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Individual differences in voluntary ethanol consumption lead to differential activation of the central amygdala in rats: relationship to the anxiolytic and stimulant effects of low dose ethanol

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Abstract

Background—Although alcohol use disorders and anxiety disorders are highly comorbid, the relationship between these two disorders is not fully understood. Previous work from our laboratory shows that anxiety-like behavior is highly variable in outbred Long-Evans rats and is related to the level of voluntary ethanol consumption, suggesting that basal anxiety state influences ethanol intake. To further examine the relationship between the acquisition of ethanol consumption and anxiety phenotype, Long-Evans rats were assessed for anxiety-like behavior and neuronal activation following voluntary ethanol consumption in a limited access drinking paradigm.

Methods—Rats were allowed to self-administer ethanol (6% v/v) for four days using a limited access drinking in the dark (DID) paradigm and divided into high and low drinking groups based on a median split of average daily ethanol intake. Immediately following the fourth drinking session, animals were tested on the elevated plus maze and evaluated for anxiety-like behaviors. Fos immunoreactivity was assessed in the central and basolateral amygdala, as well as the bed nucleus of the stria terminalis.

Results—High ethanol drinkers spent significantly more time on the open arms of the plus maze than low ethanol drinkers. High ethanol drinkers also had increased locomotor activity as compared to both low ethanol drinkers and water drinkers. Fos immunoreactivity was positively correlated with ethanol consumption in all brain regions examined, although Fos positive cell counts were only significantly different between high and low ethanol drinkers in the central amygdala.

Conclusions—Our findings demonstrate that outbred rats will voluntarily consume behaviorally effective doses of ethanol in a short-term access model and ethanol consumption is positively correlated with increased neuronal activation in the central amygdala.

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Keywords

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Introduction

Alcohol use disorders are frequently associated with comorbid anxiety disorders, with 75% of individuals that abuse alcohol having a current or previous diagnosis of an anxiety disorder (Kushner et al. 2000; Kushner et al. 2011; Swendsen et al. 2010). The prevalence with which alcohol use disorders co-occur with anxiety disorders suggests that the development and expression of these disorders may share overlapping neurobiological mechanisms. A number of studies have shown that acutely administered ethanol is anxiolytic in both humans and animal models (Eckardt et al. 1998; Kushner et al. 2000), and preclinical studies suggest that elevated anxiety states may contribute to increased drinking in two bottle choice paradigms (Primeaux et al. 2006; Spanagel et al. 1995). Moreover, repeated ethanol exposure and ethanol withdrawal can produce anxiety-like symptoms (Kliethermes 2005; Valdez et al. 2002; Zhang et al. 2007), and relieving withdrawal-induced anxiety may promote abusive drinking behavior and relapse to heavy ethanol drinking in abstinent alcoholics (Koob 2003). Although these studies indicate that ethanol can both reduce and enhance anxiety, it is unclear how individual differences in pre-existing anxiety levels contribute to ethanol use and abuse.

Clinical studies suggest that increased anxiety is associated with increased quantity and frequency of ethanol consumption, as well as coping-motivated ethanol consumption (Conrod et al. 1998; Demartini and Carey 2011). A number of preclinical studies also suggest that individual differences in unconditioned anxiety-like phenotype are associated with differences in ethanol-related behaviors. Previous work from our laboratory has shown that outbred Long-Evans rats show highly variable anxiety-like behavior on the elevated plus maze (EPM) (Primeaux et al. 2006). Rats characterized as having a high anxiety phenotype had higher preference scores for ethanol in a two bottle choice paradigm, as compared to low anxiety animals. Wistar rats show a similar relationship, with high anxiety animals having higher ethanol intake and ethanol preference than low anxiety animals (Spanagel et al. 1995), suggesting that higher basal anxiety may contribute to higher ethanol consumption. Some of the rodent lines selectively bred for high or low ethanol consumption and preference also show differences in anxiety-like behaviors. Both alcohol preferring (P) rats and Sardinian alcohol preferring (sP) rats demonstrate more anxiety-like behavior on the EPM than the associated non-preferring (NP and sNP) lines (Colombo et al. 1995; Stewart et al. 1993). Taken together, these studies indicate that the relationship between anxiety and ethanol use and abuse is not only behavioral, but likely due to individual neurobiological differences, as well.

However, not all the data point to a positive correlation between increased anxiety-like behavior and elevated ethanol consumption. Some selectively bred lines, such as the high alcohol drinking HAD and low alcohol drinking LAD rats do not show differences in anxiety-like behavior (Badia-Elder et al. 2003), while alcohol avoiding ANA rats show

increased anxiety-like behavior as compared to the alcohol preferring AA rats and the parent Wistar line (Moller et al. 1997). Additionally, in rat lines selectively bred for high and low anxiety-like behaviors, high anxiety HAB animals consumed less ethanol and had lower ethanol preference scores than the low anxiety LAB animals (Henniger et al. 2002). These discrepancies may be the result of selection for different neurobiological mechanisms controlling ethanol preference and intake in the different lines that are not well described. Additionally, previous work from our laboratory indicates that the relationship between anxiety and ethanol consumption may be influenced by the type of ethanol consumption paradigm used. In contrast to earlier results using two-bottle choice paradigms (Primeaux et al. 2006; Spanagel et al. 1995), high anxiety animals consumed less ethanol than low anxiety animals in limited access paradigms. Therefore, it is clear that further research is necessary to elucidate the complex relationship between ethanol consumption and anxiety and to determine the extent to which anxiety contributes to the development of alcohol use disorders and dependence.

The purpose of the present study was two-fold. First, we sought to determine if out-bred Long-Evans rats will consume enough ethanol to decrease anxiety-like behavior in the elevated plus maze (EPM). For these experiments, we used a short-term limited-access drinking during the dark paradigm, similar to the drinking in the dark (DID) mouse model (Rhodes et al. 2005). The benefit of this model is that it induces higher levels of ethanol consumption in non-preferring animals and more closely mimics heavy, episodic drinking than other self-administration models. We found that Long-Evans rats show highly variable ethanol consumption using this paradigm and distinct groups of high and low drinkers emerge over the course of the four days. Second, we examined the relationship between ethanol consumption, anxiety-like behavior, and activation of brain regions known to mediate anxiety, including the central and basolateral nuclei of the amygdala and the bed nucleus of the stria terminalis (BNST). Acute ethanol treatment has been shown to alter neurotransmission in each of these regions (Kash et al. 2008; Lack et al. 2008; Roberto et al. 2003; Silberman et al. 2008; Weitlauf et al. 2004), suggesting that these regions may be of particular importance in mediating the relationship between ethanol and anxiety. Our results show that high levels of ethanol consumption are positively correlated with reduced anxiety-like behavior on the EPM. Expression of c-fos, an immediate-early gene commonly used as a marker for neuronal activation (Curran and Morgan 1995), was assessed in the amygdala and the BNST. Increased ethanol consumption was also positively correlated with increased Fos expression in the central amygdala (CeA), the basolateral amygdala (BLA), and the BNST and high ethanol drinkers had significantly higher Fos positive cell counts in the CeA than either low ethanol drinkers or water drinkers. These results further our understanding of the relationship between anxiety-like behavior and the behavioral and neurobiological effects of ethanol.

METHODS

Animals

Forty adult male Long-Evans rats (175–200g; Harlan, Indianapolis, IN) were singly housed and maintained on a reversed 12-hour light dark cycle (lights off at 10AM) with *ad libitum*

access to food and water. Animals were habituated to daily handling before the experiment, and body weight, water consumption, and food consumption were monitored for the duration of the experiment. All procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee.

Ethanol Self-administration and Elevated Plus Maze

Limited access ethanol self-administration procedure was based on (Rhodes et al. 2005). Animals were divided into two groups: water drinkers (n=20) and ethanol drinkers (n=20). On ethanol exposure days, water bottles were removed and animals were presented with either a bottle of ethanol (6% v/v) or a new bottle of water on the home cage. Animals were given free access for 2 hours. Food was available during the drinking sessions, and animals were not water deprived. Bottles were presented 1.5 to 3 hours into the dark cycle (11:30AM to 1PM) to stagger the animals for behavioral testing.

Immediately following the fourth self-administration session, animals were tested in the elevated plus maze (EPM) as described in (Wilson et al. 2004). All testing was done between 1:30PM and 3:30PM. The Plexiglas elevated plus maze apparatus consists of two open arms ($56 \times 10 \times 1 \text{ cm}^3$) and two closed arms ($56 \times 10 \times 40 \text{ cm}^3$) elevated 50 cm off of the floor. At the beginning of the session, animals were placed into the center of the maze, facing one of the open arms and behavior was video recorded for 5 minutes. Animals were immediately returned to their home cages following EPM testing. The maze was cleaned with 7% ammonium hydroxide between subjects. Behavioral data was analyzed using Ethovision XT (Noldus, Leesburg, VA). Measures for analysis included time spent in each arm and total distance travelled. Anxiety-like behavior was assessed as percent open arm time, which was calculated as the time spent in the open arm as a percentage of total time spent in all arms. Locomotor activity was assessed as distance traveled in cm.

Immunohistochemistry

Two hours after EPM testing, animals were deeply anesthetized using isoflurane inhalation. Rats were transcardially perfused with ice cold phosphate buffer (0.1 M) followed by 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Brains were removed from the skulls and post-fixed for 24 hours in 4% paraformaldehyde. Brains were transferred to 15% sucrose and stored at 4°C until sectioning. Coronal brains sections (45µm) were cut on a freezing microtome (Microm, Waldorf, Germany) and stored in cryoprotectant (30% sucrose in phosphate buffered ethylene glycol) at -20°C until processing for immunohistochemistry.

Sections were washed in Tris buffered saline prior to free-floating immunohistochemical processing as previously described (Burghardt et al., 2006). Following blocking, sections were agitated with goat Fos antibody (1:1000; Santa Cruz, Santa CA), followed by biotinylated donkey anti-goat secondary antibody (1:1000 Jackson ImmunoResearch, West Grove, PA) and HRP-streptavidin (1:1600; Jackson ImmunoResearch). Sections were visualized using nickel/cobalt-enhanced diaminobenzidine to produce a blue-black precipitate in Fos immunoreactive nuclei. Sections were viewed at 10×–40× magnification under brightfield illumination (Nikon, Melville, NY). Images were acquired using a digital

camera and Fos-positive cells were counted using NeuroLucida (MBF Biosciences, Williston, VT).

For immunohistochemical analysis, measurements were taken from the left and right side of each section and expressed as positive cell counts over area of region counted (counts/mm²). Two sections were analyzed for BNST and four sections were analyzed for amygdalar nuclei. Anatomical coordinates used for analysis were taken from the Rat Brain in Stereotaxic coordinates (Paxinos and Watson 1986). The BNST was evaluated in coronal sections ranging from -0.26 to -0.40 mm posterior to Bregma. The central (CeA) and basolateral amygdala (BLA) were evaluated in coronal section ranging from -2.12 to -3.60 mm posterior to Bregma. Measurements from individual brain regions were averaged to produce a single value for a given region in each animal. Data were analyzed within the area of interest for significant correlations and using a between-subject one-way analysis of variance. All statistical analyses were done using GraphPad.

Data Analysis and Statistics

For purposes of data analysis, ethanol drinking animals were divided into high (n=10) and low (n=10) drinking groups. Limited access consumption was averaged over days 2, 3 and 4, and animals were divided into high and low drinking groups based on a median split of average daily ethanol intake (g/kg). Consumption data for low and high ethanol drinkers are expressed either as mean consumption (g/kg) per day or mean daily consumption (g/kg) over days 2, 3 and 4. Two-way repeated measures analysis of variance (RM ANOVA) was used to compare daily consumption in low and high ethanol drinkers. One-way RM ANOVA was used to compare daily consumption within low and high ethanol drinkers. Correlation statistics were used to compare behavioral measures and Fos immunoreactivity with mean consumption and one way ANOVA was used to compare behavioral measures and Fos immunoreactivity between high ethanol drinkers, low ethanol drinkers and water drinkers. All significance measurements were set to $P < 0.05$. Water drinking animals were similarly divided into high and low anxiety phenotype groups based on median percent open arm time (%OAT) on the elevated plus maze (EPM) for some analyses. One animal had 100% open arm time on the plus maze and was excluded from all analyses. A second animal was excluded from c-fos analyses due to excessive background staining.

RESULTS

Individual differences in ethanol intake are negatively correlated with unconditioned anxiety-like behavior

Figure 1A shows the daily consumption (mean g/kg $\pm$ SEM) of high and low ethanol drinkers in a four day limited access paradigm. High ethanol drinkers consumed significantly more ethanol on days 2, 3 and 4 than low ethanol drinkers ($p < 0.05$). Two-way repeated measure ANOVA revealed a main effect of both consumption group ($F[1,18]=30.74$; $p < 0.0001$) and day ($F[3,54]=10.01$; $p < 0.0001$), as well as a consumption group $\times$ day interaction ($F[3,54]=3.44$; $p < 0.05$). Figure 1B shows the average daily consumption (mean g/kg $\pm$ SEM) for low ethanol drinkers and high ethanol drinkers for limited access sessions on days 2–4. Low ethanol drinkers consumed significantly less

during the limited access sessions than high ethanol drinkers ($P<0.0001$). No differences between water drinkers, high ethanol drinkers and low ethanol drinkers were seen in daily *ad lib* food or water consumption (Table 1).

High ethanol drinkers had significantly higher percent open arm time than low ethanol drinkers (Fig 2A; $F[2,37] = 3.495$; $P<0.05$) and mean daily ethanol consumption is positively correlated with percent open arm time (Fig 2B; $R=0.75$; $F[1,17] = 21.75$; $P<0.0005$) suggesting that higher levels of ethanol consumption reduced anxiety like behavior. Interestingly, animals consuming water had mean open arm times between high and low drinkers, and were not significantly different from either of the two ethanol groups. High ethanol drinkers also had significantly higher measures of distance traveled on the plus maze (2C; $F[2,37] = 9.340$; $p<0.005$), and distance traveled was positive correlated with mean daily ethanol consumption (2D; $R=0.78$; $F[1,17] = 26.64$; $P<0.0001$). However, no correlation was found between ethanol consumption and number of closed arm entries and closed arm entries were not significantly different between high ethanol drinkers, low ethanol drinkers, and water drinkers. Mean water consumption during limited access sessions (control animals) was not correlated with any behavior on the EPM. Water drinkers did show highly variable anxiety-like behavior on the plus-maze, as previously reported, and there was a significant difference in percent open arm time between high and low anxiety animals (Table 2), but there were no significant differences in activity (distance traveled or closed arm entries) on the plus maze between high and low anxiety groups (Table 2).

Individual differences in ethanol intake and EPM behavior are correlated with Fos expression

High ethanol drinkers had significantly more Fos positive cell counts in the CeA than either water drinkers or low ethanol drinkers (Fig 3A; $F[2,37]=5.70$; $P<0.01$). As shown in Figure 3B, mean daily ethanol consumption over day 2–4 was positively correlated with Fos positive cells counts in the CeA ($R=0.75$; $F[1,16]=21.09$; $P<0.0005$), BLA ($R=0.52$; $F[1,16]=6.069$; $P<0.05$) and BNST ($R=0.50$; $F[1,16]=5.256$; $P<0.05$), but there were no significant differences in Fos positive cell counts in the BLA or BNST between high and low ethanol drinkers (Fig 3A). Water consumption during the limited access period was not correlated with Fos positive cell counts in any brain region, nor was there any difference between Fos positive cell counts when water drinkers were divided into high and low anxiety groups (Table 2). Among ethanol drinkers, percent open arm time on the EPM was positively correlated with Fos positive cell counts in the CeA ($R=0.82$; $F[1,16]=33.62$; $P<0.0001$) and BNST ($R=0.51$; $F[1,16]=5.690$; $P<0.05$) (Fig 3C) and distance traveled was positive correlated with Fos positive counts in the CeA ($R=0.72$; $F[1,17]=19.10$; $P<0.0005$) (Fig 3D). Figure 4A shows the location of the brain regions analyzed: BNST (left panel), CeA, and BLA (right panel). Panels B-D are representative photomicrographs of c-Fos positive staining in the central amygdala of a water drinker (B), low ethanol drinker (C), and high ethanol drinker (D).

DISCUSSION

Relief of anxiety is often cited as a motivation for alcohol consumption, and it is thought that the need to relieve anxiety symptoms can promote increased alcohol consumption and the transition from moderate ethanol intake to abuse and dependence (Koob 2003). However, the relationship between anxiety and the acquisition of alcohol use is not well understood. The present study seeks to further elucidate the complex interaction of ethanol consumption and anxiety by examining individual differences in drinking behavior and the resulting differences in anxiety-like behavior and region-specific neuronal activation. Using a short-term limited access voluntary drinking paradigm in rats, we looked first at the anxiolytic effects of consumed ethanol. We found that higher levels of voluntary ethanol consumption were positively correlated with decreased anxiety-like behavior, measured as percent open arm time, in the elevated plus maze. To focus specifically on the differences resulting from high and low ethanol consumption, we divided the ethanol drinking animals into high and low drinking groups based on mean daily ethanol intake. High ethanol drinkers spent more time on the open arms of the elevated plus maze than low ethanol drinkers. High ethanol drinkers also had more Fos positive cell counts in the central amygdala than low ethanol drinkers, suggesting that a certain level of ethanol consumption and/or plus maze exposure is needed for activation of the central amygdala. Importantly, daily consumption levels and anxiety-like behaviors did not correlate with Fos expression in water drinkers, indicating that these effects are specific to animals that consumed ethanol and that individual differences in plus maze behavior alone do not cause neuronal activation of the central amygdala.

Identifying risk factors for disease is a common research goal across many fields. Although demographic and heredity factors that contribute to alcohol use disorders are often reported, more subtle behavioral and neurobiological differences also play a role in susceptibility to alcohol abuse and dependence. Outbred rat strains are useful for examining these individual differences, but studies can be limited by the fact that non-selected strains generally do not voluntarily consume high quantities of ethanol. To induce higher ethanol consumption in Long-Evans rats, we used a limited access paradigm similar to the mouse drinking in the dark model. In our procedure, animals have 2 hours of access to a 6% ethanol solution for four days, with mean daily intake ranging from 0.24g/kg to 1.86g/kg (as compared to 2.5g/kg/day in a 24 hour 2 bottle choice paradigm (Primeaux et al. 2006)). Although blood ethanol concentrations were not assessed in this study to avoid manipulations that could induce changes in Fos or change EPM behavior, previous experiments from our laboratory indicate that low ethanol drinkers have an average BEC of 19 mg% and high ethanol drinkers have an average BEC of 53 mg% at the end of the fourth drinking session (unpublished data). The four day time course is intentionally short to model acute heavy ethanol consumption, but ethanol intake consistently plateaus after two days of access (see Fig 1).

The correlation between the dose of ethanol consumed and the reduction in anxiety-like behaviors is in keeping with the behavioral effects of acute administration of low doses of ethanol. The high ethanol drinkers had a mean daily intake of 1.3g/kg, which is within the 1.0g/kg to 2.0g/kg dose range of ethanol's anxiolytic effects on the elevated plus maze (Bertoglio and Carobrez 2002; Boerngen-Lacerda and Souza-Formigoni 2000; Wilson et al.

2004). In contrast, low ethanol drinkers had a mean daily intake of 0.6g/kg, below the lower limit of ethanol's anxiolytic effects. While our data show that Long-Evans rats will voluntarily consume doses of ethanol that have been reported to produce anxiolysis, it is unclear to what extent the quantity of ethanol consumed is contributing to the differences in behavior on the elevated plus maze. The data presented here are strictly correlative, so we cannot definitively conclude that the changes in plus maze behavior are the result of ethanol exposure rather than the selection process. We have previously shown that ethanol-naïve Long-Evans rats show highly variable anxiety-like behavior on the elevated plus maze and percent open arm time positively correlates with subsequent ethanol consumption during limited access drinking (White et al. 2009). These data suggest that the low ethanol drinkers may have a higher basal anxiety phenotype than the high ethanol drinkers prior to ethanol consumption and the observed differences in anxiety-like behavior following ethanol consumption may be the result of differences in basal anxiety phenotype, rather than the quantity of ethanol consumed. Higher basal anxiety might explain why the low ethanol drinkers consumed significantly less liquid during the limited access sessions than either water or high ethanol drinkers, as the novel bottle on the cage could be aversive, but this could also be the result of increased sensitivity to the aversive effects of the ethanol itself and may represent another facet of the individual differences that contribute to alcohol use and abuse.

Although the high ethanol drinkers have a significantly higher percentage of open arm time on the plus maze than low ethanol drinkers, they do not differ from the water drinkers in this measure. This may be evidence that the high ethanol drinkers do not consume enough ethanol to express the anxiolytic effects of ethanol. However, the low ethanol drinkers also did not differ from the water drinkers in open arm time, suggesting that there is a complex interaction between trait anxiety phenotype, voluntary ethanol consumption, and the anxiolytic effects of ethanol that needs to be explored further. Ethanol consumption was positively correlated with distance traveled on the plus maze, and high ethanol drinkers have significantly higher measures of distance traveled as compared to low ethanol drinkers and water drinkers. However, closed arm entries, another measure of locomotor activity specific to the plus maze, were not correlated with ethanol consumption, nor were there any differences in closed arm entries among high drinkers, low drinkers, and water drinkers.

Fos immunoreactivity was positively correlated with ethanol consumption in the CeA, BLA and BNST, suggesting that the differences in Fos activation in these regions may be due, at least in part, to the quantity of ethanol consumed. High ethanol drinkers had significantly greater Fos-positive cell counts in the central amygdala as compared to low ethanol drinkers and water drinkers. This increase is consistent with reports that low to moderate doses of ethanol (0.5–2.0 g/kg) increase Fos immunoreactivity in the central amygdala (Bachtell et al. 1999; Chang et al. 1995; Morales et al. 1998; Thiele et al. 1997). Our previous analysis of blood ethanol levels in high and low drinkers (White et al. 2009) suggests that the low ethanol drinkers may not achieve BECs high enough to induce increases in Fos activation and that the increased Fos immunoreactivity in the central amygdala seen in these experiments is produced by a certain level of ethanol consumption, and perhaps independent of EPM exposure. Although there was a weak correlation between ethanol consumption and Fos positive cell counts in both the BLA and BNST, there was no difference in Fos positive

counts between high and low ethanol drinkers. These results are consistent with previous reports that low dose ethanol does not alter Fos induction in these regions, although higher doses of ethanol (3g/kg) do increase Fos immunoreactivity in the BNST (Bachtell et al. 1999; Chang et al. 1995; Herring et al. 2004; White et al. 2009).

Since all animals were exposed to the elevated plus maze, the changes in Fos activation may also be related to the observed behavioral outcomes in the plus maze. The central amygdala is known to play a role in both natural and pathological anxiety, as well as alcohol use and abuse, making it a prime site for examining the overlapping mechanisms that contribute to these behaviors. A single plus maze exposure does not alter Fos expression in the central amygdala (Albrechet-Souza et al. 2008; Duncan et al. 1996) and individual differences in basal anxiety phenotype alone do not appear to account for differences in Fos activation, as we found no correlation between plus maze activity and Fos immunoreactivity in water drinkers. The positive correlations between open arm time and Fos immunoreactivity in the CeA and BNST of ethanol, but not water, drinkers suggest that the increased neuronal activation seen in these regions may be related to the anxiolytic effects of ethanol, although we cannot determine causation due to the correlative nature of the data. As there was a significant difference in locomotor activity between high ethanol drinkers and water drinkers/low ethanol drinkers, the observed increase in Fos immunoreactivity may be related to the locomotor stimulating properties of ethanol. Increased sensitivity to the locomotor stimulating effects of ethanol has been shown in sP rats as compared to sNP rats (Agabio et al. 2001) suggesting that the high ethanol drinkers may be more sensitive to ethanol-induced locomotor stimulation. Furthermore, DBA/2J inbred mice which are more sensitive to the locomotor stimulating effects of low dose ethanol than C57BL/6J inbred mice show increased Fos immunoreactivity in the central amygdala, as compared to BL6, after ethanol challenge (Hitzemann and Hitzemann 1997). These data indicate that sensitivity to low dose ethanol effects, as measured by locomotor activation, may be another factor for which individual differences in response play a role in alcohol use and abuse. However, given that we found no significant group differences in closed arm entries, additional work is required to determine if individual differences in behavioral responses to low dose ethanol relate to differences in ethanol consumption and in amygdalar activation.

It has been suggested that the anxiolytic and the locomotor stimulating properties of low dose ethanol are mediated by separate neurobiological mechanisms (see Boerngen-Lacerda and Souza-Formigoni 2000), but there has been varying success identifying mechanisms that mediate one behavior and not the other (Ghozland et al. 2005; Olive et al. 2003; Tambour et al. 2005). Based on our behavioral data, the differences in Fos induction we observed may serve as a starting point for identifying potential targets for further examination. Phenotypic analysis of the Fos positive cells will allow us to determine which types of cells are activated following voluntary ethanol consumption and plus maze exposure and may clarify the role of the observed neuronal activation in the effects of low dose ethanol. Further analysis of Fos activation in other brain regions, particularly cortical regions, is also warranted based on our results.

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Figure 1.

Daily ethanol intake for high and low ethanol drinkers and average daily intake for days 2–4. A) High ethanol drinkers consumed significantly more ethanol (g/kg) than low ethanol drinkers on days 2–4. B). Low ethanol drinkers consumed significantly less average ethanol (g/kg) averaged over days 2–4 than high ethanol drinkers. N=9–10; *: P<0.05

Figure 2.

Plus maze behavior. A) High ethanol drinkers had a significantly greater percentage of open arm time (%OAT) than low ethanol drinkers. Open arm time for water drinkers was not different from either high or low ethanol drinkers. B) High ethanol drinkers travelled farther on the plus maze than either low ethanol drinkers or water drinkers, and distance travelled was positively correlated with ethanol consumption. Water drinkers N=20; low ethanol drinkers N=9; high ethanol drinkers N= 10; *:P<0.05. C) Percent open arm time and D) distance travelled are positively correlated with ethanol consumption (N=19).

Figure 3.

Fos positive cell counts in the CeA, BLA and BNST. A) High ethanol drinkers had significantly more Fos positive cell counts in the central amygdala than low ethanol drinkers or water drinkers. $N=9$; $^*P<0.05$. Fos positive cell counts were positively correlated with ethanol consumption (B) in all brain regions, with percent open arm time (C) in the CeA and BNST, and with distance traveled (D) in the CeA ($N=18$).

Figure 4.

A) Diagrams (modified from Paxinos and Watson 1986) showing the area counted as central amygdala, basolateral amygdala and BNST. Representative photomicrographs (20X) of Fos immunoreactivity in the central amygdala of a water drinker (B), low ethanol drinker (C), and high ethanol drinker (D). Scale bar =100 microns.

Table 1

Mean daily *ad lib* food (g $\pm$ SEM) and water intake (ml $\pm$ SEM) before and during limited access drinking sessions

Baseline measurements are mean water and food intake for four days before initiation of limited access sessions. Limited access measurements are mean daily water intake outside of limited access sessions (22 hours) and mean daily food intake (24 hours) on limited access days.

	Water Drinkers	Low Ethanol Drinkers	High Ethanol Drinkers
Baseline water intake	32.76 $\pm$ 1.00	33.53 $\pm$ 3.76	32.43 $\pm$ 5.65
Limited access water intake	31.31 $\pm$ 1.17	35.64 $\pm$ 4.71	33.02 $\pm$ 4.43
Baseline food intake	23.35 $\pm$ 2.25	23.84 $\pm$ 2.73	24.60 $\pm$ 3.34
Limited access food intake	23.64 $\pm$ 2.54	23.01 $\pm$ 3.43	23.91 $\pm$ 3.12

Table 2

Plus maze behaviors ($\pm$ SEM) and c-fos immunohistochemistry ($\pm$ SEM) for high and low anxiety water drinkers

	High Anxiety	Low Anxiety
% open arm time	1.4* ($\pm$ 0.6)	18.5* ($\pm$ 3.5)
Closed arm entries	15.9 ($\pm$ 1.7)	19.7 ($\pm$ 2.0)
Distance (cm)	2179 ($\pm$ 137)	2345 ($\pm$ 128)
c-fos (counts/mm ²)		
CeA	80.0 ($\pm$ 8.6)	82.3 ($\pm$ 6.9)
BLA	76.4 ($\pm$ 7.6)	80.3 ($\pm$ 7.2)
BNST	88.8 ($\pm$ 9.6)	93.4 ($\pm$ 6.0)