*Neuroscience* 224 (2012) 202–209

# MODERATE DRINKING? ALCOHOL CONSUMPTION SIGNIFICANTLY DECREASES NEUROGENESIS IN THE ADULT HIPPOCAMPUS

**M. L. ANDERSON,[a] M. S. NOKIA,[b] K. P. GOVINDARAJU[c] AND T. J. SHORS[c]***

[a] *Department of Neuroscience, Rutgers University/UMDNJ – Robert Wood Johnson Medical School, 683 Hoes Lane, RWJSPH 363, Piscataway, NJ 08854, USA*

[b] *Department of Psychology, P.O. Box 35, 40014 University of Jyvaskyla, Finland*

[c] *Department of Psychology and Center for Collaborative Neuroscience, Rutgers University, 152 Frelinghuysen Road, Piscataway, NJ 08854, USA*

**Abstract—Drinking alcohol in moderation is often considered a health-conscious behavior, associated with improved cardiovascular and brain health. However, ''moderate'' amounts of alcohol include drinking 3–4 alcohol beverages in a day, which is closer to binge drinking and may do more harm than good. Here we examined how daily drinking of moderate-high alcohol alters the production of new neurons in the adult hippocampus. Male and female adult Sprague–Dawley rats were provided free access to a liquid replacement diet that was supplemented with either 4% ethanol or Maltodextrin for a period of 2 weeks. Proliferating cells were labeled with 5-bromo-2-deoxyuridine (BrdU) and the number of BrdU-positive cells in the hippocampus was assessed after the final day of drinking. A subset of rats was also exposed to a motor skill or associative learning task to examine the functional effects of alcohol consumption. The drinking regime resulted in an average blood alcohol concentration of approximately 0.08%, which is comparable to the human legal driving limit in many countries. This level of intoxication did not impair motor skill learning or function in either sex, nor did the alcohol consumption disrupt associative learning 2 days after drinking. Therefore, moderate alcohol consumption did not disrupt basic sensory, motor or learning processes. However, the number of cells produced in the dentate gyrus of the hippocampus was reduced by nearly 40%. Thus, even moderate consumption of alcohol for a relatively short period of time can have profound effects on structural plasticity in the adult brain.** 

**Key words: alcohol, adult neurogenesis, trace eyeblink conditioning, BrdU, hippocampus, sex differences.**

## INTRODUCTION

Light-moderate alcohol consumption is associated with improved cardiovascular and brain health (Ngandu et al., 2007; Ronksley et al., 2011). As such, some guidelines for health and wellness advocate drinking one or two alcoholic beverages each day. Conversely, it is widely accepted that large amounts of alcohol are detrimental to our health. This information has been gleaned, in part, from animal studies, which tend to focus on the consumption of large amounts of alcohol in models of addiction and binge drinking. Still, many individuals are more likely to consume alcohol at a level and frequency between the two categories of ''light/moderate'' and ''chronic binge drinking''. Moderate drinking defined as having as many as 3–4 drinks per day and no more than 7–14 drinks per week (depending on your sex) is generally socially acceptable and considered safe. Some research even suggests that moderate levels of alcohol intake are beneficial to our health. However, the definition of ''moderate consumption'' varies greatly across social circles, cultures, and most importantly, scientific literature. The reported effects of moderate consumption on brain and mental health vary as much as does the definition. For example, moderate alcohol consumption in middle-aged men and women is associated with improved cognitive function later in life (Ngandu et al., 2007), but also correlates negatively with brain volume in both sexes (Verbaten, 2009). Thus, it is not clear whether moderate drinking is beneficial or harmful to overall brain health and function.

Some of the effects of alcohol on brain health depend on sex/gender differences. For example some studies report a connection between alcohol consumption and cognitive performance in women but not in men (Dufouil et al., 1997; Edelstein et al., 1998; Au Yeung et al., 2012). Stampfer et al. (2005) found that elderly women drinking up to one portion of alcohol per day outperformed nondrinkers in cognitive tasks and also had a reduced risk for cognitive decline. In a similar manner, Dufouil et al. (1997) reported a positive correlation between alcohol consumption and cognitive performance in elderly women but not in men. Moderate alcohol intake in elderly women has also been associated with a 50% reduced risk of dementia (Espeland et al., 2005). On the contrary, yet another study reported a negative correlation between alcohol consumption and memory performance specifically in women (Edelstein et al., 1998). In summary, alcohol differentially affects males and females, but whether the effects are positive or negative remains unclear, particularly in females.

*Corresponding author. Tel: +1-848-445-2576; fax: +1-732-445-2263.
E-mail addresses: megander@eden.rutgers.edu (M. L. Anderson), miriam.nokia@jyu.fi (M. S. Nokia), shors@rutgers.edu (T. J. Shors).
*Abbreviations:* ANOVA, analysis of variance; BAC, blood alcohol concentration; BrdU, 5-bromo-2-deoxyuridine; CR, conditioned response; CS, conditioned stimulus; EMG, electromyography/electromyographic; GCL, granule cell layer; US, unconditioned stimulus.

0306-4522/12 $36.00 
http://dx.doi.org/10.1016/j.neuroscience.2012.08.018

A crucial process in maintaining brain plasticity and healthy cognitive function is adult neurogenesis (Shors et al., 2012; Curlik and Shors, in press). New neurons are produced in the hippocampus of the adult mammalian brain throughout the entire lifespan. The production of new neurons is sensitive to environmental influences and the ingestion of chemicals (van Praag et al., 1999; Glenn et al., 2007; Hodes et al., 2009; Leuner et al., 2010). In turn, adult neurogenesis is crucial for certain types of learning, such as trace conditioning in which two events, separated in time, have to be associated (Shors et al., 2001; Nokia et al., in press). Moreover, in the adult brain, most new neurons die unless the individual is exposed to an effortful but successful learning experience (Curlik and Shors, 2011). Whereas convincing reports of deleterious effects of consuming large amounts of alcohol (i.e. binge drinking) on adult neurogenesis exist (Nixon and Crews, 2002; He et al., 2005), the effects of moderate consumption have not been studied to a large extent, particularly with regard to sex differences.

In the present study, we aimed to clarify the effects of moderate alcohol consumption on hippocampal neurogenesis and learning. Male and female rats consumed alcohol via a liquid diet for 14 days according to a regime that resulted in blood alcohol levels approximate to the legal driving limit in many countries including the United States, the United Kingdom and Canada (0.08%). The consequences for neurogenesis, motor skill learning, and associative learning were determined.

## EXPERIMENTAL PROCEDURES

### Subjects

Male and female Sprague–Dawley rats were bred at Rutgers University in the Department of Psychology. At 28 days after birth, animals were housed in groups of 2–3 for males and 2–4 for females in a plastic box style cage (44.5 cm long × 21.59 cm wide × 23.32 cm high) and kept on a 12-h light–dark cycle, with the lights turning on at 7 am. Before and after the experiment, animals were provided free access to water and solid food pellets. All experiments were conducted with full compliance with the rules and regulation specified by the PHS Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals.

### Diet

Adult rats (60 days or older) were handled, singly housed and randomly assigned to either an alcohol diet or non-alcohol diet. Animals assigned to the alcohol-drinking group were provided alcohol through a liquid diet for 2 weeks. The alcohol diet (approximately 4% w/v) was mixed in batches that contained 132.2 g of powder diet (Premix for Fisher's Suspendable Rat Diets, Research Diets, Inc., New Brunswick, NJ, USA), 45.3 g of 100% ethanol, and 823 g of water, which supplied 848 kcal per 1 kg consumed. An iso-volumetric and iso-caloric non-alcohol liquid diet containing 132.2 g of the same powder diet, 80 g of fine ground Maltrose Dextrin (Research Diets, Inc., New Brunswick, NJ, USA), and 788 g of water per batch was given to the no-alcohol-drinking group. Both diets were mixed and blended 1–3 days in advance, however, the alcohol was added fresh daily to prevent fermentation. Diets were mixed in a blender again immediately before administering it to the animals. Animals were given free access to approximately 150 ml of their assigned diet in a plastic bottle with a rubber stopper with ball bearing tipped sippers at 10 am (light portion of the cycle) daily. The sipper bottles containing the diet were weighed (total weight including the bottle, the diet and the sipper top) before and after to calculate the daily mass consumed for each animal. Consumption for 37 animals (male no alcohol $n = 7$, male alcohol $n = 7$, female no alcohol $n = 11$, female alcohol $n = 12$) in experiment 1 and 28 animals (female no alcohol $n = 14$; female alcohol $n = 14$) for experiment 2 was obtained and used in the analysis. Animals received all nutritional needs through this diet. Animals were weighed before starting the diet and twice more, 1 and 2 weeks after starting the liquid diet. Consumption was expressed as grams of the diet consumed per kilogram of the average body weight. The overall health of the animals was also monitored. Animals ($n = 4$) that did not acclimate well to the liquid diet were immediately removed from the study and returned to solid food. Overall, animals did not experience significant changes in weight or overall health during these experiments. Rats were provided with a surplus of diet each day. In a few instances (less than five), all of the diet given was consumed in 1 day.

### BrdU and immunohistochemistry

In experiment 1, the effect of alcohol on the number of new cells produced in the hippocampus was assessed. All 37 rats were injected intraperitoneally with 5-bromo-2-deoxyuridine (BrdU, 200 mg/kg, Sigma, Atlanta, GA, USA) in physiological saline solution 1 week after the onset of liquid diet consumption. BrdU is a thymidine analog that incorporates into the DNA of a dividing cell during the S-phase of the cell cycle and thereby marks cells that are actively proliferating at the time of the injection. Animals were sacrificed 1 week after the BrdU injection, at the end of the liquid diet regime. With this timeline, new neurons labeled with BrdU were born under the influence of alcohol and then exposed to another week of alcohol before the rats were euthanized. Rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and intracardially perfused with 4% paraformaldehyde, in 0.1 M phosphate buffer, to preserve the tissue structure. Trunk blood was collected from animals immediately before the perfusion. Brains were extracted and fixed in 4% paraformaldehyde for 24 h and then transferred to 0.1 M PBS until sectioning.

A vibratome was used to obtain 40-μm coronal sections through an entire extent of the hippocampus in one hemisphere. The hemisphere used was counterbalanced in all groups. Using one hemisphere is standard practice in our laboratory and no differences have been observed in neurogenesis in animals where samples were taken from the left versus right hemisphere (Dalla et al., 2007; Anderson et al., 2011). Tissue from one animal in the "male alcohol" group was lost due to excessive damage during sectioning, and therefore, not included in the BrdU analysis. Every 12th slide was placed onto a superfrost glass slide (Fisher, Suwanne, GA, USA) and allowed to air dry. Once dry, slides were stained with immunoperoxidase staining to visualize the cells that incorporated BrdU as described previously (Anderson et al., 2011). In summary, the tissue was pretreated with a heated 0.1 M citric acid solution (pH 6.0) for 15 min, a trypsin solution for 10 min and finally 2 N HCl for 30 min. Slides were kept overnight at 4 °C in primary mouse anti-BrdU (1:200, Becton–Dickinson, Franklin Lakes, NJ, USA), then biotinylated anti-mouse antibody (1:200, Vector Labs, Burlingame, CA, USA) for 1 h, followed by avidin–biotin–horseradish peroxidase (1:100,

Vectastain ABC Kit, Vector Labs, Burlingame, CA, USA) for 1 h, and lastly in diaminobenzidine (DAB SigmaFast tablets, Sigma, Atlanta, GA, USA).

### Quantification of BrdU-positive cells and hippocampal volume

Estimates of total number of BrdU-labeled cells were determined using a modified unbiased stereology protocol (West et al., 1991; Gould et al., 1999). BrdU-labeled cells in the granule cell layer (GCL) and hilus, on every 12th section, were counted at 1000× on a Nikon Eclipse 80i light microscope. Cells that were out of focus or beyond the boundaries of the GCL and hilus were excluded. The number of cells was multiplied by 12 to obtain an estimate of the total number of BrdU-labeled cells in the hippocampal formation of the hemisphere collected and then by 2 to obtain an estimate for the number of BrdU-positive cells in both hemispheres. Volume of the dentate gyrus was determined with high resolution microphotographs of each 40-μm slice at either 40× or 10× (depending on the size of the dentate gyrus) using a DS-Fi1 Nikon camera. Area of the dentate gyrus was calculated using ImageJ (NIH, USA) by setting the scale with a known distance and then outlining the dentate gyrus and using the measure function. From this, volume was calculated using Cavalieri's principle: Volume $= TX(\sum A_{1-m})$, where $T$ is the distance between each reference section (12 × 0.04 mm in this case), $A$ represents the area of each reference section (sections 1-$m$) and $\sum A_{1-m}$ is the sum of the area of sections 1 through $m$. The volume was multiplied by 2 to obtain volume for both hemispheres and expressed as $mm^3$. Differences in the number of BrdU-labeled cells per unit volume between the dorsal (interaural 4.8–6.3) and ventral (interaural 1.8–2.8) hippocampus were also examined (Dalla et al., 2009).

### Blood alcohol concentration (BAC)

Trunk blood was collected in heparinized capillary tubes from 16 animals (male alcohol $n = 5$, female alcohol $n = 11$) approximately 3 h after the end of the dark period and used to analyze the BAC. Blood serum was isolated by centrifuging the trunk blood at 500$g$ for 25 min and stored at −20 °C. Within 2 weeks, the BAC was determined using the QuantraChrom Ethanol Assay Kit (DIET-500; Fisher Scientific, Pittsburgh, PA, USA) following the supplied instructions. In short, solutions containing dichromate were added to prepared samples producing a color change in samples that contained ethanol. The change in color was measured using a spectrophotometer and the percentage of ethanol in the sample was calculated using a standard curve. An inadequate amount of blood was collected from 2 males and 1 female and therefore, excluded from the group for BAC analysis. The BAC was converted to and expressed as a percentage (volume/volume).

### Motor and associative learning

To obtain blood alcohol levels, animals from the first experiment were euthanized immediately after drinking. Therefore, separate groups of animals were used to examine how alcohol would affect motor learning while drinking and how it would affect associative learning after drinking. Females were selected because they are less often studied despite their reported sensitivity to alcohol consumption (see Introduction). Female rats (alcohol $n = 14$ and no alcohol $n = 14$) were exposed to the alcohol diet each day for 14 days exactly as described in experiment 1.

A subset of the these female rats (no alcohol $n = 6$, alcohol $n = 6$) were then tested on a motor learning task using a rotarod (Four-station Rotarod for rat, Med Associates, Inc., Georgia, VT, USA). In the afternoon of the 6th day of drinking (approximately 3 pm), animals were placed on a rod that rotated at a speed of 8 rpm for up to 5 min. Latency to fall from the rod was measured for three trials with an inter-trial interval of approximately 20 min.

To examine the effects of moderate alcohol consumption on cognitive performance, another set of animals (no alcohol $n = 11$, alcohol $n = 9$) was trained with trace eyeblink conditioning. These animals were trained following the end of the two-week liquid diet period, therefore, the animals were sober at the time of training. Four rats that were tested on the rotarod were also used in this portion of the experiment (no alcohol $n = 3$, alcohol $n = 1$).

Prior to any drinking, rats were implanted with electrodes to assess the eyeblink response with electromyography (EMG). To do so, rats were anesthetized with sodium pentobarbital (50 mg/kg; Henry Schein, Indianapolis, IN, USA) and supplemented with isoflurane inhalant (Baxter Healthcare, Deerfield, IL, USA) and oxygen during the surgery to attach a headstage for eyeblink conditioning. The head was shaven and a headstage, which consisted of a four-pin connector and four wires, was attached to the skull using skull screws and dental cement. The four stainless wires were implanted around and through the right upper eyelid forming two bipolar electrodes. One electrode was used to record EMG activity in order to detect eyeblinks, and the other electrode delivered periorbital stimulation to the eyelid. The animals were given a minimum of 5 days recovery before given access to the liquid diet.

Trace conditioning was conducted 2 days after the end of drinking in order to evaluate learning ability in the absence of alcohol following 2 weeks of continuous drinking. Two days after the liquid diet was removed and were returned back to the solid standard chow diet, the animals were acclimated to the conditioning apparatus. The number of spontaneous blinks was recorded 1 day before training (100 trials, ∼40 min) and the day training began (30 trials, ∼13 min). Following the acclimation periods, the animals were trained with trace eyeblink conditioning. A trace trial consisted of a 250-ms white noise conditioned stimulus (CS, 82 dB) followed by a 500-ms stimulus-free trace interval, and immediately thereafter by the unconditioned stimulus (US), a 100-ms shock (0.62–0.65 mA) to the eyelid, which always elicits an eyeblink. The inter-trial interval was 25 ± 5 s. Training was conducted for a total of 800 trials, 200 trials per day, on consecutive days. EMG activity was recorded throughout the training session. Eyeblinks were considered conditioned responses (CRs) if they occurred within the last 250-ms prior to the onset of the US, were larger than 0.5 mV in amplitude and/or larger than three standard deviations above the mean of the baseline recording (250-ms period immediately preceding the CS), and lasted longer than 10 ms were considered a conditioned response (CR). Following trace eyeblink conditioning, animals were euthanized as described above.

### Statistical analyses

Repeated measures analysis of variance (ANOVA) was used to analyze changes across time and differences between groups in each experiment. Separate ANOVAs for each group, three-way ANOVA, independent samples $t$-tests and pairwise multiple comparison using the Student–Newman–Keuls method were also used, when appropriate, to further examine differences between groups and time points. All differences were considered statistically significant if the $P$-value was less than 0.05. Error bars in all graphs represent standard error.

## RESULTS

### Experiment 1

*Sex differences in consumption.* Female and male rats were provided a liquid diet each day with or without 4%


**Fig. 1.** Liquid diet regime produces moderate blood alcohol levels. (A) Animals that received a liquid diet with alcohol drank less than with the alcohol but this difference dissipated by the second week. Females tended to drink more of the diet than males, regardless of the alcohol content. (B) After the 2-week period, blood alcohol levels were measured from trunk blood. Concentrations ranged from 54.2 to 147.6 mg/dl with a mean of 86.4 mg/dl (0.0864%) for males and 87.6 mg/dl (0.0876%) for females.

(w/v) alcohol for 2 weeks. Consumption was binned into two-day blocks and is represented in Fig. 1a. In a few cases, due to spilled diet or error in collection of the consumption data, a data point reflects a single day of consumption rather than the average of 2 days of consumption. A two-way repeated measures ANOVA (sex and diet content as independent variables and consumption of diet as the repeated measure) revealed an interaction between whether the diet contained alcohol or not and consumption, $F[6,198] = 9.52$, $p < .001$. Separate repeated measures ANOVAs for each diet group (alcohol and no alcohol) showed that animals given a nonalcoholic diet drank high levels of the diet, which decreased over time, $F[6,96] = 3.98$, $p < .05$. There were no sex differences, $F[6,96] = 0.23$, ns. Conversely, animals given a diet containing alcohol commenced drinking lower amounts than those drinking the nonalcoholic diet but their consumption increased over the 2-week experiment, $F[6,102] = 9.65$, $p < .001$. There were no sex differences, $F[6,102] = 1.67$, ns. The two-way repeated measures ANOVA revealed that females consumed more grams of diet per unit of body weight, in general, than males regardless of whether it contained alcohol or not, $F[1,33] = 31.44$, $p < .001$. By the end of the drinking period, all groups were drinking similar amounts of the diet (sex: $F[1,33] = 1.62$, ns. and alcohol condition: $F[1,33] = 0.01$, ns.).

Blood was collected approximately 3 h after the end of the dark cycle for the final night of drinking. The BAC was on average 0.08% or 86.4 mg/dl. Even though females tended to consume more diet in general and thus more alcohol, blood alcohol levels were similar between females and males, $t(14) = 0.07$, ns. (see Fig. 1b).

*Adult neurogenesis.* New cells in the dentate gyrus continue to proliferate for approximately 1 week after their birth, but many of the adult-born hippocampal cells die within 1–2 weeks (Cameron et al., 1993; Waddell and Shors, 2008). Rats were given a single BrdU injection (200 mg/kg) after 1 week of the liquid diet and sacrificed at the end of the 2-week experiment. That way, we could assess the number of new cells produced in the presence of alcohol and surviving for 1 week in animals drinking moderate levels of alcohol. Alcohol decreased the number of new cells that were produced 1 week following a BrdU injection in both males and females: $t(11) = 2.70$, $p < .05$ and $t(21) = 3.67$, $p < .05$, respectively (Fig. 2a, b).

The volume of the hippocampus is greater in males than in females. Therefore, volume of the dentate gyrus was determined for each animal to calculate the number BrdU-positive cells per unit volume. An ANOVA with sex, alcohol condition and hemisphere as the independent conditions revealed that there were significantly fewer BrdU-labeled cells in the dentate gyrus in animals that consumed alcohol compared to rats given a diet without alcohol, $F[1,28] = 21.57$, $p < .001$ (Fig. 2c, d). However, there was no difference in the number of BrdU-positive cells between sexes ($F[1,28] = 0.51$, ns.) nor an interaction between sex and alcohol condition ($F[1,28] = 0.82$, ns.). The hemisphere used to estimate the total number of BrdU-positive cells was counterbalanced and no difference in the data was detected because of this, $F[1,28] = 3.87$, ns.

The number of BrdU-positive cells per unit volume was separated to examine the effect of alcohol of dorsal versus ventral hippocampus. An ANOVA with sex, alcohol condition, hemisphere and dorsal/ventral as independent factors revealed that fewer BrdU-positive cells were located in the ventral hippocampus compared to dorsal hippocampus irrespective of sex, alcohol condition and hemisphere used to estimate the total number of BrdU-positive cells, $F[1,56] = 20.18$, $p < .001$.

To summarize, moderate alcohol consumption over a period of 2 weeks significantly decreased cell production in the hippocampus in adult rats. The robust effect was similar in both sexes, in both hemispheres and in both the dorsal and ventral regions of the hippocampus.

## Experiment 2

*Consumption.* Again, consumption was binned into two-day blocks and is represented in Fig. 3a. In a few

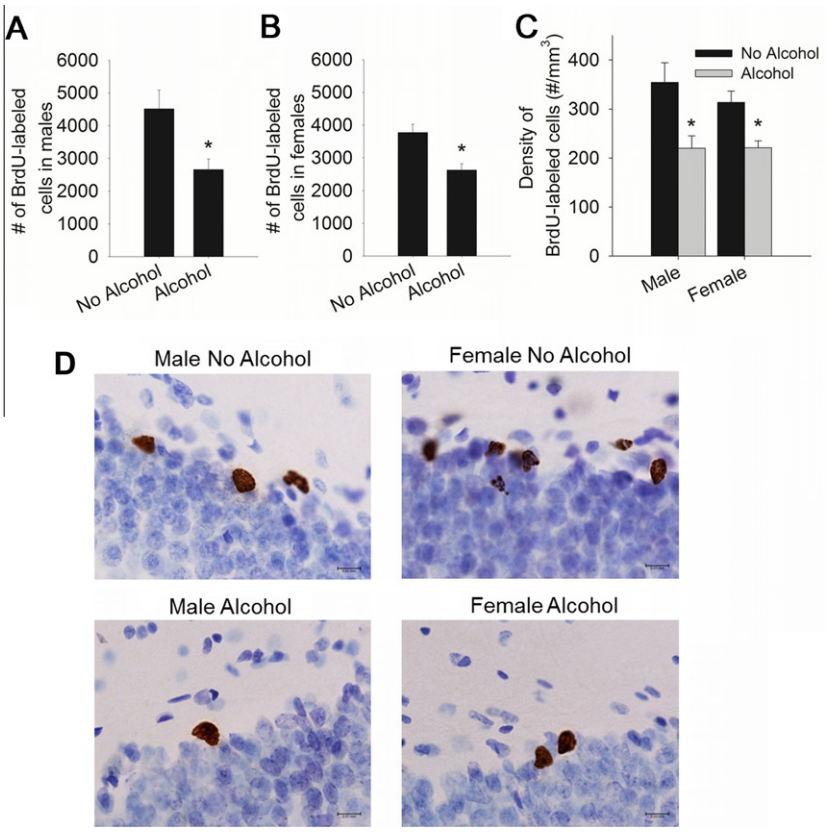


**Fig. 2.** Daily moderate alcohol consumption reduces the number of new cells made in the dentate gyrus. (A) Males that drank alcohol produced and retained fewer new cells than those that did not drink alcohol. (B) Similar effects were observed in females. (C) The number of BrdU-positive cells per unit volume was calculated to assess and account for sex differences in volume, but there were none. (D) Representative photomicrographs of BrdU-positive cells at 1000×.

cases, due to spilled diet or error in collection of the consumption data, a data point reflects a single day of consumption rather than the average of 2 days of consumption. In the second experiment, we assessed the effects of moderate alcohol consumption on motor performance and learning. To this end, female rats were subjected to a liquid diet regime identical to the first experiment. A one-way repeated measures ANOVA revealed an interaction between the alcohol condition and consumption over time, $F[6,156] = 13.53$, $p < .001$. A separate repeated measures ANOVA for the no alcohol group revealed that although animals adjusted to the liquid diet well, consumption varied somewhat from day to day, $F[6,78] = 3.77$, $p < .05$. A separate repeated measures ANOVA for the alcohol group revealed that the consumption of the alcoholic diet increased toward the end of the 14-day period, $F[6,78] = 19.05$, $p < .001$. Pairwise comparisons revealed that consumption on the last 2 days of the alcohol diet was significantly higher compared to consumption during the first 10 days ($p < .05$). At the end of the 14-day period, animals in both groups (no alcohol and alcohol) were consuming similar amounts of the diet, $t(26) = 0.79$, ns.

*Motor ability and associative learning.* On day 6, rats were trained on the rotarod test during the light portion of the light cycle. A one-way repeated measures ANOVA revealed an overall increase in performance across trials, $F[2,20] = 4.06$, $p < .05$ (Fig. 3b). Alcohol consumption did not alter performance on the task (main effect of group: $F[1,10] = 2.29$, ns.; interaction of group and trial: $F[2,20] = .79$, ns.).

After the alcohol delivery had ceased, we assessed associative learning with trace eyeblink conditioning. Training started on the third day of abstinence from the alcoholic/non-alcoholic liquid diet. Both groups made more CRs as training progressed (main effect of session of training: $F[11,198] = 13.25$, $p < .001$, Fig. 3c). The number of CRs made did not differ between animals as a function of the alcohol (main effect of group: $F[1,18] = 0.83$, ns.; interaction of group and session: $F[11,198] = 0.29$, ns.). A similar percentage of animals in each group reached a learning criterion of 60% conditioned responding during one 100-trial block of training (no alcohol 54.5% vs. alcohol 55.6%), $t(18) = .04$, ns. To summarize, moderate alcohol consumption for 2 weeks did not impair motor performance during the drinking nor did it impair associative learning days after the drinking had ceased.

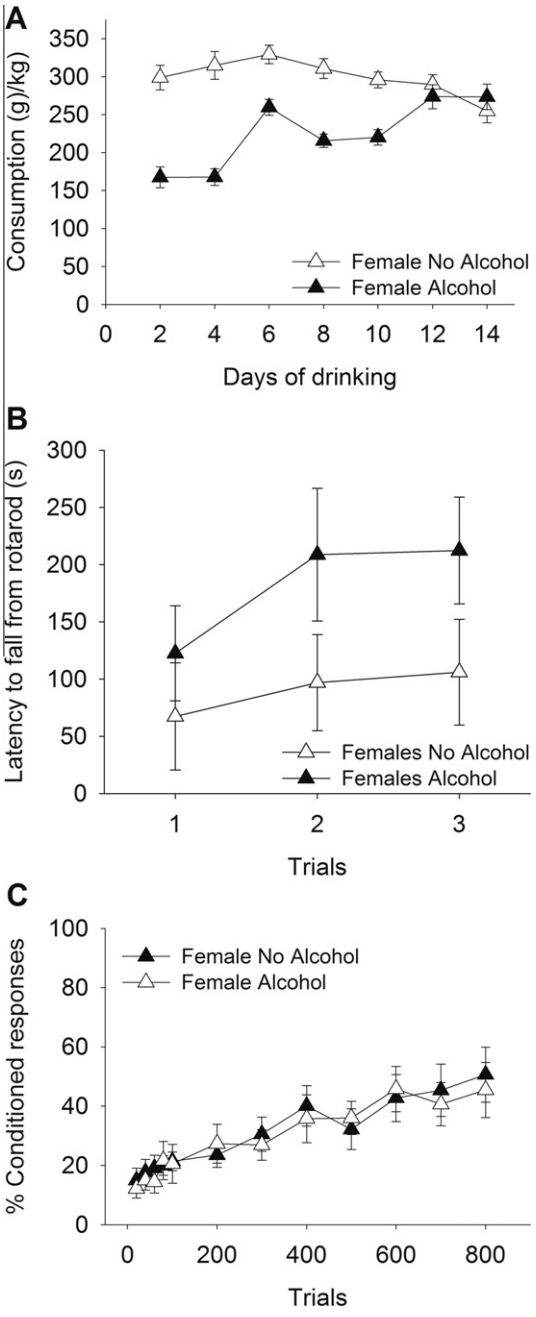


**Fig. 3.** Moderate drinking does not impair motor performance or associative learning. (A) Consumption was similar to females in experiment 1. Initially, animals that were given diets that contained alcohol drank less than animals not given alcohol. However, daily consumption was similar by the end of the two-week drinking period. (B) Females that drank alcohol performed similarly compared to females that did not drink alcohol when motor performance was tested on a rotarod on day 6 of the drinking regime. (C) Females that consumed alcohol emitted similar numbers of CRs during training as those that did not drink alcohol. Therefore, there was no persistent effect of alcohol consumption on this type of associative learning.

## DISCUSSION

New neurons generated in the adult hippocampus are presumably beneficial for brain health and may even keep the brain fit for learning in the future (Shors et al., 2012). Most studies indicate that alcohol is detrimental to neurogenesis, but tend to focus on modeling alcohol addiction or binge drinking. In the present study, we modeled humans who consume moderate levels during some days, but engage in heavier drinking on other days. Animals that engaged in similar behaviors with alcohol levels near the legal driving limit produced ~40% fewer new cells than did their abstinent counterparts. This type of drinking also did not impair motor performance. The relatively large magnitude of this effect indicates that even socially acceptable levels of alcohol consumption can have long-lasting and detrimental consequences for brain health and its structural integrity.

Men who drink 14 drinks a week and women who drink 7 are considered at-risk drinkers by the National Institute for Alcohol Abuse and Alcoholism. Moreover, binge drinking is defined as when blood concentrations reach 0.08% or above. The rats in the current study attained this level (0.08%) and probably had higher levels during some periods of time. Blood was collected during the light portion of the day at the end of the final day on the liquid alcohol diet. Even though they drank during both phases, it is presumed that most of the diet was consumed during the dark portion (between 7 pm and 7 am) when rats are more active. Arguably, higher levels of BAC may have been obtained if BAC had been measured immediately after bouts of feeding. Indeed, animals consuming similar amounts of alcohol to the animals during the second week of drinking in the present study (~8 g/kg/day) expressed BAC levels of 140 ± 12 mg/dl during the dark cycle as compared to 85 ± 5 mg/dl during the light cycle (Lukoyanov et al., 2000). Because animals in the present study did not drink as much alcohol during the first week, they would not have produced blood levels consistent with binge drinking until the second week. This type of behavior occurs often in humans. Many individuals drink smaller amounts of alcohol daily but also engaged in heavier consumption of alcohol on other days, so that their BAC reaches at least 0.08%. Of course, this concentration can be attained with only 3–4 portions of alcohol during a two-hour period, a behavior that is often considered moderate and acceptable according to societal definitions. Nonetheless, similar levels reduced cell production in the hippocampus by nearly 40%.

It is unclear whether this neurogenic response to alcohol is discrete or continuous. In other words, lower levels of alcohol more consistent with 1–2 drinks a day may not produce a deficit or alternatively, may produce less of a deficit. One study reported that lower levels (BAC of 24 mg/dl or 0.02%) increased neurogenesis (Aberg et al., 2005). Interestingly, this effect was only observed when the animals had already been drinking for 60 days prior to the BrdU injection. Drinking for 5 days after a BrdU injection did not affect neurogenesis (Aberg et al., 2005). It is thus possible that long-term consumption of alcohol producing BAC levels lower than those achieved in our current study have positive effects on adult neurogenesis. While it is clear the binge drinking is detrimental to adult neurogenesis (Nixon and

Crews, 2002), we find that level at which alcohol consumption becomes harmful to the development of new neurons is lower than previously shown. It is noted that in the current study, female rats tended to consume more of the alcohol diet in general when compared to male rats, and as a result were exposed to more alcohol. However, no sex differences in BAC or neurogenesis were detected. Females express more gastric alcohol dehydrogenase, which might explain the similar BAC levels between the sexes despite differences in intake (Mezey et al., 1992; Walker et al., 2008).

The decrease in the number of proliferating cells in response to alcohol could reflect an increase in cell death or a decrease in cell division (either by fewer proliferating cells or slower cell cycle). Others report that high levels of alcohol increase cell death (Obernier et al., 2002; He et al., 2005). In the present study, we did not observe noticeable increases in pyknotic nuclei in animal that were exposed to alcohol. Regardless, acute and chronic binge drinking of alcohol (where BAC reached 200 mg/dl and above) decreases the number of proliferating cells in addition to inducing cell death (Nixon and Crews, 2002; He et al., 2005). It is likely that even more moderate amounts can decrease the proliferating cell population and this rather than an increase in cell death more likely accounts for the 40% reduction in neurogenesis observed in the present study.

One might argue that the animals consuming alcohol were less active and thus produced fewer cells in response to decreased physical activity (see van Praag et al., 1999 for example). However, there was little opportunity for the rats in either group to engage in excessive locomotor activity and both groups performed similarly in a motor performance test using a rotarod. Thus, this explanation for the decrement seems unlikely. Furthermore, the effect of alcohol on neurogenesis did not differentially affect dorsal and ventral hippocampus. In non-alcohol consuming rats, fewer new cells were generated in the ventral compared the dorsal hippocampus, which supports previously published reports (Brummelte and Galea, 2010). Previously published data suggest that the ventral hippocampus appears to be more susceptible to factors that influence the survival of newly generated cells as opposed to the proliferation of cells (Dalla et al., 2009; Jinno, 2011). Alcohol decreased neurogenesis to the same magnitude throughout the hippocampus which supports the idea that alcohol impacted proliferation rather than the survival of new cells in the hippocampus.

Over time humans and other animals develop tolerance to daily use of moderate amounts of alcohol. As a result, the production of new neurons may return to baseline once tolerance is achieved. To our knowledge, it is not known how changes in alcohol metabolism over time would affect neurogenesis. Human studies indicate that chronic yet moderate alcohol consumption can have a negative impact on brain volume and cognition in cases where participants had likely developed some tolerance to alcohol (Edelstein et al., 1998; Verbaten, 2009). However, the contribution of neurogenesis to these observed effects has not been examined, in part because of the difficulty in assessing neurogenesis in postmortem tissue.

The effect of withdrawal from long-term moderate alcohol use has been studied. He et al. (2009) used a protocol where alcohol-preferring rats were exposed to alcohol for 7 weeks which resulted in a mean BAC of ~40 mg/dl in males and females. Abstinence for 6 weeks increased neurogenesis to levels exceeding those expressed in rats that had not received alcohol during the initial 7-week period. That is, withdrawal from alcohol had positive, rebound-like effects on neurogenesis. Therefore, alcohol appears to have a prolonged negative impact on brain health, which culminates in a rebound when alcohol is withdrawn. It should be noted that withdrawal from alcohol consumption does not exert its positive effect on neurogenesis immediately. After nearly two months of low-moderate alcohol consumption and only 3 days of abstinence, cell proliferation remained lower than in animals that had never consumed alcohol (Aberg et al., 2005). However and as noted, Aberg et al. (2005) reported that low-moderate drinking resulted in enhanced neurogenesis. Clearly, parametric studies are necessary to understand the dynamics of these relationships.

Alcohol consumption reportedly induces positive effects on cognition in women (Dufouil et al., 1997; Espeland et al., 2005; Stampfer et al., 2005; Ngandu et al., 2007). To assess cognitive performance, female rats were trained with trace eyeblink conditioning, an associative learning task that depends on the hippocampus for learning and is sensitive to changes in hippocampal neurogenesis (Shors et al., 2001, 2002; Nokia et al., in press). Female rats that had been consuming moderate-heavy amounts of alcohol for 2 weeks followed by 3 days of abstinence learned just as well as their counterparts that had been consuming a non-alcoholic diet. Thus, a substantial decrease (~40%) in cell production due to alcohol did not elicit deficits in basic processes of associative learning. Of course, learning deficits may emerge after a longer period of drinking.

## CONCLUSIONS

Moderate-heavy alcohol consumption each day over just 2 weeks reduced cell production in the hippocampus by nearly 40%. The blood concentration at the end of the drinking was 0.08% and close to the legal driving limit for humans. However, the amount of drinking varied greatly over days and thus fluctuated above and below this level throughout the time period. We suggest that this behavior may model humans who drink a few drinks every day during the work-week and more on the weekends or holidays. The data reported here underscore the fine line between "harmless" or supposedly healthy drinking and that associated with neuronal dysfunction and damage. Social and/or daily drinking may be more deleterious to brain health than commonly recognized by the general public.

*Acknowledgements*—*This work was funded by the National Institutes of Health (National Institute of Mental Health – 59970) and National Science Foundation (Integrative Organism Biology –*

*0444364) and RISE supplement from NSF to Dr. Tracey J. Shors. Our funding had no involvement in the design of the study nor the collection, analysis or interpretation of the data. The authors would like to thank George Wagner's laboratory for assisting in the setup of the alcohol diet regime used in this study.*

## REFERENCES

Aberg E, Hofstetter CP, Olson L, Brené S (2005) Moderate ethanol consumption increases hippocampal cell proliferation and neurogenesis in the adult mouse. Int J Neuropsychopharmacol 8:557–567.

Anderson ML, Sisti HM, Curlik II DM, Shors TJ (2011) Associative learning increases adult neurogenesis during a critical period. Eur J Neurosci 33:175–181.

Au Yeung SL, Jiang CQ, Cheng KK, Liu B, Zhang WS, Lam TH, Leung GM, Schooling CM (2012) Evaluation of moderate alcohol use and cognitive function among men using a mendelian randomization design in the guangzhou biobank cohort study. Am J Epidemiol. Ahead of print.

Brummelte S, Galea LA (2010) Chronic high corticosterone reduces neurogenesis in the dentate gyrus of adult male and female rats. Neuroscience 168:680–690.

Cameron HA, Woolley CS, McEwen BS, Gould E (1993) Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56:337–344.

Curlik II DM, Shors TJ (2011) Learning increases the survival of newborn neurons provided that learning is difficult to achieve and successful. J Cogn Neurosci 23:2159–2170.

Curlik II DM, Shors TJ (in press) Do mental and physical (MAP) training enhancecognition through the process of neurogenesis in the hippocampus? Neuropharmacology, in press.

Dalla C, Bangasser DA, Edgecomb C, Shors TJ (2007) Neurogenesis and learning: Acquisition and asymptotic performance predict how many new cells survive in the hippocampus. Neurobiol Learn Mem 88:143–148.

Dalla C, Papachristos EB, Whetstone AS, Shors TJ (2009) Female rats learn trace memories better than male rats and consequently retain a greater proportion of new neurons in their hippocampi. Proc Natl Acad Sci USA 106:2927–2932.

Dufouil C, Ducimetière P, Alpérovitch A (1997) Sex differences in the association between alcohol consumption and cognitive performance. EVA Study Group. Epidemiology of vascular aging. Am J Epidemiol 146:405–412.

Edelstein SL, Kritz-Silverstein D, Barrett-Connor E (1998) Prospective association of smoking and alcohol use with cognitive function in an elderly cohort. J Womens Health 7:1271–1281.

Espeland MA, Gu L, Masaki KH, Langer RD, Coker LH, Stefanick ML, Ockene J, Rapp SR (2005) Association between reported alcohol intake and cognition: results from the Womens Health Initiative Memory Study. Am J Epidemiol 161:228–238.

Glenn MJ, Gibson EM, Kirby ED, Mellott TJ, Blusztajn JK, Williams CL (2007) Prenatal choline availability modulates hippocampal neurogenesis and neurogenic responses to enriching experiences in adult female rats. Eur J Neurosci 25:2473–2482.

Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260–265.

He J, Nixon K, Shetty AK, Crews FT (2005) Chronic alcohol exposure reduces hippocampal neurogenesis and dendritic growth of newborn neurons. Eur J Neurosci 21:2711–2720.

He J, Overstreet DH, Crews FT (2009) Abstinence from moderate alcohol self-administration alters progenitor cell proliferation and differentiation in multiple brain regions of male and female P rats. Alcohol Clin Exp Res 33:129–138.

Hodes GE, Yang L, Van Kooy J, Santollo J, Shors TJ (2009) Prozac during puberty: distinctive effects on neurogenesis as a function of age and sex. Neuroscience 163:609–617.

Leuner B, Glasper ER, Gould E (2010) Sexual experience promotes adult neurogenesis in the hippocampus despite an initial elevation in stress hormones. PloS One 5:e11597.

Lukoyanov NV, Brandão F, Cadete-Leite A, Madeira MD, Paula-Barbosa MM (2000) Synaptic reorganization in the hippocampal formation of alcohol-fed rats may compensate for functional deficits related to neuronal loss. Alcohol 20:139–148.

Mezey E, Sharma S, Rennie L, Potter JJ (1992) Sex differences in gastric alcohol dehydrogenase activity in Sprague–Dawley rats. Gastroenterology 103:1804–1810.

Ngandu T, Helkala EL, Soininen H, Winblad B, Tuomilehto J, Nissinen A, Kivipelto M (2007) Alcohol drinking and cognitive functions: findings from the Cardiovascular Risk Factors Aging and Dementia (CAIDE) Study. Demen Geriatr Cogn Disord 23:140–149.

Nixon K, Crews FT (2002) Binge ethanol exposure decreases neurogenesis in adult rat hippocampus. J Neurochem 83:1087–1093.

Nokia MS, Anderson ML, Shors TJ (in press) Chemotherapy disrupts learning, adult neurogenesis and theta activity in the hippocampus. European Journal of Neuroscience, in press.

Obernier JA, Bouldin TW, Crews FT (2002) Binge ethanol exposure in adult rats causes necrotic cell death. Alcoh Clin Exp Res 26:547–557.

Ronksley PE, Brien SE, Turner BJ, Mukamal KJ, Ghali WA (2011) Association of alcohol consumption with selected cardiovascular disease outcomes: a systematic review and meta-analysis. BMJ 342:d671.

Shors TJ, Anderson ML, Curlik DMn, Nokia MS (2012) Use it or lose it: how neurogenesis keeps the brain fit for learning. Behav Brain Res 227:450–458.

Shors TJ, Miesegaes G, Beylin AV, Zhao M, Rydel T, Gould E (2001) Neurogenesis in the adult is involved in the formation of trace memories. Nature 410:372–376.

Shors TJ, Townsend DA, Zhao M, Kozorovitskiy Y, Gould E (2002) Neurogenesis may relate to some but not all types of hippocampal-dependent learning. Hippocampus 12:578–584.

Stampfer MJ, Kang JH, Chen J, Cherry R, Grodstein F (2005) Effects of moderate alcohol consumption on cognitive function in women. New Engl J Med 352:245–253.

van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270.

Verbaten MN (2009) Chronic effects of low to moderate alcohol consumption on structural and functional properties of the brain: beneficial or not? Hum Psychopharacol 24:199–205.

Waddell J, Shors TJ (2008) Neurogenesis, learning and associative strength. Eur J Neurosci 27:3020–3028.

Walker BM, Walker JL, Ehlers CL (2008) Dissociable effects of ethanol consumption during the light and dark phase in adolescent and adult Wistar rats. Alcohol 42:83–89.

West MJ, Slomianka L, Gundersen HJ (1991) Unbiased stereological estimation of the total number of neurons in the subdivisions of the rat hippocampus using the optical fractionator. Anat Rec 231:482–497.

## GLOSSARY

*Bromodeoxyuridine (BrdU):* A thymidine analog that incorporates into the DNA of a dividing cell during the S phase of the cell cycle and thereby marks cells that are actively proliferating.

*Delay eyeblink conditioning:* A type of associative learning where a tone (CS) predicts stimulation to the eyelid (US) where the CS and US are contiguous in time.

*Trace eyeblink conditioning:* A type of associative learning where a tone (CS) predicts stimulation to the eyelid (US) where the CS and US are separated in time with a stimulus-free trace interval.

*Adult neurogenesis:* The development of neurons from adult neural stem cells that reside in the brain.

*(Accepted 10 August 2012)*
*(Available online xxxx)*