ABSTRACT

Binge alcohol use is of primary concern in humans and has been associated with similar patterns of alcohol use in early adulthood. In animal models, excessive ethanol has been correlated with inhibition and decreased activity within the hippocampus. The hippocampus plays a primary role in the formation of short-term memory, cognition, and learning. These impairments are most debilitating during adolescence in which the brain undergoes rapid and substantial developmental changes. The present experiment investigated the effect of ethanol on the working memory and non-spatial learning of adolescent male and female rats treated with saline or binge ethanol. Using a novel object recognition paradigm, rats were habituated within the arena with two identical objects. After three hours, the rats were reintroduced to the arena with a novel object present. Time spent within each zone which contained the objects during habituation and test was measured. For all three groups (control saline, 1.75 g/kg, and 3.0 g/kg EtOH), females spent more time exploring the novel object during the first five minutes of the trial, however this effect was greater for the EtOH-treated animals. In male rats, there was no difference in time of exploration between habituation and test under any treatment conditions. The data suggests there are sex differences mediating changes in non-spatial learning following binge-ethanol pretreatment in adolescent rats.

Keywords: ethanol, binge-ethanol treatment, learning, memory, sex differences

INTRODUCTION
Binge-drinking is defined as the consumption of four (for women) or five (for men) or more drinks on one occasion within two hours. The episodes of binge-drinking have risen drastically among young adults in the United States. About 90% of the alcohol consumed by youth under the age of 21 in the United States is in the form of binge drinks (CDC 2010). According to the U.S. Office of Juvenile Justice and Delinquency Prevention, another appalling statistic reports that binge drinkers make up 23 percent of the population, but consume 76 percent of the alcohol (Office of Juvenile Justice and Delinquency Prevention 2008). As adolescence is a time of rapid neurological growth and development, the binge-drinking pattern is taking a serious toll on the neurological processes within adolescents.

With the escalating frequency of binge-drinking among adolescents, many research studies have proved alcohol's stiffening effect on proper neurological development (Land and Spear, 2003). Meta-analysis of previous studies has shown that chronic alcohol consumption has been correlated with neurological damage and neurotoxicity in a variety of structures within the brain (Banta and Lavenex, 2009; Cippitella et al., 2010; Ford et al., 2002; Gill, 2000; Loft et al., 1987; McCool 1021, Morris et al., 2010; Obernier 2002, Nixon, 1994, Ristuccia and Spear, 1998, Sharma et al., 1998). In addition to damage in the pre-frontal cortex, nucleus accumbens, ventral tegmental area, and cerebellum, the hippocampal cortical circuits of the brain- which includes the subgranular cell layer of the dentate gyrus- are damaged by chronic alcohol consumption. In addition, the olfactory bulb, perirhinal cortices and entorhinal cortices have been found to be negatively impaired by the detrimental effects of chronic ethanol consumption (Cippitelli et al., 2010). The hippocampus plays a pivotal role in cognition, learning, as well as the formation and retention of both short and long-term memory.
A myriad of previous research studies also reveal that binge-like alcohol consumption produce spatial learning and memory impairments (Cippitelli et al., 2010). Cippitelli and fellow researchers found that following excessive alcohol consumption, there was permanent, irreversible damage to the spatial memory of rat subjects (Cippitelli et al., 2010). Studies also show that excessive alcohol consumption leads to the degeneration of neurons within the hippocampus (Morris et al., 2009). This neurodegeneration is caused by alcohol induced inhibition of neural stem cells as well the dampening neural cell survival. The inhibition of neurogenesis does not allow the components within the hippocampus to develop properly, ultimately leading to cognitive and memory retention deficits (Morris et al., 2009). In addition, studies even suggest that only short-term exposure to binge-like consumption is needed to cause neurotoxicity and cognitive impairments (Obernier et al., 2001).

There has been profound evidence that binge-ethanol consumption negatively impacts cognitive skills and short-term memory (Banta and Lavenex, 2009; Cippitelli et al., 2010; Land and Spear, 2004; McCool 2010; Morris 2010, Obernier et al., 2002; Nixon, 1994, Sutcliffe et al., 2007). However, there has been little research conducted on the sex-based disparities on non-spatial learning and working memory between male and female rat subjects following binge-pattern ethanol exposure. In humans, studies have revealed that women are more susceptible to the negative effects of alcohol than men, ranging from liver damage to cognitive functions and memory tasks (Loft et al., 1987; Mumenthaler et al., 1999; Cahill, 2006; Sharma et al., 2007). These sex disparities are contributed to the effect of female sex hormones and the oestrous cycle (Cahill, 2006; Sutcliffe et al., 2007).

The present experiment further investigates sex differences in adolescent male and female rats using a novel object recognition (NOR) paradigm after treatment with various doses
of ethanol. Novel object recognition is a suitable, simple test which allows researchers to test non-spatial learning and working memory of rat subjects (Ennaceur, 1988). By using a NOR paradigm, rats were habituated to two familiar novel objects and during the testing phase were prompted to explore a novel object in place of one of familiar objects, three hours after habituation. The test measured non-spatial learning skills as well as working memory. Duration of exploration of the novel object and preference of exploring the novel object over the familiar object during the testing phase served as the simple yet accurate indicator of working memory and cognitive skills in action (Ennaceur, 1988). The present study aimed to investigate how various doses of ethanol affected this duration of exploration as well as any sex-based disparities between the male and female rats.

**METHODS**

*Subjects*

Fifty-eight adolescent male and female Sprague-Dawley rats bred at the University of South Florida, Tampa were utilized in the present experiment. The subjects were divided into six group categorized by sex and dose of ethanol or saline administered through intraperitoneal injection. Group 1 consisted of eight male rats treated with an isovolumetric volume of saline (0.9% NaCl) per day. Group 2 consisted of eleven female rats treated with an isovolumetric volume of saline. Group 3 consisted of nine male rats treated with 1.75g/kg/ipEtOH (17% v/v EtOH diluted from 95% EtOH in saline). Group 4 consisted of ten female rats treated with 1.75 g/kg/ipEtOH. Group 5 consisted of nine male rats treated with 3.0 g/kg/ipEtOH. Group 6 consisted of eleven female rats treated with 3.0g/kg/ipEtOH. For the purpose of this study, all rat subjects were adolescents starting treatment at postnatal day (PND) 30. The date of birth was designated at PND 0 and litters were sexed and culled on PND 1. On PND 21, the rats were
weaned and tri-housed with same-sex littermates. All subjects were treated under normal conditions with free access to water and a proper diet under a 12-hour light/dark cycle (lights on at 07:00 AM).

Procedure

The present experiment was divided into three phases. The first phase involved handling the rat subjects, the second phase involved acclimating the rats to the arena followed by three cycles of four-day binge treatments, the final stage involved re-acclimation to the arena, habituation, and testing. In order to acclimate the subjects to the experimenter, all rats were handled on PND 27-28 prior to binge EtOH treatment. During the handling phase, rats explored and moved freely about the experimenter hands for five minutes. During this time frame, the rats were placed in the intraperitoneal (i.p.) injection position for thirty seconds to get accustomed to subsequent treatment. Using a novel object recognition paradigm, on PND 29, the rats were introduced to the testing arena, a square black box containing four quadrants designated by numbers 1, 2, 3, and 4, for ten minutes. Equidistant parameters for objects zones were marked within each quadrant. On PND 30, the treatment phase began, in which the rats were administered with their respective treatments of saline (0.9% NaCl), low dose ethanol: 1.75g/kg i.p. EtOH (17% v/v EtOH diluted from 95% EtOH in saline), or high-dose ethanol: 3.0g/kg i.p. EtOH. The adolescent rats were transported to the lab, weighed, and administered with the calculated dosage of their respective treatment. Injections were administered between 1200-1400 hr every 24 hours. Treatment phase took place over the course of eighteen days involving repeated binge treatment cycles. Each binge cycle comprised of four days of chronic saline or ethanol treatment followed by three days of abstinence. On PND 30—33, 37—40, and 44-47, the adolescent rats were treated with saline, low-dose ethanol, or high-dose ethanol. On
intermittent days, rats were not administered ethanol or saline. On PND 48, the rats were re-acclimated to the arena for ten minutes. On PND 49, the rats were habituated within the arena for ten minutes with two identical objects placed in alternating zones of either 1 & 3 or 2 & 4. After a three hour time span, the test took place in which the rats were reintroduced to the arena with the novel object in the place of one of the familiar objects. Using NoldusEthovision XT tracker, the duration of exploration within the zones of the two identical objects during habituation was measured as well as the duration of exploration of the familiar object and novel object during the testing phase of the experiment. Total time spent within each zone which contained the objects during habituation and test was measured. Rats were returned to colony room after experimentation.

**DESIGN AND ANALYSIS**

After treatment phase, time spent within each zone - which contained the objects during habituation and test - was measured for each rat (N=58). For all three groups (control saline, 1.75 g/kg, and 3.0 g/kg EtOH), the duration of exploration was measured and compared for each of the six categorized group designated by sex and treatment. Preferential exploration and increased duration of exploration of the novel object over familiar object indicated formation of memories which allowed the subjects to differentiate between the novel object and familiar object. Differentiation between the novel object and familiar object was based on the successful identification of the characteristic shaped and texture of the object as well as recognition of novel object within a particular zone location in the NOR paradigm.

**RESULTS**
When the novel object was introduced it was found that for all three treatment groups (control saline, low dose: 1.75 EtOH, and high dose: 3.0 EtOH) female rats spent more time exploring the novel object during the first five minutes of the trial during the testing phase, in which the novel object was present. This revealed there was a significant difference in time of exploration during the testing phase compared to the habituation phase. This effect was also greater for the female, adolescent rats treated with both the low-dose ethanol and high-dose ethanol compared to the control, saline-treated group. However, in male rats, there was no significant difference between habituation and test time of exploration under any treatment conditions.

![Figure 1](image1.png)

*Figure 1:* Compares the time of exploration during habituation to the time of exploration during testing phase during the first five minutes and last five minutes of trial. The bars represent duration of exploration corresponding to gender and treatment expressed in g/kg/min. Overlapping standard error bars indicate insignificant difference in time of exploration between the two phases.
DISCUSSION

It is evident from the proposed study that there is a profound difference on cognitive learning and short term memory retention due to sex disparities. The data reveal that female adolescent rats showed a significant difference in time of exploration during habituation and testing phase (See Figure 1). This indicates that the female rats, under saline as well as low-dose and high-dose treatment, recognized the novel object and as a result were inclined to explore the novel object. However, adolescent male rats did not have a greater time of exploration during the testing phase (See Figure 1 & 2). This resulting data coincides with Sutcliffe’s research which found that female rats performed significantly better than male rats in non-spatial Novel Object Recognition paradigm (Sutcliffe et al., 2007). In addition, male rats performed better on spatial NOR test than female rats. However, in Flannery’s experiment involving human subjects, it was found that alcoholic female subjects exhibited a more debilitating effect on tests of visual working memory, spatial planning, problem solving, and cognition compared to alcoholic male
subjects (Flannery et al., 2007). Despite the contradicting outcomes, these research studies all suggest gender influences spatial learning, non-spatial learning and working memory.

Research studies reveal how these sex disparities in non-spatial learning and memory are specifically influenced by the gonadal hormones, such as estrogen, progesterone, and testosterone. In Luine and Rodriguez’s study, estradiol was administered to male and female adolescent and adult rats to see how memory performance was affected. It was seen that males treated with or without the estradiol performed better than females on an eight-arm radial maze designed to test spatial memory (Luine and Rodriguez, 2006). The study also reported that the administration of estradiol improved the performance of male rates only. In addition to this study, many others reveal that how hormone fluctuations have been seen to alter memory performance without any neurotoxic substance such as alcohol (Ford et al., 2002; Luine and Rodriguez, 2006; Juraska and Rubinow, 2008). Research studies have also shown that fluctuations in hormones in the estrus cycle in female rats result from chronic ethanol consumption (Ford et al., 2002). However, in the present study, the various treatments and doses did not produce an observable effect on the male rates. This may be due to the fact that males are more resistant to the cognitive detrimental effects of ethanol compared to females (Nixon 1999; Mumenthaler et al., 1999; Flannery et al., 2007). In the present study, it was hypothesized that the high dose ethanol would have a more detrimental effect on the non-spatial learning and memory task compared to the low-dose ethanol and saline. This hypothesis, however, proved null as there was no significant difference in time of exploration among the various treatment conditions for male or female rats.

The present study and past research findings, further strengthen the conclusion that sex hormones influence working memory and cognitive learning abilities as well as the ethanol’s
effects on the body upon metabolism. However, further research must be conducted to investigate the underlying hormonal fluctuations that coincide with cycles of repeated binge-like ethanol consumption and how these hormonal fluctuation influence non-spatial learning, novel-object recognition, and working-memory tasks.


Increased severity of alcoholic liver injury in female verses male rats: a microarray analysis.
