CAN EXERCISE REVERSE BINGE ALCOHOL-INDUCED BRAIN DAMAGE?

A Thesis

Presented to

The Faculty of the Department

of Psychology

University of Houston

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

By

Mark E. Maynard

May, 2013

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ABSTRACT

Binge pattern drinking is characterized by excessive alcohol intake, combined with multiple periods of intoxication and withdrawal. This excessive consumption produces gray and white matter cell loss and cognitive impairments, leading to a cycle of further alcohol consumption. Animal models implicate the hippocampus, important for learning and memory, as being particularly sensitive to alcohol induced-neurodegeneration. Furthermore, binge alcohol has been shown to induce cell death and inhibit adult neurogenesis. Voluntary running-wheel exercise has been shown to increase hippocampal neurogenesis and promote overall brain health. The current study investigates the effects of repeated binge alcohol exposure and the potential of voluntary exercise to aid in recovery during abstinence. Female Long Evans rats were exposed to 1 or 2 four-day binge alcohol exposures before exercising voluntarily for four weeks. Rats were sacrificed 35 days after their last dose of alcohol and examined for histological markers of neurogenesis (doublecortin (DCX+)) and cell survival, (number of remaining granule cells) in the dentate gyrus. Binge exposure resulted in a decrease in granule cells, an effect that was reversed by exercise. A single binge had no effect on DCX+ cells; however, a second binge significantly reduced the number of DCX+ cells. Exercise increased the number of DCX+ cells in controls, however there was no increase in single binge animals demonstrating a binge induced suppression of the neurogenic effect of exercise. We conclude that while exercise can restore binge alcohol induced loss of granule cells, repeated binge alcohol exposure decreases neuronal differentiation in the hippocampus after 5 weeks of abstinence. Exercise, which promotes neurogenesis in the alcohol-naïve brain, may have limited ability to do so in the binge-exposed brain.

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Can Exercise Reverse Binge Alcohol-Induced Brain Damage?

Alcohol abuse and alcoholism continue to be both prevalent and problematic, resulting in numerous consequences to health and well-being. In fact, over 30% of Americans meet the diagnostic criteria for an alcohol use disorder (AUD) within their lifetime, with very few ever seeking treatment (Hasin, Stinson, Ogburn, & Grant, 2007). The consumption of dangerous amounts of alcohol, the characteristic feature of an AUD, is associated with changes in brain physiology, and structural and functional abnormalities in the brain and other organs (Courville, 1955; Victor, Adams, & Mancall, 1959). Postmortem analysis of alcoholic brains and *in vivo* imaging of brain morphology reveal significant volume loss in cortical and subcortical brain structures that include both gray and white matter shrinkage (Harper, 1985; Pfefferbaum et al., 1992; Sullivan & Pfefferbaum, 2005). Animal models have supported a direct link between excessive alcohol intake, neurotoxicity, and behavioral impairments and have begun to reveal the various mechanisms involved in the net loss of cells (Collins, Corse, & Neafsey, 1996; Crews & Nixon, 2009; Crews, Braun, Hoplight, Switzer, & Knapp, 2000; Obernier, Bouldin, & Crews, 2002a; Walker, Barnes, Zornetzer, Hunter, & Kubanis, 1980). The frontal lobe, responsible for many complex cognitive skills, such as working memory, judgment, motivation, and risk taking, is particularly vulnerable to alcohol (Kubota et al., 2001; Sullivan & Pfefferbaum, 2005). The hippocampus is another area particularly sensitive to neurodegeneration as a result of excessive alcohol consumption, leading to deficits in memory and cognition (Obernier et al., 2002a; Crews, Nixon, & Wilkie, 2004; Townsend & Duka, 2005). It is common for chronic alcoholics to demonstrate impaired judgment, deficits in impulse control, poor insight, and reduced motivation (Oscar-

Berman & Hutner, 1993; Sullivan et al., 2000a; Sullivan & Pfefferbaum, 2005). It is thought that reduced behavioral control due to alterations in brain structure (in the frontal lobe and hippocampus in particular) and the development of tolerance and dependence associated with excessive alcohol intake, promotes a cycle of further alcohol abuse and neurodegeneration (Bechara, 2005; Crews et al. 2004; Crews & Nixon, 2009).

Binge Pattern Drinking

Interestingly, recent evidence, such as lifetime consumption of alcohol not correlating with neurodegeneration (Tivis, Beatty, Nixon, & Parsons, 1995), supports the fact that pattern of intake is an important factor involved in the neurodegeneration resulting from excessive alcohol consumption. Studies on the neurocognitive functioning of adolescents with a history of an AUD reveal deficits in verbal and spatial working memory, verbal and nonverbal retrieval, and attention (Brown, Tapert, Granholm, & Delis, 2000; Tapert, Granholn, Leedy, & Brown, 2002; Tarter, Mezzich, Hsieh, & Parks, 1995). Additionally, neuroimaging studies of adolescents with an AUD show signs of alcohol neuropathology in the brain, including decreased hippocampal volume (De Bellis et al., 2000; Medina, Schweinsburg, Cohen-Zion, Nagel, & Tapert, 2007; Nagel, Schweinsburg, Phan, & Tapert, 2005). This neuropathology may be due to the fact that adolescents are more likely to drink in a binge pattern (Zeigler et al., 2005). In fact, binge pattern drinking is more predictive of neurodegeneration than total lifetime intake amounts (Hunt, 1993). Binge consumption, defined as a pattern of drinking that brings the blood alcohol concentration (BAC) above the legal limit of .08 (at least 5 drinks) on more than one occasion within 6 months, is responsible for approximately half of the annual 85,000 alcohol related deaths in the U.S. each year (NIAAA, 2004; Courtney &

Polich, 2009; Morbidity and Mortality Weekly Report, 2009). Binge drinking is also the most common pattern of alcohol intake, with 40-60% of alcohol abusers drinking in this manner (Robin et al., 1998). A study looking at the amount of alcohol consumed between binge and chronic drinkers, found that even though both consumed equal amounts in a month, binging alcoholics averaged 83 days between drinking episodes compared to .7 days in chronic alcoholics (McMahon, Davidson, Gersh, & Flynn, 1991). This demonstrates that alcoholics drinking in a binge pattern drink large quantities in short periods of time. It is thought that these episodes of sustained elevated intoxication are responsible for the greater degree of cognitive and structural dysfunction seen in binge consumers.

Binge pattern drinking is not only characterized by multiple periods of intoxication, but also multiple periods of detoxification, or withdrawal. Several studies indicate that these successive periods of high alcohol intake and detoxification lead to increasingly more severe cognitive dysfunction, neurodegeneration, and withdrawal symptoms, including increased susceptibility to seizures (Becker & Hale, 1993; Branchey, Rauscher, & Kissin, 1971; Carrington, Ellinwood, & Krishnan, 1984; McCown & Breese, 1990, Duka, Townshend, Collier, & Stephens, 2003). The increased susceptibility to seizures includes both an increase in incidence and intensity (Duka, et al., 2004). Multiple periods of withdrawal is also associated with increased feelings of anxiety and cravings for alcohol, leading to further excessive consumption with the purpose of avoiding withdrawal symptoms (Duka et al., 2011). The further perpetuation of the excessive drinking cycle results from dysfunction of the frontal lobes and

amygdala, which is associated with multiple detoxifications (Stephens & Duka, 2008; Stephens et al., 2005).

Binge Model of Alcohol Dependency and Neurodegeneration

The binge model used in the present study is a reliable model of ethanol-induced brain damage in rodents that is consistent with and characteristic of the human condition (Collins et al., 1996; Crews et al., 2000; Crews et al., 2004). Animals receive ethanol 3 times a day over four days via intragastric gavage, resulting in blood levels between 250 and 400 mg%, mimicking the high blood ethanol levels and heavy alcohol consumption common among binge drinking alcoholics (Crews & Nixon, 2009). The binge ethanol-induced brain damage is also consistent with human pathology, with significant damage in the frontal lobes and limbic association areas (Crews et al., 2004; Obernier et al., 2002a). During the binge, brain damage progressively increases after 2 days, starting in the frontal olfactory bulb before spreading to additional brain regions over time, with peak damage occurring after the last of the 4-day doses (Crews & Nixon, 2009). The cognitive impairments in rats after the binge measured in the Morris water maze are consistent with that of the disrupted hippocampal function in alcoholics (Obernier et al., 2002b).

Two of the key mechanisms responsible for gray and white matter loss are inhibition of neurogenesis and disruption of supportive glia cells, caused by ethanol (Crews et al., 2004; He & Crews, 2008; Kimelberg & Aschner, 1994; Miguel-Hidalgo, 2005.). The finding that ethanol inhibits neurogenesis by retarding cell proliferation and increasing cell death has been widely supported (Crews et al., 2004). It is believed that

this inhibitory effect is contributing to the significant gray matter loss in the hippocampus, because new neurons are not being creating to replace those being lost through apoptosis (Crews & Nixon, 2009). Binge consumption has also been shown to decrease numbers of astrocytes and oligodendrocytes, responsible for the maintenance and myelination of white matter tracts in the brain (Korbo, 1999). Alcohol causes astroglia to degenerate, leaving a void in trophic and metabolic support, which leads to further neuronal degeneration (Kimelburg & Aschner, 1994).

Abstinence and Brain Recovery

The withdrawal and abstinence period following the binge, namely the first week after, is key for the brain's ability to recover from ethanol-induced damage. Previous studies of this binge model found that abstinence resulted in increased cell proliferation after just one day (Nixon et al., 2008). Two bursts of cell proliferation occur during the first week of abstinence, with the first happening just two days after the last dose of alcohol. Cells generated during this first burst have been shown to primarily differentiate into microglia, and is thought of as a means for the brain to begin the recovery by secreting factors that stimulate brain regeneration (Nixon et al., 2008). Cells generated during the second burst primarily differentiate into neurons. This second burst occurs on the seventh day after the last dose, and is region-specific to the dentate gyrus subgranular zone (Nixon & Crews, 2004).

Findings about recovery during abstinence in the rodent model mirror those in human studies, which provide substantial evidence that sobriety results in improvement to structural and functional deficits (Carlen et al., 1978; Sullivan, Rosenbloom, &

Pfefferbaum, 2000b). After just one month of sobriety, improvements are seen in cognition, such as nonverbal memory and attention (Sullivan et al., 2000a, 2000b), as well as increases in cortical gray matter volume (Pfefferbaum et al., 1995). Increases in white matter tracts and reversal ventricular enlargement will also occur with longer abstinence (O'Neill, Cardenas, & Meyerhoff, 2001).

Potential of Exercise to Enhance Recovery

Exercise has various psychological and physiological benefits, which may provide potential mechanisms for benefiting alcohol abstinent patients, and, in particular, those who drank in a binge pattern. Exercise induces many physiological changes that promote healthy living. Exercise increases cell proliferation and neurogenesis in the dentate gyrus, promoting the health of the hippocampus (van Praag et al., 1999a; Trejo, Carro, & Torres-Aleman, 2001; Kim et al., 2002; Bjornebekk, Mathe, & Brene, 2005; Leasure & Jones, 2008). This is important for the brain's ability to respond to damage and is one of the key mechanisms behind exercise's ability to enhance memory and cognition (Crews et al., 2004). In fact, exercise can also block the inhibition of cell proliferation caused by ethanol (Crews et al., 2004). It has previously been shown that voluntary access to exercise prior to binge alcohol exposure will protect vulnerable brain areas from alcohol induced cell death (Leasure & Nixon, 2010).

Exercise benefits the brain by increasing the density of dendritic spines, which aid in signal transmission, and enhancing LTP (Farmer et al., 2004; van Praag, Kempermann, & Gage, 1999b). Another way that exercise helps maintain a healthy brain is to increase neurotrophin levels, the fertilizer for the brain that is necessary for maintaining the health

of neurons and other essential cells (Asano et al., 1998; Carro, Trejo, Busiguina, & Torres-Aleman, 2000). Additionally, anti-inflammatory signals are increased by exercise (Skalicky & Viidik, 1999). Exercise has been shown to have vast benefits to the cardiovascular system, including increased blood flow capacity, decreased baseline blood pressure, an increase in cardio protective proteins, and angiogenesis (Pescatello et al. 2004; Noble et al., 1999; Black, Isaacs, Anderson, Alcantara, & Greenough, 1990; Swain et al. 2003). Exercise will help develop and maintain cardiorespiratory fitness, in addition to musculoskeletal and neuromotor fitness in healthy adults (Garber, 2011). Additionally, exercise will increase muscle mitochondrial proteins (Moraska, Deak, Spencer, Roth, & Fleshner, 2000) and help bolster antioxidant activity (Alessio et al. 2005).

Current Study

Whereas ethanol inhibits neurogenesis and produces neurodegeneration in various brain areas (Crews & Nixon, 2009; Nixon & Crews, 2002; Obernier et al., 2002a), exercise increases neurogenesis and promotes the health of the hippocampus and overall functioning of the brain in general (van Praag et al., 1999b; Asano et al., 1998; Alessio et al., 2005). Because of exercise's opposing effects to ethanol and previous work supporting exercise as neuroprotective of binge-induced damage (Leasure & Nixon, 2010), we investigated the potential of exercise to aid the brain in recovery after binge alcohol-induced damage. Animals experiencing either one or two binge alcohol exposures were given access to voluntary exercise post-binge for four weeks to determine whether exercise will be neurorestorative. It was expected that exercise would be neurorestorative of the brain following binge alcohol exposure by increasing

neurogenesis and creating a post-binge environment that is better suited for recovery and cell survival.

Further, because binge pattern drinking in humans is characterized by multiple periods of withdrawal and intoxication, we will repeat the binge paradigm a second time. Although significant recovery occurs during abstinence from ethanol, binge pattern consumers typically drink to intoxication on multiple occasions, potentially preventing the brain from having ample time for recovery from the first binge. The second binge will be timed on the sixth day following the last dose of alcohol in an attempt to suppress the neuroproliferative burst that is important in neural recovery during abstinence (see Figure 2). To compare long term outcomes of animals exposed to a single or two alcohol binges and the effect of exercise, tissue will be stained for doublecortin (DCX), a marker for proliferating cells in the dentate gyrus that have committed to a neuronal fate. It was expected that alcohol will decrease the number of DCX+ cells 35 days after the last dose of alcohol, and that a second binge exposure will result in fewer DCX+ cells compared to a single binge exposure. We expected that animals given access to voluntary exercise will have a greater number of DCX+ cells compared to their sedentary binge drinking counterparts. It has previously been shown that animals that exercised prior to a single binge exposure had significantly more granule cells in the dentate gyrus than animals that were sedentary (Leasure & Nixon, 2010). Therefore, we expected exercised animals to have a greater number of dentate gyrus granule cells compared to those that were sedentary, and animals exposed to a second binge will have fewer granule cells than animals exposed to a single binge.

For behavioral measures, it was expected that animals would develop tolerance to alcohol after a single binge exposure, and therefore, require more alcohol to get to the same levels of intoxication during a second binge exposure. This tolerance was indicated by a larger volume of doses for second binge animals compared to that of the single binge animals, and similar behavior intoxication scores between the two groups. Additionally, consistent with the literature on human alcohol withdrawal that demonstrates the more periods of withdrawal, the more severe the symptoms, it was expected that animals going through a second binge exposure would experience more severe withdrawal symptoms and a greater incidence of seizure like behavior than animals exposed to a single binge episode.

Finally, the prevalence rates of binge drinking are increasing in young woman (Keyes, Grant, & Hasin, 2008) and women with AUDs have higher rates of medical problems and a significantly higher death rate than men with AUDs (Smith & Weisner, 2000). Additionally, women are more sensitive to the neurotoxic effects of alcohol and neurodegeneration than males (Hommer, Momenan, Kaiser, & Rawlings, 2001; Prendergast, 2004) and as such, cognitive and structural impairments emerge faster in the course of the disease for women than for men (Mann, Batra, Gunthner, & Scroth, 1992; Nixon, Tivis, & Parsons, 1995); therefore the current study investigated the potential beneficial effects of exercise following repeated binge ethanol exposure in female rats.

Method

Animals and Housing Conditions

All experimental procedures were conducted in accordance with the National Institutes of Health's (NIH) Public Health Service Policy on Humane Care and Use of Laboratory Animals (NRC, 1996), and were approved by the Institutional Animal Care and Use Committee of the University of Houston. For this study, 48 female Long-Evans rats (Harlan) weighing 175 to 200 grams and aged two months at the beginning of the experiment were randomly divided into 6 groups in a 3 x 2 design comparing Diet (Control diet, One binge, Two binges) and Activity (exercise versus sedentary controls). Each group consisted of 8 animals: sedentary control (S0) which were sedentary and received an isocaloric control diet; sedentary one binge (S1) which were sedentary and received the ethanol diet over one four-day binge; sedentary two binge (S2) which were sedentary and received the ethanol diet over two four-day binge periods; exercise control (E0) which exercised daily and received an isocaloric control diet; exercise one binge (E1) which exercised daily and received the ethanol diet over one four-day binge; and exercise two binge (E2) which exercised daily and received the ethanol diet over two four-day binge periods. Animals were group housed in clear Plexiglas cages and given 1 week to acclimate to vivarium conditions, which include *ad libitum* rat chow and water, and a reversed light/dark cycle (lights off at 9:00/ on at 21:00). Prior to beginning the experiments, all rats were tamed by gentle handling to acclimate them to the experimenters and make them amenable to gavage. Animals began the experiment two weeks after arriving (one week of acclimation, one week of taming). Female rats were chosen because of their consistent running behavior and have not been studied extensively in this model of an AUD.

Binge Consumption Paradigm

The 4-day binge model is a well-accepted model of alcohol abuse with a characteristic and consistent pattern of cell death in male rats and is a reliable model for ethanol-induced brain damage (Crews et al. 2000; Crews & Nixon, 2009). As the majority of those diagnosed with an AUD drink in a binge pattern, and because a binge pattern of drinking is predictive of neuropathology (Hunt, 1993), this model was chosen to best mimic the human condition. During the period of alcohol administration, food was removed, but water was always available. Ethanol was administered via intragastric gavage according to a paradigm modified from Majchrowicz (1975), which maintains consistent intoxication while avoiding mortality. Rats were gavaged with ethanol diet (25% ethanol w/v in vanilla Ensure™; Abbot Laboratories, Columbus, OH) or isocaloric control diet (Dextrose w/ vanilla Ensure™) every 8 hours for 4 days, starting on the first day of the experiment (12 doses total). The initial dose for each animal was 5g/kg and caused significant intoxication; further doses were determined based on a 6-point behavioral intoxication scale that corresponds to an accompanying dose of ethanol (see Table 1). Thus, the greater the observed behavioral intoxication, the smaller the subsequent dose. Animals in the two binge exposure groups began their second binge 6 days after their last dose of alcohol (see Figure 2).

Blood Ethanol Concentration

Blood ethanol concentration (BEC) was determined from tail blood samples taken 90 minutes after the morning dose on day 3. Samples were centrifuged, and then stored at -20°C until further analysis. Serum was extracted and BEC determined using a GM7 Analyzer based on external standards (Analox, Waltham, MA).

Voluntary Exercise

On the seventh day following the last dose of alcohol or isocaloric diet, rats in the exercise groups were given access to exercise wheels for a total of five and a half hours a day, for four weeks (start at 9:00/ end at 14:30). In order to precisely monitor distance travelled, rats were removed from home cages and placed into individual running wheels equipped with counters. During the exercise period, animals had access to food and water *ad libitum*. After exercise, animals were returned to their home cages in the vivarium. Although rats in exercise groups were socially isolated while exercising, this occurred for only 5.5 hours daily, leaving these animals group-housed the majority of the time. Sedentary animals remained in their home cages in the vivarium.

Monitoring of Withdrawal Symptoms

Eight hours after the last dose of ethanol, food was replaced in the animal's home cages. Spontaneous withdrawal behavior in all ethanol treated rats was monitored starting 10 hours after their last dose through 26 hours after their last dose (see Figure 2). This range corresponds to the peak period of withdrawal (Faingold, 2005; Majchrowicz, 1975; Penland, Hoplight, Obernier, & Crews, 2001). During the 17 hours withdrawal was monitored, rats were observed in their home cages and scored for spontaneous withdrawal behaviors in 30-minute intervals. Red lamps were used during the dark cycle so as to not disturb the Circadian rhythms of the rats. Behaviors were scored based on the withdrawal scale of Penland et al. (2001) that was modified from Majchrowicz (1975; See Table 2). At every time point, the spontaneous withdrawal symptoms of each animal were observed; the most severe symptom was recorded so that a mean withdrawal

severity score and peak withdrawal severity score could be determined, consistent with the methods of Morris et al. (2010). The mean withdrawal score refers to the average of the scores observed and recorded for each 30-minute interval over the 17 hours monitoring period. The peak withdrawal score on the other hand, refers to the average of the highest and most severe withdrawal symptom observed in each animal during the entire 17-hour period.

Histological Procedures

After four weeks of exercise (35 days after the last dose of alcohol) each rat was given an overdose of anesthetic and intracardially perfused with cold saline, followed by 4% paraformaldehyde until the upper body was stiff. Brains were removed and post-fixed overnight in paraformaldehyde, and then refrigerated in 30% sucrose. Brains were cut in 50 µm coronal sections on a microtome from prefrontal cortex through approximately Bregma -6.6. Sections were stored in cryprotectant in 96-well microtiter plates at -20°C until processed.

DCX Immunohistochemistry

Doublecortin (DCX) is expressed in proliferating progenitor cells and newly generated neuroblasts (Brown et al. 2003), making it a reliable early neuronal marker. For immunohistochemistry, every sixth section was rinsed with 0.1 M tris-buffered saline (TBS) three times at room temperature for 10 minutes each. Sections were quenched for 30 minutes at room temperature in 0.6% hydrogen peroxide (to exhaust the activity of endogenous peroxidases) followed by three 10-minute washes in TBS. These sections were then blocked for 60 minutes in 3% normal donkey serum (Sigma-Aldrich, St. Louis,

MO, USA), followed by incubation at 4° C for 72 hours in primary antibody (goat anti-DCX, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; 1:100). After two TBS rinses for 15 minutes each and 15 minutes blocking in 3% normal donkey serum, sections were incubated overnight at room temperature in secondary antibody (biotinylated donkey anti-goat, Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:250). Next, sections were rinsed three times in TBS for 10 minutes each, then treated for 90 minutes in avidin-biotin complex (ABC, Vector Labs, Burlingame, CA, USA) and then rinsed three times in TBS for 10 minutes each. Sections reacted and were visualized with diaminobenzidine (DAB) and then rinsed four times in TBS for 10 minutes each, before being mounted onto gelatinized slides and allowed to dry overnight. Once dried, all brain sections were counterstained with Methyl Green, cleared in xylene and coverslipped using Protexx.

Stereology

The number of doublecortin-positive (DCX+) cells in the dentate gyrus was estimated using the optical fractionator method applied via an automated stereology system (StereoInvestigator, MicroBrightField, Williston, VT, USA) as the means of unbiased estimation of the total cell population. Using a Nikon Eclipse 80i upright microscope, the region of interest was traced using the 10x objective, and cells were counted within two-dimensional counting frames using a 40x oil objective. The average mounted section thickness was approximately 37μm, thus top and bottom guard zones were set at 5μm each, for an optical dissector height of 27μm. DCX+ cells within the granule cell layer were counted in every sixth section in a single hemisphere beginning at the earliest emergence of the dentate gyrus at Bregma -1.80mm and ending at Bregma -

6.1mm (See Figure 2). The counting frame for the granule cell layer of the dentate gyrus was 40 x 40 μm and the grid size was 60 x 60 μm. The total number of granule cells was quantified using the same method, with the exception of a 100x oil objective used for counting, with counting frames of 40 x 40 μm, and a 200 x 200 μm grid. The total number of immunopositive profiles for each cell count was then used to estimate the total number of granule cells and DCX+ cells per dentate gyrus ± SEM. Coefficients of error (CE) will be calculated using the method of Gunderson and were less than 0.05 for all groups (Gunderson, Jensen, Ki^u, & Nielsen, 1999).

Statistical Analyses

All values presented are expressed as mean ± standard error of the mean. Running distance, body weight, behavioral intoxication, ethanol dose, and mean withdrawal scores were analyzed using repeated measures ANOVA. The Greenhouse-Geisser correction was used in all instances where the sphericity assumption was violated in repeated-measures ANOVAs. All other data, including granule cells and DCX+ cells, were analyzed with two-way ANOVA using the variables Activity, Diet, and the Activity by Diet interaction. All two-group comparisons between a single and two binge exposure, e.g. BECs (mg/dl), ethanol dose (g/kg), peak intoxication and peak withdrawal behaviors were analyzed by *t*-test. Pearson correlations were performed for the variables behavioral intoxication and mean withdrawal score, and dose of ethanol and BEC. Significance of the correlations were determined using the critical value table for Pearson's Correlation Coefficient for all statistical analyses, a *p*-value of less than 0.05 was deemed significant.

Results

Body Weights and Distance Run

At the start of the experiment, there was no difference between each group of rats and their starting body weight (see Fig.3). Repeated-measures ANOVA comparing each group to one another across the entire experiment revealed a significant main effect of Group [$F(5,42) = 3.62$, $p = .008$], and a significant Group x Time interaction using the Greenhouse-Geisser (GG) correction [$F(19.413, 163.065) = 13.183$, $p < .0005$]. The main effect of group is not significant when just comparing animals with a single binge exposure [$F(3,28) = 1.301$, $p = .294$], revealing the difference in groups primarily lies in the difference between the body weights of animals during their second binge exposure compared to single binge exposure animals that had abstained for over a week at this point. All animals gained a significant amount of weight over Time [$F(3.883, 163.065) = 675.268$, $p < .0005$]. Post hoc comparisons revealed that on day 5 after the binge, the animals receiving alcohol (E1, S1, E2, S2) lost 19% of their starting body weight, significantly more compared to 6% loss in body weight of animals receiving the isocaloric control diet (E0, S0) [$t(46) = -10.184$, $p < .0005$]. Additionally, following seven days of abstinence, control animals gained 9% above pre binge body weights, significantly more than single binge exposed animals who still remained 4% below pre binge levels. T-tests revealed that there was no difference in the percentage of body weight lost for alcohol consumers during the first binge exposure compared to the second binge exposure [($t(30 = -.685$, $p = .499$].

In order to assess whether exercise or being sedentary effected body weights, a repeated-measures ANOVA was run to investigate the effects of Group, Time and the Time x Group interaction over the four weeks post binge. There was a significant main effect of Time [$F(2.608, 78.204) = 259.305$, $p < .0005$], but the Group main effect [$F(1,30) = .274$, $p = .605$] and Group x Time interaction were not significant [$F(2.608, 78.240) = 2.730$, $p = .057$]. This means that both sedentary and exercise groups continued to gain a significant amount of weight over time, yet there was no difference in the weight gained between the two.

For rats in the exercise groups, there was no main effect of Diet for distance run [$F(2,21) = .781$, $p = .471$] and no significant Diet x Time interaction [$F(4.314,45.295) = .880$, $p = .49$]. There was a significant effect of Time (See Fig. 4), indicating that exercising animals increased the distance covered during the four weeks of the exercise period [$F(2.157,45.295) = 49.250$, $p < .0005$]. This finding is consistent with what we have previously observed (Leasure & Decker, 2009; Leasure & Jones, 2008; Leasure & Nixon, 2010).

Behavioral Response to Repeated Binge Alcohol Administration

In order to compare behavioral intoxication from a single binge exposure to second binge exposure, a repeated-measures ANOVA was run using Binge, Time and the Binge x Time interaction. Rats that experienced a second binge alcohol exposure behaved significantly less intoxicated compared to rats that experienced a single binge alcohol exposure [$F(1,46) = 10.99$, $p = .002$] (See Fig. 5). A significant effect of Time [$F(6.96, 320.167) = 48.540$, $p < .0005$] and a significant Binge x Time interaction [$F(6.96,$

320.167) = 48.540, $p < .0005$] indicate that all rats given alcohol acted progressively more intoxicated (behavioral signs of CNS depression) during each of the four-day binges. The greater intoxication scores for the first two days for the single binge animals compared to the second binge animals demonstrates that tolerance to the behavioral effects of alcohol developed between the two binges. A two group t-test comparing mean intoxication collapsed across four days between the two binges also revealed that animals were significantly more intoxicated during the first binge than the second [$t(44.328) = 3.898$, $p < .0005$] (See Fig. 6). There was no difference between the peak intoxication (highest intoxication score achieved across four days) between the two binges [$t(46) = .102$, $p = .919$] (See Fig.7).

Despite appearing more behavioral intoxicated, animals exposed to a second binge actually received more alcohol than animals exposed to a single binge [$F(1,46) = 10.99$, $p = .002$] (See Fig. 8). This finding also demonstrates the development of tolerance to the effects of alcohol in animals experiencing a second binge. Furthermore, there was a significant main effect of Time [$F(6.96, 320.167) = 48.540$, $p < .0005$] and the Binge x Time interaction [$F(6.96, 320.167) = 6.136$, $p < .0005$], indicating that dose of alcohol initially decreased as animals became more intoxicated and then increased as acute tolerance developed. The effect of Binge was further supported by a significantly greater mean dose of ethanol per day for second binge animals compared to single binge animals [$t(44.555) = -3.912$, $p < .0005$] (See Fig. 9). However, despite second binge animals receiving more alcohol the second time around, there was no difference between blood ethanol levels for single binge and second binge animals [$t(45) = -.312$, $p = .757$] (See Fig. 10). Finally, there was no significant correlation for the average intoxication to

blood ethanol concentration for the first or second binge respectively (data not shown, $r^2 = .17$, $p > .05$), (data not shown, $r^2 = .4$, $p > .05$).

Multiple Withdrawal Periods and More Severe Withdrawal Symptoms

In order to compare withdrawal symptoms from a single binge exposure to second binge exposure, a repeated-measures two-way ANOVA was run using Binge, Time and the Binge x Time interaction. A main effect of Diet revealed that animals experiencing a second period of withdrawal displayed significantly more severe symptoms of withdrawal than did animals experiencing a single period of withdrawal [$F(1,46) = 60.194$, $p < .0005$] (See Fig.11). Additionally, a main effect of Time [($F(7.371, 339.079) = 32.415$, $p < .0005$] and significant interaction [$F(7.371, 339.079) = 2.297$, $p = .024$] showed that the spontaneous withdrawal symptoms became more severe over time, until dropping off slightly near the end of the observation period. Overall, average withdrawal score collapsed across the 17h period were more severe in second binge animals compared to single binge animals [$t(46) = -7.734$, $p < .0005$] (See Fig.12), and the peak withdrawal severity (highest withdrawal score across 17 hours) was significantly greater in animals experiencing a second period of withdrawal [$t(44.108) = -4.693$, $p < .0005$] (See Fig.13). Convulsions, the most severe category of spontaneous withdrawal symptoms, were examined separately. The percentage of rats observed having convulsions was significantly greater during the second period of withdrawal (31%) than it was during the first period of withdrawal (13%).

For both the single and second binge, the mean intoxication was significantly correlated to the mean withdrawal (See Fig. 14, $r^2 = .617$, $p < .05$) (See Fig. 15, $r^2 = .442$,

$p < .1$) which has been previously reported (Morris et al., 2010). Finally, the mean withdrawal score for binge one was significantly correlated to the BEC from binge one (data now shown, $r^2 = .35$, $p < .05$) but the mean withdrawal score for binge two was not significantly correlated to the BEC from binge two (data now shown, $r^2 = .38$, $p > .05$)

Number of Granule Cells in the Dentate Gyrus

The effect of repeated binge alcohol exposure and exercise on the number of granule cells in the dentate gyrus was determined by stereological methods (See Fig. 16 A). Coefficients of error ranged from 0.03 to 0.04, averaged 0.03 in all groups, and were calculated according to the methods of Gundersen (Gundersen et al., 1999). Two-way ANOVA revealed a significant main effect of both Diet [$F(2,42) = 10.605$, $p < .0005$] and Activity [$f(1,42) = 23.144$, $p < .0005$], but no significant Activity x Diet interaction [$F(2,42) = .558$, $p = .577$] (See Fig. 17). Post hoc comparisons revealed that exercise did not lead to more granule cells in the control diet condition (E0 vs S0) [$F(1,42) = 3.683$, $p = .062$], however, exercise did result in significantly more granule cells for single binge (E1 vs S1) [$F(1,42) = 10.745$, $p = .002$] and two binge animals (E2 vs S2) [$F(1,42) = 9.832$, $p = .003$].

Post hoc comparisons groups by activity revealed that sedentary controls (S0) had significantly more granule cells in the dentate gyrus compared to second single (S1) and two binge (S2) animals respectively ($p < .05$; $p < .0005$) and no significant difference between number of granule cells between sedentary single and two binge animals ($p = .196$). Exercise on the other hand, revealed only a significant difference between exercise controls (E0) and exercise two binge (E2) animals ($p < .05$), and no difference between

E0 and exercise single binge (E1) animals ($p = .244$), nor a difference between E1 and E2 ($p = .153$). There was no significant correlation between number of granule cells and mean distance run ($r^2 = -.097$, $p > .05$) (See Fig. 18).

Binge Alcohol's Effects on DCX Expression in the Dentate Gyrus

The number of DCX+ cells in the dentate gyrus was determined by stereological methods 35 days following the last dose of ethanol. A high frequency of DCX stained cells were present within the subgranular zone of the dentate gyrus and with extensive processes extending through the granule layer (See Fig. 16 B, C). Two-way ANOVA revealed a main effect of Diet [$F(2,42) = 63.627$, $p < .0005$], a main effect of Activity [$F(1,42) = 61.412$, $p < .0005$], and a significant Diet x Activity interaction [$F(2,42) = 16.814$, $p < .0005$] (See Fig. 19). The results indicate that exercise had a differential effect depending on the diet of the animal. Post hoc analysis revealed that exercise led to significantly more DCX+ cells in exercise controls compared their sedentary counterparts (E0 vs S0) [$F(1,42) = 79.494$, $p < .0005$], a finding consistent with a bulk of literature demonstrating an increase in neurogenesis from exercise hippocampus (van Praag et al., 1999a; Trejo et al., 2001; Kim et al., 2002; Bjornebekk, et al., 2005; Leasure & Jones, 2008). Exercise also lead to an increased number of DCX+ cells in two binge compared to sedentary two binge animals (E2 vs S2) [$F(1,42) = 14.913$, $p < .0005$], revealing that exercise was neurorestorative following a second binge. However, there was no difference between number of DCX+ cells in exercising single binge compared to sedentary single binge animals (E1 vs S1) [$F(1,42) = .633$, $p = .431$], revealing a binge induced suppression of the neurogenic effects of exercise following a single binge.

Post hoc comparisons of groups by activity revealed there was no significant decrease in the number of DCX+ following a single binge for sedentary animals (S0 vs S1) ($p = .346$). However, there were significantly fewer DCX+ in sedentary two binge (S2) animals compared to their control (S0) and single binge (S1) counterparts respectively ($p < .0005$; $p < .0005$). This demonstrates that the sedentary two binge animals fared the worst on the number of new surviving neurons remaining. There was a significantly greater number of DCX+ cells in exercise controls (E0) compared to single binge (E1) and two binge (E2) exercisers respectively ($p < .0005$; $p < .0005$). However, there was no difference between number of DCX+ cells in E1 compared to E2 ($p = .189$). There was no significant correlation between number of DCX+ cells and mean distance run (See Fig. 20, $r^2 = -.002$, $p > .05$). Finally, there was a significant positive correlation between the number of DCX+ cells and number of granule cells (See Fig. 21, $r^2 = .452$, $p < .01$).

Discussion

This study is the first to repeat the four-day binge model of alcohol dependence on the sixth day following an initial four-day binge period. Additionally, this study is also the first to investigate voluntary wheel exercise as a means for aiding brain recovery following repeated binge alcohol exposure. Finally, the current study was the first to investigate chronic, binge alcohol induced changes to the hippocampus following repeated binge alcohol exposure 35 days following the last dose of alcohol.

The present study yielded several novel findings. First, we show that despite a single binge episode having no effect on the early neuronal marker doublecortin (DCX), a

second binge significantly reduced the number of DCX+ cells compared to single-binge animals and controls. Second, as has been previously shown, exercise increased the number of DCX+ cells in control animals (Uda, Ishido, Kami, & Masuhara, 2006), however there was no increase in DCX+ cells between sedentary and exercising single binge animals 35 days after the last dose of ethanol, demonstrating a binge induced suppression of the neurogenic effect of exercise. Third, we show that two binge episodes had no effect on exercising animals, indicating that exercise returned the number of DCX+ cells to control levels. Fourth, we show that sedentary animals had a detectable loss of granule cells in the dentate gyrus 35 days after a single 4-day binge alcohol exposure. We had previously reported a significant loss of granule cells immediately following 4 days of binge alcohol consumption (Leasure & Nixon, 2010), however this is the first time a significant loss of fully differentiated dentate gyrus granule cells five weeks following the last dose of alcohol has been reported. A second binge episode also resulted in a significant loss of granule cells compared to controls, although despite what we predicted, there was no significant further loss of granule cells compared to a single binge alcohol exposure. Finally, we show that moderate voluntary exercise following both a single and second binge alcohol exposure was neurorestorative of the loss of dentate gyrus granule cells. Previously we had reported that exercise prior to 4 days of binge alcohol consumption was neuroprotective of dentate gyrus granule cells (Leasure & Nixon); here we demonstrate exercise is neurorestorative of granule cells in the dentate gyrus.

Neurodegeneration from alcohol consumption is widely reported to occur during intoxication in this model (Crews et al., 2004; Leasure & Nixon, 2009), as well as various

other models of AUDs using different indicators of cell death such as FluoroJade B (FJB), amino cupric silver stain, necrosis, and pyknosis (Collins et al., 1996; He et al., 2005; Herrera et al., 2003, Obernier et al., 2002a, b). In binge models, brain damage increases progressively during intoxication, with peak damage occurring just after the last dose of alcohol (Obernier et al., 2002a; He et al., 2005). Neurodegeneration occurring during intoxication is also supported in human studies showing that the recency and frequency of heavy drinking are the best indicators of alcohol-induced brain damage (Parsons, 1987; Sullivan & Pfefferbaum 2005). The damage resulting from binge alcohol consumption has been found to be caused by its effect on cell death and cell birth; inhibition of proliferation and survival of neural progenitor cells residing in the subgranular zone (SGZ) of the dentate gyrus (Nixon & Crews, 2002, 2004; He et al., 2005; Morris et al., 2010). The disruption of neurogenesis in the hippocampus has been associated with deficits in hippocampal dependent learning and memory seen in chronic alcoholics (Shors et al., 2001), and implicated in clinical disorders such as major depression (Santarelli et al., 2003).

Despite damage occurring during intoxication, significant improvements are seen during abstinence from alcohol in both cognitive functioning, and increases in gray and white matter (Sullivan et al., 2000a, 2000b; Carlen et al., 1978; Pfefferbaum et al., 1995; O'Neill et al., 2001). The regeneration that occurs during abstinence is believed to be a direct response to the cell death and inhibition of cell genesis that occurs during intoxication. In the current model of binge consumption model, the first day following intoxication has increases in cell proliferation in the hippocampal dentate gyrus, with two large proliferative bursts occurring on the second and seventh day following the last dose

of ethanol, which have been shown to primarily differentiate into microglia and new neurons respectively (Nixon et al., 2008). This reactive recovery has been shown in other models of brain insult, where lesions caused increases in cell proliferation in damaged areas but also in projection areas of the dying neurons (Hailer, Grampp, & Nitsch, 1999; Ernst & Christie, 2006).

Despite previous work indicating significant recovery occurring during the first week following binge alcohol consumption, the present study discovered alcohol induced chronic deficits persist over a month after the last dose of alcohol. Where we previously reported a 16% loss of dentate gyrus granule cells immediately following a single binge episode (Leasure & Nixon, 2010), the current study showed that the binge-induced loss of granule cells persisted 35 days later. We show that ethanol administration produced a significant 10% and 15% loss of dentate gyrus granule cells in S1 and S2 animals, respectively, versus S0 rats. It is possible that even after five weeks there was not enough time for newly generated granule cells to fully differentiate and replace those lost during intoxication. Comparing the 16% loss of granule cells we previously reported immediately following the four-day binge to the 10% loss we see in the current study five weeks post-binge shows some recovery was made, however not enough to return to pre-binge levels. Although not significant, a second binge episode resulted in fewer remaining dentate gyrus granule cells than a single binge episode. This may be an indication that a second binge episode disrupted the on-going recovery process following the first episode, resulting in an increased amount of brain damage. It is also possible that a single binge weakens existing progenitor cell's ability to divide and mature into mature hippocampal neurons, and a subsequent second binge impairs them further. This is

supported by the presence of significantly fewer DCX+ cells in the dentate gyrus of S2 compared to S1 and S0 rats. In fact, the second binge was purposely timed on the sixth day following the last dose of alcohol from the first binge in an attempt to disrupt on-going recovery, specifically to inhibit the neurogenic burst known to occur on the seventh day of abstinence. The neurogenic burst on the seventh day following the first binge may be vital for the recovery process, and thus if it was inhibited in rats experiencing a second binge, they would have even fewer newly generated neurons to migrate and replace those lost after repeated binge episodes. If and how the second binge disrupted recovery and the seventh day neurogenic burst after a single first binge requires further investigation. Additionally, it is possible that the typical recovery period after a single binge may not follow the same timeline after second binge and thus requires further investigation.

The focus of the current study was to investigate the effects of exercise on repeated binge alcohol induced damage in the hippocampus. The brain displays a remarkable ability for self-recovery during abstinence from alcohol, therefore it was believed exercise would provide an effective method of enhancing the recovery process. In fact, exercise before and after brain injury in many different models of neurodegeneration, has been shown to be both protective and restorative of the brain (Carro et al., 2001). It is important to note that too much or too vigorous exercise can actually be counterproductive to brain health (Leasure & Jones, 2008), immediately following an injury in particular (Griesbach et al., 2004), and can actually lead to fewer remaining granule cells (Leasure & Nixon, 2010). In the current study, animals were monitored to ensure distance ran remained at moderate, beneficial levels to prevent overexertion. Moderate exercise is particularly effective in situations where hippocampal

neurogenesis is disrupted or inhibited because of pro-neurogenic properties. For example, exercise has been shown to be neuroprotective when it occurs prior to stroke (Hayes et al., 2008; Stummer et al., 1994), and neurodegenerative diseases such as Parkinson's (Zigmond et al., 2009; Smith & Zigmond, 2003), Alzheimer's (Laurin et al., 2001; Friedland et al., 2001), Amyotrophic Lateral Sclerosis (ALS) (Kaspar et al., 2005; McCrate & Kaspar, 2008), and Multiple Sclerosis (Prakash et al., 2009). Additionally, exercise is neurorestorative when it occurs after insults to the brain such as traumatic brain injury (TBI) (Griesbach et al., 2004; Itoh et al., 2011), irradiation (Naylor et al., 2008), and even dysfunction associated with HIV (Lee et al., 2011).

For alcohol specifically, exercise rehabilitates various neurological outcomes in rodent models of fetal alcohol syndrome (Christie et al., 2005; Thomas et al., 2008) and rescues reductions in hippocampal cell proliferation after prenatal ethanol exposure (Redila et al., 2006) and when done concurrently with alcohol self-administration (Crews et al., 2004). We have previously shown that exercise prior to binge alcohol exposure was neuroprotective of the loss of dentate gyrus granule cells, reduced cell death in the dentate gyrus and entorhinal-perirhinal cortex, and decreased behavioral sensitivity to ethanol intoxication (Leasure & Nixon, 2010). In the present study, we found that 4 weeks of voluntary exercise restored the dentate gyrus after repeated binge-induced loss of granule cells. Exercise alone did not increase the number of granule cells in the hippocampus, consistent with what we have previously found (Leasure & Nixon, 2009). Exercise lead to a large increase in DCX+ cells in control animals as was expected, however, a single binge episode resulted in a binge-induced suppression of the neurogenic effect exercise. Interestingly though, exercise was beneficial in animals

experiencing a second binge episode, resulting in similar numbers of DCX+ cells to single binge exposed animals. Our results suggest that mild exercise may be best for restoring the granule cell layer to control levels after repeated binge alcohol exposure, however, exercise only bolsters the survival of newly divided immature neurons in the dentate gyrus to control levels when significant cell loss has already occurred.

We have previously shown that social isolation suppresses exercise-induced proliferation of hippocampal progenitor cells in female rats (Leasure & Decker, 2009), an effect only reversed after 48 days of exercise (Stranahan et al., 2006) however, exercise occurred concurrently with the social isolation. The possibility that an increased number of days exercising would reverse the binge-induced suppression of neurogenesis warrants further investigation. A similar alcohol induced suppression of neurogenic benefits has been shown after introducing environmental enrichment in a model of moderate fetal alcohol exposure (FAE) (Choi et al., 2005). Environmental enrichment, like exercise, leads to an increase in on-going production of new neurons from progenitors within the adult dentate (Kempermann et al., 1997). While Choi and colleagues (2005) found no effect of FAE on neurogenesis and progenitor survival, it blocked both the neurogenic response to an enriched environment and impaired the survival of progenitor cells four weeks later, an effect found in both female and male mice. Unlike the current study, environmental enrichment did not lead to increases in granule neurons after FAE, however FAE alone did not lead to the significant loss of mature granule cells that binge alcohol exposure did. Llorens-Martín and colleagues (2006) previously found that increases in the number of newly generated immature neurons created after exercise was directly correlated to the number of existing mature granule neurons; more immature

neurons were found after exercise in animals with a larger dentate gyrus, whereas no changes were observed in those with a smaller DG. It is possible that at the time exercise generated the new immature neurons labeled by DCX, the granule cell layer of binge treated animals had yet to be restored to the levels of control animals.

The mechanism by which binge alcohol exposure suppresses the neurogenic benefits of exercise is unknown at this time. The formation of new neurons relies on four processes of hippocampal progenitors: proliferation, differentiation, migration and survival (Kempermann et al., 2004). It is widely reported that binge alcohol exposure not only impairs progenitor cell proliferation during intoxication, but also significantly reduces progenitor cell survival and blunts the growth of the dendritic arbor (Nixon & Crews, 2002, 2004; He et al., 2005; Crews et al., 2006). Thus it is possible that a single binge exposure disrupted the availability of both competent progenitors and the microenvironmental cues that control the progenitor fate needed for neurogenesis (Fabel et al., 2003), resulting in an environment not conducive for exercise to exert its beneficial effects on neurogenesis. It is now thought that the neurodegeneration and atrophy that occurs during alcohol intoxication is due to the loss of pro-health neurotrophic signaling combined with the induction of oxidative stress proinflammatory signals (see Crews & Nixon, 2009 for review). Ethanol activated proinflammatory enzymes result in oxidative stress, which sensitizes neurons to insults (Crews et al., 2004). The transcription factor nuclear factor *k*B (NF-*k*B) plays a significant role in inflammatory and immune response signaling, in addition to control of cell division and apoptosis (O'Neill & Kaltschmidt, 1997). In response to ethanol, NF-*k*B initiates a prolonged, pathological proinflammatory cascade that results in oxidative stress (Zou & Crews, 2005). On the other hand, the

transcription factor cAMP responsive element-binding protein (CREB) promotes neuronal survival, protecting neurons from excitotoxicity and apoptic cell death by regulating transcription of pro-survival factors (Lonze & Ginty, 2002; Mantamadiotis et al., 2002). Alcohol activates oxidative stress, which coincides with a loss of CREB prosurvival transcription (Crews et al., 2004). The 4-day binge model used in the present study results in a decrease in pCREB immunoreactivity during alcohol intoxication, with the most marked decreases occurring in brain regions showing degeneration (Bison & Crews, 2003). Alcohol induced reductions in levels of the CREB-regulated brain-derived neurotrophic factor (BDNF) in particular have been implicated as playing a role in alcohol-induced brain damage and coincides with sensitivity to neurodegeneration (Zou & Crews, 2006; Pandey et al., 2005a,b). The reductions of CREB and by association BDNF, and increases to proinflammatory signals, like NF-kB, are believed to be responsible for oxidative stress that results in alcoholic neurodegeneration (Crews & Nixon, 2009). The hippocampus in particular appears to be particularly vulnerable to alcohol-induced oxidative stress degeneration (Renis et al., 1996). This proinflammatory, anti-survival environment resulting from binge alcohol exposure could potentially be responsible for the impairment of the neurogenic response to exercise.

Nonetheless, exercise was able to restore the loss of dentate gyrus granule cells resulting from binge alcohol consumption, likely because its ability to reverse some of the negative effects of alcohol just mentioned. Previously, exercise has been shown to have the ability to attenuate oxidative damage (Radak et al., 2001), in addition to increase expression of pro-health and survival neurotrophins, such as BDNF, and their receptors (Farmer et al., 2004; Gomez-Pinilla et al., 1997; Li et al., 2008; Liu, et al., 2008; Neeper

et al., 1995; Oliff et al., 1998; Russo-Neustadt et al., 2000; Widenfalk., et al., 1999). Alcohol-induced decreases in BDNF have been correlated with alcohol-induced effects on new granule cell survival (Herrera et al., 2003; Nixon & Crews, 2002). Therefore it is possible that the increase in BDNF caused by exercise was able to enhance survival of new granule cells that were able to then mature and integrate into the existing hippocampal network, explaining the restoration of the granule cell layer in exercising binging animals in the current study. Conversely, despite these positive effects of exercise, it might not have been enough to overcome binge exposure's impairment of progenitor proliferation that resulted in the binge-induced suppression of the neurogenic effects of exercise. This is consistent with our previous findings that prior exercise was neuroprotective of the binge-induced loss of dentate gyrus granule cells, but not protective against the binge-induced decrease in progenitor cell proliferation. Future studies should examine the relationship between exercise and binge alcohol exposure and the contribution of BDNF.

In the current study, some interesting behavioral effects were also observed. First, animals going through a second binge alcohol exposure on average behaved significantly less intoxicated than during the first binge alcohol exposure, despite receiving more alcohol per day. Additionally, it took animals going through a second binge more doses to reach the same levels of intoxication than it did during the first binge. This demonstrated not only acute tolerance within the binge, but also the development of behavioral tolerance to the depressive effects of alcohol after a single binge. Despite appearing less intoxicated and receiving more alcohol, there was no difference in blood ethanol concentrations (BEC) between the first and second binge when assessed after the

morning dose on the third day of the binge. The lack of a significant difference between the BEC may be because on average, animals appeared similarly intoxicated before the first dose on the third day. This could also be explained by difference in body weight between the start of the binges, as when animals began the second binge, they were on average 6-7% lighter than at the start of the first binge. Ethanol absorption and distribution is affected by differences in body fat, with lower fat tending to cause lower BECs (Leasure & Nixon, 2009). Thus, it is possible that animals in the second binge actually were more intoxicated and had just developed behavioral tolerance to the sedative effects of alcohol. The development of tolerance is consistent with human patterns of excessive drinking which help promote dependence and further excessive drinking (Crews & Nixon, 2009).

The second interesting behavioral observation was that a second period of withdrawal resulted in the observation of significantly more severe spontaneous withdrawal symptoms. This was indicated by an observation of significantly higher mean and peak withdrawal symptoms, as well as a withdrawal period that started both sooner and lasted longer than the first withdrawal period. Additionally, seizure-like behavior was observed in 31% of animals experiencing a second withdrawal period, compared to 13% during the first withdrawal period. This is consistent with the human literature which indicates that successive periods of high alcohol intake and detoxification leads to increasingly more severe neurodegeneration and withdrawal symptoms, as well as the susceptibility to seizures (Becker & Hale, 1993; Branchey et al., 1971; Carrington et al, 1984; McCown & Breese, 1990, Duka et al., 2003). For both binges and subsequent period of withdrawal, mean intoxication score correlated with mean withdrawal, thus the

more intoxicated the animal was over 4 days, the higher the withdrawal severity on average. This was similar to previous reports of withdrawal severity and mean intoxication (Morris et al., 2010). Because withdrawal symptoms were observed at 30 minute intervals, it is possible that if a single seizure occurred it was missed, however our percentages of animals exhibiting seizure-like convulsions after a single binge was similar to those previously reported (Morris et al., 2010). This increased severity of withdrawal symptoms is also associated with increased feelings of anxiety and cravings in humans, contributing to the cycle of excessive drinking with the purpose of avoiding withdrawal symptoms (Duka et al., 2011).

We conclude that repeated binge alcohol consumption can result in a progressive loss dentate gyrus granule cells up to 35 days after the last dose of alcohol, an effect that can be reversed by exercise. However, binge consumption can suppress the neurogenic effects of exercise, potentially impairing both the proliferation and survival of neural progenitor cells in the hippocampus. Despite this, exercise has the potential to restore hippocampal integrity and the deficits to memory and cognition that result from alcohol-induced neurodegeneration. In particular, exercise has the potential to help cure depression and anxiety (McAuley, 1991), which are related to deficits in hippocampal integrity (Fuchs et al., 2004; Revest et al., 2009) and are highly comorbid with AUDs (Schuckit & Monteiro, 1988). It is possible that improving these negative mood states could help feelings of helplessness and the lack of control associated with alcoholism and potentially reduce alcohol consumption (Ussher et al., 2004). In fact, alcoholism treatment programs that incorporate aerobic exercise reduce alcohol consumption in number of drinks per day and percentage of days abstinent, while maintaining a high rate

of adherence to the program (Brown et al., 2009). In summary, the current study has implications in understanding the effects alcohol has on hippocampal integrity and the potential of exercise as a novel method for restoring deficits and treatment of AUDs.

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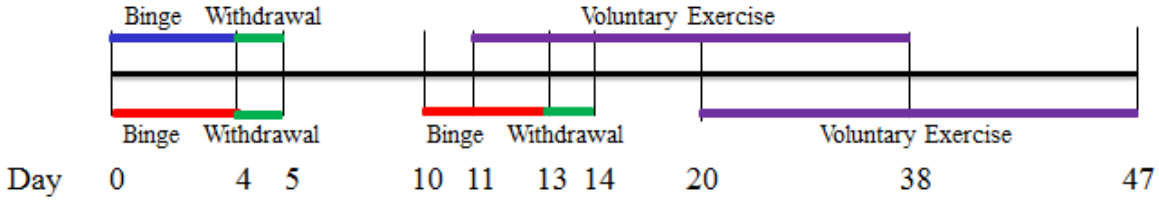

Figure 1. Timeline of the Study. Starting at time point 0, all animal began their respective diet regimens for four days. After the fourth day, binge animals are monitored for spontaneous withdrawal symptoms 10 hours to 26 hours after their last dose of alcohol. On day 10 (6 days after their last dose of alcohol), animals in the two binge group begin their second 4-day binge alcohol exposure, followed by a second 17 hour period of withdrawal monitoring. On day 11, control and single binge group animals begin 4 weeks of either voluntary exercise (5.5 hours a day) or remain sedentary in home cages. On day 20, two binge group animals behind 4 weeks of either voluntary exercise (5.5 hours a day) or remain sedentary in home cages. All animals are sacrificed 35 days after their respective final dose of alcohol.

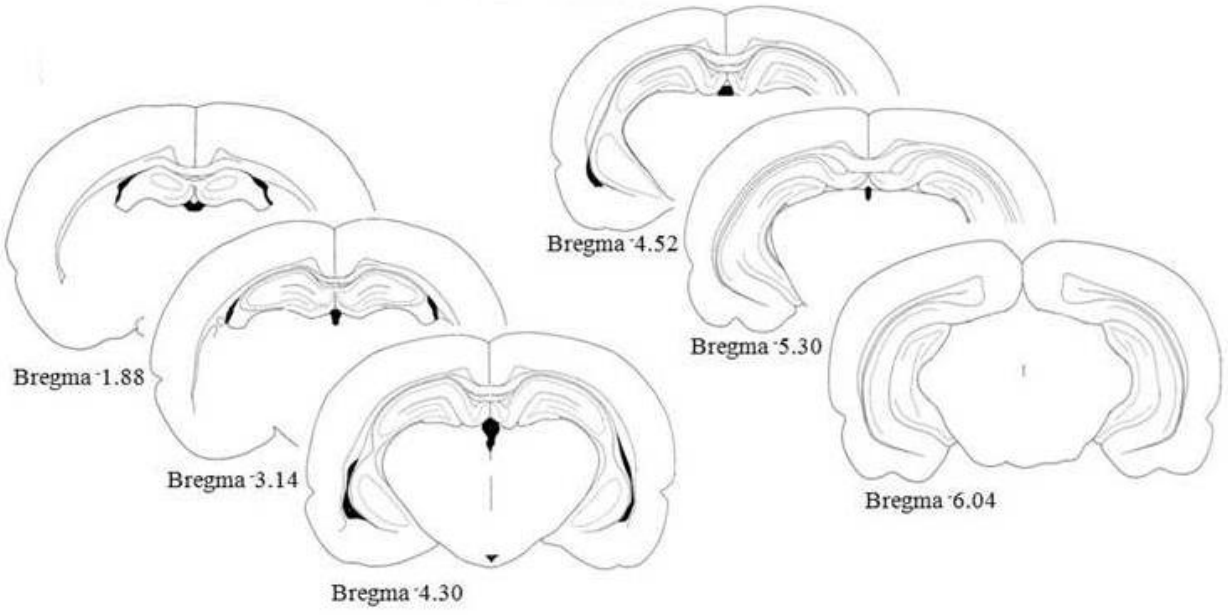

Figure 2. Coronal sections of the hippocampus illustrating where stereology was performed in the dentate gyrus of the hippocampus. The dentate gyrus was separately quantified for DCX+ cells and granule cells beginning at Bregma -1.88mm and ending at Bregma -6.10mm. These drawings were adapted from Paxinos and Watson (1998).

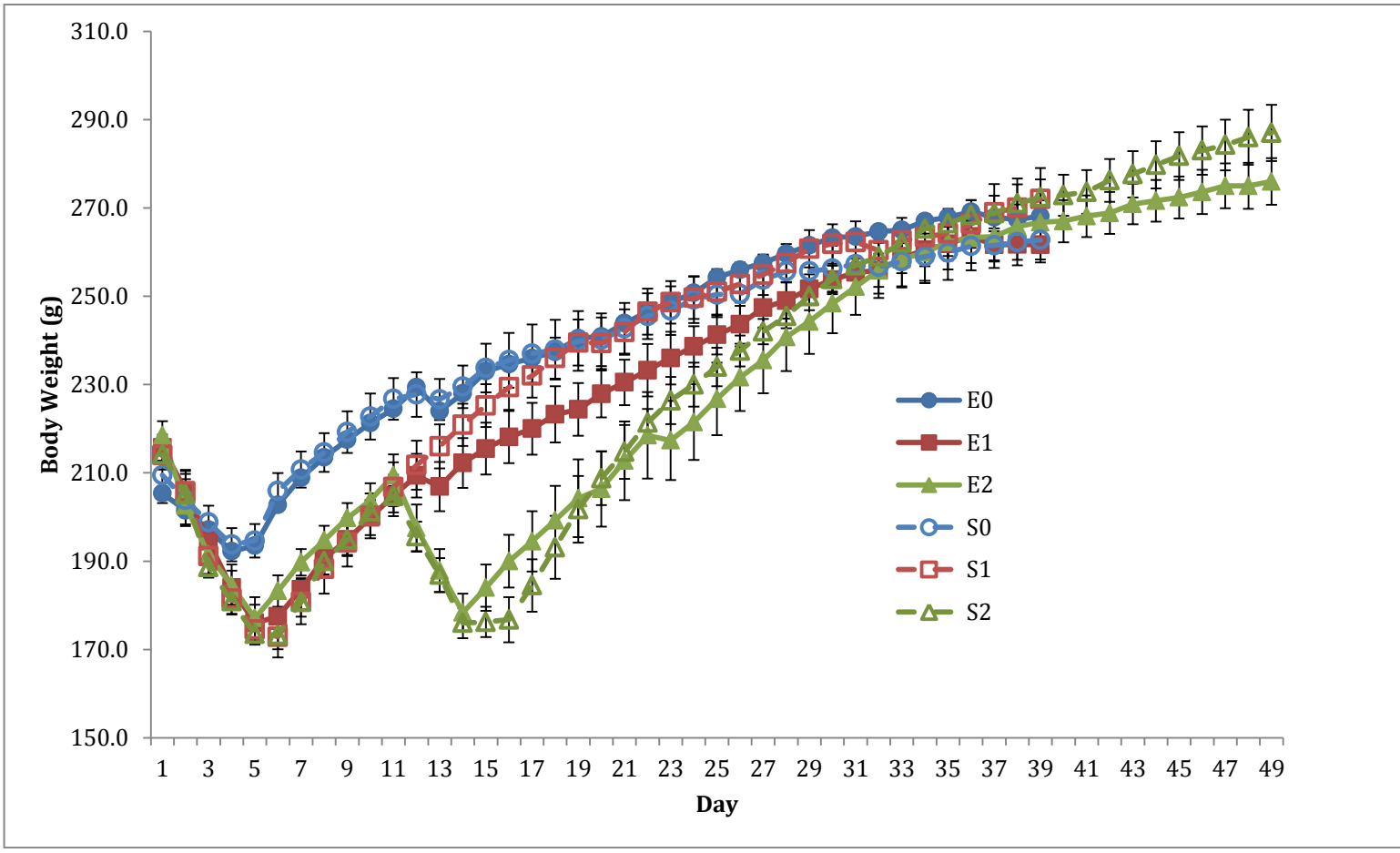

Figure 3. Body weights of each group across the entire experiment. All rats gained weight across the course of the experiment. At day 5 is the large dip in body weight following the first binge, with the loss in weight being significantly greater in alcohol treated animals compared to controls. At day 14 is the dip in body weight for S2/E2 animals following the second binge, significantly less than control and single binge animals at the same time.

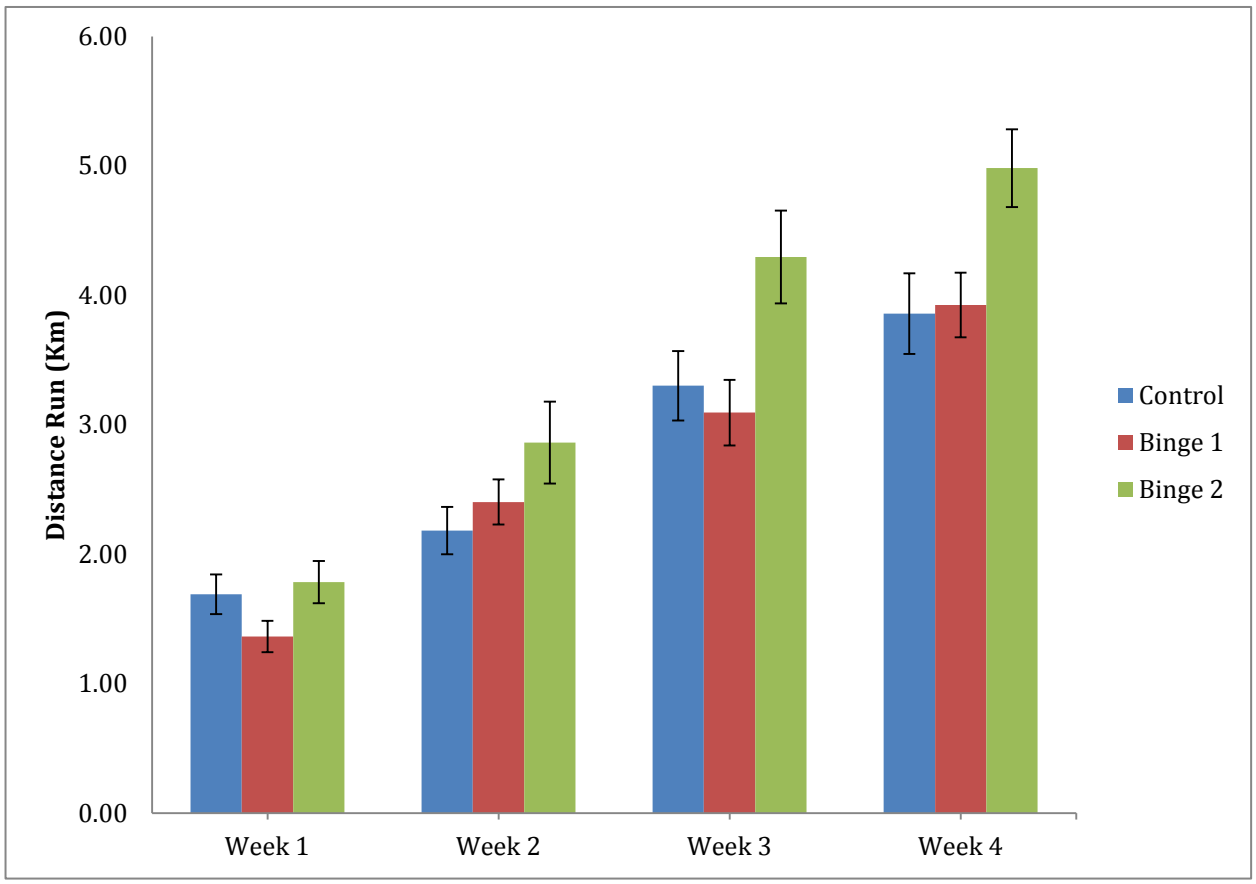

Figure 4. Distance run by exercise groups over the course of the experiment. There was no difference for distance travelled between exercising rats that received isocaloric control diet and those receiving a single or two binge exposures. All rats increased distance run overtime.

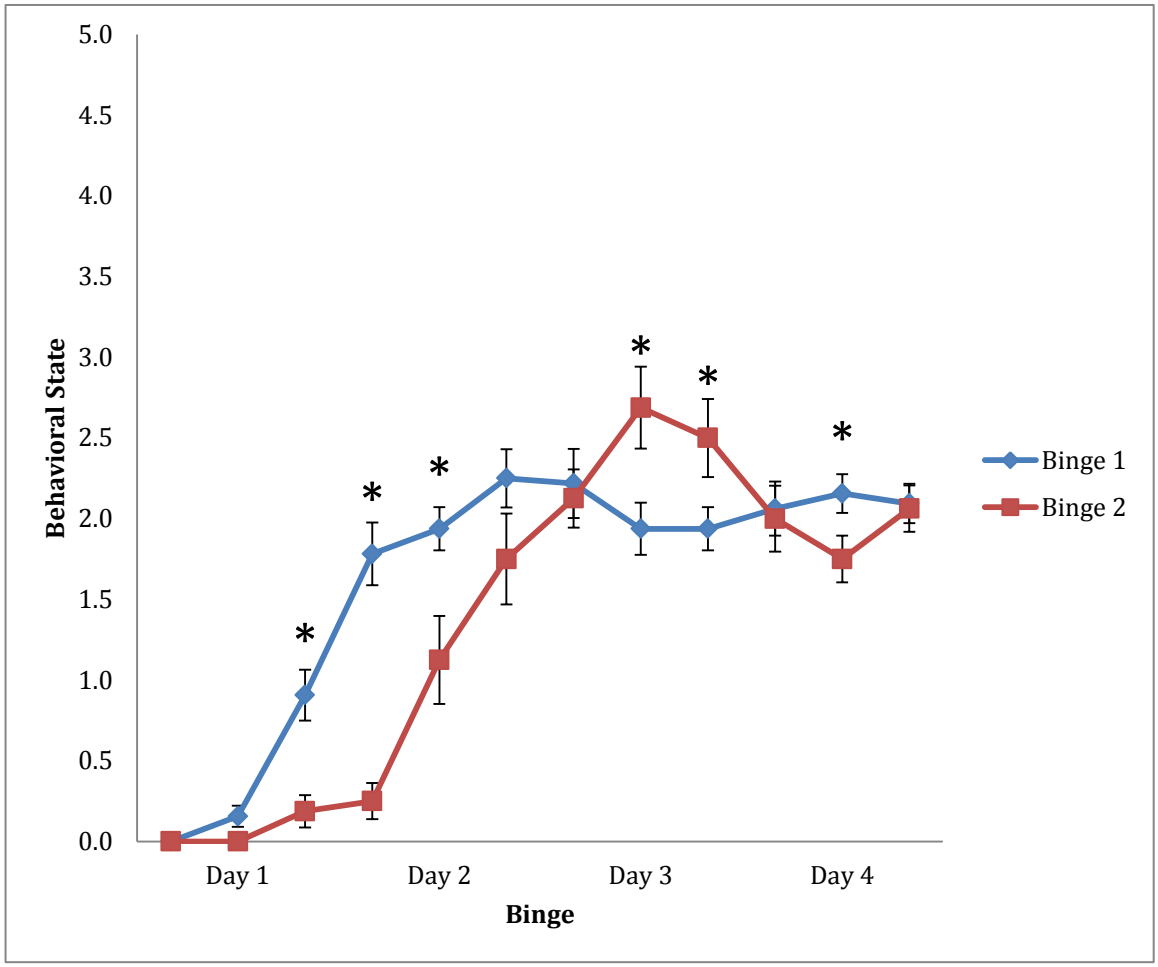

Figure 5. Behavioral intoxication across the four days of binge 1 and binge 2. Across the four days, binge 1 animals displayed significantly greater intoxication behaviors than binge 2 animals. Intoxication increased over time. * $p < 0.05$, significantly different from binge 1.

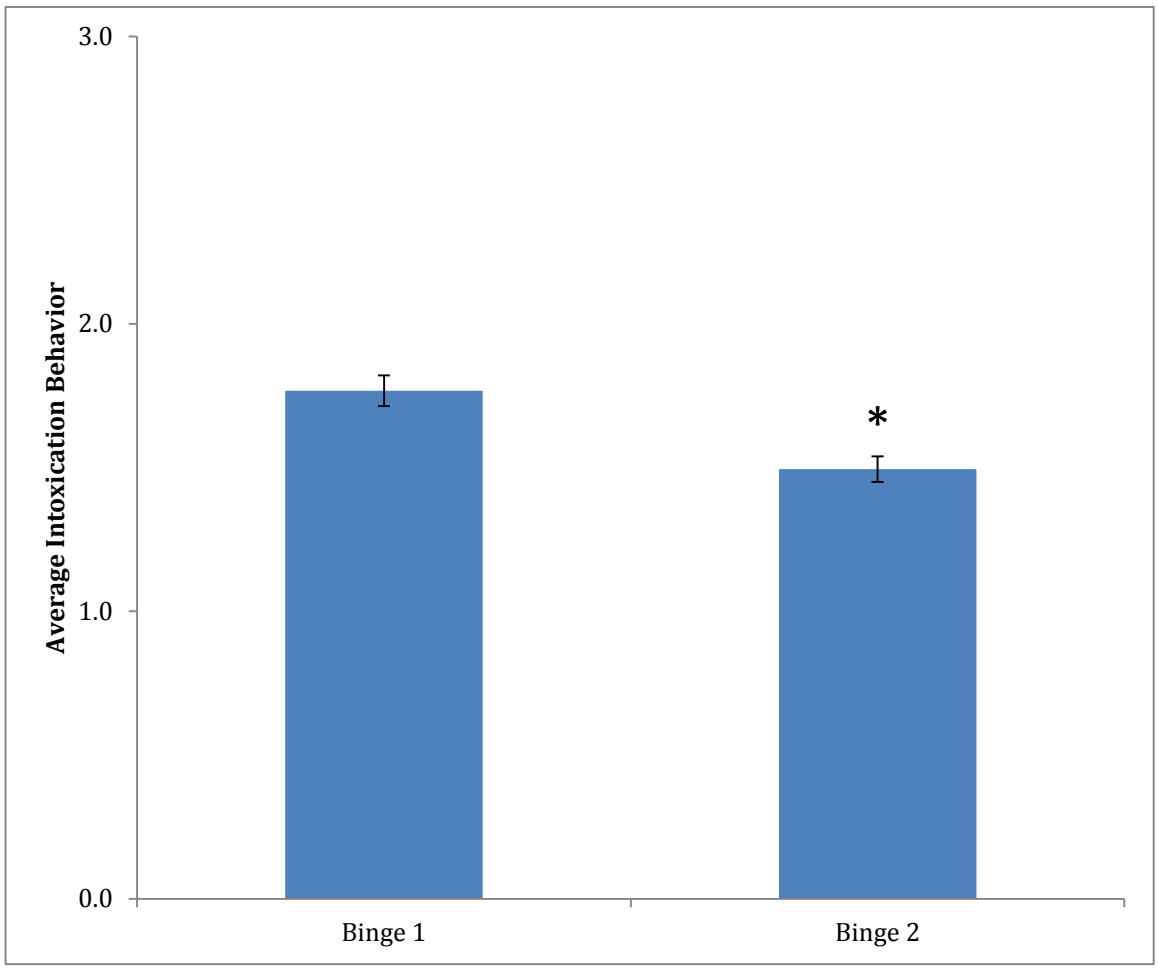

Figure 6. Mean intoxication collapsed across four days. Across the four days, binge 1 animals displayed significantly greater intoxication behaviors than binge 2 animals. * $p <$ 0.0005, significantly different from binge 1.

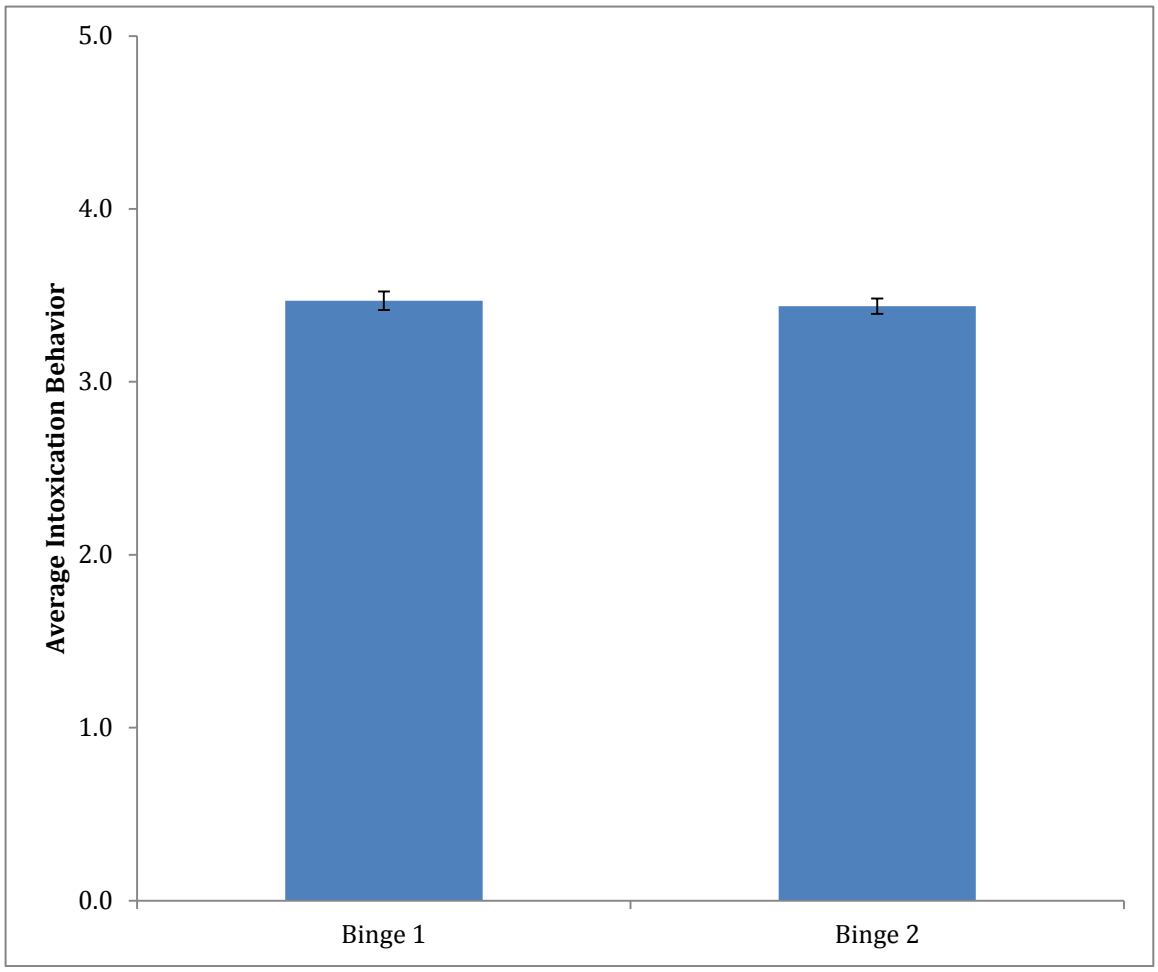

Figure 7. Peak intoxication (highest intoxication score achieved across four days) over four days. There was no difference between the peak intoxication score between Binge 1 and Binge 2.

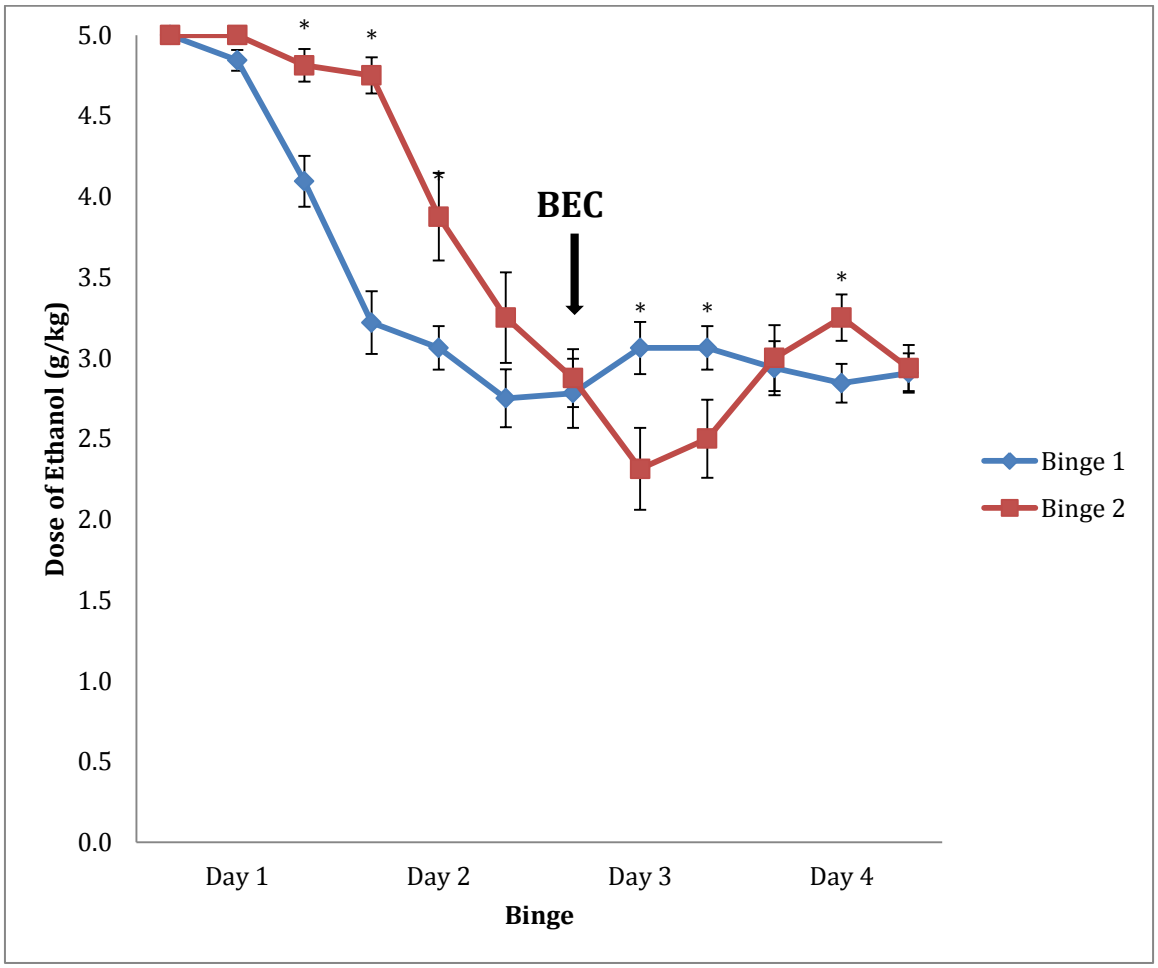

Figure 8. Dose of alcohol across the four days of binge 1 and binge 2. Across the four days, binge 1 animals consumed less alcohol than binge 2 animals, despite behaving significantly more intoxicated. The gradual decrease in dose over time signifies the animals becoming more intoxicated over time, and then the increase in dose demonstrates the development of acute tolerance. Arrow shows the point at which tail bloods were taken. * $p < 0.05$, significantly different from binge 1.

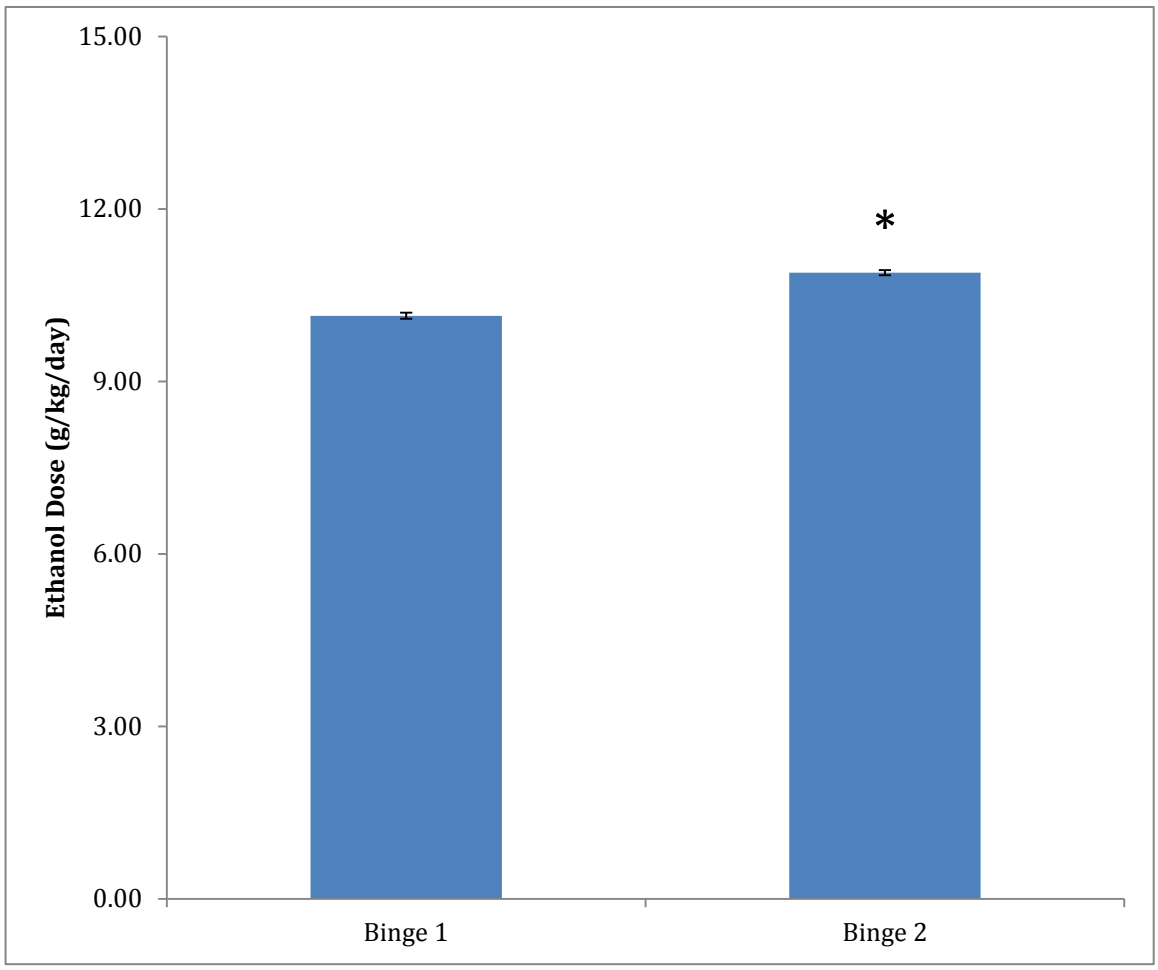

Figure 9. Mean dose of ethanol per day. Despite appearing less intoxicated, binge 2 animals received significantly more alcohol per day than binge 1 animals. * $p < 0.0005$, significantly different from binge 1.

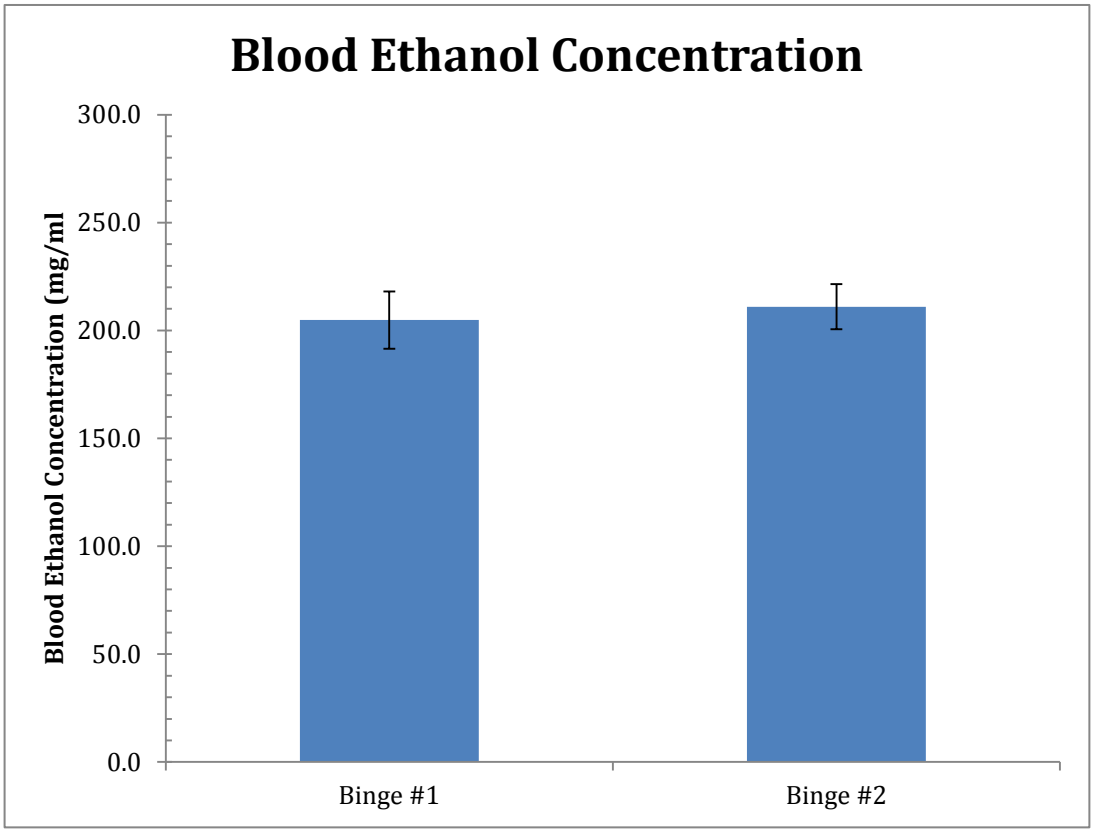

Figure 10. Mean blood ethanol content. Despite binge 1 animals appearing more intoxicated, and binge 2 animals actually receiving more alcohol per day, there was no significant difference between binge 1 and binge 2 for blood ethanol content assessed on the third day of each respective binge.

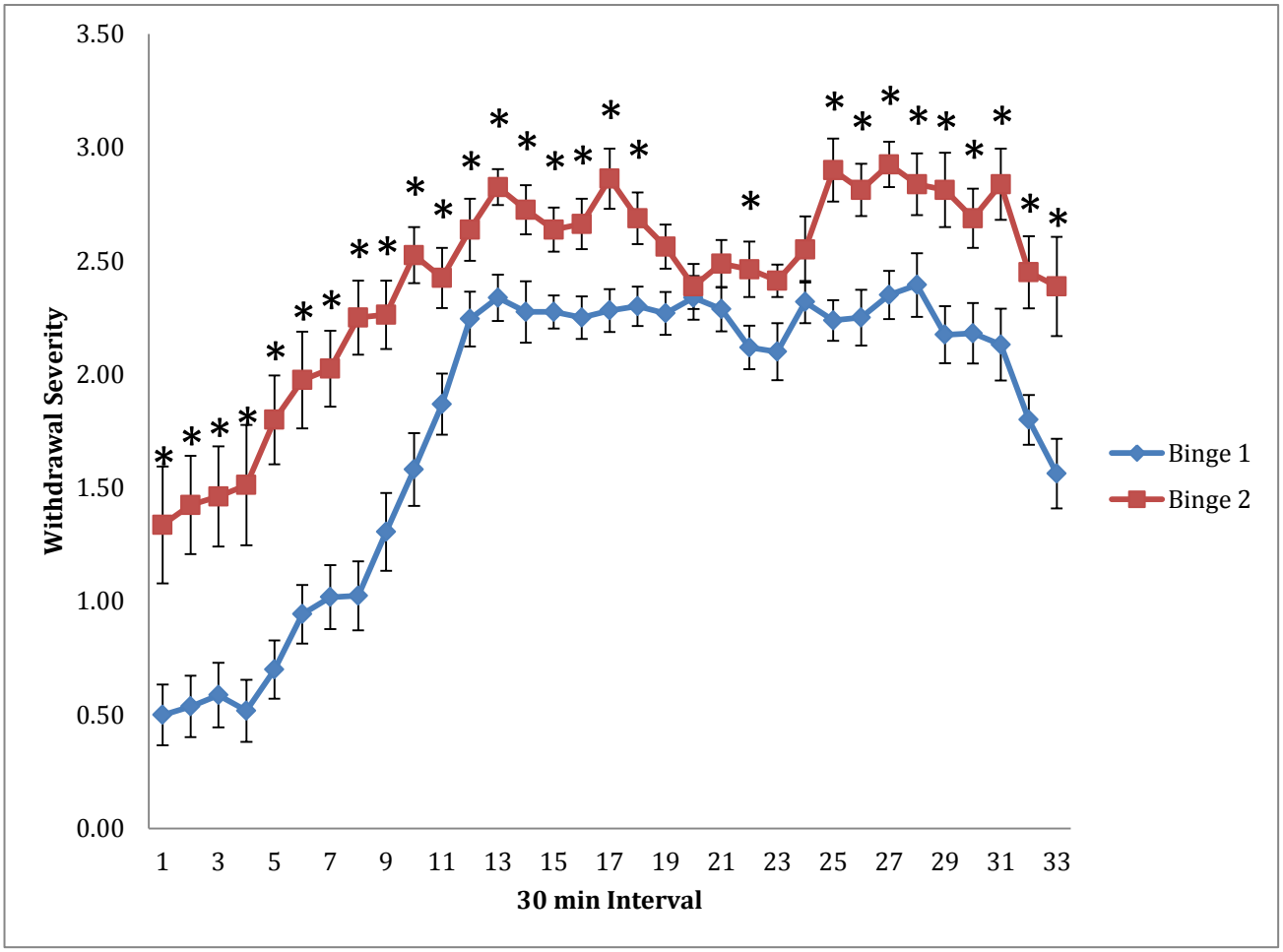

Figure 11. Spontaneous withdrawal behaviors monitored over 17 hours. Across the 17 hours withdrawal behavior was monitored, binge 2 animals displayed significantly more severe symptoms than binge 1 animals. The severity of withdrawal symptoms also increased over time. * $p < 0.05$, significantly different from binge 1.

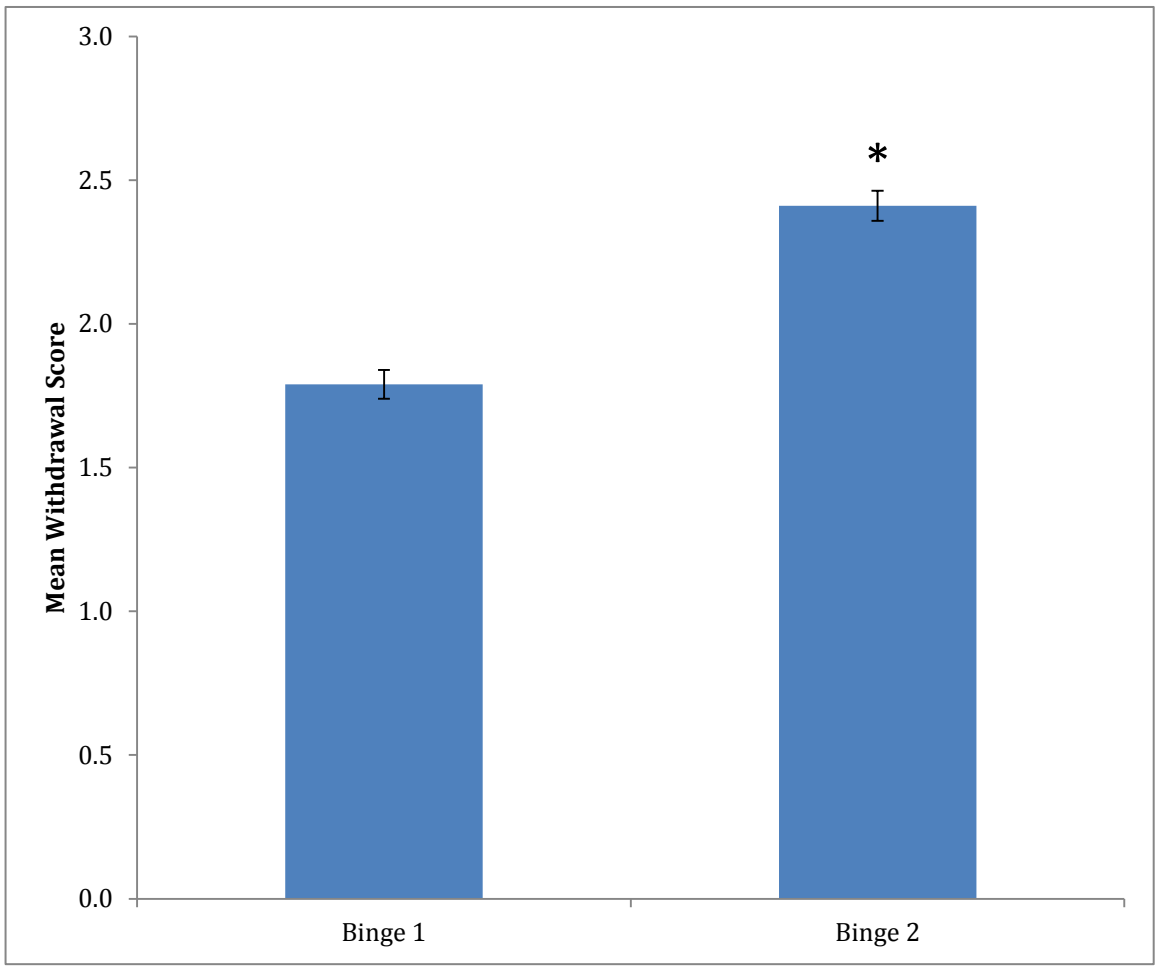

Figure 12. Average withdrawal score collapsed across 17 hours. The average withdrawal score collapsed across 17 days was significantly greater in binge 2 compared to binge 1. * $p < 0.0005$, significantly different from binge 1.

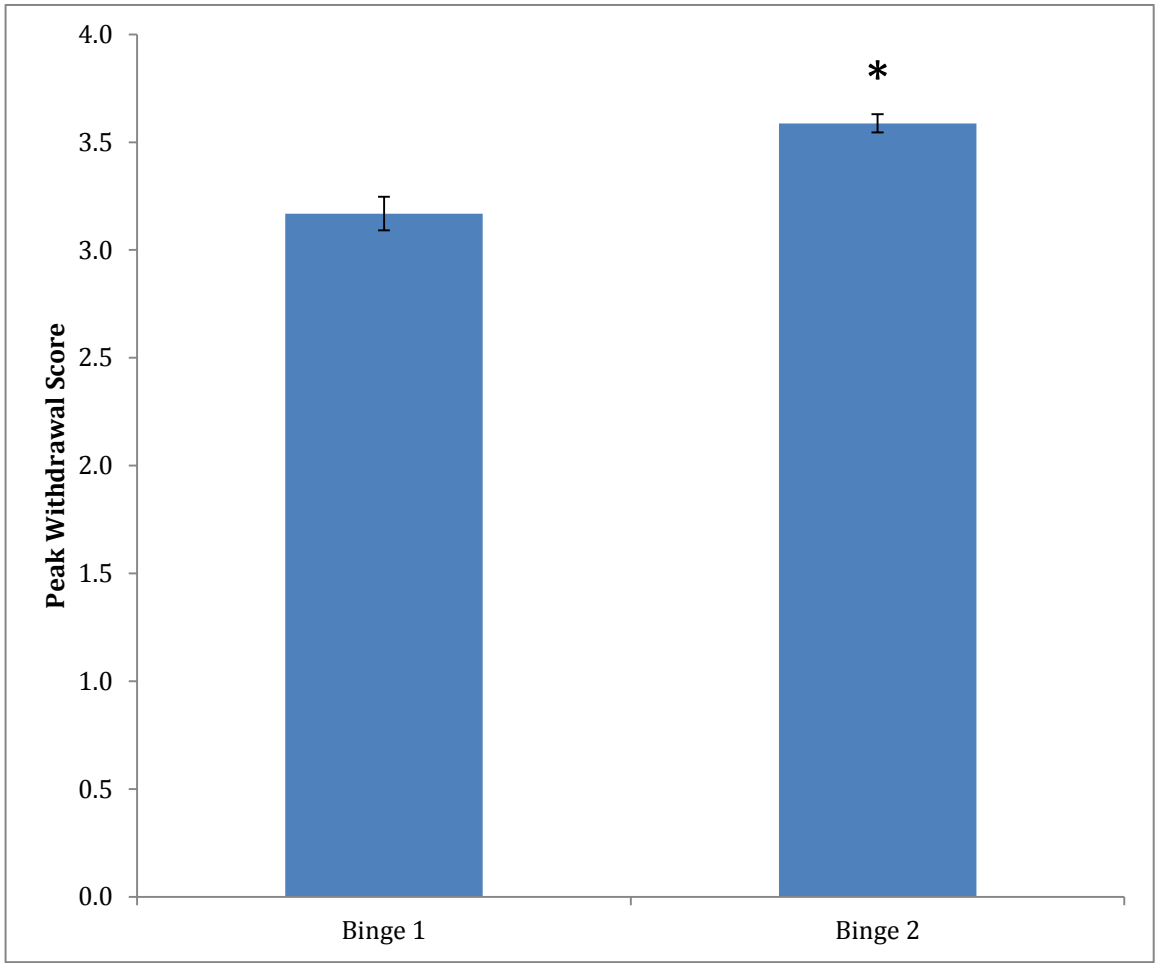

Figure 13. Peak withdrawal score (highest withdrawal score achieved across 17 hours). The peak withdrawal score was significantly greater in binge 2 animals compared to binge 1 animals. * $p < 0.0005$, significantly different from binge 1.

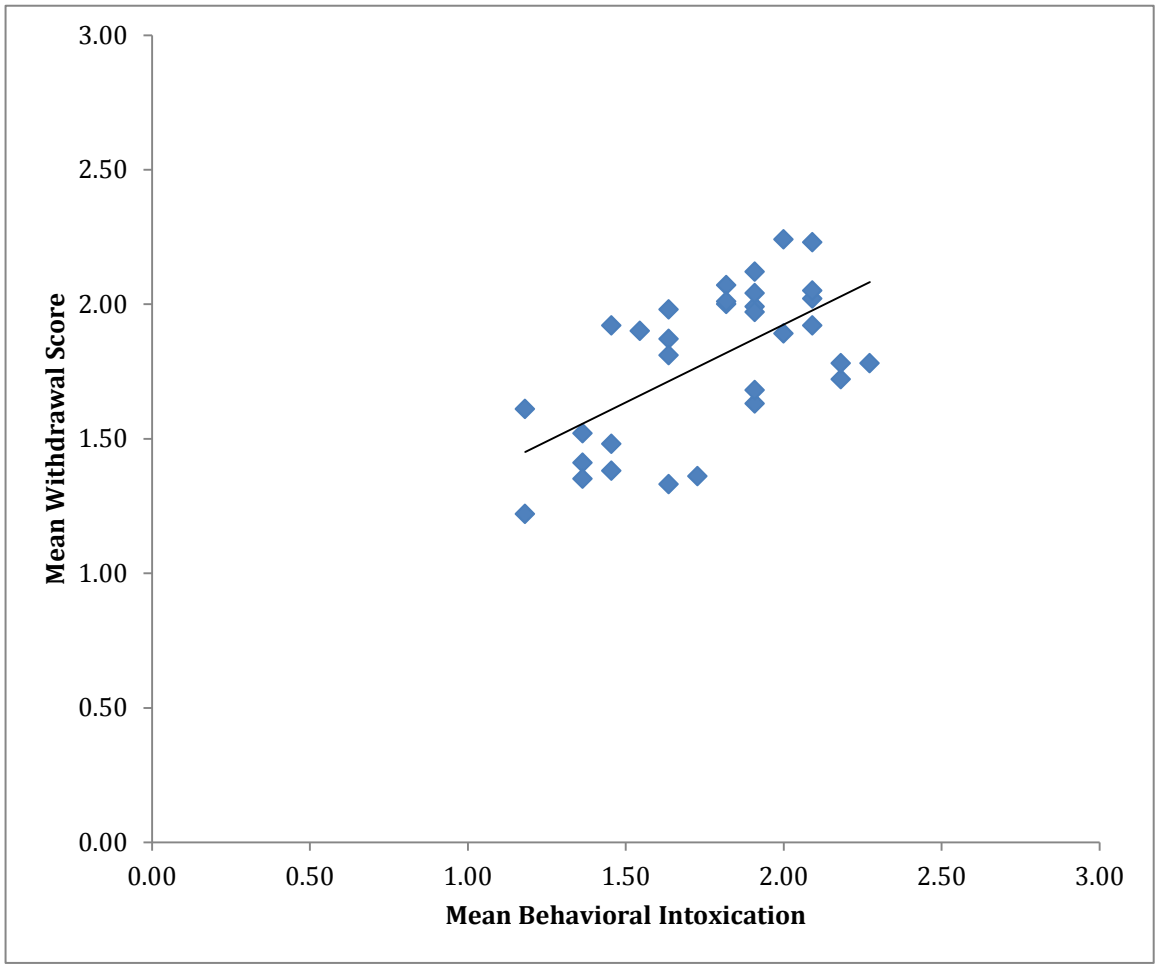

Figure 14. Correlation between average intoxication and average withdrawal for binge 1. The average intoxication and average withdrawal scores for binge 1 were positively correlated $r^2 = .617$, $p < 0.05$.

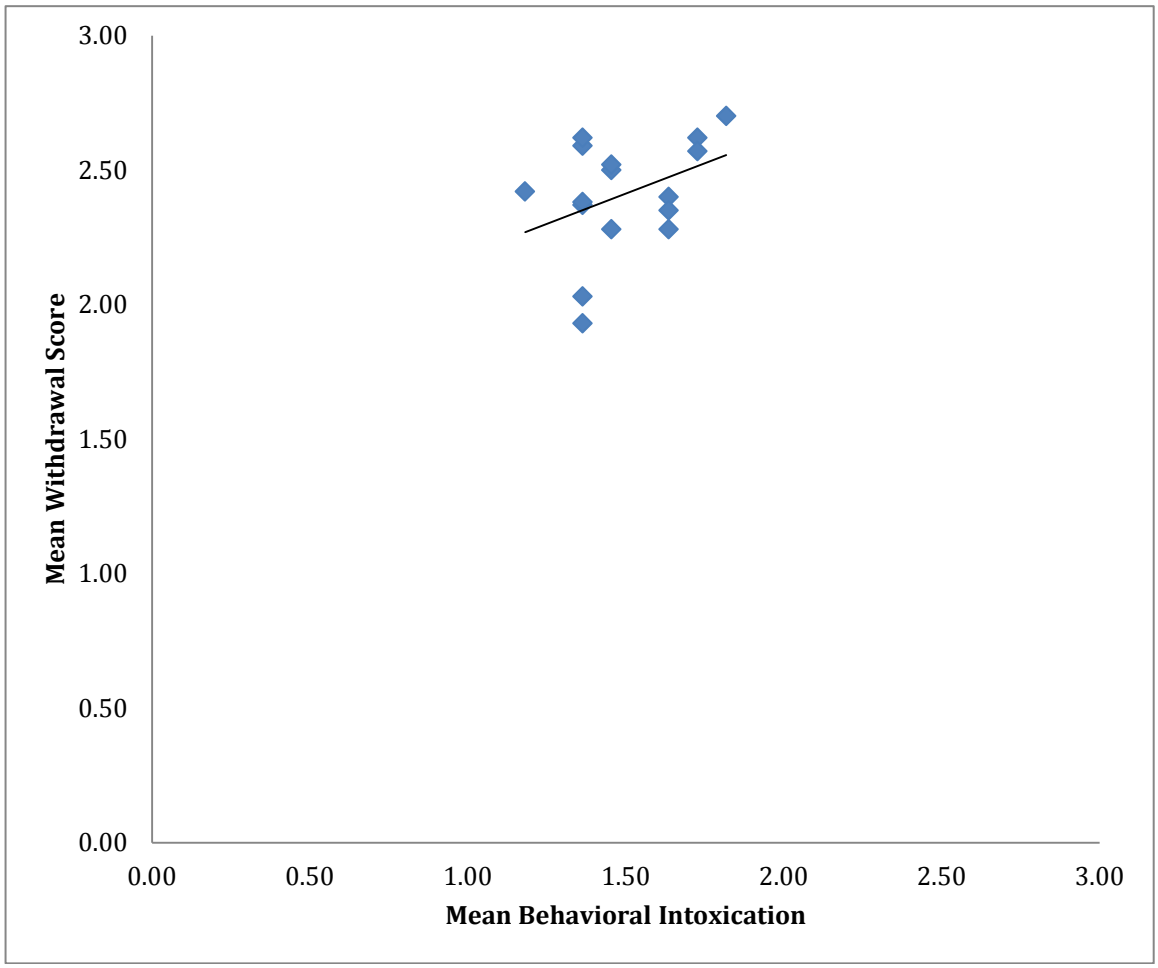

Figure 15. Correlation between average intoxication and average withdrawal for binge 2. The average intoxication and average withdrawal scores for binge 2 were positively correlated $r^2 = .442$, $p < 0.1$.

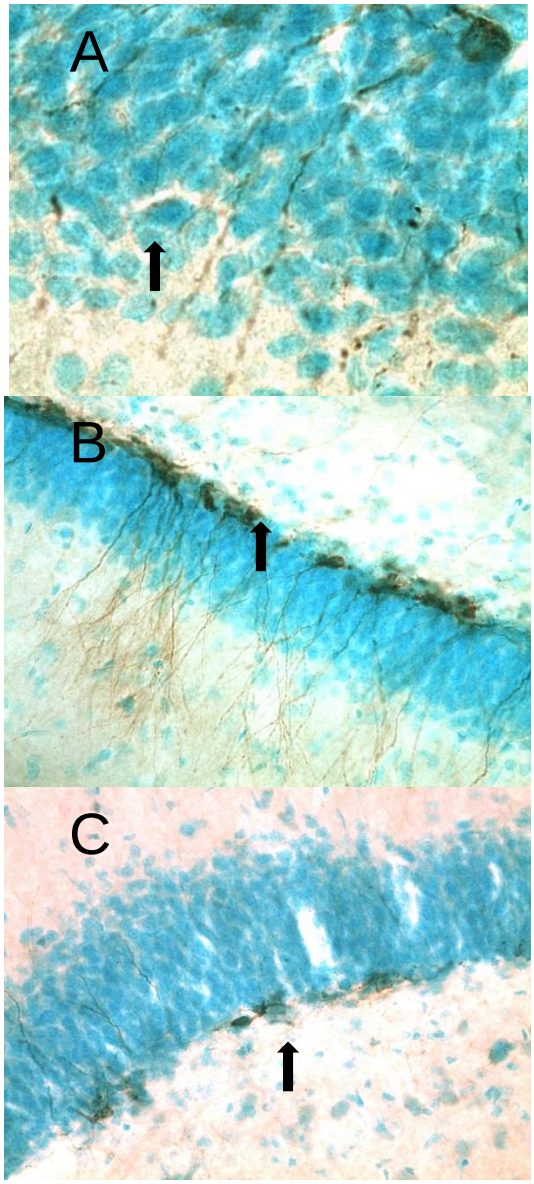

Figure 16. Granule (A) and DCX+ (B, C) cells as counted via stereological methods. Representative photograph of the granule cell layer of the dentate gyrus; arrow indicates a single granule cell (A). Exercise resulted in a significant increase in DCX+ cells (B) in control diet animals, while second binge alcohol exposure lead to a significant decrease in DCX+ cells in sedentary animals (C) compared to sedentary controls.

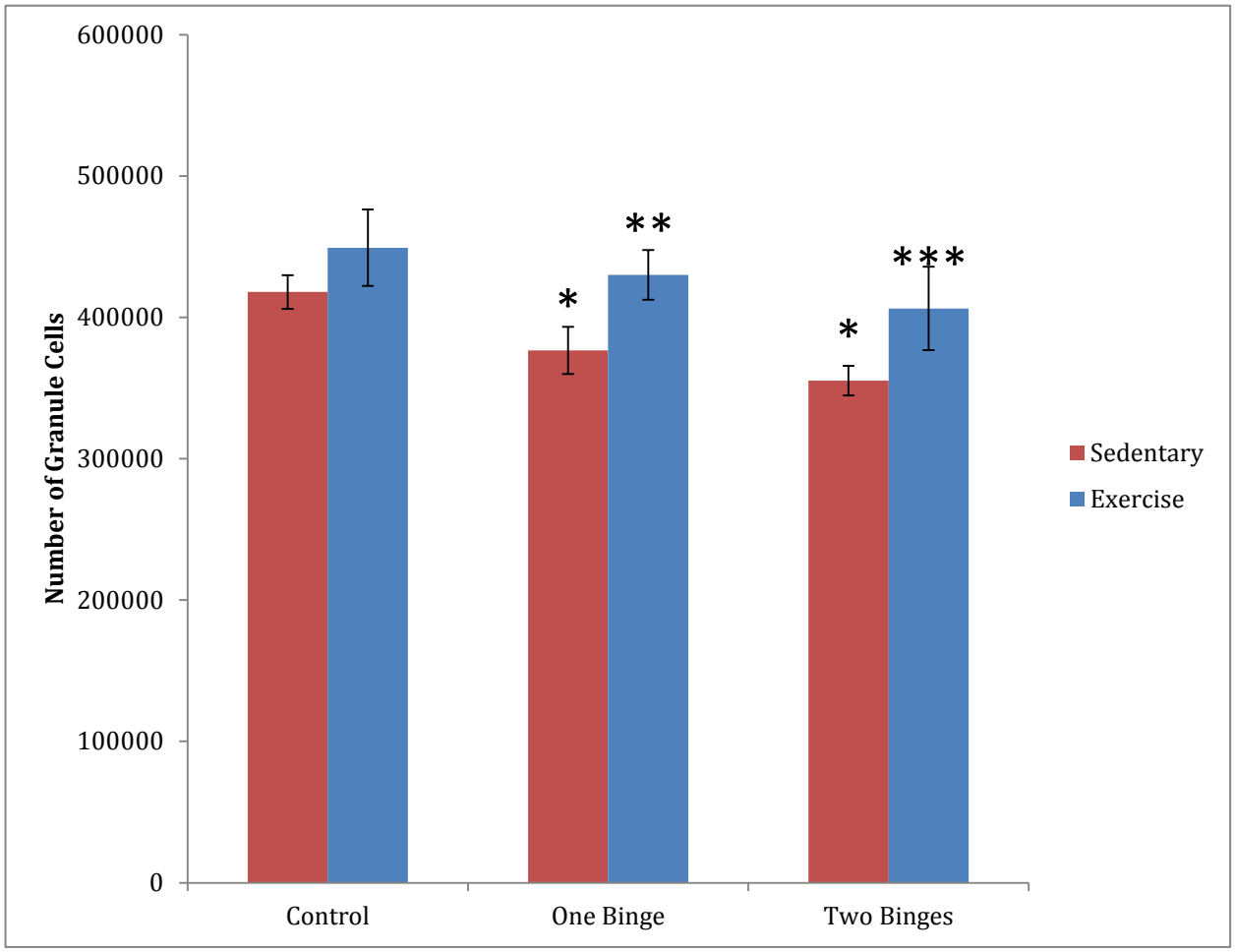

Figure 17. Repeated binge alcohol consumption and exercises effects on granule cells. Repeated binge alcohol exposure led to a decrease in the number of granule cells between S0 and S1, and between S0 and S2. Exercise was protective of the granule cell layer in a single and second binge. * $p < 0.05$ post hoc comparison to S0, ** $p < 0.005$ post hoc comparison to S1, *** $p < 0.005$ post hoc comparison to S2.

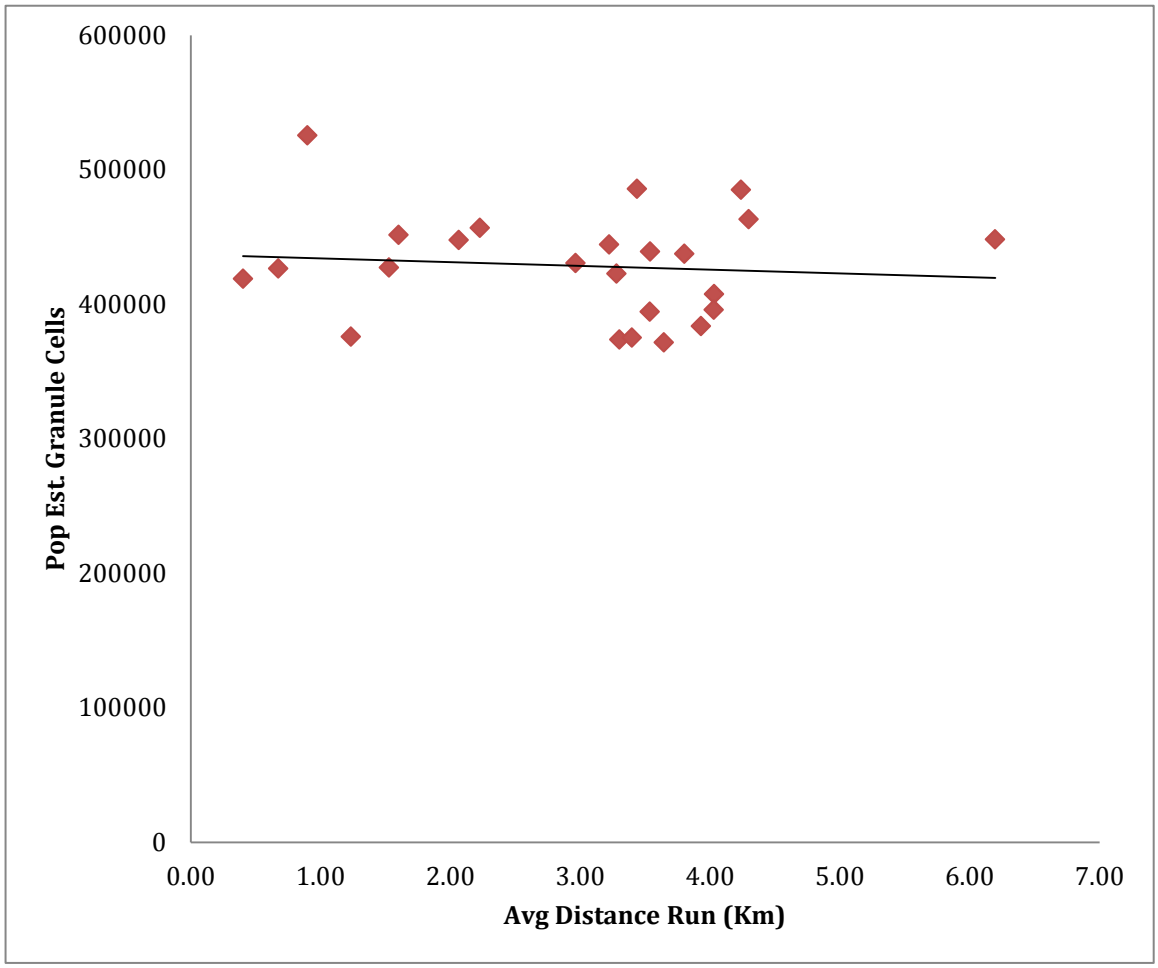

Figure 18. Correlation between mean distance run and number of granule cells. There was no significant correlation between mean distance run and number of granule cells r^2 = -.097.

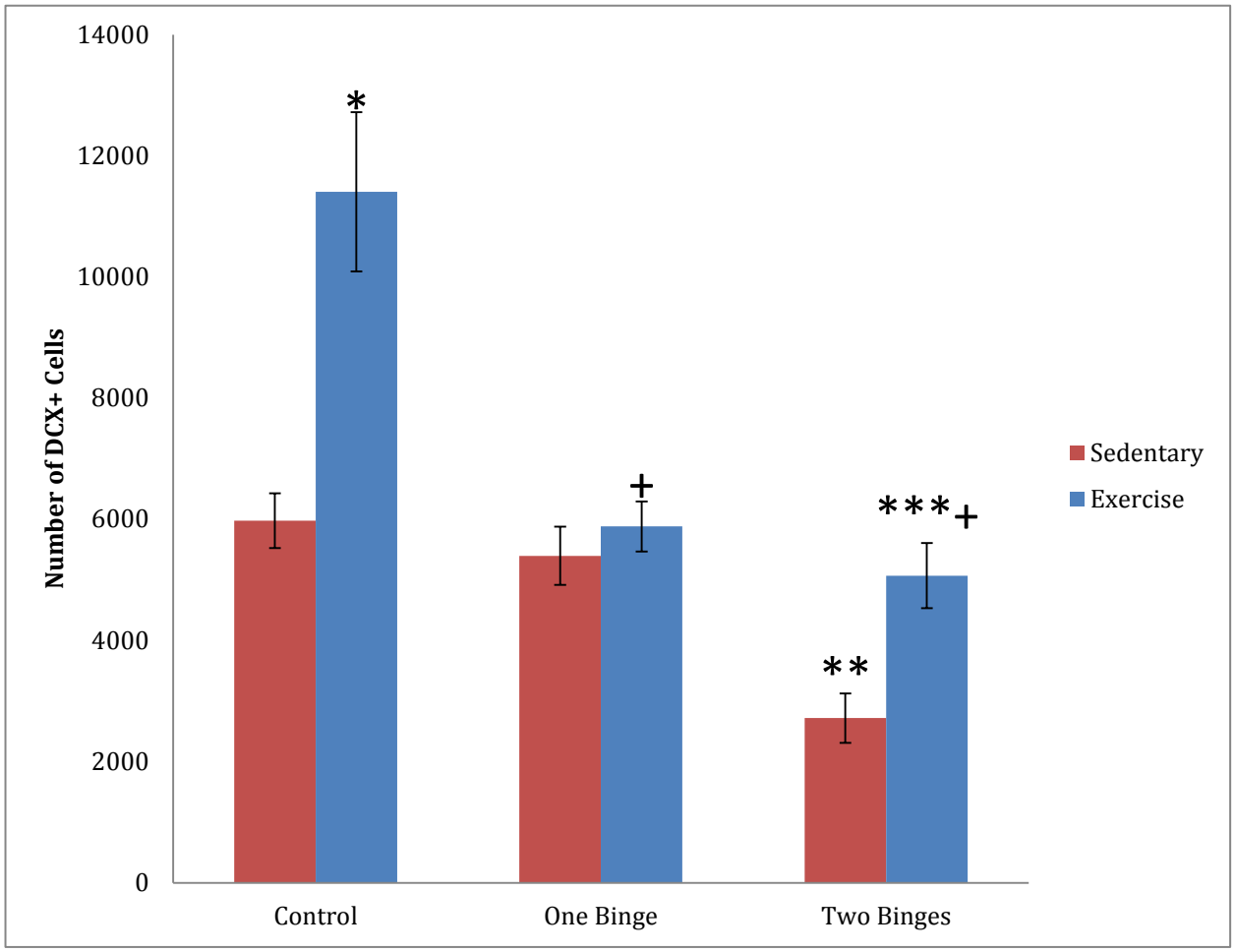

Figure 19. Repeated binge alcohol consumption and exercises effects on DCX+ cells. Exercise lead to an increase in DCX+ cells in control (E0 and S0) and two binge (E2 and S2) animals. However there was no difference between single binge animals (E1 vs S1) demonstrating a binge induced suppression of the neurogenic effects of exercise. A single binge episode had no effect on DCX+ in sedentary animals (S0 vs S1), however a second binge lead to a significant decrease (S1 vs S2). * $p < 0.0005$ post hoc comparison to S0, ** $p < 0.0005$ post hoc comparison to S1, *** $p < 0.005$ post hoc comparison to S2, + $p < 0.0005$ post hoc comparison to E0.

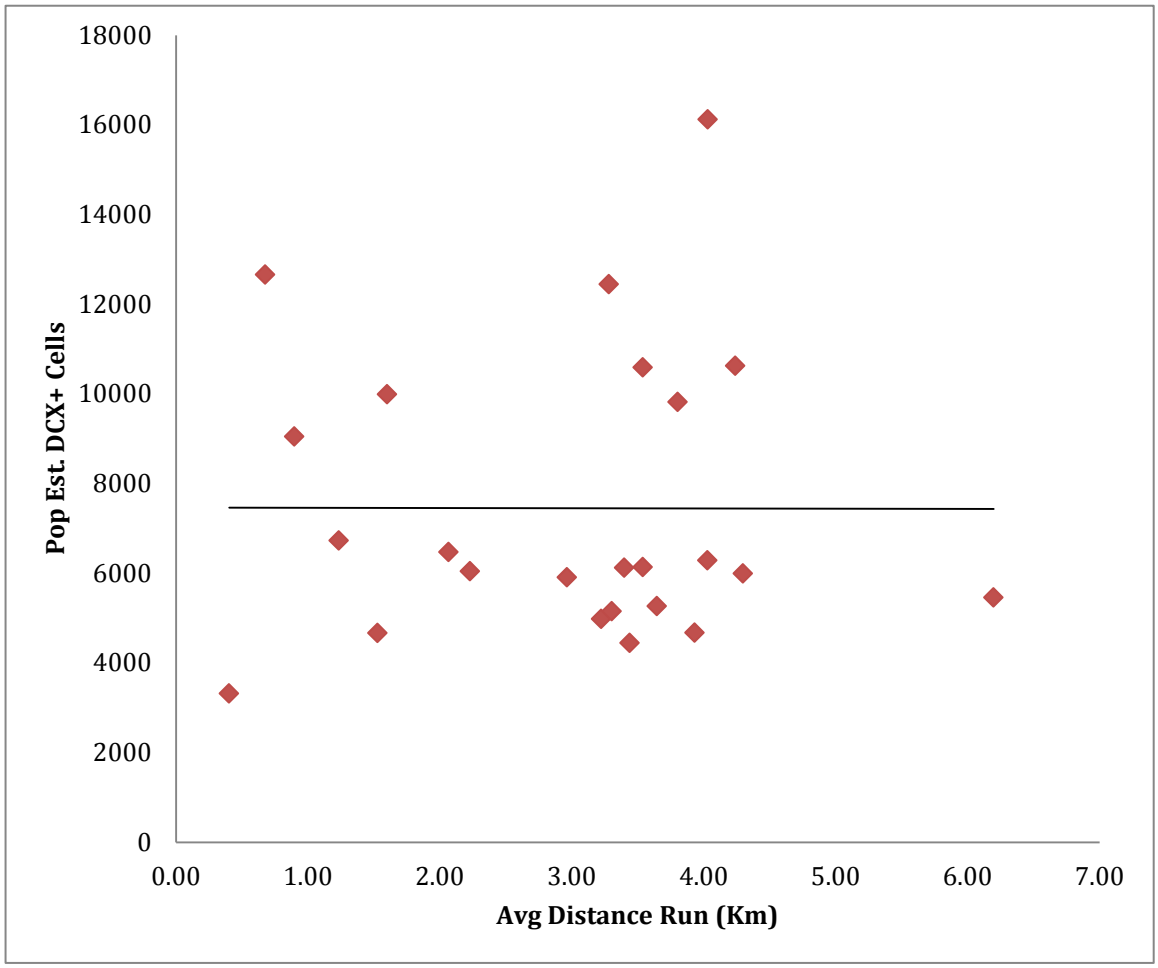

Figure 20. Correlation between mean distance run and number of DCX+ cells. There was no significant correlation between mean distance run and number of DCX+ cells r^2 = -.002.

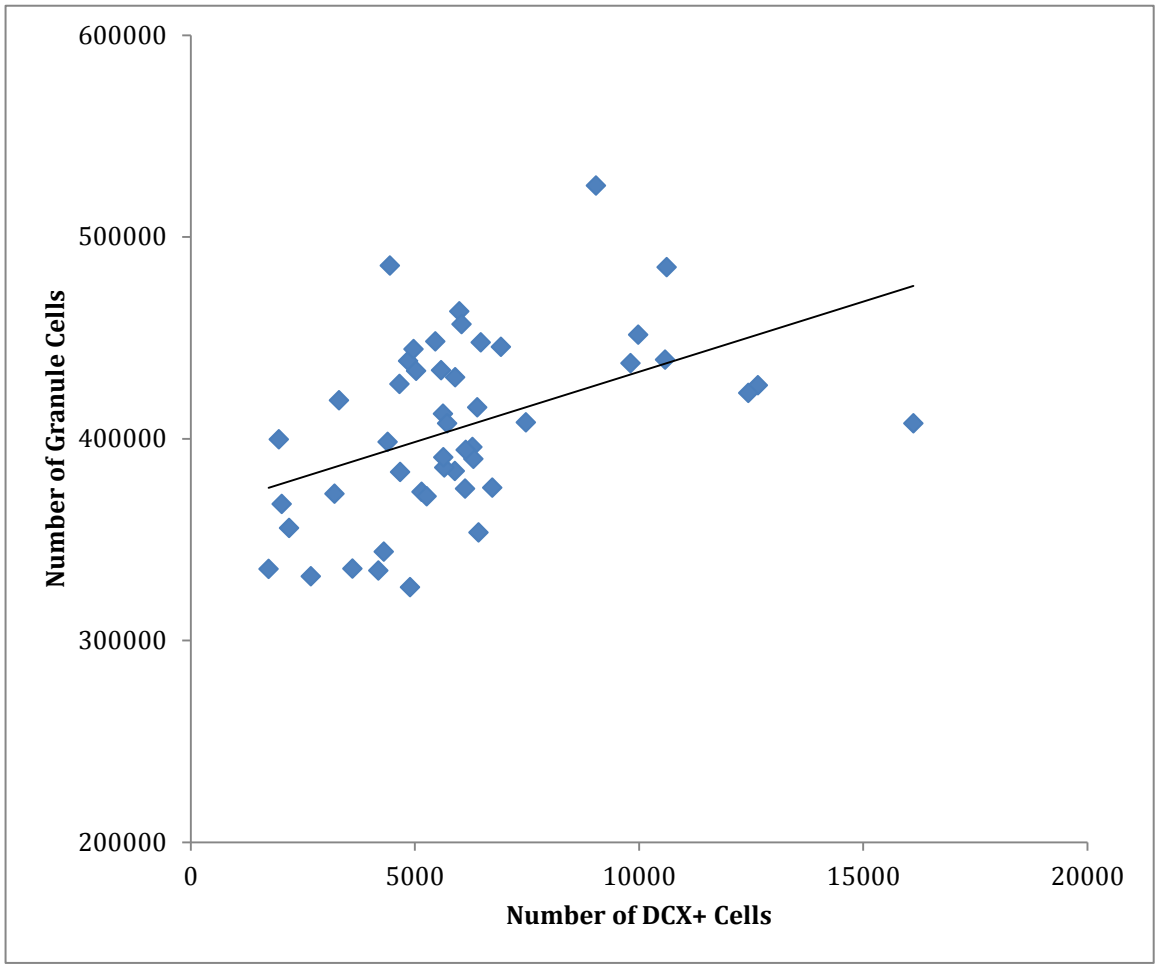

Figure 21. Correlation between number of granule cells run and number of DCX+ cells. There was a significant correlation between number of granule cells and number of DCX+ cells $r^2 = .452$, $p < 0.01$.

Table 1. Modified Majchrowicz Scale

Intoxication score	Indications	Dose (g/kg)
0	Normal rat	5
1	Hypoactive, mild ataxia	4
2	Ataxic, abdomen elevated	3
3	Delayed righting reflex, ataxic with no abdominal elevation	2
4	Loss of righting reflex, retains eye blink reflex	1
5	Loss of righting reflex, loss of eye blink reflex	0

Table 1. Behaviors used to assess intoxication levels during ethanol administration are shown

Table 2. Spontaneous Withdrawal Scale

Behavior Score	Indications
0	Normal rat
1.0	Hyperactivity
1.4	Tail tremor
1.6	Tail spasticity
2.0	Caudal tremor
2.4	Splayed limbs
2.6	General tremor
3.0	Head tremor
3.4	Wet dog shakes
3.6	Chattering teeth
3.8	Convulsion

Table 2. The scale used to assess withdrawal behavior is shown.