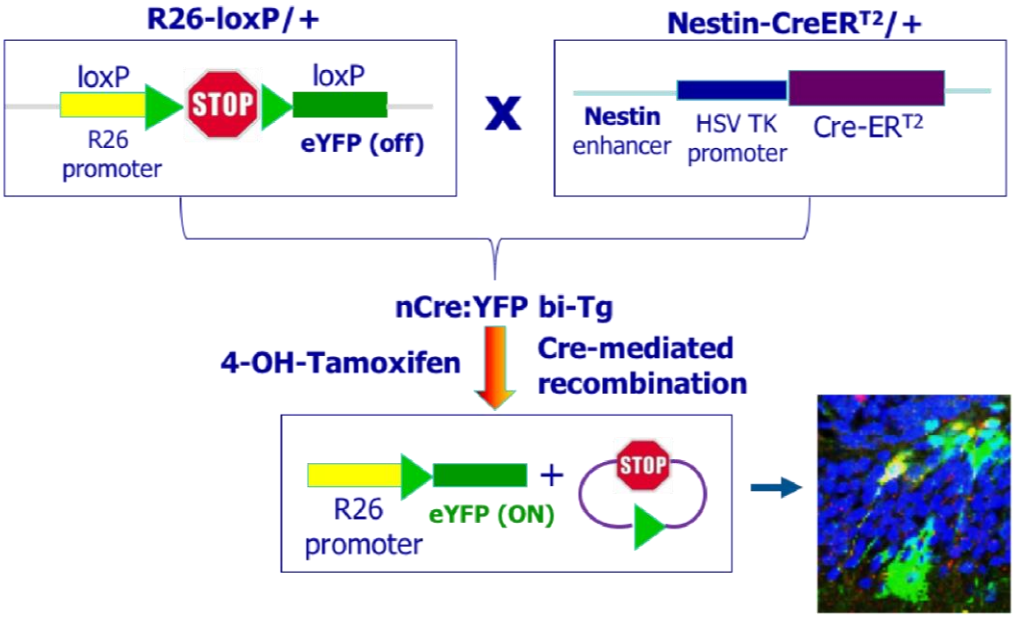



Illustration 5: Cre-Lox inducible fate tracing mouse model. The above schematic diagram illustrates how activated Cre-recombinase cleaves loxP sites flanking reporter gene to enable lineage tracing of a *Nestin* expressing cell population. Image is modified from (Lagace, 2007).

Use of these inducible transgenic models provides unique insight into NSC behavior, even to the point of elucidating unique stages of NSCs previously unexplored, such as the presence of *Nestin* positive cells that maintain the NSC population in the SVZ

(Lagace, 2007). Decarolis and colleagues recently characterized new contributions of *Nestin* and GLAST expressing cells to neurogenesis in the adult hippocampus through the use of transgenic lineage tracing mouse models (Decarolis, 2013). Additionally, Gebara describes further classification of NSCs in the adult SGZ as either α or β based on their morphology made evident by use of transgenic mouse models (Gebara, 2016).

## NSCs: A Translational Perspective

NSCs play pivotal roles in everything from infection to psychiatric disorders. Understanding achieved through advancements in technology, both *in vitro* and *in vivo*, pave the way for translational application of NSCs. In addition to increased efficacy of stem cell transplantation in the field of traumatic brain and spinal cord injury (Gao, 2006; Wu, 2002; Tarasenko, 2007; Zhu, 2005), there are a number of studies focused on modulating endogenous NSC activity in a broad range of psychiatric disorders. Use of FDA-approved antidepressants, such as lithium and aripiprazole, have been shown to increase neurogenesis, and subsequently ameliorate depressive-like symptoms in mice (Yoneyama, 2014; Yoneyama, 2014). In the addiction field, it is known that drugs of abuse such as alcohol and cocaine can significantly reduce NSCs and neurogenesis. These reductions in neurogenesis contribute to neurodegeneration and behavioral pathology such as depression and drug seeking (Yamaguchi, 2004; Noonan, 2010; Golub, 2015). Most treatments for substance use disorders focus on prevention, however there are currently no therapies or treatments to reverse the damage incurred following chronic substance use. NSCs are a promising target in reversing this damage and also attenuating cognitive and behavioral deficits. Pharmacologically blocking endogenous cannabinoid receptors has proven to protect NSC populations in the SGZ following cocaine administration, as well as reduced cocaine-conditioned movements (Blanco-Calvo, 2014).

Interventions as simple as exercise have also been shown to increase neurogenesis and decrease drug seeking behaviors in models of alcohol abuse (Crews, 2004; Leasure and Nixon, 2010)

Adult NSCs continue to be promising targets for therapeutic intervention in a number of mental health and neurological disorders. Recent advances in culture techniques have enabled expansion of iPSCs, primary human fetal NSCs, and adult NSCs isolated from specific brain regions. Culturing NSCs is a valuable tool for screening drugs and elucidating critical regulators of NSC survival and differentiation. Inducible genetic lineage tracing mouse models have created a means for selectively studying a population of NSCs in response to various treatments. Repurposing of FDA-approved drugs as well as novel therapeutic targets has shown efficacy in pre-clinical studies to improve NSC survival and neurogenesis, which leads to behavioral improvement. There is hope that advances in technology to study and monitor NSCs will bridge the gap between pre-clinical and clinical research.

# CHAPTER 2: SPATIAL, TEMPORAL, AND SEX DEPENDENT RESPONSES OF ADULT ENDOGENOUS NEURAL STEM CELLS TO ALCOHOL CONSUMPTION

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, 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, 2011; Vetreno and Crews, 2015; Staples and Mandyam, 2016).

Several factors affect the response of the brain to alcohol consumption, such as age, sex, duration, and quantity of alcohol consumed (Oscar-Berman and Marinkovic, 2003; Squeglia, 2014). There are also regional patterns of degeneration and glial pathology observed in patients with a history of chronic alcohol abuse (Pfefferbaum, 1992; Pfefferbaum, 1993; Cullen and Halliday, 1994; Agartz, 1999; Zahr, 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, 2005; Crews, 2006; Morris, 2010; Winner, 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 brain function and neurogenesis throughout life, and possibly in mediating neuro-regeneration after insults (Nixon and Crews, 2004; Leasure and Nixon, 2010; Maynard and Leasure, 2013).

The two most commonly studied NSC-containing regions in the adult brain are the subventricular zone of the lateral ventricles (SVZ) and the subgranular zone of the dentate gyrus (SGZ) (Crews and Nixon, 2003; Kempermann, 2015). In these regions, alcohol consumption impairs NSC proliferation and neurogenesis (Crews, 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 neural stem cell properties, however the impact of alcohol on this cell population has not been investigated (Robins, 2013). While studies have investigated the effect of alcohol in the SVZ and SGZ, a comparative examination of how 4 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 also 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 new tool to study longitudinal changes of endogenous NSC populations in the adult brain (Lagace, 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. Additionally, 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 bi-transgenic 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 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 difference between the body weights of control or ethanol groups (Figure 1B-C). Male mice in both groups had a higher average daily diet consumption compared to 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 to females; however, females consumed 13% more grams of ethanol per kilogram of body weight (Figure 1E-F). There were no significant changes in body weight gains, but mice in the ethanol group tended to have smaller body weight gain (Figure 1G).

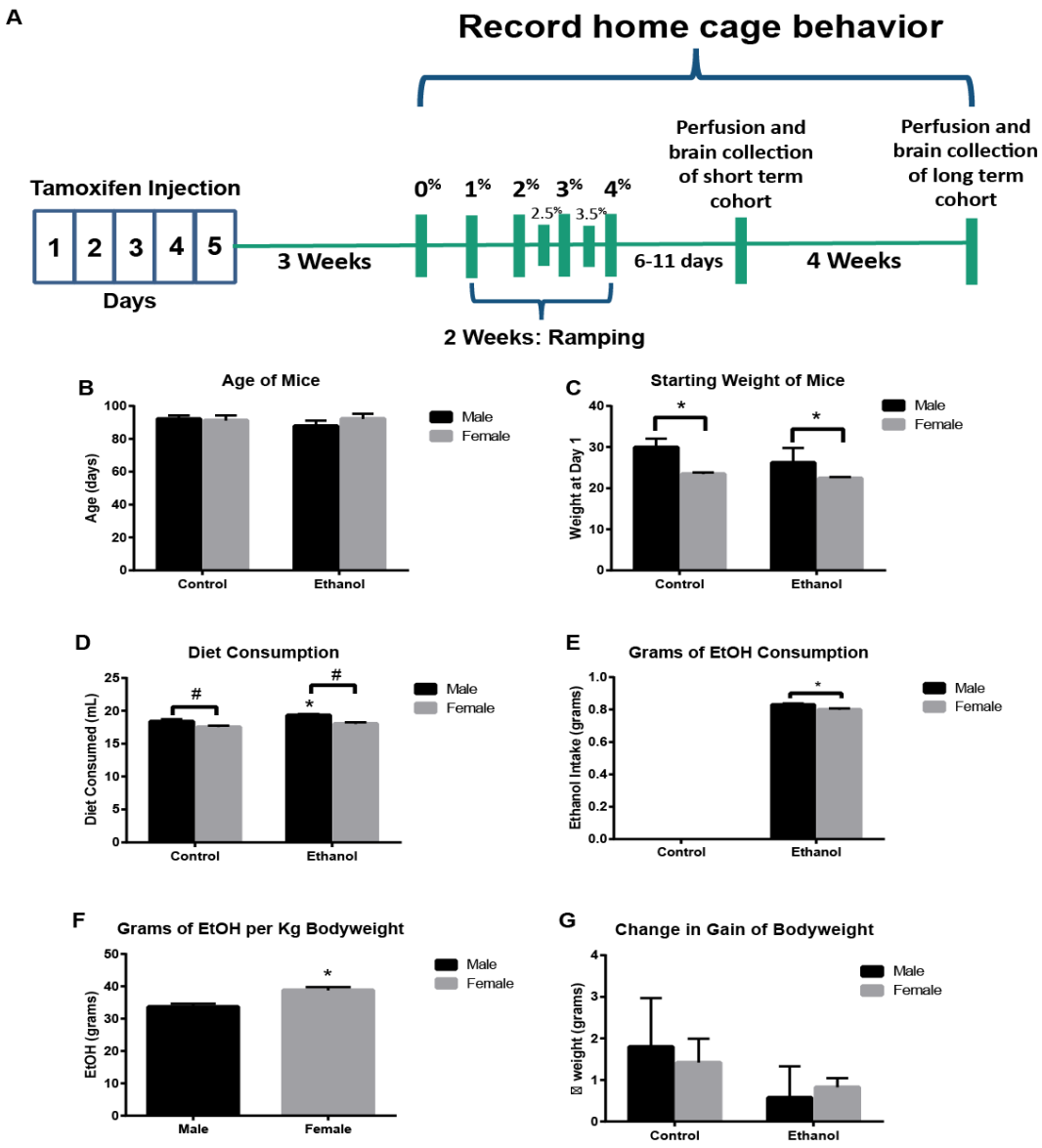



Figure 1: Experimental alcohol model. (**A**) Schematic of experimental paradigm. (**B**) Average age of mice at start of experiment. (**C**) Average bodyweight of mice at the start of experiment. (**D**) Average daily diet consumption. (**E**) Average grams of ethanol consumed daily. (**F**) Average grams of ethanol consumed per kilogram of mouse bodyweight. (**G**) Average changes in gain of bodyweight. Values are shown as mean±SEM, *p<0.05 compared to control, #p>0.05 compared to other sex in same group, male mice n=13 female mice n=12

Similar to humans, mice showed individual variations in response to alcohol intake, ranging from ataxia, tremors, 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 2A). These behaviors have previously been associated with intoxication in mice (Crabbe et al., 2008). All animals in the ethanol group tolerated the two-week ramping stage without abnormal behaviors. Among 25 mice fed with 4% ethanol, four males (30.8%) and four 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” pathological study. On the other hand, nine males (69.2%) and eight females (66.7%) showed severe symptoms of intoxication reaching a score of 4 between 6-24 days with 4% ethanol. These included five males and four females that died between 6-24 days of 4% ethanol. The mice with severe symptoms were euthanized between 6-11 days after consumption of 4% ethanol diet and grouped into the “short-term” study. Ethanol treated females displayed more severe symptoms of intoxication that escalated in severity at a higher rate than males ($p=0.009$) (Figure 2B). Blood alcohol analysis showed a wide range of blood alcohol concentrations (BAC) and blood acetaldehyde concentrations that did not correlate with behavioral severity for both sexes (Figure 3). One source of this variability may be due to that blood samples were collected in the morning, while mice typically feed in the first few hours of the dark cycle. Additionally, since mice have unrestricted access to the diet it is possible that mice with higher BAC consumed diet more recently than mice with lower BAC.

A

| # | 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. |

B

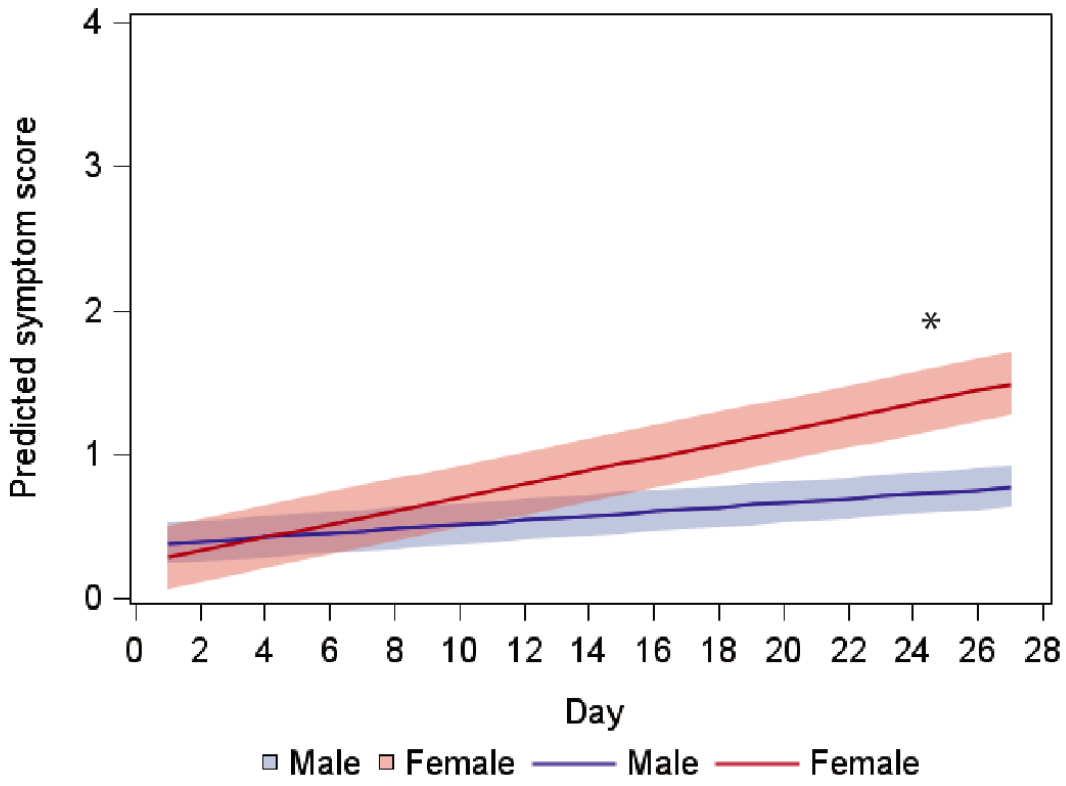


Figure 2: Home cage behavior scoring. (A) Chart showing the scoring system evaluation criteria. (B) Graph comparing the severity of alcohol-induced symptoms over time. Red line is female, pink shadow is female error bar. Blue line is male, blue shadow is male error bar. Values are medians in each day evaluated by a random slope model, *p<0.05, male n=13, female n=12. See also Figure S1.

**A**

| **Treatment** | Short Term | Long Term | Long Term | Long Term | Long Term |
|---|---|---|---|---|---|
| **Sex** | M | M | M | M | M |
| **Body Weight** | 23.4 | 25.6 | 30 | 27.9 | 23 |
| **Average diet consumed** | 23.44 | 19.64 | 18.84 | 20.70 | 18.38 |
| **Ethanol mg%** | **20.13** | < 2 | < 2 | **36.8** | **200.9** |
| **Acetaldehyde mg%** | **0.22** | **0.37** | **0.44** | **0.52** | **1.18** |
| **Behavior** | Shaking, ataxia, unresponsive, unhealthy fur | Slow, ataxia, unresponsiveness and eye squinting | Slow, no major changes | Messy fur, Slow, no major changes | Ataxia progressing to severe ataxia |

**B**

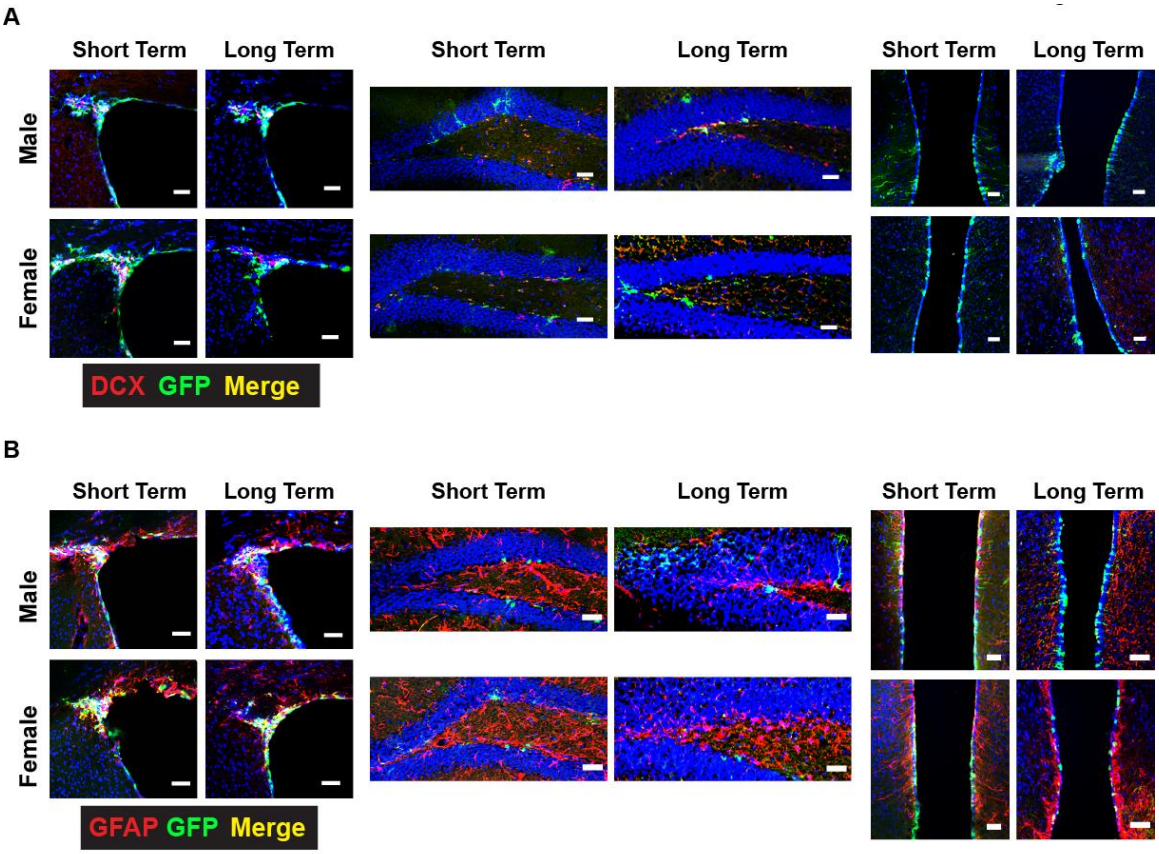
| **Treatment** | Short Term | Long Term | Long Term | Long Term |
|---|---|---|---|---|
| **Sex** | F | F | F | F |
| **Body Weight** | 18.5 | 23 | 23.5 | 22 |
| **Average diet consumed** | 20.06 | 18.98 | 19.15 | 20.66 |
| **Ethanol mg%** | **370.73** | **282.39** | < 2 | < 2 |
| **Acetaldehyde mg%** | **0.3** | **1.25** | 0.06 | 0.06 |
| **Behavior** | Slow, ataxia, shaking unresponsive | Ataxia, Shaking, slow, unresponsive | No noticable changes | Hyper active |

Figure 3: Blood alcohol and acetaldehyde concentration analysis. (A) Chart showing the blood alcohol and acetaldehyde analysis (highlighted in yellow) of 5 male mice (1 short term, 4 long term) including individual bodyweight, average daily diet consumption, blood ethanol and acetaldehyde concentration, and behavioral characteristics. (B) Chart showing the blood alcohol and acetaldehyde analysis (highlighted in yellow) of 4 female mice (1 short term, 3 long term) including individual bodyweight, average daily diet consumption, blood ethanol and acetaldehyde concentration, and behavioral characteristics. . Highlighted in yellow are values higher than those detected in control blood.

**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-CreERT2;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, 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 green fluorescent protein (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 the population of neural stem cells and their progeny in response to alcohol consumption. This genetic tracing technique allowed us examine three phases of NSCs, including 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 differentiated prior to tamoxifen induction and no

longer expressed Nestin would not be colabeled with GFP. We found no significant differences in GFP+ cell populations when comparing short- and long-term control mice (Figure 4). As shown in Figure 5A, male and female mice on the control diet had comparable numbers of GFP+ NSCs in the SVZ region (Figure 5A, 5D). Mice on the ethanol diet exhibited significant alterations of SVZ NSCs in a temporal- and sex-dependent manner. In the SVZ region, short-term consumption of ethanol diet drastically reduced the number of GFP+ NSCs by 96% in females but only 37% in males (Figure 5B, 5D), whereas long-term treatment decreased GFP+ cells by 98% in females and 89% in males (Figure 5C, 5D). The total number of GFP+ cells, both within the SVZ and the 7 origin of the rostral migratory stream (oRMS), decreased by 90% in females but only 21% in males after short-term ethanol exposure (Figure 5B, 5E). Long-term treatment further reduced total GFP+ cells by 94% in females and 74% in males (Figure 5C, 5E).


A
Short Term
Long Term
Short Term
Long Term
Short Term
Long Term
Male
Female
DCX GFP Merge
B
Short Term
Long Term
Short Term
Long Term
Short Term
Long Term
Male
Female
GFAP GFP Merge

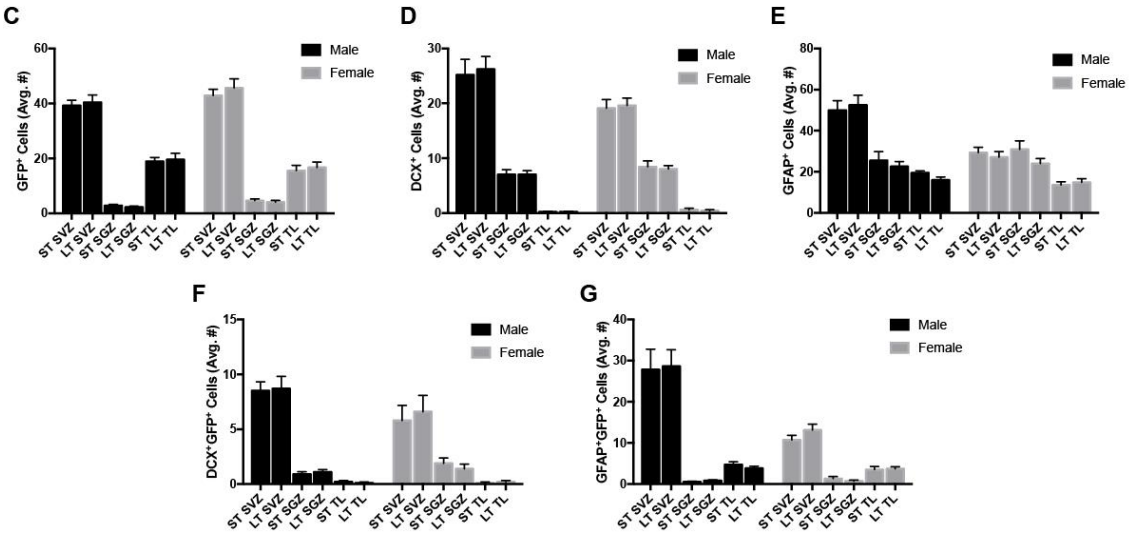

C
GFP⁺ Cells (Avg. #)
Male
Female
D
DCX⁺ Cells (Avg. #)
Male
Female
E
GFAP⁺ Cells (Avg. #)
Male
Female
ST SVZ
LT SVZ
ST SGZ
LT SGZ
ST TL
LT TL
F
DCX⁺GFP⁺ Cells (Avg. #)
Male
Female
G
GFAP⁺GFP⁺ Cells (Avg. #)
Male
Female

**Figure 4:** Comparison of control mice at short- and long-term time points. Related to Figure 3-7. (**A**-**C**)Representative images of $DCX^+$ and $GFP^+$ cells in the SVZ, SGZ, and TL of control male and female mice at short- and long-term time points. (**D**-**F**)Representative images of $GFAP^+$ and $GFP^+$ cells in the SVZ, SGZ, and TL of control male and female mice at short- and long-term time points. Scale bars measure 45μm, n=3 mice per sex per group.

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, 2011). Without ethanol treatment, males and females had similar numbers of DCX+ and DCX+GFP+ cells in the oRMS (Figure 5A, 5F-G). DCX+GFP+ accounted for 35% and 22% of the total GFP cells in control males and females, respectively (Figure 5G). Short-term ethanol significantly reduced the number of DCX+ cells in males and females by 29% and 57%, respectively (Figure 5B, 5F). Furthermore, many of the DCX+GFP+ cells were retained in the SVZ, indicating a reduced migration. Long-term ethanol consumption also significantly decreased DCX+ expression by 54% in males and 66% in females (Figure 5C and 5F). Following short-term treatment, males only had a slight trend of reduction in the number of DCX+GFP+ cells, while females had an 85% reduction in this population (Figure 5B and 5G). In the long term group, DCX+GFP+ cells were reduced by 82% in males and 87% in females (Figure 5C, 5G). Neither males nor females demonstrated significant reduction in the percent of GFP+ cells becoming DCX+ following both short- and long-term ethanol consumption, although males had a trend of decrease ($p=0.06$) (Figure 5H). The greater loss of DCX+GFP+ cells (Figure 5G) 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, a dual immunolabeling with GFP and NeuN (a mature neuronal marker) was conducted in the olfactory bulb. As shown in Figure 6, 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.

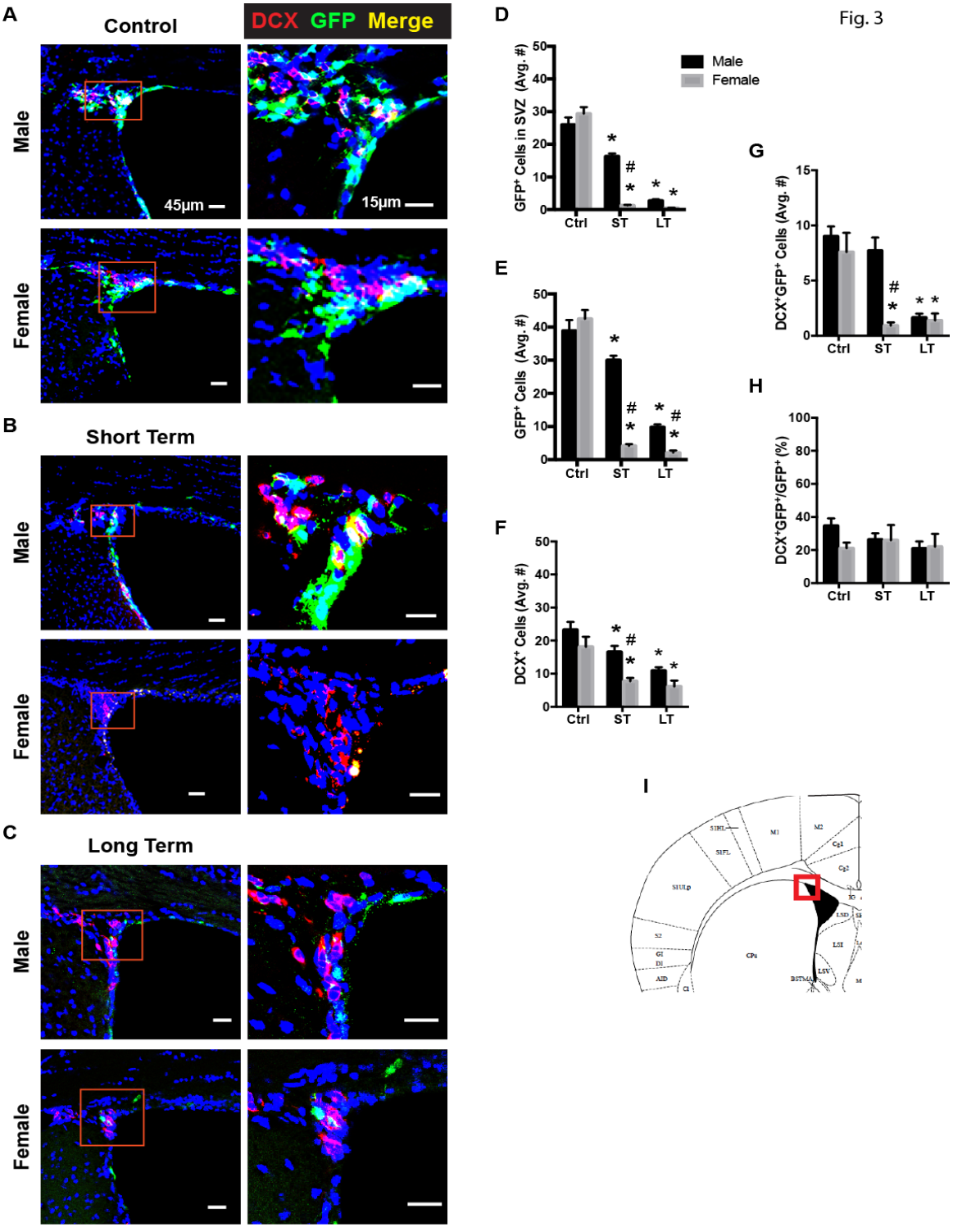

A
Control
DCX GFP Merge
Male
Female
45μm
15μm
B
Short Term
C
Long Term
D
Male
Female
GFP⁺ Cells in SVZ (Avg. #)
Ctrl
ST
LT
E
GFP⁺ Cells (Avg. #)
F
DCX⁺ Cells (Avg. #)
G
DCX⁺GFP⁺ Cells (Avg. #)
H
DCX⁺GFP⁺/GFP⁺ (%)
I

Fig. 3

Figure 5: Neurogenesis in the SVZ following ethanol consumption. (A-C) Representative images of control, short and long term male and female mice, respectively, brain stained with stem cell marker (GFP green), neuronal marker (DCX red), and merged with nuclear maker Dapi. Region in red box is shown enlarged on right. (D-H) Quantification of $GFP^+$ within the SVZ, $GFP^+$ cells in SVZ and origin of the rostral migratory stream, $DCX^+$, $DCX^+GFP^+$ average cells per section, and the percent of $DCX^+GFP^+$ over total $GFP^+$ cells. (I) Schematic image of representative region quantified (Bregma 0.5 through 1.08). Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, n=3 mice per sex per group. Scale bars, 45μm and 15μm in enlarged images.

.

SFig. 3

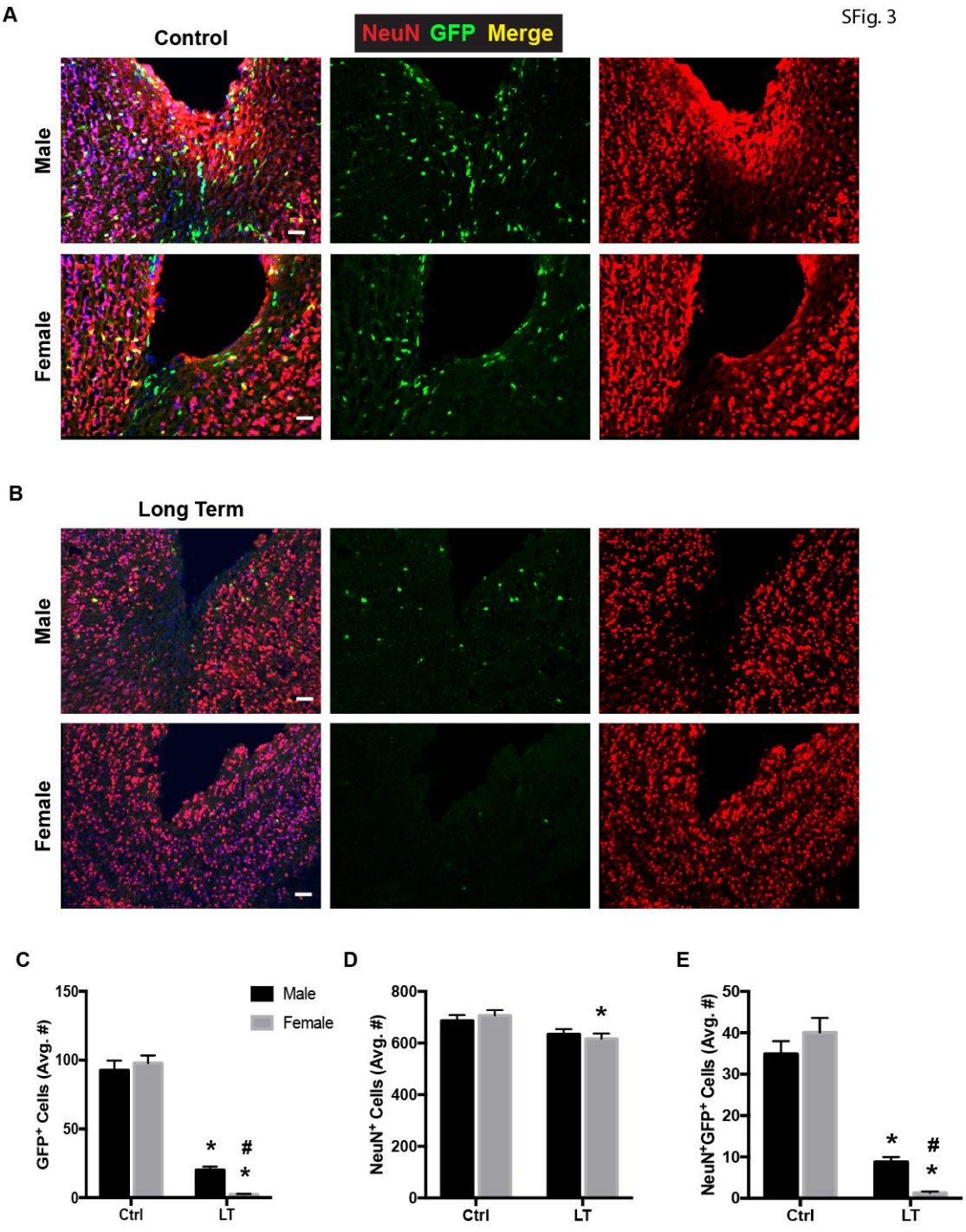

A
Control
NeuN GFP Merge
Male
Female
B
Long Term
Male
Female
C
GFP⁺ Cells (Avg. #)
150
100
50
0
Male
Female
*
#
*
Ctrl
LT
D
NeuN⁺ Cells (Avg. #)
800
600
400
200
0
*
Ctrl
LT
E
NeuN⁺GFP⁺ Cells (Avg. #)
50
40
30
20
10
0
*
#
*
Ctrl
LT

**Figure 6:** Neurogenesis in the olfactory bulb following ethanol consumption. (**A-B**) Representative images of control and long term male and female mice, respectively, brain stained with stem cell marker (GFP green), mature neuronal marker (NeuN red), and merged with nuclear maker Dapi. Scale bars measure 32μm 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 to control, #$p<0.05$ compared to other sex in the same group, n=3 mice per sex per group.

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 (Figure 7A). To determine astrogliogenesis from the SVZ NSCs expressing GFAP, we quantified those GFP and GFAP co-labeled cells in the oRMS (Liu, 2010). No significant differences were found in the number of GFP+ cells or GFAP+GFP+ /GFP+ between males and females on control diet (Figure 7A, 7D, 7G), whereas female exhibited significantly lower numbers of total GFAP+ cells and newly differentiated astrocytes (GFAP+GFP+ )(Figure 7E-F). Following short-term ethanol consumption GFP+ cells in the oRMS decreased by 30% in males and 81% in females (Figure 7B, 7D). However, both males and females in the short-term group had an increase of total GFAP+ cells by 41% and 42% in the oRMS, respectively (Figure 7B, 7E). Female mice exhibited a 98% decrease in newly differentiated GFAP+GFP+ cells, whereas males only had a 14% decrease (Figure 7B, 7F). Interestingly, there was a significant decrease in percent of GFP+ cells expressing GFAP (GFAP+GFP+ /GFP+) in females (92%) but not in males (3%) in the short-term group (Figure 7B, 7G). In long-term treated mice, the population of GFP+ cells was reduced by 84% in males and 93% in females (Figure 7C-D). Females continued to display an increased GFAP+ population compared to controls, whereas males showed a significant reduction (Figure 7E). GFAP+GFP+ cells decreased by 90% and 97% in males and females, respectively (Figure 7C, 7F).

Furthermore, the percent of GFP+ cells expressing GFAP+ was significantly reduced in males (47%) and females (92%) (Figure 7G). Individual immunohistochemical stain images can be found in the Figure 8.

In summary, these data show the NSCs in the SVZ are sensitive to both short and 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 rostral migratory stream and a subsequent loss of NeuN+GFP+ in the olfactory bulb. Also, alcohol consumption decreased the survival of newly differentiated astrocytes. Interestingly, 9 males and females had a similar increase of glial cells (GFAP+ cells) in the oRMS after short-term alcohol exposure.

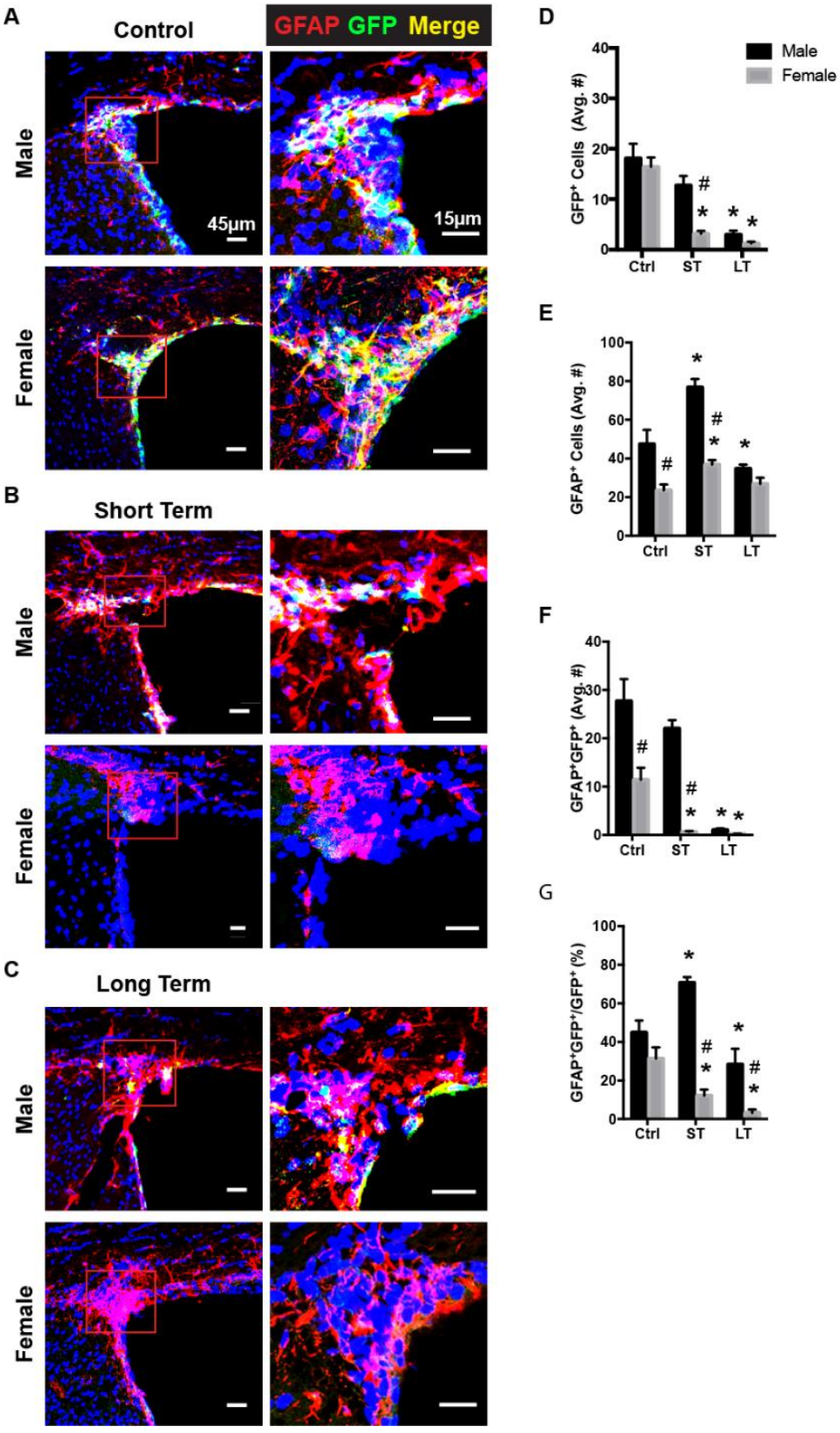

A
Control
GFAP GFP Merge
Male
Female
45μm
15μm
B
Short Term
C
Long Term
D
Male
Female
GFP⁺ Cells (Avg. #)
Ctrl
ST
LT
E
GFAP⁺ Cells (Avg. #)
F
GFAP⁺GFP⁺ (Avg. #)
G
GFAP⁺GFP⁺/GFP⁺ (%)

Figure 7: Astrogliogenesis in the SVZ following ethanol consumption. (A-C) Representative images of control, short- and long-term male and female mouse brains stained with stem cell marker (GFP green), astrocyte marker (GFAP red), and merged with nuclear maker DAPI (blue). Region in red box is shown enlarged on right. (D-G) Quantification of $GFP^+$ cells in SVZ and origin of the rostral migratory stream, $GFAP^+$, average $GFAP^+GFP+$ cells per section, and the percentage of $GFAP^+GFP^+$ over total GFP+ cells. Scale bars, 45μm and 15μm in enlarged images. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, n=3 mice per sex per group.

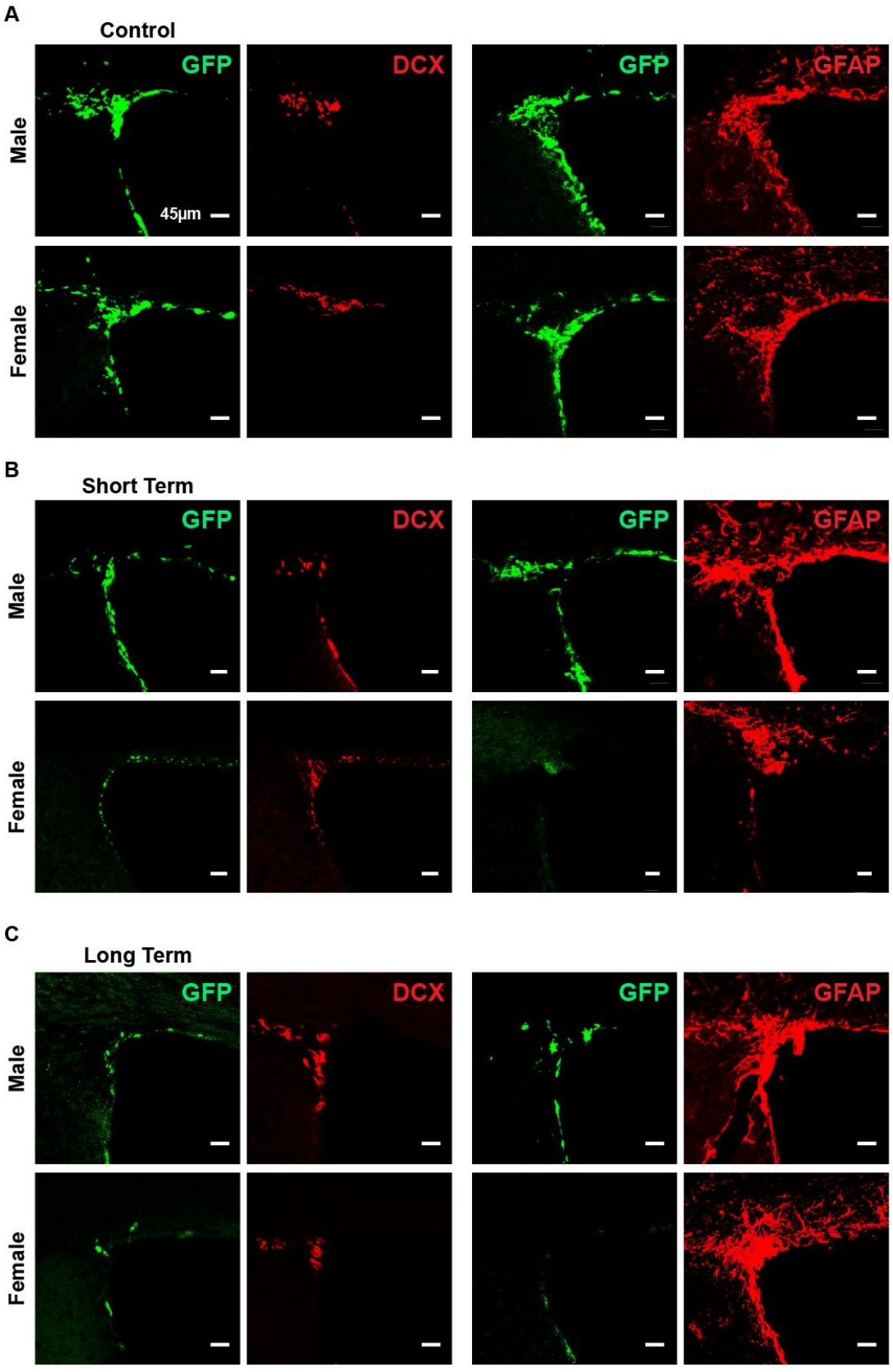

A
Control
GFP
DCX
GFP
GFAP
Male
45µm
Female
B
Short Term
GFP
DCX
GFP
GFAP
Male
Female
C
Long Term
GFP
DCX
GFP
GFAP
Male
Female

**Figure 8:** Individual channels of SVZ immunohistochemical stains. (**A**) Single channel of representative images of control male and female mice stained with stem cell marker (GFP green), neuronal marker (DCX red), and astrocyte marker (GFAP). (**B**) Single channel of representative images of short-term treated male and female mice stained with stem cell marker (GFP green), neuronal marker (DCX red), and astrocyte marker (GFAP). (**C**) Single channel of representative images of long-term treated male and female mice stained with stem cell marker (GFP green), neuronal marker (DCX red), and astrocyte marker (GFAP). Scale bars, 45µm.

In summary, these data show the NSCs in the SVZ are sensitive to both short and 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 rostral migratory stream and a subsequent loss of $NeuN^{+}GFP^{+}$ in the olfactory bulb. Also, alcohol consumption decreased the survival of newly differentiated astrocytes. Interestingly, males and females had a similar increase in glial cells ($GFAP^{+}$ cells) in the oRMS.

**Effect of alcohol consumption on SGZ NSCs in the hippocampus**

Next, we examined the SGZ in the dentate gyrus of the dorsal hippocampus, given its role in cognitive function and active adult neurogenesis (Kempermann, 2015). The SGZ showed intrinsic sex differences, with female mice in the control group having approximately twice the number of GFP+ cells as their male counterparts (Figure 9A, 9D). The SGZ GFP+ cells also showed a sex- and time-dependent response to ethanol consumption. After short-term consumption, only male mice exhibited a robust 1.80-fold increase in GFP+ cells (Figure 9B, 9D). However, long-term alcohol treatment significantly reduced the numbers of GFP+ cells in both male and female mice (Figure 9C, and 9D).

Next we evaluated neurogenesis in the SGZ. Control male and female mice had similar levels of DCX+ and DCX+GFP+ cells. The DCX+GFP+ cells accounted for 14%

and 16% of the DCX+ population in males and females, respectively (Figure 9A, 9E); and the percentage of GFP+ cells expressing DCX (DCX+GFP+ /GFP+ ) was 41% in control males and 26% in females (Figure 9A and 9G). We observed that neurogenesis in the SGZ was affected in a time-dependent manner in response to ethanol consumption. The short-term treatment in male and female mice displayed no significant changes in numbers of DCX+ or DCX+GFP+ cells, however males had a significant decrease (71%) in the percentage of GFP+ cells expressing DCX (DCX+GFP+ /GFP+ )(Figure 9B and 9E-G). This is likely due to the increase of GFP+ NSC proliferation without changes in the DCX+ population. In the long-term ethanol cohort, both DCX+ and DCX+GFP+ cells were significantly decreased in males and females. Males exhibited a 49% and 75% decrease in DCX+ and DCX+GFP+ cells, respectively; whereas females had a 65% and 53% decrease in DCX+ and DCX+GFP+ cells, respectively (Figure 9C, 9E-F). Males in 10 the long-term group exhibited a significant reduction (63%) in the percentage of GFP+ cells labeled with DCX (Figure 9G).

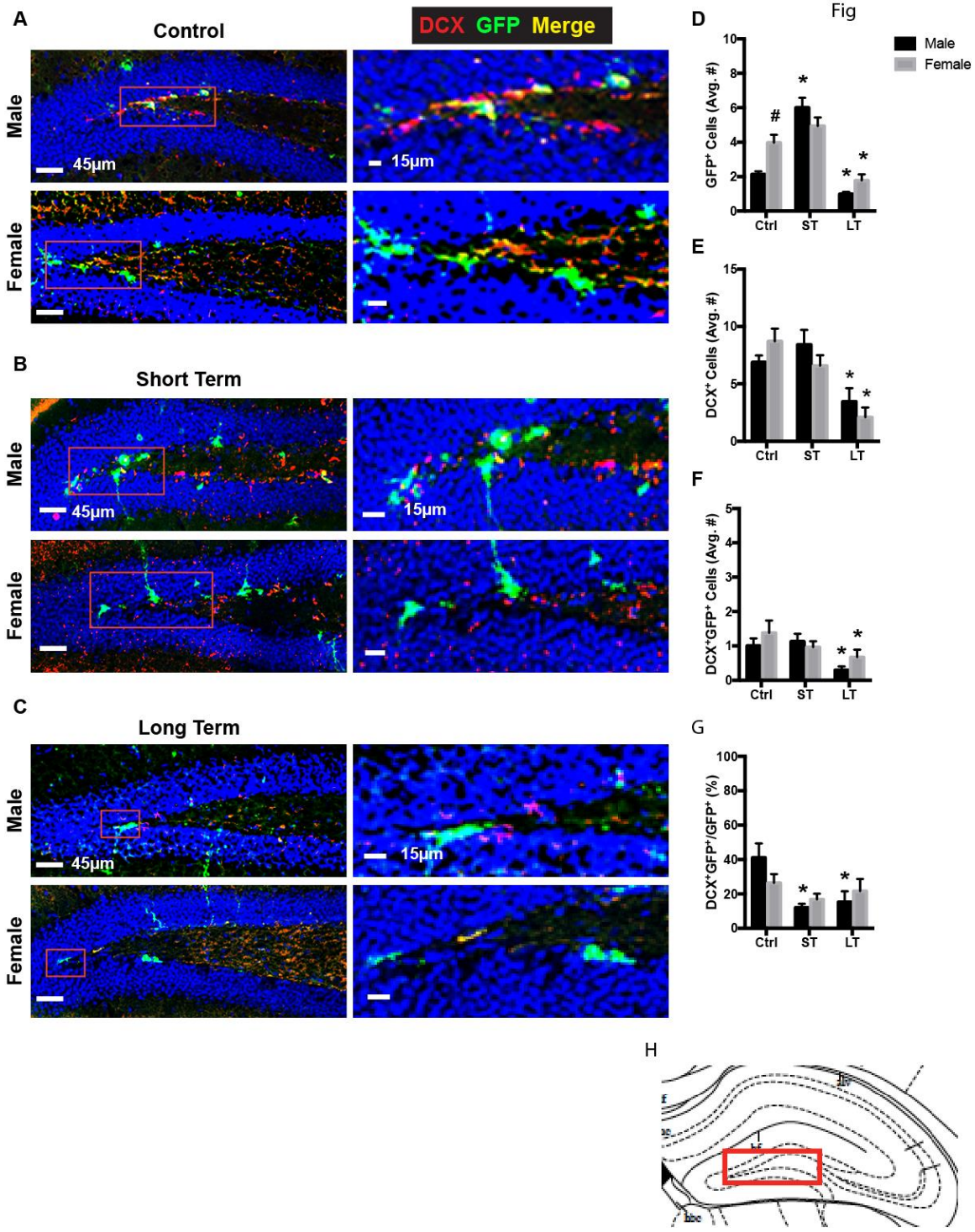

A
Control
DCX GFP Merge
Male
Female
45μm
15μm
B
Short Term
C
Long Term
D
Fig
Male
Female
GFP+ Cells (Avg. #)
Ctrl
ST
LT
E
DCX+ Cells (Avg. #)
F
DCX+GFP+ Cells (Avg. #)
G
DCX+GFP+/GFP+ (%)
H

**Figure 9:** Neurogenesis in the SGZ following ethanol consumption. (**A-C**) Representative images of control, short and long term male and female mice, respectively, brain stained with stem cell marker (GFP green), neuronal marker (DCX red), and merged with nuclear maker Dapi. Region in red box is shown enlarged on right. (**D-G**) Quantification in SGZ of average $GFP^+$, $DCX^+$, $DCX^+GFP^+$ cells, and percent of $DCX^+GFP^+$ over total $GFP^+$ cells. (**H**) Schematic image of representative region that was quantified (Bregma -1.58 through -2.16). Scale bars, 45μm and 15μm in enlarged images. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, n=3 mice per sex per group. See also Figure S5.

We then examined astrogliogenesis in the SGZ. There were no significant differences in GFAP+ or GFAP+GFP+ cells between control males and females (Figure 10A, 10D-E). GFAP+GFP+ cells were 2.7% and 3.1% of the total GFP+ population in control males and females, respectively (Figure 10A and 10E). The percentage of GFP+ cells expressing GFAP in the SGZ was also similar in control male and female mice (26% in males, 21% in females) (Figure 10A and 10F). Short-term ethanol consumption reduced GFAP+ cells in the SGZ by 32% in females, but had little effect on males (Figure 10B, 10D). Interestingly, the reduction in GFAP+ cells was roughly the same in short-term and long-term treated females. In the long-term group, female mice had a 36% reduction, and again, males were relatively unaffected (Figure 10C-D). Short and long-term ethanol consumption did not have an impact on GFAP+GFP+ cells in males, or females (Figure 10B-C, 10E). Additionally, there was a significant decrease in the percentage of GFP+ cells expressing GFAP in short-term treated females (48%), but not long-term (Figure 10BC, 10F). These data show that female GFAP+ populations in the SGZ are more sensitive to changes following ethanol consumption compared to males, and that GFAP+ populations show a different response in the SGZ compared to the SVZ. Individual immunohistochemical stain images can be found in Figure 11.

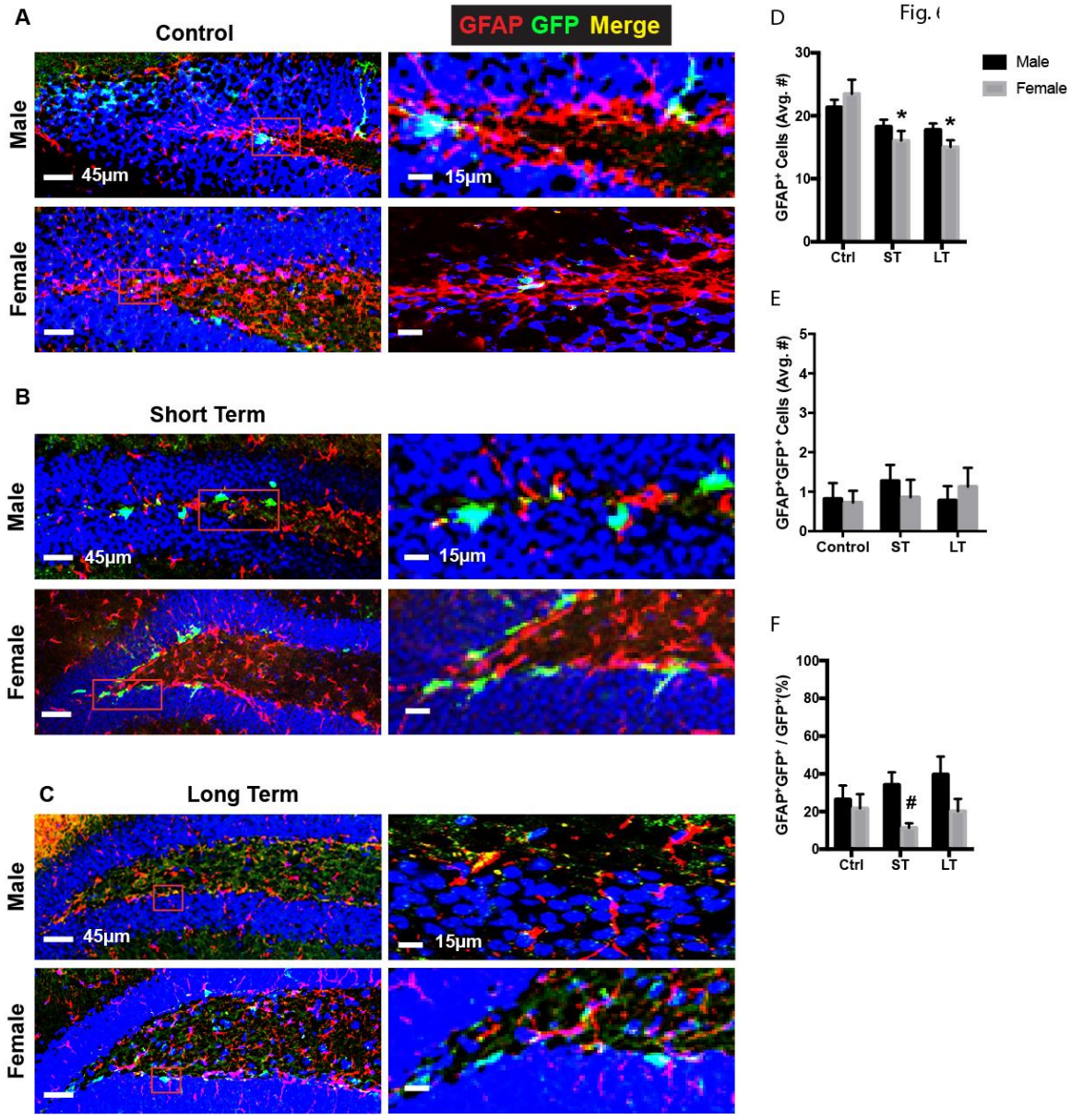

A
Control
GFAP GFP Merge
Male
Female
45µm
15µm
B
Short Term
C
Long Term
D
Fig. (
Male
Female
GFAP⁺ Cells (Avg. #)
Ctrl
ST
LT
E
GFAP⁺GFP⁺ Cells (Avg. #)
Control
F
GFAP⁺GFP⁺ / GFP⁺(%)

**Figure 10:** Astrogliogenesis in the SGZ following ethanol consumption. (**A-C**) Representative images of control, short and long term male and female mice, respectively, brain stained with stem cell marker (GFP green), neuronal marker (GFAP red), and merged with nuclear maker Dapi. Region in red box is shown enlarged on right. (**D-F**) Quantification in SGZ of average $GFAP^+$, $GFAP^+GFP^+$ cells, and percent of $GFAP^+GFP^+$ over total $GFP^+$ cells. Scale bars, 45µm and 15µm in enlarged images. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, n=3 mice per sex per group. See also Figure S5.

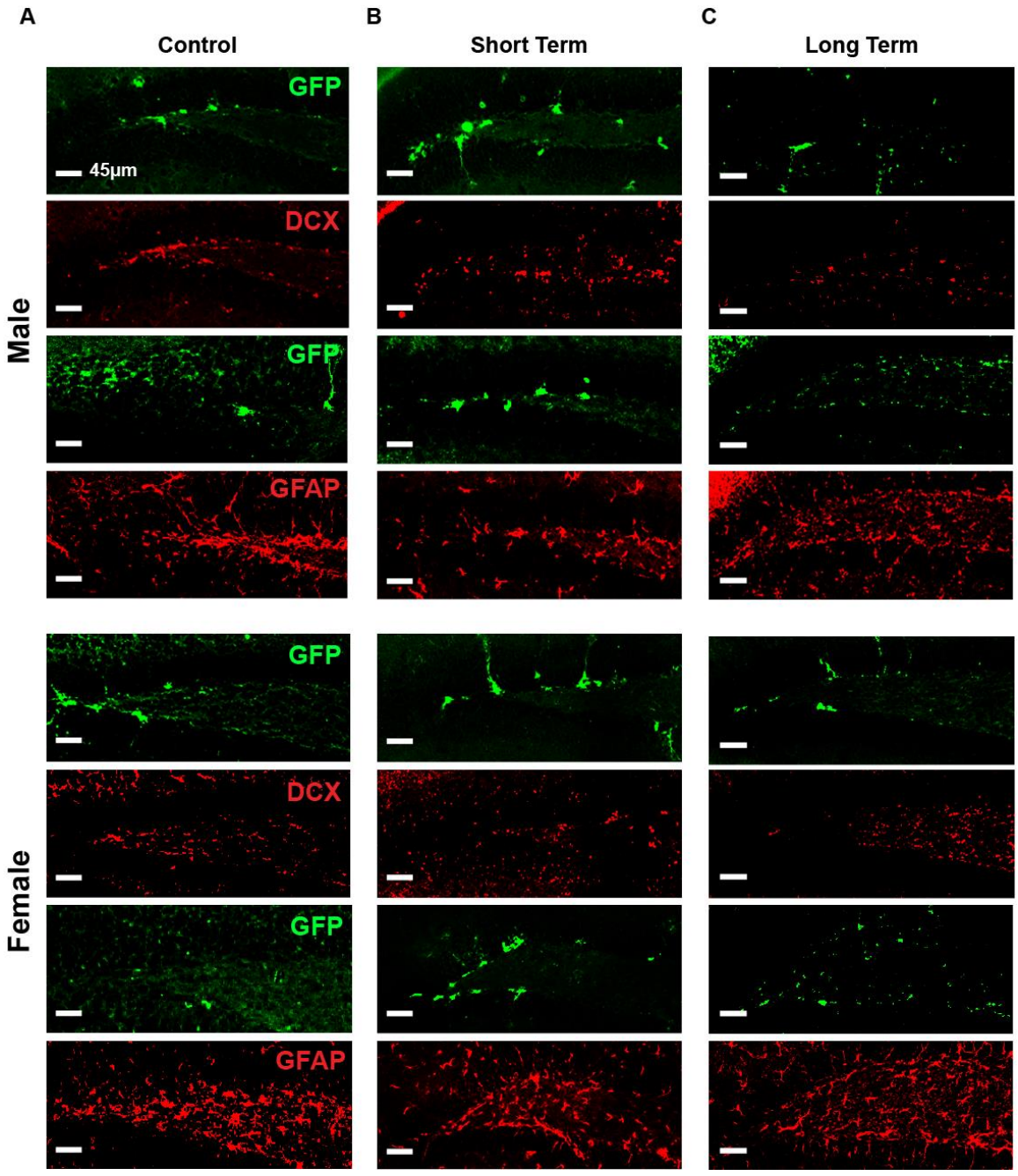

A
B
C
Control
Short Term
Long Term
Male
Female
GFP
DCX
GFP
GFAP
GFP
DCX
GFP
GFAP
45µm

**Figure 11:** Individual channels of SGZ immunohistochemical stains. (**A**) Single channel of representative images of control male and female mice stained with stem cell marker (GFP green), neuronal marker (DCX red), and astrocyte marker (GFAP). (**B**) Single channel of representative images of short-term treated male and female mice stained with stem cell marker (GFP green), neuronal marker (DCX red), and astrocyte marker (GFAP). (**C**) Single channel of representative images of long-term treated male and female mice stained with stem cell marker (GFP green), neuronal marker (DCX red), and astrocyte marker (GFAP). Scale bars, 45µm.

In summary, short-term alcohol consumption increased SGZ NSCs in males, but decreased neurogenesis. Astrogliogenesis was reduced in females following short-term consumption. Long-term alcohol consumption reduced neurogenesis in both males and females. Additionally, females, but not males, had a significant reduction in GFAP+ cells in the long-term cohort.

**Effect of chronic alcohol consumption in the TL NSCs in the third ventricle**

The third region we evaluated was the tanycyte layer (TL) of the third ventricle. This region was selected because it has recently been shown to contain cells with NSC potential (Robins, 2013; Haan, 2013). However, in our study we found little evidence of newly differentiating neurons (Figure 12), 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, 2005; Robins, 2013). The primary role of tanycytes is to communicate chemical signals from the ventricles to the hypothalamus (Rodriguez, 2005). Control mice had similar numbers of GFP+ , GFAP+ and GFAP+GFP+ cells between both sexes (Figure 13A, 13D-G).

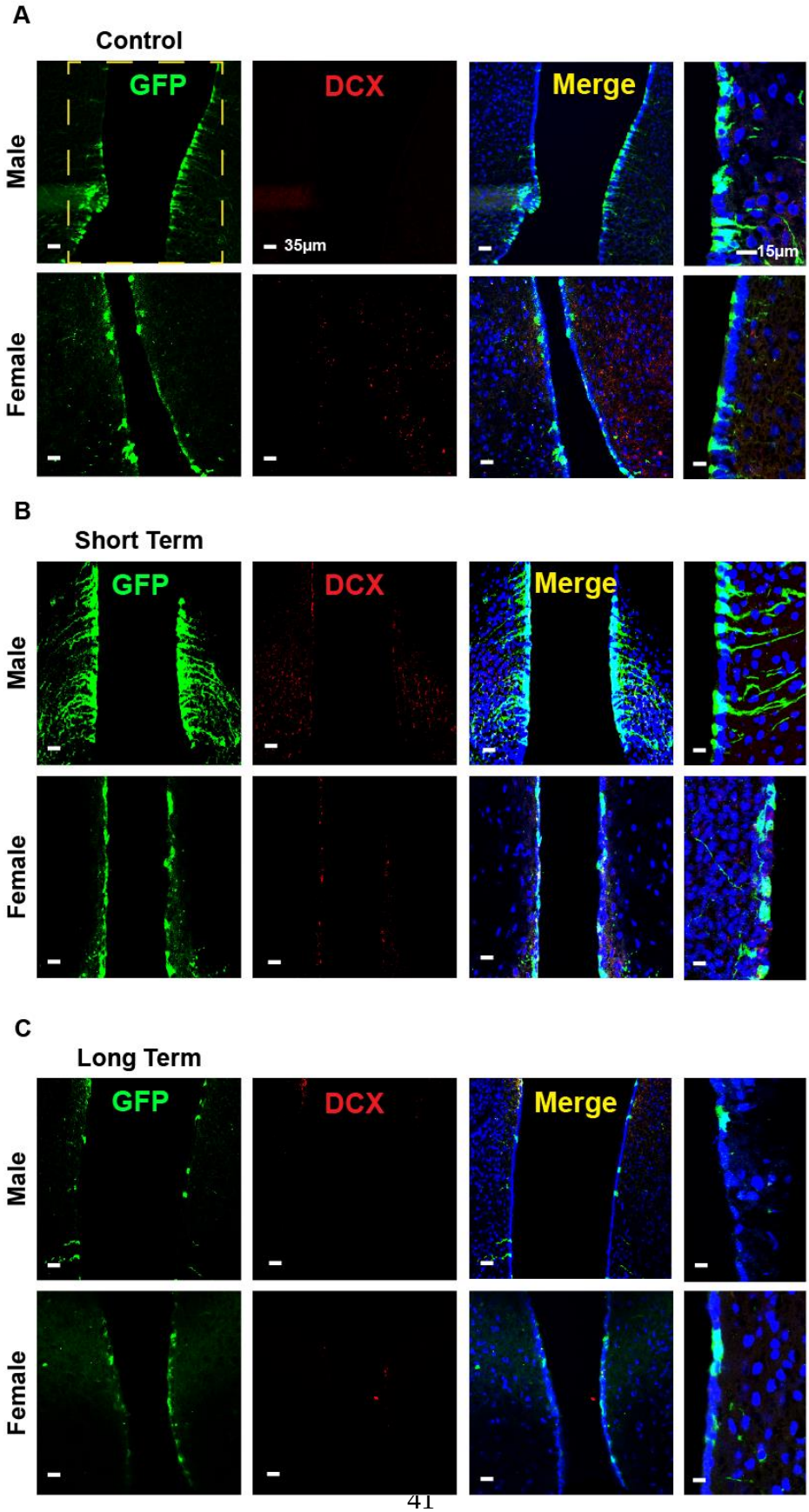

A
Control
GFP
DCX
Merge
Male
Female
35μm
15μm
B
Short Term
GFP
DCX
Merge
Male
Female
C
Long Term
GFP
DCX
Merge
Male
Female

**Figure 12:** Lack of neurogenesis in the TL in mice older than 18 weeks. (**A**) Representative images of control male and female mice stained with stem cell marker (GFP green), neuronal marker (DCX red), and merged with nuclear maker Dapi. (**B**) Representative images of short-term short-term ethanol male and female mice with same stains as control. (**C**) Representative images of long-term ethanol male and female mice with same stains as control. n=3 mice per sex per group. Scale bars, 45μm and 15μm in enlarged images; n=3 mice per sex per group.

Following short-term ethanol consumption, both males and females had an increase in tanycyte GFP+ cells (Figure 13B, 13D). Males had the most robust response of a 139% increase in GFP+ cells, while females had an 87% increase (Figure 13E). Interestingly, while both males and females had an increase in GFP+ and GFAP+ cells, females did not have a change in the number of GFAP+GFP+ cells after short-term consumption (Figure 13F). Males, on the other hand, had a 167% increase in the number of GFAP+GFP+ cells after short-term consumption. (Figure 13F). Neither males nor females had a change in the percent of GFP+ cells differentiating into GFAP+ following short- and long-term alcohol consumption (Figure 13G). Following long-term ethanol consumption, both males and females exhibited significant decreases of GFP+ in the TL region by 56% and 40%, respectively (Figure 13C-D). Interestingly, females had an increase in GFAP+ cells (75%), whereas males experienced a 67% decrease in GFAP+ cells (Figure 13E). Similar to the short-term cohort, females did not have a significant change in the number of GFAP+GFP+ cells (Figure 13F). However, males in the long-term cohort experienced a 67% decrease in GFAP+GFP+ cells (Figure 13F). Individual immunohistochemical stain images can be found in Figure 14.

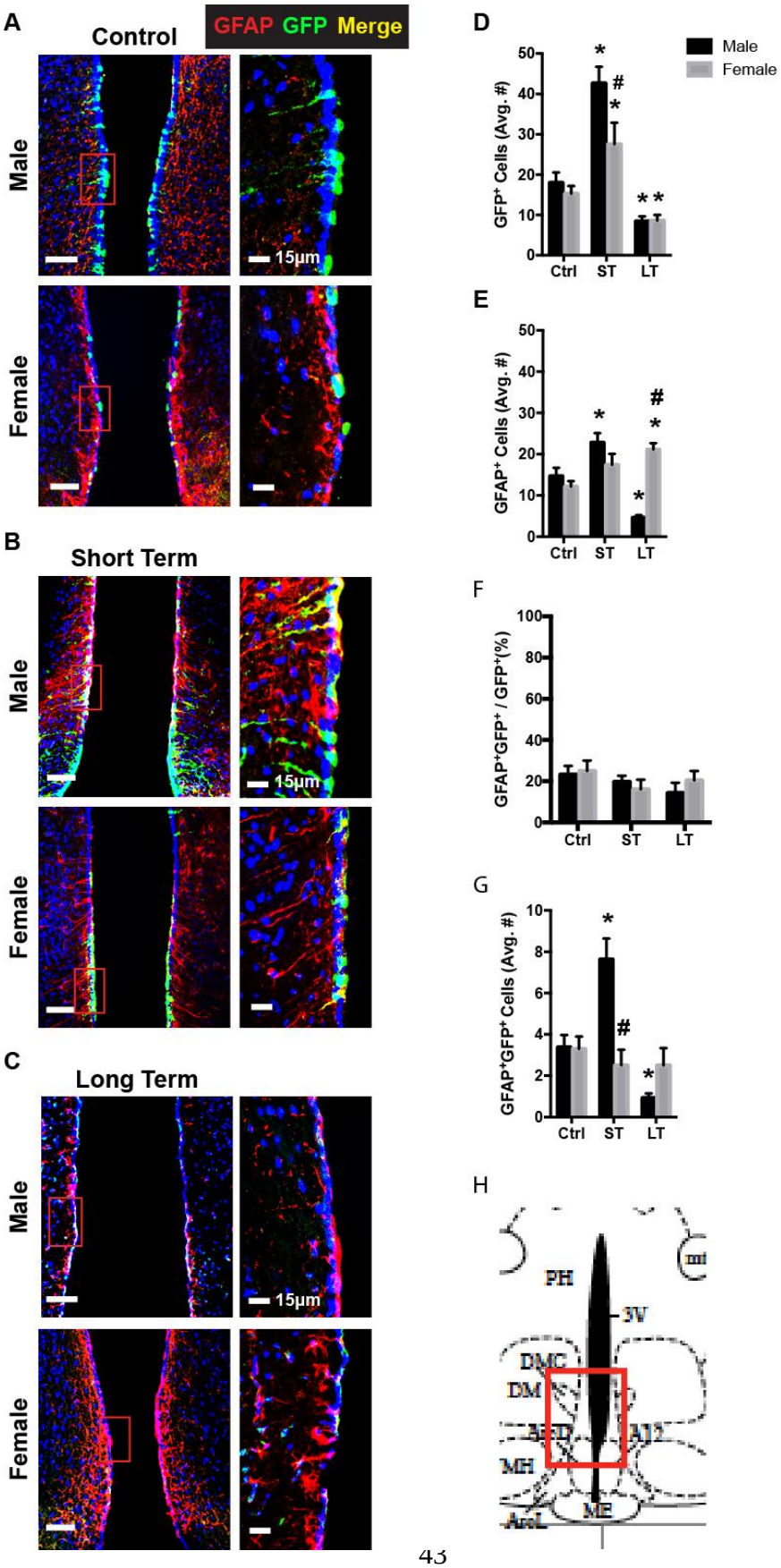

A
Control
GFAP GFP Merge
Male
Female
15μm
B
Short Term
C
Long Term
D
Male
Female
GFP⁺ Cells (Avg. #)
Ctrl
ST
LT
E
GFAP⁺ Cells (Avg. #)
F
GFAP⁺GFP⁺ / GFP⁺(%)
G
GFAP⁺GFP⁺ Cells (Avg. #)
H
PH
3V
DMC
DM
A11
A12
MH
ME
ArcL

**Figure 13:** Astrogliogenesis in the TL following ethanol consumption. (**A-C**) Representative images of control, short and long term male and female mice, respectively, brain stained with stem cell marker (GFP green), neuronal marker (GFAP red), and merged with nuclear maker Dapi. Region in red box is shown enlarged on right. (**D-G**) Quantification in the TL of average $GFP^+$, $GFAP^+$, $GFAP^+GFP^+$ cells, and percent of $GFAP^+GFP^+$ over total $GFP^+$ cells. (**H**) Schematic representative image of region quantified (Bregma -1.58 through -2.16). Scale bars, 45μm and 15μm in enlarged images. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, n=3 mice per sex per group. See also Figure S6-7.

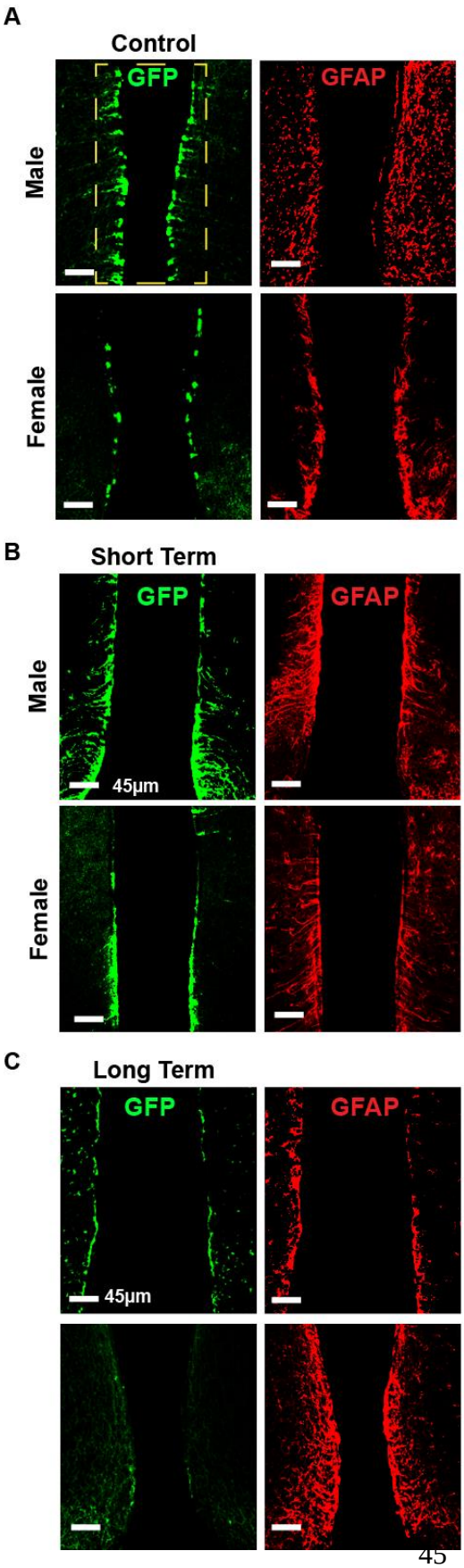

A
Control
GFP
GFAP
Male
Female
B
Short Term
GFP
GFAP
45µm
Male
Female
C
Long Term
GFP
GFAP
45µm

**Figure 14:** Individual channels of TL immunohistochemical stains. (A) Single channel of representative images of control male and female mice stained with stem cell marker (GFP green), and astrocyte marker (GFAP). (B) Single channel of representative images of short-term treated male and female mice stained with stem cell marker (GFP green), and astrocyte marker (GFAP). (C) Single channel of representative images of long-term treated male and female mice stained with stem cell marker (GFP green), and astrocyte marker (GFAP). Scale bars, 45μm.

In summary, these data show different behavior of NSCs in the TL compared to both SVZ and SGZ. Short-term alcohol promoted TL NSC proliferation in males and females, but only increased TL astrogliogenesis in males. Long-term alcohol decreased NSCs in males and females, and increased total GFAP+ cells without changes of astrogliogenesis in females.

### DISCUSSION OF ETHANOL CONSUMPTION RESULTS

Our study is the first 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. Also, to the best of our knowledge, this is the first study to evaluate adult astrogliogenesis in the TL in response to alcohol consumption.

In terms of astrocytes, we observed regional, sex, and durational differences in the astrocyte responses following alcohol consumption. The net changes of GFAP+ cells were resulted from a combination of NSC astrogliogenesis and astrocyte reactivation, and varied among different brain regions. Previous studies have evaluated regional and durational differences in GFAP expression (Franke, 1995; Franke, 1997; Dalcik, 2009), however our study is the first to examine both these variables together with the sex differences in adult astrocyte response to alcohol consumption. Furthermore, using the genetic induced fate mapping technology, we were able to distinguish the newly NSC-

differentiated astrocytes from the reactivated astrocytes. Some studies have reported increases in GFAP immune-labeled cells in the hippocampus following alcohol consumption (Dalcik, 2009; Kane, 2014). In contrast, we found no alteration of GFAP in males but decreased GFAP in female in the hippocampal dentate gyrus. Using a similar dosing paradigm and in agreement with us, Franke et al. also observed similar GFAP responses (Franke, 1995; Franke, 1997). These suggest that both ethanol dosing regimen and sex play key roles in astrocyte responses.

Use of the transgenic fate tracing mice 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, 2011; Kempermann, 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, 2003; He, 2005, Crews, 2006; Hansson, 2010; Golub, 2015). We also showed a greater impact of ethanol on SVZ than SGZ, which has previously been done in a vapor ethanol administration model (Hansson, 2010).

However, one distinguishing aspect of our study is that by using the genetic inducible fate tracing mouse model, we were able 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 the 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 NSCs lining the SVZ of the rostral lateral ventricles were most susceptible to the effects of ethanol, both short- and long-term. The significant reduction in the number of GFP+ NSCs, and particularly the absence of GFP labeling in regions lining the lateral ventricle, 14 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, 2015; Hansson, 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. One reason may be that the insult of ethanol and metabolites on the SVZ initiates an injury response leading to local astrocyte reactivation (Anderson, 2014; Brahmachari, 2006). The mechanism and extent to which astrocyte reactivation contributes to changes in SVZ neurogenesis presents a unique opportunity for future studies.

An interesting phenomenon that occurred in the male SGZ of short-term treated mice was the significant increase in NSCs. This indicates a transient increase of NSC

proliferation. While the finding seems contradictory to some previously published literature (Nixon and Crews, 2002; Crews, 2006; Morris, 2010; Campbell, 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 and colleagues who also observed an increase in NSC proliferation in the dentate gyrus following 14 days of ethanol liquid diet consumption (Pawlak, 2002). Additionally, moderate ethanol consumption has also been shown to increase adult NSC proliferation in the dentate gyrus (Aberg, 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, 2010; Petrenko, 2015). On the other hands, in agreement with the literature on chronic alcohol consumption (Golub, 2015), we found the long-term cohort (males and females) had a reduction in NSCs in addition to a significant reduction in neurogenesis.

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, 2013; Haan, 2013; Rodriguez, 2005). Tanycytes are the primary cells found lining the tanycyte layer 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, 2013; Rodriguez, 2005). Robins and colleagues 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 (Robins, 2013). 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, 2012; Haan, 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, 2012; Haan, 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 weeks old mice in our study vs. 8-12 weeks old in others (Rojczyk-Golebiewska, 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. Even more interesting was the fact that following short-term alcohol consumption, both males and females had an increase in GFP+ NSCs. This is opposite to what was observed in the SVZ, and was more similar to the behavior of the NSCs in the SGZ. Since NSCs in the TL 16 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 detected in males in the short-term ethanol group, but in females in the long-term group. 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, 2005). Since the morphology of the cells in our study look similar to tanycytes, and

GFAP and Nestin is expressed in tanycytes, further studies are needed to fully validate the identity of this cell population.

In addition to the differentiation phase, duration of ethanol consumption, regional location and sex all played a pivotal role in determination of NSC response to ethanol consumption. Behaviorally, females showed more severe symptoms of intoxication over a shorter period of time compared to 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 more and in less time compared to males. However, similar responses to ethanol were seen in the SGZ in both males and females. In the TL, short-term ethanol consumption elicited greater NSC proliferation in males compared to females, whereas long-term consumption initiated a greater increase in GFAP+ cells in females compared to 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 central nervous system more quickly than males. Additionally, it would be of great value to further interrogate the role of the endocrine system in NSC response to ethanol; especially given the new 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.

# CHAPTER 3: SPATIAL AND SEX DEPENDENT EFFECTS OF CHRONIC COCAINE AND ALCOHOL CO-ADMINSTRATION ON ENDOGENOUS ADULT NEURAL STEM CELLS

Typically, substances such as ethanol and cocaine are studied individually. While this approach has yielded significant information and contributed to our understanding of the relationship between drugs and neurogenesis, there is an urgent need to study poly-drug exposure. Alcohol and cocaine are two commonly co-abused substances. In a 1990 survey, 96% of cocaine users reported alcohol use within the same month (concurrently), and 85% reported using the two drugs simultaneously (Grant and Harford, 1990). In 1994 the Drug Abuse Warning Network (DAWN) declared cocaine and alcohol the third most common fatal substance combination (DAWN, 1994). Since then, numerous studies have shown that co-abuse of alcohol and cocaine is more fatal than either drug alone(Busse and Riley, 2003) (Church, 1988; Cittadini, 2015; Farooq, 2009; Lacoste, 2010; Patel, 2009; Pennings, 2002; Randall, 1992).

What is it that makes this combination so deadly? One of the major factors is that when taken together, cocaine and alcohol form a unique metabolite in the body called cocaethylene (CE) (Brzezinski, 1994; Chen and West, 1998; Farre, 1993; York and MacKinnon, 1999). In the first step of cocaine metabolism, carboxylesterase-1 breaks typically breaks cocaine into benzoylecgnine, which is pharmacologically inactive and excreted in the urine. In the presence of alcohol, CE is formed by the addition of an ethyl group onto the benzoylecgnine molecule. Unlike benzoylecgnine, CE is pharmacologically active and has a strong affinity for the dopamine transporter (Hearn, 1991).

CE is also far more cardio-toxic than cocaine, and has been shown to have more severe neurological effects as well (Sachpekidis and Vogiatzis, 2007; Jatlow, 1996;

McCance-Katz, 1998). Co-abuse of alcohol and cocaine has been shown to produce psychotic episodes, increase suicidal and homicidal ideation, and enhance symptoms of some psychiatric disorders such as depression (Garlow, 2007; Goldstein, 2004) (Salloum, 1996; Velasquez, 2007; Salloum, 2004).

Despite the strong negative consequences, cocaine and alcohol are still widely co-abused. Studies in rats show alcohol and cocaine co-administration creates prolonged stimulatory effects (Masur, 1989). In clinical studies, individuals report a sustained “high” when using cocaine and alcohol simultaneously. Most commonly, individuals report that using alcohol ameliorates the negative side effects of “coming down” after using cocaine (Magura and Rosenblum, 2000; Graziani, 2014). Interestingly, females reported stronger euphoric and well-being feelings compared to males when using alcohol and cocaine simultaneously (Graziani, 2014).

Individuals who co-abuse cocaine and alcohol show poorer prognosis after treatment and have a higher risk for relapse (Lyne, 2010; Mengis, 2002; Schmitz, 1997). Cocaine taking has been shown to enhance alcohol-seeking and relapse in animal models (Hauser, 2014). In clinical studies cocaine increases alcohol preference, although drinking alcohol typically reduced the amount of cocaine individuals took at one time. (Brady, 1995; Hedaya and Pan, 1996; Hedaya and Pan, 1997; Pan and Hedaya, 1999a; Pan and Hedaya, 1999b; Uemura, 1998; Vanek, 1996). In addition to drug seeking and administration behavior, concurrent alcohol and cocaine use impacts various learning such as place preference and aversive taste learning (Busse and Riley, 2002;  Etkind, 1998). When taken together, cocaine and alcohol also decrease neurocognitive function more than either drug separately (Bolla, 2000; Higgins, 1992; Horowitz and Torres, 1999).

Aside from clinical studies evaluating cognition and self-reported experiences, very little work has been done evaluating the chronic effect of alcohol and cocaine co-abuse in animal models. To the best of our knowledge, there are currently no animal studies using a chronic dosing treatment of both cocaine and ethanol. This leaves a very large gap in knowledge as to the mechanistic and neuropathological changes that occur in the brain following chronic co-abuse of cocaine and alcohol.

It is known that both alcohol and cocaine individually result in neurodegeneration and changes to the endogenous NSC population. These changes are associated with cognitive and behavioral deficits. The effect of alcohol on endogenous NSCs has been covered in Chapter 2 of this dissertation. However, to date, there is nothing known about the combined use of alcohol and cocaine on NSCs. Considering that the majority of cocaine abusers also use alcohol, it is clinically relevant to evaluate the impact of simultaneous drug use on the NSC populations in order to advance the translational opportunities to treat substance use disorders. Understanding the impact of alcohol and cocaine co-abuse on NSC survival, proliferation, and differentiation is the first and most fundamental step in developing effective therapeutics to treat neurodegeneration incurred by chronic poly-drug abuse.

To address these gaps in knowledge, we utilized the transgenic mouse model described in Chapter 2 to trace the fate of endogenous NSC populations in the adult brain in response to chronic alcohol, cocaine, or combination treatment. We conducted behavioral analysis to evaluate cognitive and behavioral outcomes in addition to analyzing the SVZ, SGZ, and TL for markers of neurogenesis and astrogliogenesis. Additionally, we conducted western blot analysis to interrogate the presence of drug metabolizing enzymes in the brain.

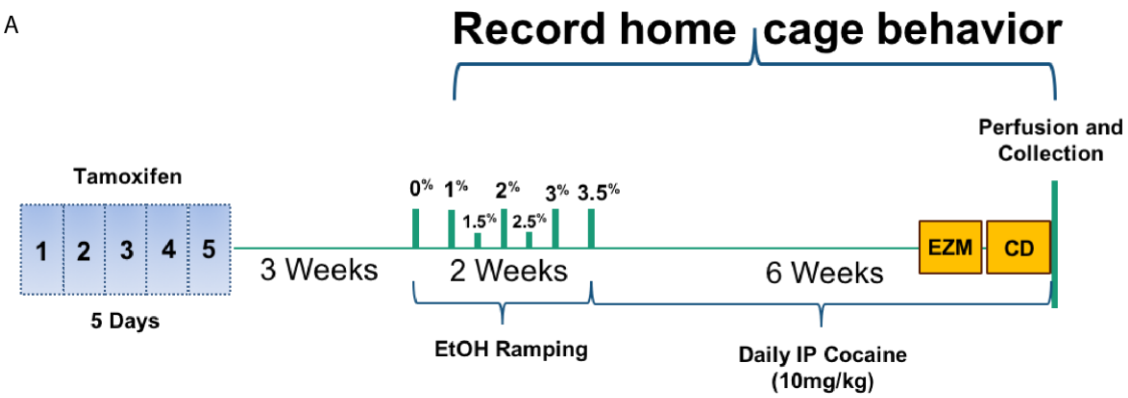
## RESULTS

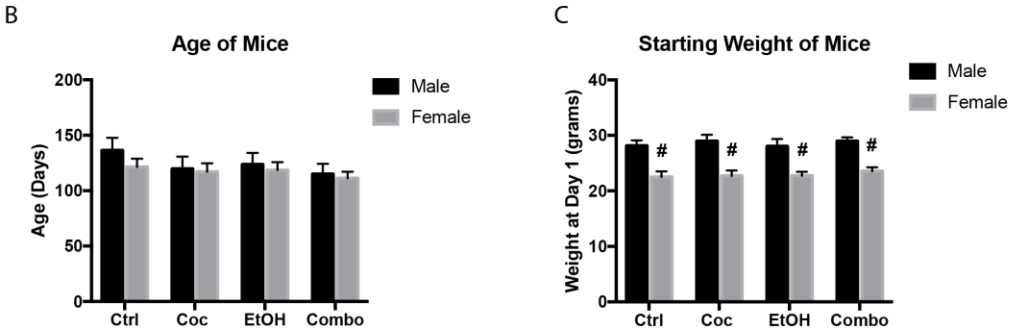
### Differences in tolerance to alcohol consumption

Adult *Nestin*-CreER$^{T2}$:R26R-YFP bi-transgenic mice were used to trace the fate of endogenous NSCs following tamoxifen induction in response to chronic ethanol feeding and/or cocaine administration (Figure 1A). As described in Chapter 2, pair fed control and ethanol-fed mice were given *ad libitum* access to their respective diets and water. The amount of ethanol in the liquid diet of ethanol and combination mice was slowly increased from 1% to 3.5% over the course of two weeks. Mice in the ethanol and combination group were then maintained on 3.5% ethanol in the liquid diet for six weeks. On the first day of 3.5% ethanol in diet, the combination and cocaine mice received daily intraperitoneal injections (i.p.) of cocaine every day for six weeks. Mice in the ethanol only group received daily injections of saline starting on the first day of 3.5% ethanol and continued for 6 weeks. All behavioral experiments were conduced on in the fifth week of treatment.

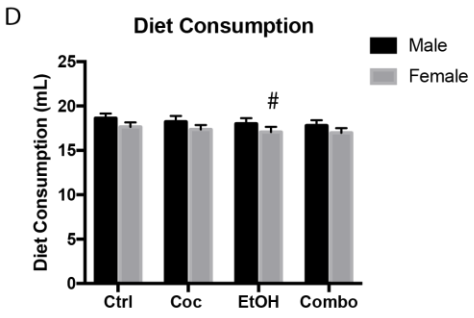
Caloric value of ethanol and control diets was balanced using maltose-dextrin. There were no significant differences in the age of mice used in this study (Figure 1B). Male mice weighed more than females however, there were no significant differences between body weights of control or treatment groups (Figure 1C). Male mice tended to have higher average daily diet consumption compared to their female counterparts, and in the ethanol group, males consumed significantly more than females (Figure 1D). Due to the greater consumption, male mice consumed more grams of ethanol compared to females in the ethanol group; however, females in both the ethanol and combination treatment groups consumed significantly more grams of ethanol per kilogram of body weight (Figure 1E-F). Interestingly mice in the control and cocaine groups did not experience a significant change in gain of bodyweight. However, in the ethanol and

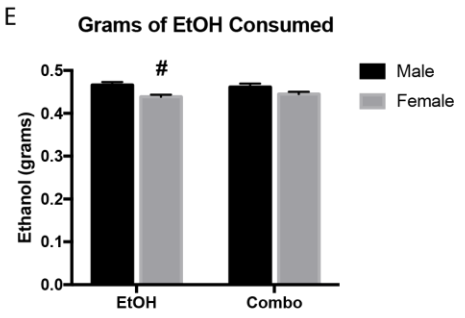
combination groups both males and females had a significant reduction in gain of bodyweight (Figure 1G). In other words, throughout the course of the study, mice in the ethanol and combination groups gained less weight than control and cocaine.

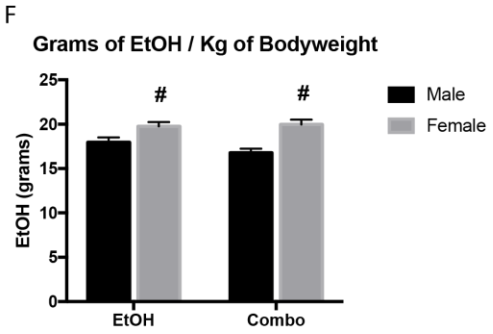

A
Record home cage behavior
Perfusion and Collection
Tamoxifen
1 2 3 4 5
5 Days
3 Weeks
0% 1% 1.5% 2% 2.5% 3% 3.5%
2 Weeks
6 Weeks
EZM
CD
EtOH Ramping
Daily IP Cocaine (10mg/kg)


B
Age of Mice
Age (Days)
Male
Female
Ctrl Coc EtOH Combo


C
Starting Weight of Mice
Weight at Day 1 (grams)
Male
Female
Ctrl Coc EtOH Combo


D
Diet Consumption
Diet Consumption (mL)
Male
Female
Ctrl Coc EtOH Combo


E
Grams of EtOH Consumed
Ethanol (grams)
Male
Female
EtOH Combo


F
Grams of EtOH / Kg of Bodyweight
EtOH (grams)
Male
Female
EtOH Combo

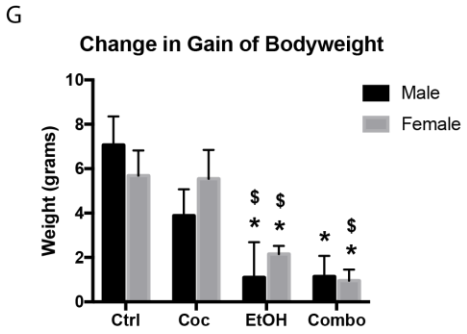

G
Change in Gain of Bodyweight
Weight (grams)
Male
Female
Ctrl Coc EtOH Combo

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. Values are shown as mean±SEM, *$p<0.05$ compared to control, #$p>0.05$ compared to other sex in same group, control male n=7, control female n=13, ethanol male n=10, ethanol female n=13, cocaine male n=10, cocaine female n=14, combination male n=10, combination female n=15.

**Behavioral assessment of combination drug use.**

To evaluate the behavioral effects of chronic cocaine and alcohol administration we ran a panel of behavioral tests to assess, anxiety-like behavior, contextual memory, and feeding preferences. Due to the number of mice available for behavior, sexes were analyzed together. On the first day of Week 5, mice underwent an elevated zero maze (EZM) test to assess anxiety like behaviors. Reductions in olfactory bulb neurogenesis have been linked with decreased exploratory behavior. In an attempt to correlate behavioral outcomes with immunohistochemical analysis mice was assessed to see if drug treatment reduced time in the open sections of an elevated zero maze. Mice were allowed to acclimate to the testing room an hour before testing began. EZM testing took place between 7am and 12pm. Mice were randomly chosen for the maze and the maze was cleaned with 70% isopropyl alcohol between each subject. Mice in all groups spent the fast majority of their time in the closed arms. In the cocaine and combination group, most data had to be excluded due to mice jumping or falling off of the maze. There were no significant differences among the groups in time spent in either the closed or open arms (Figure 2).

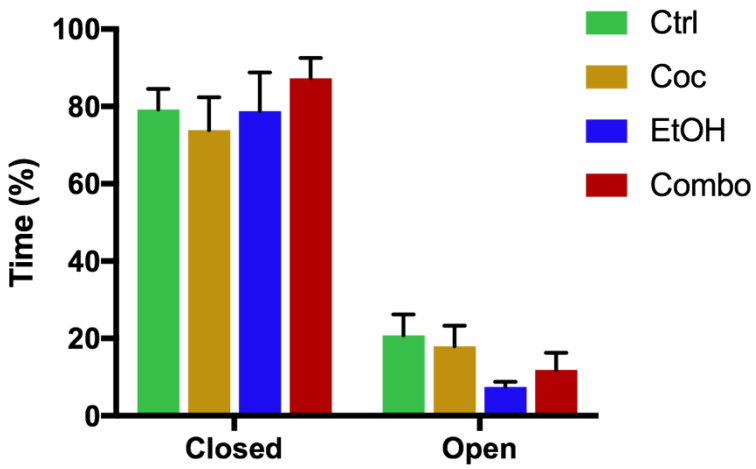



Figure 2: Elevated zero maze. The above chart shows percent time in closed and open arms of maze. No significant differences were observed among groups. All animals spent significantly more time in the closed arms of the maze. Values shown as average ± SEM. Control n=20, Ethanol n=21, Cocaine n=23, Combination n=22.

Next we conducted context discrimination in a fear conditioning paradigm. To assess whether changes in context discrimination would correlate with immunohistochemical analysis as far as reduction in neurogenesis, mice were tested using fear conditioning context discrimination. This method of context discrimination is a hippocampal-dependent memory test and requires that neurogenesis be undisrupted (Kemperman, 2015a). Significant discrimination was not seen in the control group until day 4 (Figure 3). None of the drug treatment groups were able to significantly discriminate between the contexts during the duration of this test; however, the ethanol mice had a trend of discrimination on Day 4 (p=0.08) (Figure 3C)**.** Although none of the treatment groups were able to discriminate, the cocaine and combination groups had a greater range of discrimination ratios individual mice (even at day 4). This suggests that mice in the cocaine and combination groups have a greater difficulty discriminating compared to ethanol mice. While only mice in the control group had a significant increase in freezing time in the shock context on Day 4, ethanol mice had a trend of

increased freezing and followed the same linear pattern as control mice (Figure 3E). Cocaine and combination mice, on the other hand, had an increase in percent freezing in the shock context on Days 1 and 2 but this effect plateaued for days 3 and 4 (Figure 3E). A schematic diagram of the contexts used can be seen in Figure 3F.

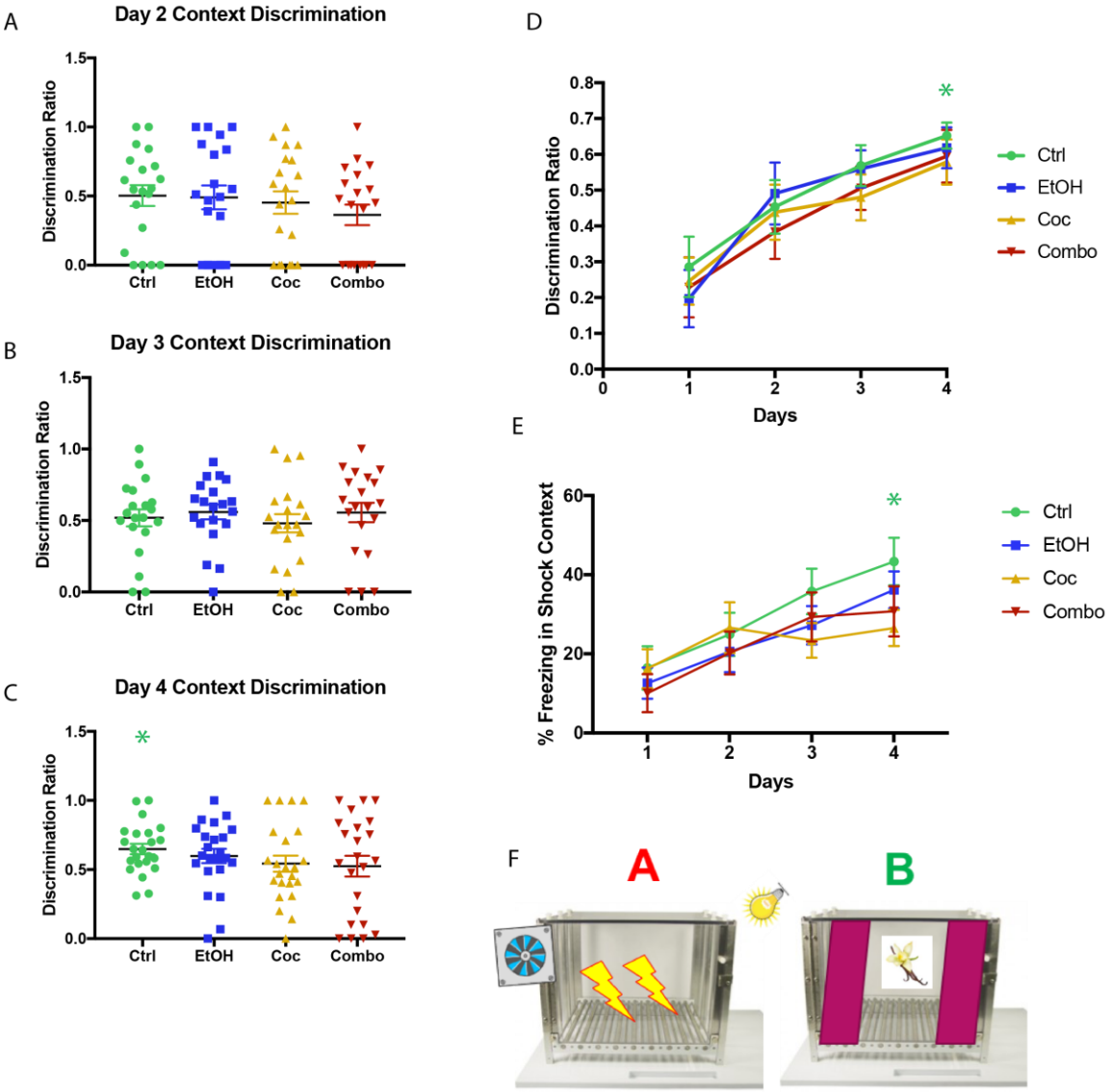


Figure 3: Context discrimination. (A-C) Discrimination ratio plots of individual mouse values on Days 2-4 of context discrimination testing. Black bar represents mean of each group. (D) Discrimination ratio plotting average of each group over the course of testing. (E) Graph showing the mean percent freezing in the shock context over the course of testing. (F) Illustration of two contexts. 1-way repeated measures ANOVA was used to evaluate significance. Mean values are shown ±SEM, *p<0.05, Control n=20, Ethanol n=21, Cocaine n=23, Combination n=22.

The final behavioral test was a modified form of the sucrose preference task. Benford and colleagues recently showed that tanyctes in the TL (including the ones with neural stem cell potential) have sweet receptors similar to those on the tongue (Benford, 2017). These sweet receptors sense glucose and other sweeteners, and relay signals to the hypothalamus. in response to other artificial sweeteners (Benford, 2017). This paper suggests that alterations in the tanycyte population may affected taste preferences based on communication between the tanyctes and hypothalamus. In order assess if changes observed in cell populations in the TL correlated with behavioral changes, a sucrose preference test was employed. In order to assess if drug treatment affected taste preference for sucrose compared to maltose-dextrin, sucrose was slowly increased in the liquid diets over the course of 8 days. Sucrose was increased incrementally every 2 days in increments of 0.05%, 1%, 10%, and 20%. The equivalent amount of maltose-dextrin was removed from the liquid diet corresponding to the amount of sucrose added. Mice were given two bottles, one containing only maltose-dextrin, the other containing the substituted percentage of sucrose. Diet consumption was measured daily and used to calculate the ratio of sucrose:maltose-dextrin consumption.

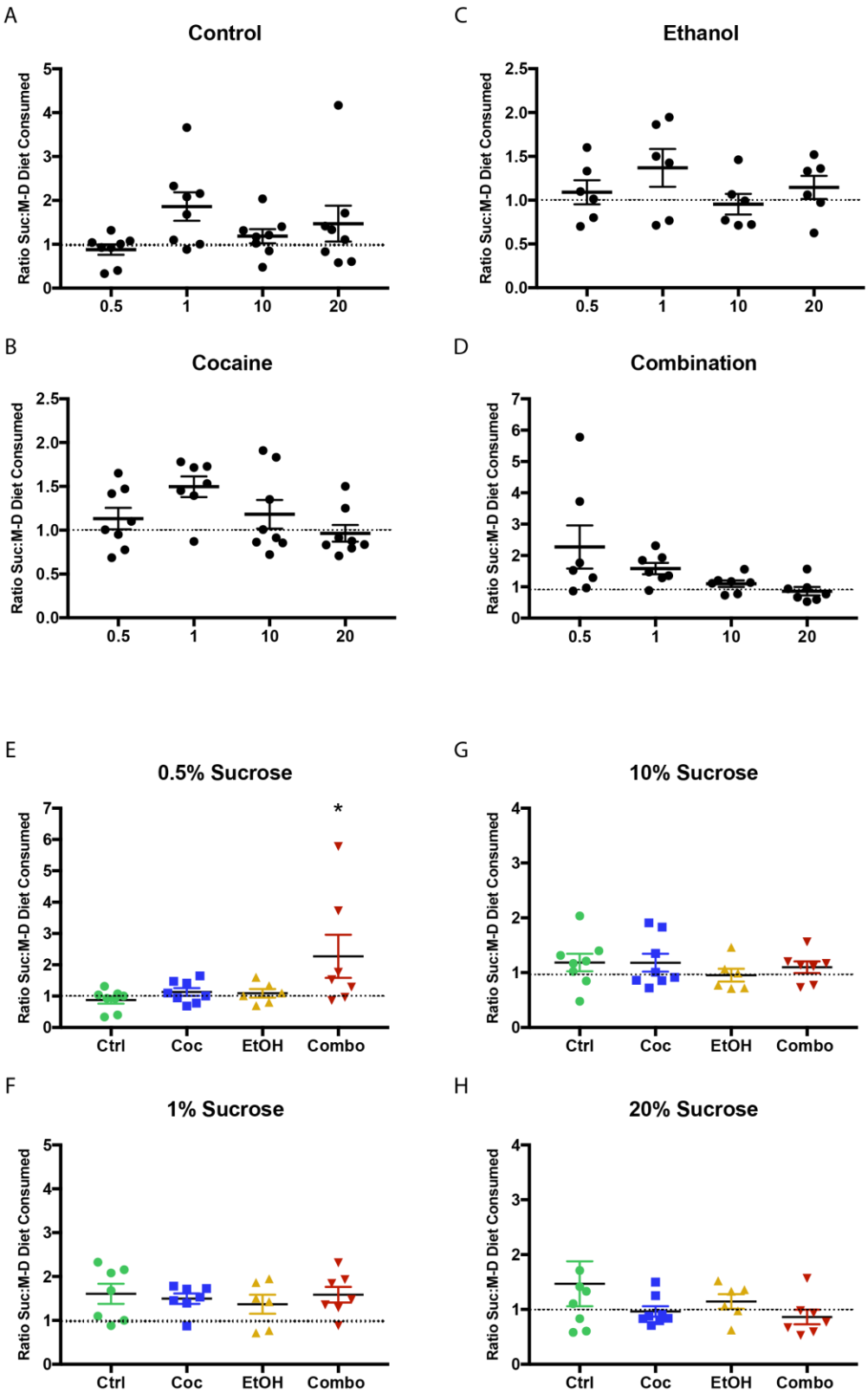

A
Control
Ratio Suc:M-D Diet Consumed
0.5
1
10
20
C
Ethanol
B
Cocaine
D
Combination
E
0.5% Sucrose
*
Ctrl
Coc
EtOH
Combo
G
10% Sucrose
F
1% Sucrose
H
20% Sucrose

Figure 4: Sucrose Preference. (A-D) Graphs showing individual values and mean for sucrose preference in control, cocaine, ethanol, and combination group, respectively. (E-H) Graphs showing individual values, and mean, for all treatment groups in response to 0.5%, 1%, 10%, and 20% sucrose, respectively. Individual values and means are shown with ±SEM. Data analyzed using 1-way repeated measures ANOVA *p<0.05, control n=8, ethanol n=6, cocaine n=8, combination n=7.

Control, ethanol and cocaine groups did not show any preference for sucrose diet at 0.05% sucrose (Figure 4A-C, 4E). Interestingly, the combination group consumed significantly more sucrose diet compared to the maltose dextrin diet at 0.05% sucrose (Figure 4D, 4E). This suggests that combination mice were able to detect a difference in sugar substitution at a lower percent than other treatments, and preferred it. At 1% sucrose, control mice showed significant preference for the sucrose diet, whereas ethanol, cocaine, and combination mice had an increase in sucrose diet consumption, but this was not significant (Figure 4A-D, 4F). With 10% sucrose in diet, control and cocaine mice consumed slightly more sucrose diet on average but this was not significant (Figure 4A-B, 4G). Ethanol and combination mice, on the other hand consumed almost equal amounts of sucrose and maltose-dextrin diet on average (Figure 4C-D, 4G). Finally, with 20% sucrose in diet, females consumed significantly more than maltose-dextrin diet, while cocaine, ethanol and combination groups did not have a significant preference (Figure 4A-D, 4H). Interestingly, the combination group started with significant preference for sucrose diet, but this preference seemed to taper as the percent of sucrose increased. One possible explanation for the lack of significance in this behavioral paradigm is that very few mice were used in each group. Control and Cocaine groups had an n=8, while ethanol and combination groups had an n=6 and n=7 respectively.

Altogether these data suggest that combination treatment enhances sensitivity to sucrose when substituted for maltose dextrin at low percentages.

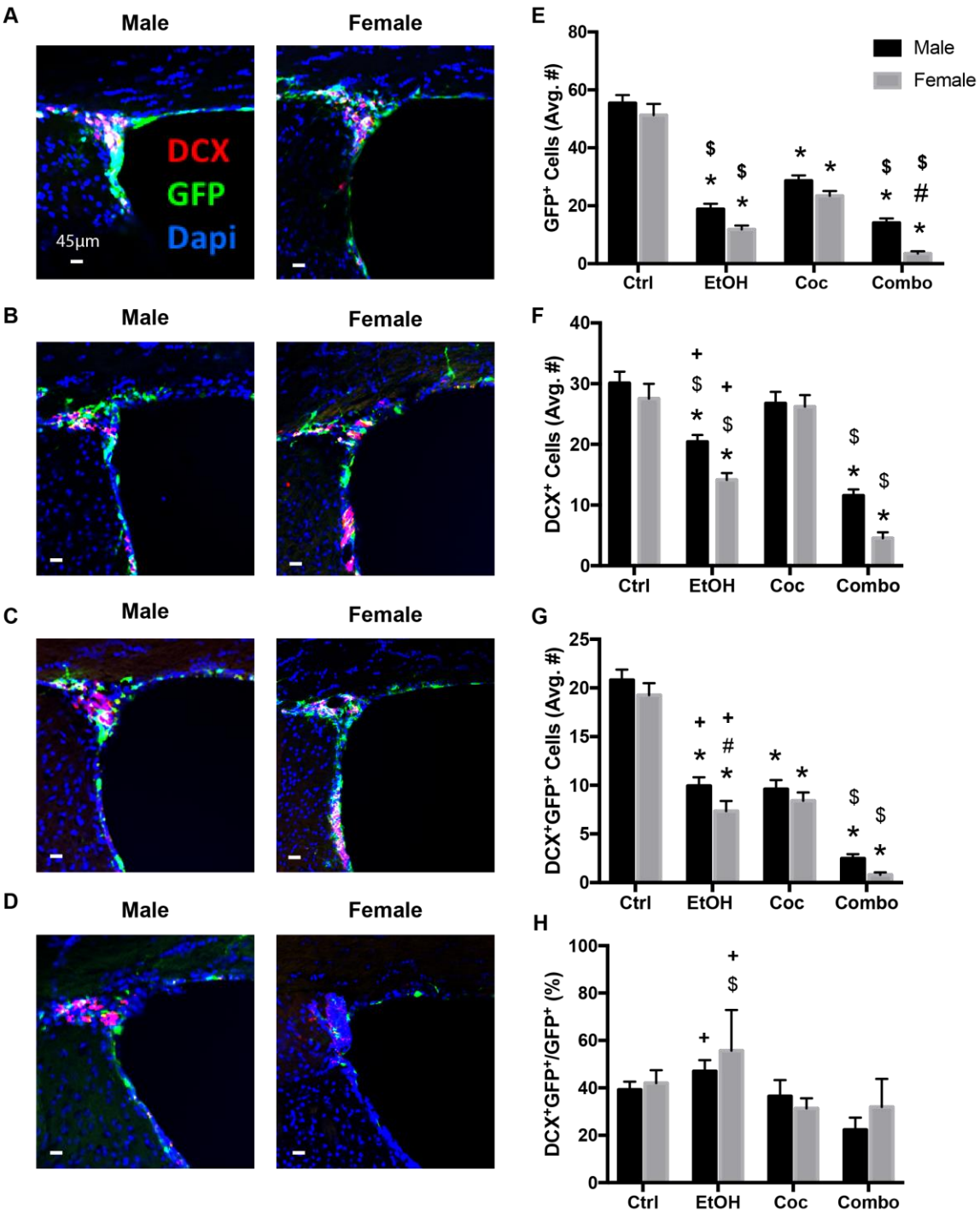
**Effect of drug treatment on SVZ NSCs in the lateral ventricle**

We still focused on the rostral lateral ventricle for the reasons discussed in Chapter 2 (Lim and Alvarez-Buylla, 2014). The Nestin-CreERT2;R26R-YFP transgenic mice were also used to to trace the NSCs that were present at the time of tamoxifen injection by their expression of yellow fluorescent protein (YFP) (Lagace, 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 green fluorescent protein (GFP) antibody. We examined three phases of NSCs, including 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 differentiated prior to tamoxifen induction and no longer expressed Nestin would not be co-labeled with GFP.

We found no significant differences in GFP+ between control male and female mice (Figure 5A, 5E). All drug treatments resulted in a significant reduction of GFP+ cells (Figure 5). Similar to the first study using 4% ethanol, both male and female mice in the ethanol group had a significant reduction in GFP+ cells (Figure 5B, 5E). This difference was not as dramatic as the previous study, however, this could because the lower percent of ethanol was better tolerated. Males in the ethanol group exhibited approximately a 66% decrease in GFP+ cells, while females had an 82% decrease (Figure 5B, 5E). While males had slightly more GFP+ cells following ethanol treatment, they were not significantly different from their female counterparts. Cocaine treated mice

had a decrease of approximately 48% and 50% in males and females, respectively. Again, no sex differences were seen between male and female cocaine mice, however, both males and females in the cocaine group had significantly more GFP+ cells compared to the ethanol group (Figure 5B, 5C, 5E). Combination drug treatment had the impact on GFP+ cells, with male and female mice exhibiting a 83% and 91% reduction, respectively. Females in the combination group also had fewer GFP+ cells than their male counterparts (Figure 5E). The combination group had significantly less GFP+ positive cells compared to the cocaine group (67% in males and 82% in females), but not the ethanol group (Figure 5B, 5C, 5E).

Next we examined the neurogenic capacity of SVZ NSCs after drug treatment using DCX. Control male and female mice had similar DCX expression (Figure 5A, 5F). Ethanol treatment reduced DCX+ cells by approximately 30% and 48% in males and females, respectively (Figure 5B, 5F). Interestingly, cocaine treatment did not reduce the DCX+ population in the SVZ or oRMS (Figure 5C, 5F). Combination treatment had the greatest impact on DCX+ cells and caused an approximate 60% and 82% reduction in males and females, respectively (Figure 5D, 5F). Mice in both the ethanol and combination groups had significantly few DCX+ cells than the cocaine mice. Additionally, mice in the combination group had a greater reduction in DCX+ cells compared to the ethanol group (40% in males and 67% in females) (Figure 5B-D, 5F).


A
Male
Female
DCX
GFP
Dapi
45µm
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GFP⁺ Cells (Avg. #)
Ctrl
EtOH
Coc
Combo
F
DCX⁺ Cells (Avg. #)
Ctrl
EtOH
Coc
Combo
G
DCX⁺GFP⁺ Cells (Avg. #)
Ctrl
EtOH
Coc
Combo
H
DCX⁺GFP⁺/GFP⁺ (%)
Ctrl
EtOH
Coc
Combo

Figure 5: Neurogenesis in the SVZ. (A-D) Representative brain images of control, ethanol, cocaine, and combination treated mice, respectively, stained with stem cell marker (GFP green), neuronal marker (DCX red), and merged with nuclear maker Dapi (Bregma 0.5 through 1.08). (E-H) Quantification of $GFP^+$, $DCX^+$, $DCX^+GFP^+$, and the percent of $DCX^+GFP^+$ over total $GFP^+$ cells in the SVZ and origin of the rostral migratory stream. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, \$$p<0.05$ compared to cocaine group, +$p<0.05$ compared to combination group, n=3 mice per sex per group. Scale bars, 45μm.

All drug treatments significantly reduced the number of DCX+GFP+ cells (Figure 5G). Ethanol and cocaine treatment reduced DCP+GFP+ cells in males by approximately 48%. In females, ethanol treatment reduced DCX+GFP+ cells by approximately 63%, and cocaine reduced this population by 53% (Figure 5B, 5C, 5G). Females in the ethanol group had a greater reduction of DCX+GFP+ cells compared to ethanol males (Figure 5G). In the combination group, there were significantly less DCX+GFP+ cells compared to control, ethanol, and cocaine groups (5D, 5G). Males in the combination group experienced an 87% decrease, while females had a reduction of approximately 98% (Figure 5G). Combination drug treatment resulted in a 73% greater reduction in DCX+GFP+ cells compared males treated with ethanol. Females in the combination group had 87% less DCX+GFP+ cells compared to their ethanol counterparts. In addition to having the greatest reduction in DCX+GFP+ cells, the combination group also had a significant reduction in the percent of GFP+ cells becoming DCX+ compared to their ethanol counterparts (DCX+GFP+/GFP+) (Figure 5H). Females in the cocaine group also had significantly fewer GFP+ cells becoming DCX+ compared to the ethanol females. However, none of the treatment groups had a significant change in this percentage compared to controls (Figure 5H).

To further verify a reduction in neurogenesis, the olfactory bulb of control and combination mice were stained for GFP and NeuN (Figure 6). There was a significant

decrease in both GFP+ and NeuN+ cells, which was more pronounced in the females. Additionally, there was a dramatic reduction in NeuN+GFP+ cell populations (Figure 6).

In summary, these data show the NSCs in the SVZ are sensitive chronic alcohol and cocaine treatment, but most sensitive to combination drug treatment. Additionally, female NSCs cells are more sensitive to combination drug treatment compared to males. DCX+ cells are not affected by cocaine treatment, and are most sensitive to combination drug treatment. Furthermore, newly differentiated neurons (DCX+GFP+) seem to be the most vulnerable to combination drug treatment, compared to GFP+ and DCX+ populations.

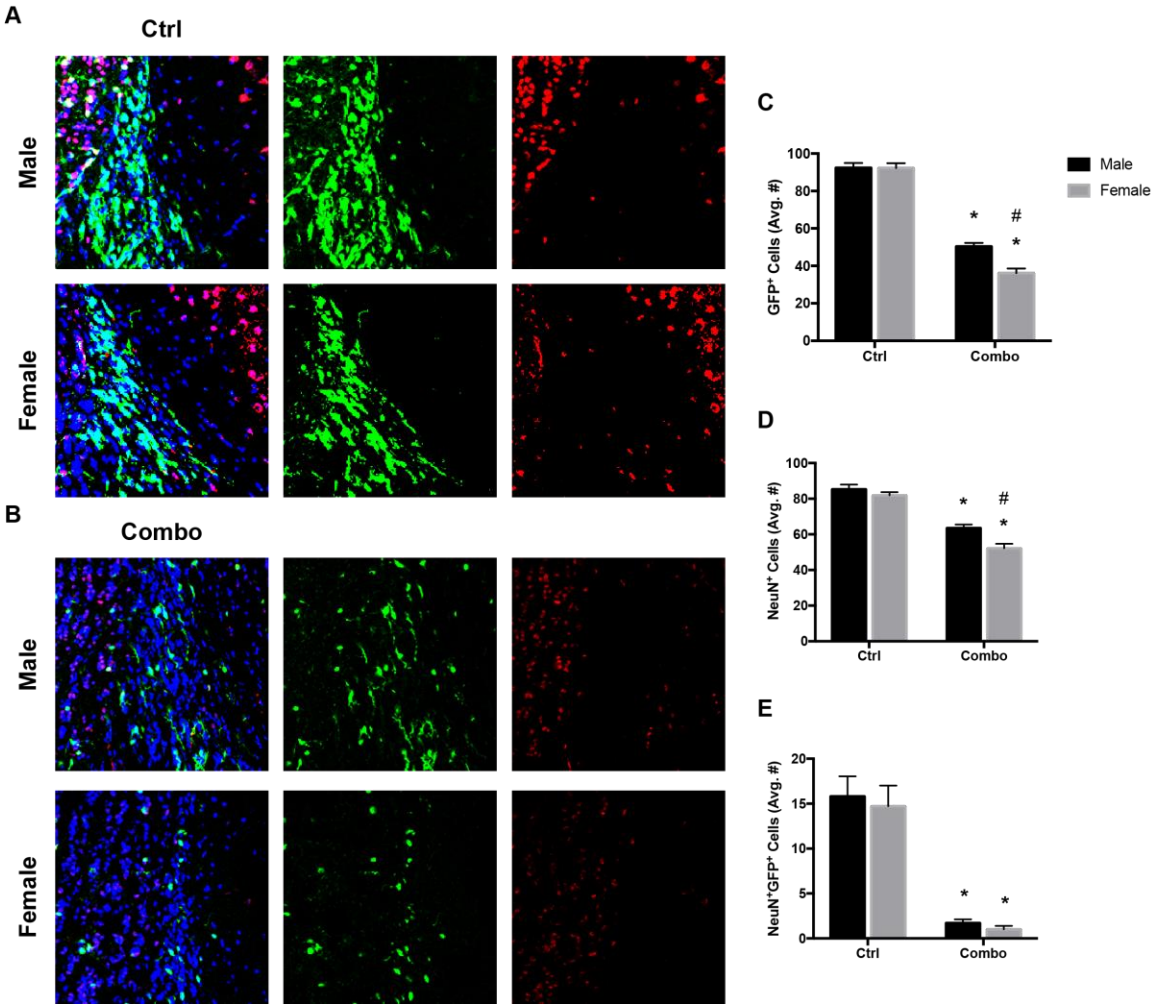
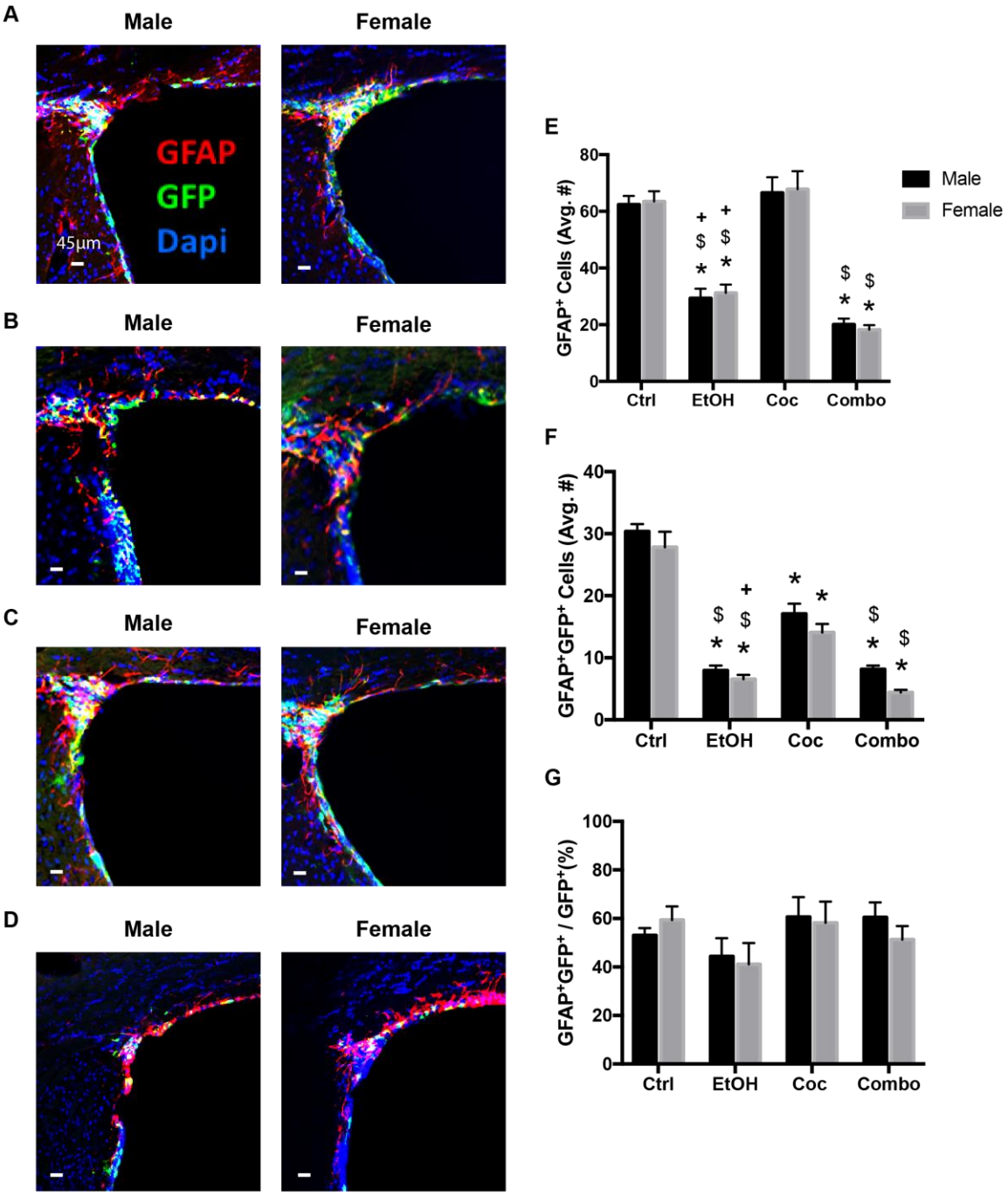


Figure 6: Neurogenesis in the olfactory bulb. (A-B) Representative olfactory bulb images of control and combination treated mice, respectively, stained with stem cell marker (GFP green), mature neuronal marker (NeuN red), and merged with nuclear maker Dapi. (C-E) Quantification of $GFP^+$, $NeuN^+$, and $NeuN^+GFP^+$ cells Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group.

We further assessed the effect of ethanol consumption on NSC astroglial differentiation by using GFAP as a glial marker. In the SVZ region of the control mice, the majority of GFP+ cells were co-labeled with GFAP (Figure 7A). No significant differences were found in the number of GFAP+ or GFAP+GFP+ in control mice (Figure 7A, 7E). Ethanol and combination treatment significantly reduced the number of GFAP+ cells in the SVZ and oRMS. This reduction was greater in the combination group. Males and females in both the ethanol and combination groups responded with similar reductions in GFAP+ cells. Males and females had approximately a 48% and 64% reduction in GFAP+ cells in the ethanol and combination groups, respectively (Figure 5B, 5D, 5E). Cocaine had no effect on GFAP+ cell populations in either males or females (Figure 5C, 5F). Cocaine did reduce the number of GFAP+GFP+ cells by 38% in males and 45% in females (Figure 5C, 5F). Ethanol reduced GFAP+GFP+ cells by approximately 73% in males and 76% in females (Figure 5B, 5F). Combination treatment reduced this population by 70% and 86% in males and females, respectively (Figure 5D, 5F). Combination females also had approximately 45% fewer GFAP+GFP+ cells compared to their ethanol counterparts (Figure 5F). Both ethanol and combination treatments resulted in a greater reduction in GFAP+GFP+ cell compared to cocaine alone (Figure 5B-D, 5F). There were so significant changes among the groups on the percent of GFP+ cells becoming GFAP+ (GFAP+GFP+/GFP+) (Figure 5G).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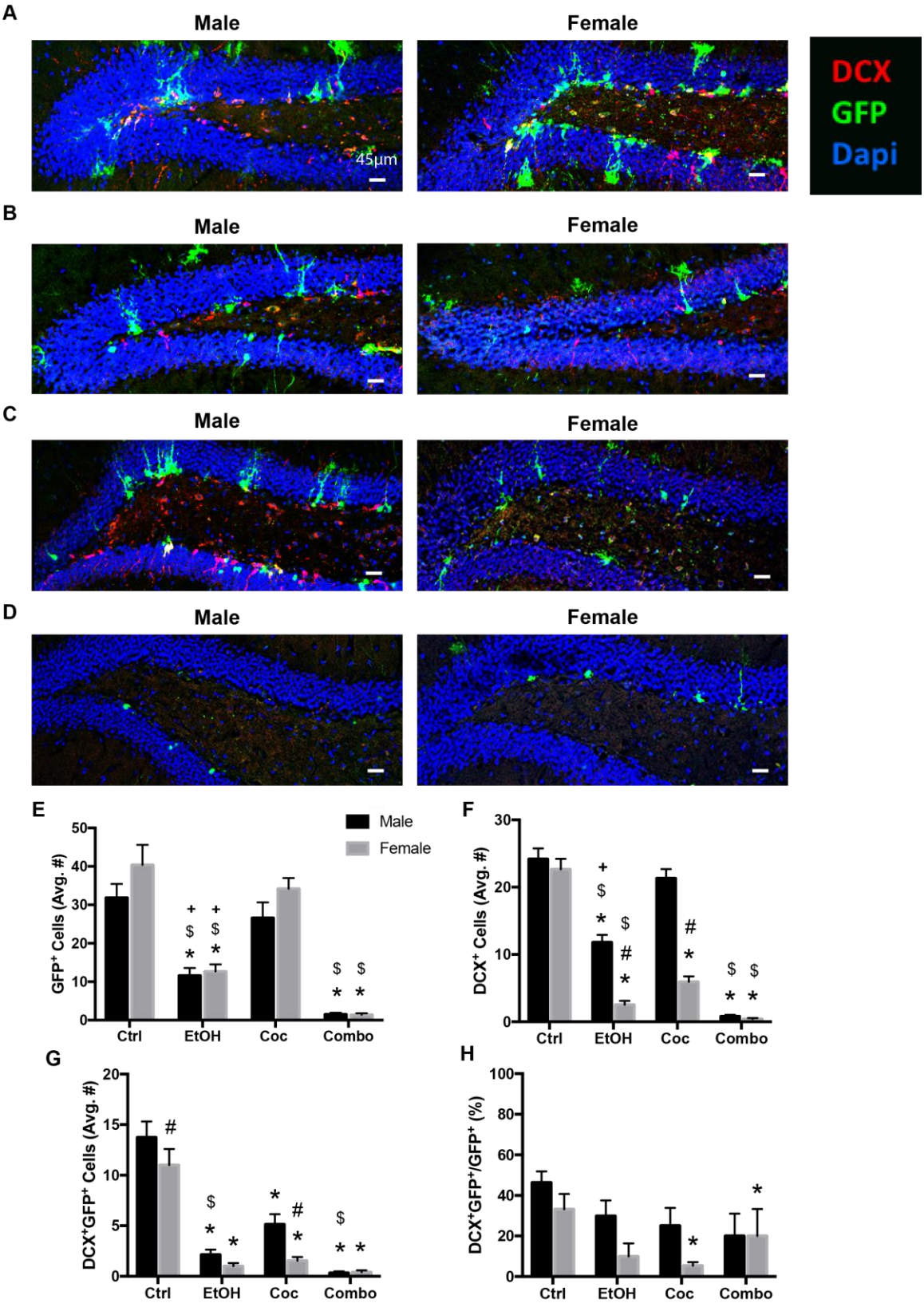
GFAP+ Cells (Avg. #)
Male
Female
Ctrl
EtOH
Coc
Combo
F
GFAP+GFP+ Cells (Avg. #)
Ctrl
EtOH
Coc
Combo
G
GFAP+GFP+ / GFP+(%)
Ctrl
EtOH
Coc
Combo

Figure 7: Astrogliogenesis in the SVZ. (A-D) Representative brain images of control, ethanol, cocaine, and combination treated mice, respectively, stained with stem cell marker (GFP green), astrocyte marker (GFAP red), and merged with nuclear maker Dapi (Bregma 0.5 through 1.08). (E-G) Quantification of $GFAP^+$, $GFAP^+GFP^+$, and the percent of $GFAP^+GFP^+$ over total $GFP^+$ cells in the SVZ and origin of the rostral migratory stream. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, \$$p<0.05$ compared to cocaine group, +$p<0.05$ compared to combination group, n=3 mice per sex per group. Scale bars, 45μm.

**Effect of drug treatment on SGZ NSCs in the hippocampus**

Next, we examined the SGZ in the dentate gyrus of the dorsal hippocampus. Control females tended to have more GFP+ cells compared to their male counterparts (Figure 8A). Ethanol treatment significantly reduced the GFP+ population in both male and female mice by 61% and 68%, respectively (Figure 8B, 8E). Cocaine did not significantly impact the number of GFP+ cells in the SGZ; however, there was an interesting change in the morphology of GFP+ cells (Figure 8C, 8E). GFP+ cells lost their arborization after cocaine treatment in both males and females, though they retained long processing stretching through the granular layer (Figure 8C). Combination treatment resulted in an almost 100% reduction in GFP+ cells in both males and females (Figure 8D, 8E). Males and females in the combination grou0 had a 73% and 80% reduction in GFP+ cells compared to males and females in the ethanol group, respectively (Figure 8B, 8D, 8E).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Ctrl
EtOH
Coc
Combo
F
DCX⁺ Cells (Avg. #)
Ctrl
EtOH
Coc
Combo
G
DCX⁺GFP⁺ Cells (Avg. #)
Ctrl
EtOH
Coc
Combo
H
DCX⁺GFP⁺/GFP⁺ (%)
Ctrl
EtOH
Coc
Combo

Figure 8: Neurogenesis in the SGZ. (A-D) Representative brain images of control, ethanol, cocaine, and combination treated mice, respectively, stained with stem cell marker (GFP green), neuronal marker (DCX red), and merged with nuclear maker Dapi (Bregma 0.5 through 1.08). (E-G) Quantification of $GFP^+$, $DCX^+$, $DCX^+GFP^+$, and the percent of $DCX^+GFP^+$ over total $GFP^+$ cells, in the SGZ. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, \$$p<0.05$ compared to cocaine group, +$p<0.05$ compared to combination group, n=3 mice per sex per group. Scale bars, 45μm.

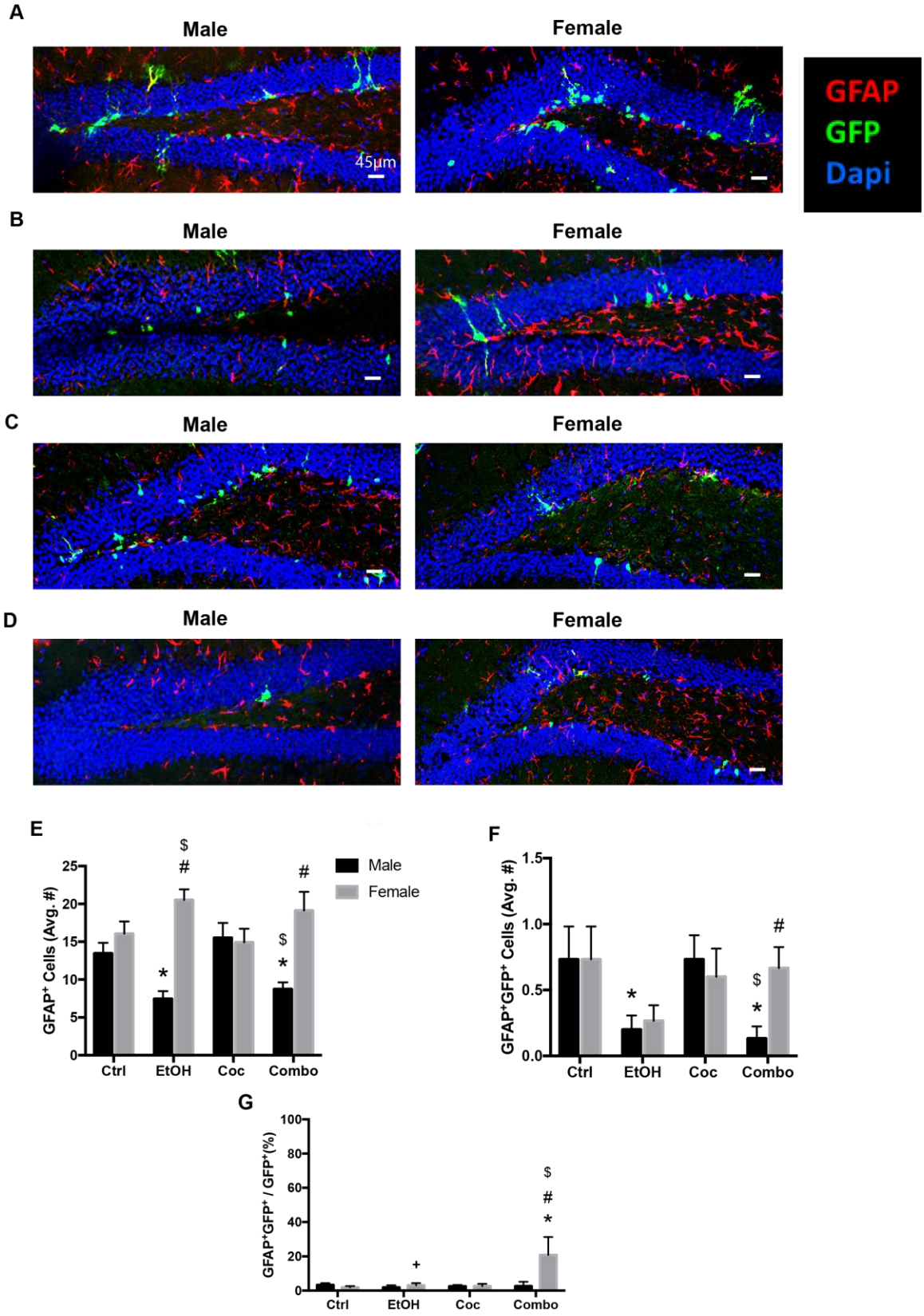
We further evaluated neurogenesis in the SGZ. Ethanol, cocaine and combination treatment significantly reduced DCX+ cells in female mice by 89%, 77%, and almost 100%, respectively (Figure 8B-D, 8F). Female mice in the ethanol and cocaine groups had significantly fewer DCX+ cells compared to their male counterparts. Males in the cocaine group did not have a change in DCX+ cells, whereas males in the ethanol and combination group experienced a 43% and almost 100% decrease, respectively (Figure 8B-D, 8F). Both male and female combination mice had significantly less DCX+ cells compared to the cocaine group, but only combination males had significantly less than ethanol males (Figure 8F).

Although control female mice tended to have more GFP+ cells than males, they had significantly fewer DCX+GFP+ cells. All drug treatments significantly reduced DCX+GFP+ cell populations in both male and female mice. Ethanol reduced DCX+GFP+ cells by 86% in males and 83% in females (Figure 8B, 8G). Cocaine treatment resulted in a 57% and 82% decrease in males and females respectively. Females had a greater reduction in DCX+GFP+ cells following cocaine treatment compared to males Figure 8C, 8G). Combination drug treatment resulted in an almost 100% reduction in males and females respectively (Figure 8D, 8G). Cocaine treatment significantly reduced the percent of GFP+ cells differentiating into DCX+ cells in female

mice (Figure 8H). Additionally, combination treatment significantly reduced this percent in male mice (Figure 8H).

In summary, combination drug treatment almost completely obliterated NSCs, newly differentiated neurons and more mature DCX+ neurons in the SGZ of both males and females. Newly differentiated neurons are most susceptible to drug treatment, and the SGZ neurons of female mice are more sensitive to cocaine treatment compared to males.

We then examined astrogliogenesis in the SGZ. There were no significant differences in GFAP+, GFAP+GFP+, or percent of GFP+ cells becoming GFAP+ cells between control males and females (Figure 9A, 9E-G). Ethanol treatment significantly decreased GFAP+ cells in males (50%) while females tended to have an increase (Figure 9B, 9E). Similarly, combination treatment reduced GFAP+ cells in males (38%), but females had a slight trend of increase (Figure 9D-E). Females in both the ethanol and combination group had significantly more GFAP+ cells compared to their male counterparts, but not compared to controls (Figure 5B, 5D-E). Cocaine did not impact GFAP+, or GFAP+GFP+ expression (Figure 5C, 5E-F). Ethanol treatment significantly reduced GFAP+GFP+ cells in males by approximately 50% (Figure 9B, 9F). Females had a trending ($p=0.07$) reduction of 43% (Figure 9B, 9F). Combination treatment also significantly reduced GFAP+GFP+ cells in males (75%). Interestingly, combination treatment did not have an impact on GFAP+GFP+ cells in females (Figure 9D, 9F). Furthermore, combination treatment increased the percent of GFP+ cells becoming GFAP+ (GFAP+GFP+/GFP+) (Figure 5g). Other treatments did not impact this percentage.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Coc
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GFAP⁺GFP⁺ Cells (Avg. #)
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EtOH
Coc
Combo
G
GFAP⁺GFP⁺ / GFP⁺(%)
Ctrl
EtOH
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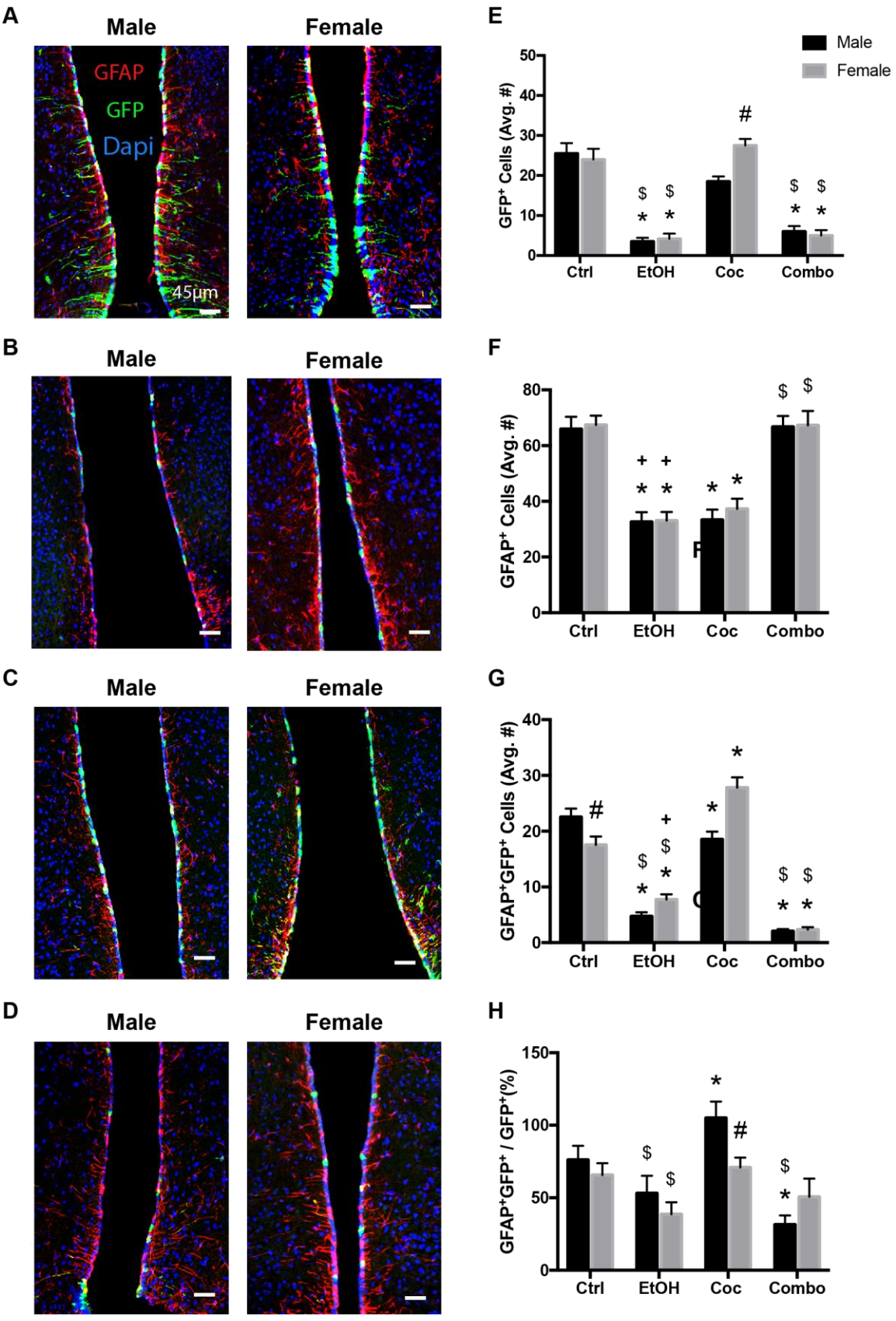
Figure 9: Astrogliogenesis in the SGZ. (A-D) Representative brain images of control, ethanol, cocaine, and combination treated mice, respectively, stained with stem cell marker (GFP green), astrocyte marker (GFAP red), and merged with nuclear maker Dapi (Bregma 0.5 through 1.08). (E-G) Quantification of $GFAP^+$, $GFAP^+GFP^+$, and the percent of $GFAP^+GFP^+$ over total $GFP^+$ cells, in the SGZ. Values are shown as mean ±SEM, *p<0.05 compared to control, #p<0.05 compared to other sex in the same group, $p<0.05 compared to cocaine group, +p<0.05 compared to combination group, n=3 mice per sex per group. Scale bars, 45μm.

In summary males appear more sensitive to ethanol and combination treatment-induced reduction of GFAP+ cells. Additionally, combination treatment may shift NSCs in the female SGZ toward an astroglial lineage.

**Effect of drug treatment on TL NSCs in the third ventricle**

Finally, we evaluated the tanycyte layer (TL) of the third ventricle. As discussed in Chapter 2, we found little evidence of newly differentiating neurons (Chapter 2 Figure 12), therefore our primary focus remained on GFP+ and GFAP+ cell populations. Control mice had similar numbers of GFP+ and GFAP+ cells, although male mice had more GFAP+GFP+ (Figure 10A, 13E-G). Ethanol and combination treatment significantly reduced GFP+ cells in the TL by 88% and 81% in males, and 87% and 83% in females, respectively (Figure 10B, 10D, 10E). Cocaine did not significantly alter GFP+ cell populations compared to control, however females had significantly more GFP+ cells compared to males following cocaine treatment (Figure 10C, 10E). Ethanol and cocaine treatment reduced the number of GFAP+ cells in both males and females. Ethanol reduced male GFAP+ cells by 48% and female by 49% (Figure 10B and 10E). Cocaine reduced GFAP+ cells by 48% and 39% in males and females, respectively (Figure 10C, 10E). Interestingly, combination treatment did not impact GFAP+ cells in the TL (Figure 5D, 5E). This is notably different from the effects of combination treatment in the SVZ.

Ethanol reduced GFAP+GFP+ populations by 77% and 56% in males and females respectively (Figure 10B, 10G). Cocaine, on the other hand, decreased GFAP+GFP+ cells in males (20%), but increased these cells in females (40%) (Figure 10C, 10G). Combination treatment reduced GFAP+GFP+ cells in both males and females by 93% and 89%, respectively (Figure 10D, 10G). Combination treatment resulted in significantly lower GFAP+GFP+ cells compared to the cocaine group. Interestingly, female combination mice had fewer GFAP+GFP+ cells compared to ethanol females (Figure 10G). Despite the reduction in GFAP+GFP+ cells in male cocaine mice, they had an increase in percent of GFP+ cells becoming GFAP+ cells (Figure 10H). As a result, cocaine males had a significantly higher percentage of GFP+ cells becoming GFAP+ cells compared to their female counterparts, despite females having more GFAP+GFP+ cells. Males in the combination group had a reduction in this percentage compared to both cocaine and control mice (Figure 10H).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EtOH
Coc
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F
GFAP⁺ Cells (Avg. #)
Ctrl
EtOH
Coc
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G
GFAP⁺GFP⁺ Cells (Avg. #)
Ctrl
EtOH
Coc
Combo
H
GFAP⁺GFP⁺ / GFP⁺(%)
Ctrl
EtOH
Coc
Combo

Figure 10: Astrogliogenesis in the TL. (A-D) Representative brain images of control, ethanol, cocaine, and combination treated mice, respectively, stained with stem cell marker (GFP green), astrocyte marker (GFAP red), and merged with nuclear maker Dapi (Bregma 0.5 through 1.08). (E-G) Quantification of $GFP^+$, $GFAP^+$, $GFAP^+GFP^+$, and the percent of $GFAP^+GFP^+$ over total $GFP^+$ cells, in the SGZ. Values are shown as mean ±SEM, *$p<0.05$ compared to control, #$p<0.05$ compared to other sex in the same group, \$$p<0.05$ compared to cocaine group, +$p<0.05$ compared to combination group, n=3 mice per sex per group. Scale bars, 45μm.

In summary, combination treatment reduced GFP+ and GFAP+GFP+ cell populations while not impacting GFAP+ cell populations. This indicates that NSCs and newly differentiated astrocytes are much more sensitive to cocaine and alcohol co-administration compared to more mature astrocyte populations. Also, GFP and GFAP+GFP+ cells in female TL are more resilient than males to cocaine treatment. However, NSCs in the male TL may undergo an increase in astrogliogenesis, unlike females, as observed by the increase in GFP+ cells becoming GFAP+.

**Alterations in drug metabolizing enzymes in the hippocampus following drug treatment.**

We have shown the cocaine and alcohol administration significantly alters NSCs and their progenies. In order to elucidate a possible mechanism of action for these changes we conducted western blot analysis to evaluate the presence of, and changes in, enzymes which play a critical role in drug metabolism. Cocaine and alcohol readily pass through the blood brain barrier, and interact with cells in the brain. While most drug metabolism occurs in the liver, it is of interest to know whether NSCs or other cell populations in the brain possess the ability to produce these metabolizing enzymes, and further assess if they may play a role in alterations seen in neuro- or astrogliogenesis following drug consumption. We elected to examine hippocampal samples to ensure we

had adequate amounts of protein and could observe a region with active neurogenesis and adult NSC population. We evaluated two enzymes critical for the metabolism of ethanol, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase-1 (ALDH1). ADH is responsible for breaking down alcohol into acetaldehyde and ADLH1 breaks down the toxic acetaldehyde into acetate. We also examined carboxylesterase-1 (CES1) and Cytochrome-P450-3A4 (CYP3A4). These two enzymes are essential for the metabolism of cocaine, importantly, CES1 is the enzyme responsible for the production of cocaethylene when cocaine is taken in the presence of alcohol.

We found no significant changes in ADH expression among the groups, however we observed dramatic increases in ALDH1 in cocaine, combination, and female ethanol groups (Figure 11A-B). Females in the ethanol group had a 78% increase in ALDH1. Males and females in the cocaine group had a 72% and 81% increase, respectively. In the combination group, males had 70% increase while females had a 78% increase (Figure 11B). Males in the cocaine group had a significant increase in CES1 (82%), while females had a trending increase of 74% ($p=0.06$) (Figure 11C). Both males and females had significantly higher CES1 expression compared to their ethanol counterparts (Figure 11C). Despite the increase in CES1 in cocaine groups, the combination group did not have a significant increase (Figure 11C). Additionally, there were no significant increases in CYP3A4 in either cocaine or combination treated mice (Figure 11D). Interestingly, females in the ethanol group had significantly higher CYP3A4 compared to controls (54%) and their male counterparts (69%) (Figure 11D).

These data show that cells within the hippocampus contain drug metabolizing enzymes and up regulate ALDH1, CES1, and CYP3A4 in response to drug treatment.

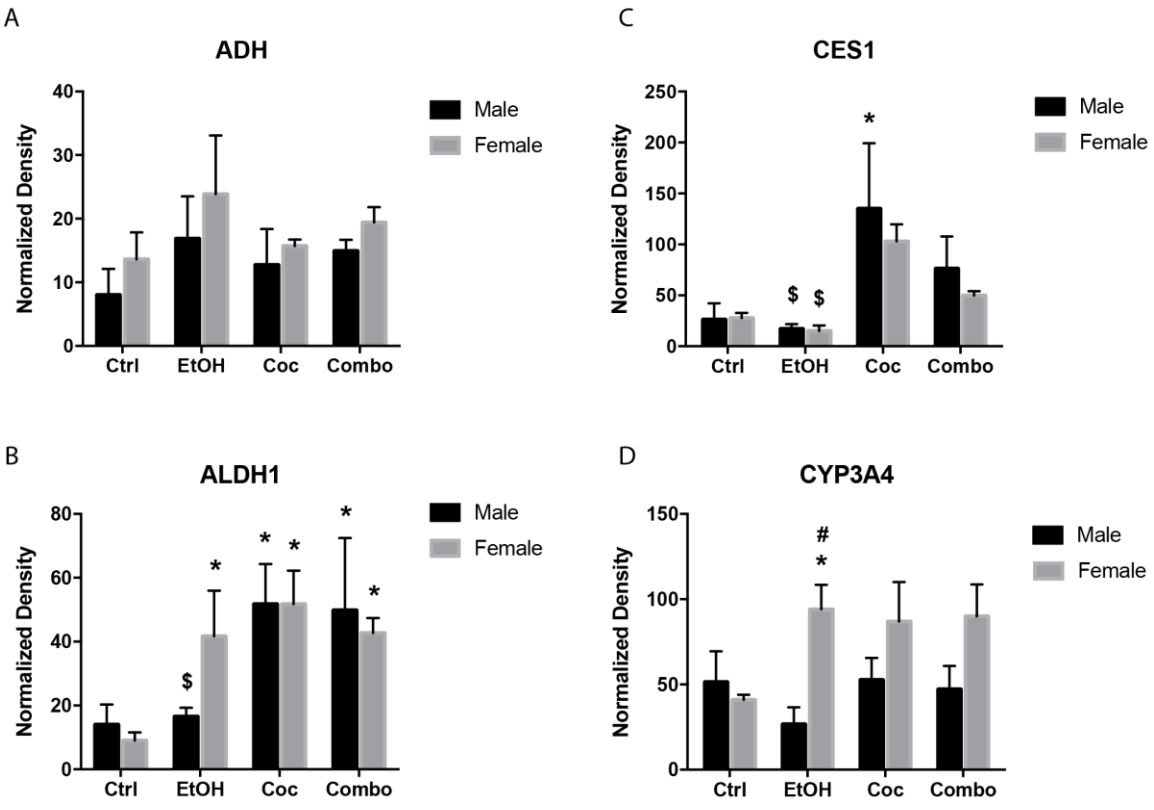


Figure 11: Alcohol and cocaine metabolic enzymes in the hippocampus. (A-D) Western blot densitometry analysis for ADH, ALDH, CES1, and CYPE3A4, respectively. Values are shown as mean ±SEM, *p<0.05 compared to control, #p<0.05 compared to other sex in the same group, $p<0.05 compared to cocaine group, n=4 per sex per group.

## DISCUSSION OF COMBINATION DRUG STUDY

This study is the first to employ a genetic inducible fate mapping model to study the effects of chronic alcohol and cocaine co-administration on behavior and NSCs and their progeny. We compared three main brain regions in both male and female mice. Also, this is the first study to evaluate chronic co-administration on astrocytes, newly formed neurons, and drug metabolizing enzymes in the hippocampus.

Behaviorally, we observed decreases in context discrimination that correlated with the decreases in neurogenesis observed in the SGZ. All drug treated mice were unable

to distinguish between the shock and safe context, and all mice had significant decreases in newly formed neurons (DCX+GFP+). We also observed changes in sucrose preference in combination mice. It is well established that individuals with substance use disorders frequently have vitamin deficiencies and poor diets. To interrogate whether this could be related to changes in the tanycyte layer we conducted sucrose preference testing. Benford and colleagues recently showed that tanycytes in the TL, including the ones with NSC phenotypes have sweet taste receptors, like those found on the tongue. These receptors are capable of gluco-sensing and relaying the "sweet" signals to the hypothalamus. They also play a role in dietary preferences (Benford, 2017). Furthermore, Fox et al. demonstrated sex-specific changes in the HPA response to alcohol and cocaine intake, while it was not conjectured in Fox's paper that these changes could be related to sex-specific changes in the tanycyte population, it warrants further investigation (Fox, 2009). A study conducted by Jones and colleagues in 2006 shows that there were sex-dependent differences on alcohol and cocaine induced taste aversions (Jones, 2006). When taking these three studies into consideration (Benford, Fox, and Jones), there is a decent rational for using sucrose preference as a behavioral correlate for changes in the tanycyte layer.

Interestingly, combination treatment significantly reduced NSCs and newly differentiated astrocytes in this region, however mature astrocyte populations (GFAP+) were not altered. While further experiments are needed to fully understand the impact of NSCs in the TL, one possible explanation could be that since tanycytes contribute to gluco-sensing there is a heightened sensitivity of these sweet receptors to sucrose following combination drug treatment. The offers one reason why combination mice consumed significantly more sucrose at low doses than tapered off when sucrose was

increased. It is possible that when the sucrose level was steadily increased, the diet lost its novelty and mice exhibited no preferences.

It is of interest, moving forward, to conduct this same test using glucose instead of sucrose since it may yield more sensitive results. Additionally, behavioral studies should be conducted to assess aversive taste learning, anxiety, and conditioned place preference in response to chronic administration (Busse, 2004; Busse, 2005a; Busse, 2005b; Chaplin, 2010)

In terms of immunohistochemical data, we observed regional and sex dependent responses as well as different responses among NSCs, neurons, and astrocytes at different stages of differentiation. In the SVZ, neurogenesis was significantly reduced, however combination treatment resulted in the most dramatic reduction of newly differentiated neurons. Additionally, female NSCs were more sensitive to the toxic effects of combination drug treatment. One interesting finding in this study was that unlike the alcohol study described in Chapter 2, combination drug treatment was almost equally detrimental to NSCs and newly differentiated neurons in the SVZ and SGZ. Additionally, DCX+ neurons formed before tamoxifen induction were more sensitive to combination drug treatment in the SGZ compared to the SVZ. This was not observed in the first ethanol study. It is well established that alcohol interacts with cocaine, and forms cocaethylene which is a strong dopamine receptor agonist (Althobaiti, 2016; Bunney,2001). It has also been shown that alcohol does not change cocaine pharmacokinetics, suggesting that the enhanced effect seen in combination drug use can be attributed to a synergistic effect of both drugs taken simultaneous and/or the cocaethylene metabolite (Fowler, 1992). Over activation of dopaminergic signaling by cocaethylene in conjunction with alcohol and cocaine in the brain can cause cytotoxicity globally in the brain. This effect is more exaggerated than either drug taken individually

and can account for the greater reduction in SGZ NSCs and neurogenesis compared to just alcohol or cocaine alone.

Yet, despite the dramatic decrease in neurogenesis, a study conducted in 1998 showed that abstinent cocaine and alcohol co-abusers displayed normal MRI volumes; however still suffered from cognitive impairment (Di, 1998). It may be possible that during abstinence GFAP cells can proliferate which could explain the typical MRI volume but lack of cognitive improvement. In this study we observed that females did not have a decrease in astrocyte populations following combination use, and males only had a slight reduction. These changes to astrocytes were not as dramatic as those observed in the neural populations. Further studies would be needed to determine if astrocytes play a role in decreased cognitive function or if neurons are able to recover but not functionally integrate in abstinence. Interestingly, even though there was reduction in NSCs in the TL following combination treatment, the reduction was not quite as dramatic as that seen in the SGZ or SVZ. Further studies are needed to determine if there may be protective factors in the neurogenic niche of the TL. Another interesting facet of the immunohistochemical findings was the the GFAP+ cell population was more resilient than the DCX+ population in the SGZ and TL, particularly in female SGZ. Even newly differentiated astrocytes in the SVZ were less vulnerable than newly differentiated neurons. The heightened resiliency of astrocyte populations following combination drug treatment, especially in the female SGZ, warrants further investigation into the mechanisms of their resilience.

Examination of the response of NSCs and their progeny to chronic drug administration is important for understanding how the brain is affected, however identification of potential mechanisms underlying these responses is critically important for translational applicability. Identification of metabolic enzymes in the hippocampus

may provide drugable targets for treatment of neurodegeneration associated with chronic drug intake. Our western blot analysis revealed significant changes in the hippocampus, primarily in the enzymes that metabolize acetaldehyde and cocaine (ALDH1 and CES1). While most drug metabolism occurs in the liver, it is possible that local drug metabolism can occur in the brain. Local drug metabolism in the brain can result in neurotoxicity due to homeostatic disruptions in metabolic processes.

Additionally, toxic metabolites can be produces that the brain is not capable of effectively processing. It is unknown whether local metabolism of alcohol and cocaine in the brain is harmful or beneficial in removing drug. Interestingly, in this study only ALDH1 was up regulated in combination treatment in the hippocampus. ALDH antagonism has been used clinical for the treatment of alcohol use disorders for decades, in the form of disulfram (Carroll, 2000; Carroll,1998). Disulfram has also been used to treat concurrent use of alcohol and cocaine, and has shown some efficacy in reducing consumption (Carroll, 2000). Yao and colleagues have also evaluated efficacy of ALDH-2 antagonists to decrease cocaine seeking behavior (Yao, 2010). It is of interested to further investigate ALDH antagonism to see if improvements in NSC survival and differentiation occur in addition to drug seeking behaviors. Another interesting therapeutic that warrants further investigation is topiramate (Johnson, 2005; Kampman, 2013). Topiramate has been used to treat bi-polar disorder and substance use disorders. Interestingly, it is a weak inhibitor of CYP3A4. Whether this inhibition of CYP3A4 is enough to be neuro-protective in addition to decreasing drug seeking should be investigated.

Altogether our findings demonstrate unique regional and sex dependent responses of NSCs and their progeny to chronic alcohol and cocaine co-administration.

Furthermore, we have evidenced the possible implication of drug metabolizing enzymes in the hippocampus as therapeutic targets.

# CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS FOR UTILIZING ENDOGENOUS ADULT NEURAL STEM CELLS AS THERAPEUTIC TARGETS FOLLOWING CHRONIC SUBSTANCE USE

In the studies covered in this dissertation, the effect of alcohol and cocaine on endogenous NSC survival and differentiation was assessed. In Chapter 2, 4% ethanol by volume was administered to male and female mice over the course of 4 weeks. This dosing is similar to a human consuming between 15-20 standard drinks per day, taking into account that the mouse metabolism is approximately seven times greater than that of a human. This dosing parading strongly models that of a patient who is a chronic heavy drinker. Dramatic changes in NSCs were observed in three key regions, the SGZ, SGZ, and TL. Females displayed a greater sensitivity to the intoxicating effects of ethanol, from a behavioral perspective. Additionally, female NSCs had heighted sensitivity to the neurotoxic effects of ethanol in the SVZ. Interestingly, in the SGZ and TL, males and females had similar responses in terms of NSC survival following long term consumption. Short term consumption (6-11 days) resulted in a wide array of responses. In the SVZ, there was a reduction in NSCs in both males and females; however, in the SGZ and TL there were robust increases in NSCs compared to control (particularly in males). This could indicate a compensatory proliferation of NSCs that can be observed after an injury. The fact that this occurred in the short term, as opposed to long term treatment, indicates that initially, NSCs may proliferate to deal with the insult of ethanol and other metabolites. Long term treatment may provide enough of an insult to inhibit NSC survival and proliferation.

In the second chapter study, there were significantly high mortality rates of the mice (about 50%). This was most likely due to the high dose of ethanol. Moving forward

to the study presented in Chapter 3, a lower dose of ethanol was chosen (3.5% by volume). This dosing was reflective of a human taking in between 13-17 standard drinks a day. Using 3.5% alcohol by volume significantly reduced the mortality to the point where only 4 mice out of 30 had unanticipated deaths. We were able to observe similar intoxication behavioral patterns and similar NSC trends, however the reduction in NSCs and neurogenesis was not quite as severe in this study as it was in the first study. In chapter 3 we also addressed the clinically relevant issue of poly-substance abuse by designing and implementing the first chronic co-administration model of alcohol and cocaine. In addition to the alcohol being administered in the complete nutrient liquid diet, cocaine was administered via and intraperitoneal injection daily, at a dose of 10mg/kg. The dose of cocaine was relatively low, however it was chosen to represent a more chronic cocaine user as opposed to a binge user. Furthermore, when cocaine is administered through intraperitoneal injections, over time it will accumulate in the brain. Therefore, over the course of six weeks it was anticipated that cocaine levels in the brain would most likely double, reflecting an individual who consumes varying amounts of cocaine over time.

Interestingly, we observed that alcohol had greater negative impacts on NSCs and neurogenesis compared to cocaine in the three brain regions. This suggests that ethanol and its metabolites are more neurotoxic compared to cocaine. The combined use, however, was significantly more detrimental than either drug alone. There was almost a complete obliteration of NSCs and newly differentiated neurons in both the SVZ and SGZ following combined administration. However, the TL showed amazing resiliency to combined substance administration. While there was a significantly reduction of NSCs in the TL, the $GFAP^+$ population was maintained at levels similar to control mice, and there were still some NSCs that persisted in this region (more than the

SVZ). This raises important research questions about the role of tanycytes in response to drug and resiliency factors to molecules in the CSF.

Finally, at the end of Chapter 3 we investigated metabolic enzymes in the hippocampus related to alcohol and cocaine metabolism. We found that in response to drug ALDH1 was significantly increased in cocaine and combination mice, as well as female ethanol mice. While ALDH1 is known for breaking down acetaldehyde into acetate, in the brain it is also responsible for break down of dopyl, a bi-prodcut of dopamine metabolism. Therefore, when there is an increase in dopamine, induced by alcohol and cocaine, there will be an increase in dopyl and consequently an increase in ALDH1. Interestingly, male mice only treated with ethanol did not have an increase in this enzyme in the hippocampus. Furthermore, there was a significant increase in CYP3A4 in female mice in response to alcohol, cocaine, and combination treatment. CYP family members are well known players in drug metabolism and can be promiscuous in their effects. In the brain, it is possible that CYP3A4 not only plays a key role in breakdown of cocaine, but in breakdown of ethanol as well. CYP2E1 is another cytochrome family member that is known for ethanol metabolism as well as retinoic acid metabolism. It is possible that upregulation of CYP3A4 could play a similar role to CYP2E1 and also break down retinoic acid, a key neurogenic cue. This could explain greater reduction in neurogenesis in females compared to males. While further work needs to be done to investigate the extent to which disruption of metabolic enzymes in the hippocampus contributes to NSC behavior, the first step has been made in this dissertation to show that indeed metabolic enzymes exist in the brain and are locally changed in response to drug.

## FUTURE DIRECTIONS

The data in this dissertation paves the way for a number if interesting studies. It is of interest, in the future, to interrogate the mechanisms behind sex-dependent response to cocaine and alcohol administration, particularly with regards to hormonal contribution. The TL presents a unique opportunity examine the impact of drug intake on endocrine function in the hypothalamus and vice versa. The role of estrogen and testosterone in survival and differentiation of TL NSCs would be valuable in elucidating sex differences observed in this region. It may also contribute to our understanding of the role of tanycytes in communicating drug-related signals from the cerebrospinal fluid to the hypothalamus.

Additionally, behavioral studies should be conducted to evaluate a broader range of behavioral assessment, including novel object recognition, fear conditioning, taste preference, and drug self-administration. Self-administration studies will be a vital task in future experiments. Since alcohol is known to attenuate the negative effects of coming down off cocaine and produces a sustained high, it would be of interest to model drug seeking in the context of an animal model (Kampman, 2002; Martin, 2014). Additionally Lau-Barraco and colleagues showed that patients with dual cocaine and alcohol required a higher monetary reward before choosing money over drug (Lau-Barraco, 2008). Use of a fixed-ratio behavioral test would be of interest to evaluate how motivation for drug in animals. Combination drug use has also been shown to enhance cocaine raving, but not cue-related attentional bias (Marks, 2015). The mechanism behind this behavioral phenomenon could be addressed using a combination drug treatment animal model and investigating reward neuro-circuitry. Furthermore, patients with combined substance use have defects in cerebral perfusion while actively taking drug and during abstinence

(Gottschalk and Kosten, 2002) (Kosten, 1998). It would be worthwhile to examine the impact of defective cerebral perfusion on survival and differentiation of NSCS.

Moving forward, it will also be of interest to conduct abstinence studies. Now that we have shown the impact of drugs on NSCs, it will be critical to evaluate their recovery potential in various abstinence situations. Also, treatments can be tested for their ability to promote neurogenesis and NSC survival and differentiation. Opiod receptor antagonists have shown some promise in promoting abstinence (Calleja-Conde 2016; Schmitz, 2004; Schmitz, 2009; Suh, 2008). It is of value to determine whether opioid receptor antagonists could also enhance neurogenic recovery. Use of transcriptome and epigenetic studies would also be a highly valuable next step in evaluating changes to NSCs in response to drug treatment, as epigenetic variants have been shown to impact drug preference in global gene analysis (Farris, 2015). These mechanistic studies can elucidate new targets for neurogenic recovery.

In conclusion, endogenous adult NSCs are significantly impacted following chronic alcohol and cocaine abuse in a regional and sex-dependent manner. Given the adverse effects of neurodegeneration in substance using populations, targeting NSCs regeneration and neurogenic repair is a promising approach to improve clinical efficacy of treatments and patient quality of life.

# CHAPTER 5: MATERIALS AND METHODS

## ANIMALS

*Nestin*-CreERT$^2$:R26R-YFP bi-transgenic 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, 2007). Mice were genotyped by PCR using genomic DNA from tail snip to confirm presence of Cre and YFP trans-genes. 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 hour light/dark cycle.

## ANIMAL GENOTYPING

In order to verify the genotype of mice used in the study, mouse tail DNA was analyzed by PCR. CreERT2 sequences was detected using primers TK139 (5'-ATTTGCCTGCATTACCGGTC-3') and TK141 (5'-ATCAACGTTTTCTTTTCGG-3') (Indra et al., 1999). To detect the R26R sequence primers for (5´−CCTAAAGAAGAGGCTGTGCTTTGG−3´) and (5´−CATCAAGGAAACCCTGGACTACTG−3´) were used (Soriano, 1999).

## BLOOD ALCOHOL AND ACETALDEHYDE ANALYSIS

Both, blood alcohol and acetaldehyde levels were analyzed by head space gas chromatography (GC) using Hewlett Packard 6980N Gas Chromatograph equipped with a flame ionization detector (FID, Agilent Technologies) and 5975 GC-mass spectrometer (GC-MS, Agilent Technologies) as described previously (Strassnig and Lankmayr, 1999;

Kaphalia et al., 2014). Briefly, 50 µl blood was heated at 100°C for 5 min in a vial sealed with Teflon lined cap, and 50 µl from the head space was injected into the column (DB-ALC1, 30 m × 0.53 mm ID, J & W Scientific, Folsom, CA) preheated at 35°C. The column temperature was increased to 150°C at 15°C/min after 2 min. The temperature of injector port and FID was maintained at 250 and 300°C, respectively. The GC was operated at a 50:1 spit ratio with 1 ml/min flow rate using helium as carrier gas. Ethanol and acetaldehyde were further confirmed by GC-MS operated under single ion monitoring mode with a dwell time of 150 ms for each ion. The temperatures set for the MS detector were 250°C for transfer line, 230°C for the source and 150°C for the quadruple. Concentrations of ethanol and acetaldehyde were determined using external standards of ethanol and acetaldehyde and isopropanol as an internal standard. Recoveries for ethanol and acetaldehyde from the culture medium were >90%, and the data was corrected for their respective recoveries.

### TAMOXIFEN-INDUCED RECOMBINATION

Stock solutions of Tamoxifen (Sigma-Aldrich, St. Louis, MO) were prepared at 20 mg/ml in corn oil (Sigma). Mice, 8-10 weeks old, received i.p. injections of 2mg of tamoxifen once a day for 5 consecutive days. A 3-week interval was left before ethanol ramping 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, 2007).

### ETHANOL TREATMENT AND HANDLING

Mice, 3-4 months 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 randomized group assignment, mice were given ad libitum access to water and a Lieber-DeCarli liquid diet containing complete nutrients for rodents (Dyets Inc., Bethlehem, PA, CAT # 710260) and has been previously described (Brandon-Warner, 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 two weeks. Mice in the ethanol group were then maintained on a 4% ethanol in the liquid diet for up to four weeks. Both the control diet and ethanol containing liquid diet were made fresh daily. Each day, between 9:00-11:00am, 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 8:00-11:00am.

### COCAINE AND ETHANOL STUDY TREATMENT AND HANDLING

Mice, 3-4 months old, were randomly assigned using the "randomize" (RAND) function in Microsoft Excel to one of four groups: control (male n=7, female n=13), cocaine (male n=10, female n=14), ethanol (male n=10, female n=13), or cocaine-plus-ethanol (male n=10, female n=15). Mice in each group were age and sex matched to control for variability. Following randomized group assignment, mice were given *ad libitum* access to water and a Lieber-DeCarli liquid diet containing complete nutrients for rodents (Dyets Inc., Bethlehem, PA, CAT # 710260) and has been previously described (Brandon-Warner, 2012). Pair feeding was done in which ethanol calories replaced the equivalent calories of maltose-dextrin in the diet for ethanol and combination mice. The amount of ethanol in the liquid diet was slowly increased from 1% to 3.5% over the course of two weeks. Mice in the ethanol and cocaine-plus-ethanol groups were maintained on 3.5% ethanol liquid diet for 6 weeks. All diet was made fresh daily. Each day, between 9:00-11:00am, the feeding bottles were removed from the cage, the

remaining liquid diet was measured and subtracted from the original diet administered. (−)-Cocaine (National Institute on Drug Abuse), dissolved in 0.9% NaCl, was administered during bottle changes via daily intraperitoneal injections of 10mg/kg to mice in the cocaine and cocaine-plus-alcohol groups for 6 weeks. Mice in the ethanol group received vehicle injection of 20µL of 0.9% saline. Control mice were pseudo-injected by pressing on lower abdomen with index finger. Animals were weighed once a week.

### HOME CAGE BEHAVIOR SCORING

Mice were observed daily in home cages during exchange of liquid diet tube. Behavior was recorded and scored on a 0-4 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 early and not used for this study.

### ELEVATED ZERO MAZE

Elevated zero maze (EZM) testing was conducted to measure anxiety-like and exploratory behaviors. A standard elevated zero maze (elevated 50cm off the ground) was used and mice were placed into the center of the arena and allowed to freely explore for 5 minutes. Percent time in either the open or closed sections was measured using TopScan digital video-based data capture and analysis software (Clever Sys).

### CONTEXT DISCRIMINATION

Context discrimination was conducted as previously described (Cortez, 2017). All behavioral procedures were carried out between 7:00 a.m. and 6:00 p.m. Each day

before context discrimination testing, mice were allowed to acclimate to the testing room for 1 hour. Control (n=20), ethanol (n=21), cocaine (n=23), and combination (n=22) were exposed to the shock context on training day (Day 0), before being returned to their home cage. Starting Day 1 through the rest of the experiment, mice were placed in both the shock (Context A) and a safe (Context B) context. There was no foot shock in Context B. Percent freezing was recorded in each context. Because contextual fear-discrimination is a hippocampal-dependent task, it measures an animal's ability to distinguish between two environmental contexts. Each context used a standard mouse fear conditioning chamber (MedAssociates). Context A had no modifications to the chamber. Context B also had a grid floor, however it also contained carboard inserts, vanilla extract, and the chamber light and fan were shut off. Mice spend equal amounts of time in both chambers, and were randomly assigned to which context they would experience first. A two-hour interval was left in between contexts for each subject on each day of testing. Chambers were cleaned with 70% ethanol in between each subject. Digital video-based data capture and analysis with FreezeFrame software (Actimetrics) was used to assess freezing behavior. Discrimination ratios (% freezing in Context B ÷ % freezing in Context A+ B) were calculated for each group on each test day.

### SUCROSE PREFERENCE

Sucrose preference was assessed by incrementally increasing the percent of sucrose (Sigma-Aldrich, Cat#: S0389) in the liquid diet from 0.5%, 1%, 10%, and 20% (weight/volume). The caloric equivalent of maltose-dextrin was removed from the diet, so that diets remained calorically balanced. In this way, taste preference for sucrose was tested, as opposed to the preference for an overall sweeter and higher caloric value option.

### IMMUNOHISTOCHEMISTRY

At the termination of the experiments, mice were anesthetized with ketamine/xylazine (90mg/kg and 10mg/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 media. Tissue was serially sectioned at a thickness of 30µm using a Leica cryostat machine. Detection of induced-YPF was enhanced by immunohistochemical staining with chicken anti-GFP antibodies (Aves Labs, Tigard, OR, CAT# GFP-1020). Additionally, sections were incubated with antibodies against GFAP (Thermo Fisher Scientific, Waltham, MA, CAT# PA110019), DCX (San Francisco, CA, Abcam, CAT# ab18723), or NeuN (Millipore, Billercia MA Cat# MAB377). Following primary antibody incubation at 4°C overnight, sections were incubated with AlexaFluor secondary antibodies (goat-anti-chicken 488, CAT3# A11039; goat-anti-rabbit 568, CAT# A11011; and goat-anti-mouse 568 Cat#AB175473).

### 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 through 1.08, and both the SGZ of the hippocampus and the TL of the third ventricle were counted from bregma -1.58 through -2.16 Four sections (both hemispheres) from each region, spanning a total of 580µm longitudinally, were counted for each antibody. Average total positive cells were performed using triplicate mice for each region and antibody.

## WESTERN BLOTTING

At the termination of the experiments, mice were anesthetized with ketamine/xylazine (90mg/kg and 10mg/kg), and perfused with cold 5 mL PBS containing phenylmethylsulfonyl fluoride(PMSF) and protease inhibitor cocktail (Sigma). Tissue was flash frozen by placing in cryopreservation tubes (Cornig) then submerged in liquid nitrogen. Tissue was homogenized using manual desiccation, protein was collected by removing the supernatant following centrifugation at 20,000xg and a BCA protein concentration assay was performed to determine protein concentration (Pierce, Rockford, IL). 30µg of total protein were diluted in 4µL Nu-PAGE LDS sample buffer and 7.5µL NuPAGE reducing agent (Invitrogen, Carlsbad, CA) and heated to 75°C for10 min. Samples were briefly centrifuged, and loaded into 4–12% NuPAGE Novex Bis Tris Gels and electrophoresed at 100 V for approximately 1hr. Gels were transferred onto polyvinylidene difluoride (PDVF) membrane via electrophoretic transfer at 60 V for 2hr on ice. 5% non-fat milk (w/v in 0.1%TBS-Tween) was used for blocking 1 hour at room temperature followed by primary antibody incubation overnight at 4°C. Antibodies used in this study were anti-alcohol dehydrogenase-1 (ADH; Cell Signaling Cat#: 5295), anti-aldehyde dehydrogenase (ALDH; Abcam Cat#: ab18723), anti-carboxylesterase-1 (CES1; Abcam Cat#: ab53008), anti-Cytochrome-P450-3A4 (CYP3A4; Abcam Cat#3572), and anti-Beta-Actin (ß-actin; Santa Cruz Cat#: sc-47778). Membranes were then washed three times, for10 minutes each, with 0.1% TBS-Tween, and incubated for 1 hour at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody (SouthernBio Tech). Membranes were again washed three times, for 10 minutes each, and immunoreactive bands were detected by using a chemiluminescent ECL substrate (Lico; Cat#: NEL744B001). Visualization of immunoreactive bands was done using a Licor Oddessy system, and blots were exposed for 30 seconds, 2 minutes, and 10 minutes. All blots were normalized to ß-actin controls and control cerebellar protein

samples (male and female) were used to normalize between western blots. Densitometry analysis was conducted using ImageStudio. Each treatment group had an n=4 males and n= 4 females.

## STATISTICS

The number of mice required for analysis was calculated based on our previous results and power analyses using G*Power 3.1.7. For all analysis $p<0.05$ was required for significance with a power of 0.80. Home cage behavioral data was analyzed using a random intercept model by SAS9.4 (Tom A.B.Snijders, 2011). Elevated zero maze was analyzed using a 2-Way-ANOVA with Fisher's LSD correction to determine significance. A One-Way repeated measures ANOVA was used to determine statistically significant discrimination of mice compared to a theoretical discrimination value (0.5). Ratios above 0.5 indicate successful discrimination. Students t-tests were used to evaluate significance of sucrose preference compared to maltose-dextrin preference. For morphological analyses, two- way ANOVA with a post hoc uncorrected Fisher's LSD test assisted by GraphPad Prism v6 software were applied to analyze the effects of ethanol on brain cells both individually and combined. Each group had three mice being used for each immunostaining, 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. Western blot analysis was conducted using a 2-Way-ANOVA with a Fisher's LSD correction.

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