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Role for the rostromedial tegmental nucleus in signaling the aversive properties of alcohol

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Abstract

Background—While the rewarding effects of alcohol contribute significantly to its addictive potential, it is becoming increasingly appreciated that alcohol's aversive properties also play an important role in the propensity to drink. Despite this, the neurobiological mechanism for alcohol's aversive actions is not well understood. The rostromedial tegmental nucleus (RMTg) was recently characterized for its involvement in aversive signaling and has been shown to encode the aversive properties of cocaine, yet its involvement in alcohol's aversive actions have not been elucidated.

Methods—Adult male and female Long-Evans rats underwent conditioned taste aversion (CTA) procedures where exposure to a novel saccharin solution was paired with i.p. administration of saline, lithium chloride (LiCl), or ethanol (EtOH). Control rats underwent the same paradigm except that drug and saccharin exposure were explicitly unpaired. Saccharin consumption was measured on test day in the absence of drug administration and rats were sacrificed 90–105 min following access to saccharin. Brains were subsequently harvested and processed for cFos immunohistochemistry. The number of cFos labeled neurons was counted in the RMTg and the lateral habenula (LHb) – a region that sends prominent glutamatergic input to the RMTg.

Results—In rats that received paired drug and saccharin exposure, EtOH and LiCl induced significant CTA compared to saline to a similar degree in males and females. Both EtOH- and LiCl-induced CTA significantly enhanced cFos expression in the RMTg and LHb but not the hippocampus. Similar to behavioral measures, no significant effect of sex on CTA-induced cFos expression was observed. cFos expression in both the RMTg and LHb was significantly correlated to CTA magnitude with greater cFos being associated with more pronounced CTA. In addition, cFos expression in the RMTg was positively correlated with LHb cFos.

Conclusions—These data suggest that the RMTg and LHb are involved in the expression of CTA and are consistent with previous work implicating the RMTg in aversive signaling. Furthermore, increased cFos expression in the RMTg following EtOH-induced CTA suggests that this region plays a role in signaling alcohol's aversive properties.

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Keywords

conditioned taste aversion; lateral habenula; addiction; sex differences; cFos

Introduction

For many years, research on the neurobiology of alcohol addiction has focused on a mechanistic understanding of the rewarding properties of alcohol, which clearly contribute to its addictive potential. A number of studies, however, have also established a prominent role for alcohol's aversive properties in the propensity to drink. For example, individuals who are family history positive for an alcohol use disorder (AUD), and therefore at greater risk for the development of an AUD, are less sensitive to the impairing effects of alcohol (Schuckit et al., 2004; Trim et al., 2009). This is particularly true when subjects are evaluated during the descending limb of the blood alcohol curve when the aversive properties of alcohol are most salient (Morean and Corbin, 2010). Similarly, heavy drinkers frequently report experiencing greater stimulant-like effects and fewer sedative-like and aversive effects of alcohol than light drinkers (Holdstock et al., 2000; King et al., 2002). Collectively, these data and related studies are supportive of a link between sensitivity to alcohol's subjective effects and the propensity to drink (Morean and Corbin, 2010; Quinn and Fromme, 2011). While causality of this relationship has been difficult to establish in human populations, recent work has shown that the reduced sensitivity to alcohol's aversive properties observed in heavy drinkers is in fact predictive of future problem drinking and AUD diagnosis when assessed during follow up two years later (King et al., 2011). This suggests that differences in subjective response to alcohol predate drinking. These data are also supported by work in rodent models indicating that results from behavioral assays measuring reward and aversion correlate similarly with levels of ethanol intake (Green and Grahame, 2008; Cunningham, 2014). These findings have prompted a re-conceptualization of the theoretical framework for drug and alcohol use as a function of the balance between reward and aversion. Within this framework a drug's rewarding properties serve to promote its use while its aversive properties serve to limit use (Verendeev and Riley, 2012).

Despite an increasing appreciation for the role of aversion in alcohol use and misuse, the neurobiological basis for the aversive response to alcohol is not well understood. One brain region that has garnered growing interest due to its involvement in aversive signaling is a midbrain area known as the rostromedial tegmental nucleus (RMTg). The RMTg is located immediately posterior to the ventral tegmental area (VTA) and consists primarily of GABAergic projection neurons that synapse onto dopaminergic neurons of the VTA and substantia nigra (SN) (Kaufling et al., 2009; Jhou et al., 2009b; Balcita-Pedicino et al., 2011). Early work suggested that neurons in this region encode aversive stimuli as evidenced by enhanced cFos expression following aversive, but not rewarding, stimuli such as footshock and food deprivation and their predictive cues (Jhou et al., 2009a). In addition, lesions of this region have been shown to significantly reduce a wide range of behaviors indicative of fear, anxiety and pain (Jhou et al., 2009a, 2012).

The RMTg receives dense glutamatergic input from the lateral habenula (LHb) (Kaufling et al., 2009; Jhou et al., 2009b; Balcita-Pedicino et al., 2011), a region previously shown to encode negative reward prediction errors (Hikosaka et al., 2008). Recently, Hikosaka and colleagues extended this work by demonstrating a critical role for the RMTg in facilitating negative reward prediction error through a functional circuit made up of glutamatergic afferents from the LHb to RMTg, which in turn sends GABAergic efferents to the VTA and SN (Hong et al., 2011). Collectively these data have prompted increased interest in the involvement of the RMTg in the response to the aversive properties of drugs of abuse. Indeed, recent work has implicated the RMTg in signaling the aversive properties of cocaine (Jhou et al., 2013), very little is known regarding its role in signaling the aversive properties of alcohol.

The present study was designed to investigate the role of the RMTg in signaling the aversive properties of alcohol by assessing cFos expression following ethanol-induced conditioned taste aversion (CTA). Because the magnitude of aversion to alcohol has previously been shown to differ between the sexes (Cailhol and Mormède, 2002; Vetter-O'Hagen et al., 2009; Sherrill et al., 2011; Morales and Spear, 2013; but see Morales et al., 2014; Schramm-Sapota et al., 2014) we undertook this study in both male and female rats to determine if sex differences in behavioral response were also reflected in our neurobiological measures. The resulting data demonstrated a significant inverse relationship between cFos expression in the RMTg and magnitude of ethanol-induced CTA in both males and females. These data are the first to suggest a role for the RMTg in CTA expression and its potential involvement in signaling alcohol's aversive properties.

Materials & Methods

Animals

Adult male and female Long-Evans rats (P60) were purchased from Harlan Laboratories and housed individually in standard polycarbonate cages for the duration of the study. The animal colony room was maintained on a 12:12 reverse light-dark cycle with lights extinguished at 09:00. After at least one week of habituation to the new housing facilities, rats were briefly handled daily for at least one week in preparation for the experiments described below. All procedures were conducted with the approval of the Medical University of South Carolina's Institutional Animal Care and Use Committee and abided by the National Research Council's Guide for the Care and Use of Laboratory Animals (National Research Council, 2011).

Drugs

All drugs and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. The saccharin drinking solution was made up of saccharin sodium salt hydrate diluted to 0.1% in tap water. Ninety-five percent ethanol (Ultra Pure, Darien, CT) was diluted in saline (Hospira, Lake Forest, IL) to a final concentration of 20%. Lithium chloride hydrate was dissolved in saline at a concentration of 0.15 M.

Conditioned taste aversion

The aversive properties of saline, EtOH and LiCl were assessed in male and female rats using a standard conditioned taste aversion (CTA) protocol according to previously published methods (Rinker et al., 2008). In brief, after at least one week of daily handling, water access was restricted to 30 min a day for seven days to habituate rats to the restricted access schedule. Beginning on the eighth day, rats underwent three conditioning trials where they were provided with a novel 0.1% saccharin solution instead of water. In the Paired condition, this was immediately followed by an i.p. injection of either ethanol (EtOH; 1.5 g/kg), lithium chloride (LiCl; 127 mg/kg) or saline (EtOH equivalent volume) (n=6–10 rats/group). Each conditioning trial was followed by three water recovery days to facilitate the return of fluid consumption to preconditioning levels (Figure 1A). Following the last conditioning trial and water recovery period, the rats were once again provided access to the saccharin solution but this time in the absence of drug administration. Control rats, in the Unpaired condition, underwent the same procedure except that saccharin and drug exposure were explicitly unpaired with i.p. drug injection following water exposure on the first of the three water recovery days (n=6 rats/group; Figure 1B). Fluid consumption was measured daily following the 30 min access period by comparing pre- and post-access bottle weights for each animal. Rats were sacrificed 90–105 min following their final saccharin exposure. Prior to daily fluid access, rats were weighed and handled. During this time, both male and female rats were habituated to a restraint hold that allows for both the administration of an i.p. injection and collection of a vaginal smear. All procedures were performed daily between 10:00 and 11:00.

Estrous cycle measurements

Prior to daily fluid access as described above, vaginal smears were collected from females using a non-invasive procedure as previously described with minor modifications (Marcondes et al., 2002). In brief, female rats were restrained and 40 µl of sterile water was gently pipetted onto the vaginal opening and then immediately aspirated back into the pipette tip. The sample was then transferred to a plain glass microscope slide, allowed to air dry overnight and then stored at room temperature until processing for histological analysis. Male rats were similarly restrained and briefly touched to mimic the contact experienced by the females. Vaginal smears were visualized using Quick-Dip™ stain (Mercedes Medical, Sarasota, FL). Estrous cycle phase was subsequently identified by visual inspection according to previously published methods (Cora et al., 2015) at 40X magnification using an infinity plan achromatic compound microscope (AmScope, Irvine, CA).

Immunohistochemistry

cFos expression was determined in slices containing the RMTg, LHb and hippocampus from saline, LiCl and EtOH groups following previously described methods (Trantham-Davidson et al., 2014) with minor modifications. In brief, 90–105 min following the onset of final saccharin exposure on test day, rats were transcardially perfused with PBS (pH 7.4) followed by perfusion with 4% paraformaldehyde. The brain was then rapidly removed from the skull and immediately immersed in 4% paraformaldehyde overnight followed by immersion in 30% sucrose for 48–72 hr. Brains were subsequently frozen on dry ice and sectioned

coronally at 40 μm using a cryostat maintained at -20°C . Free floating sections were stored in cryoprotectant at -20°C until ready for immunohistochemical analysis. Sections containing the RMTg, LHb and hippocampus were processed together for cFos expression using standard avidin-biotin complex (ABC) methods (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Tissue was first rinsed in PBS followed by incubation in PBS containing 0.4% Triton-X for 1 hr. The tissue was then incubated overnight at room temperature in the same solution with the addition of cFos primary antibody (rabbit polyclonal, 1:50,000; PC38 or 1:20,000 PC05; Millipore, Billerica, MA) followed by incubation in biotinylated donkey anti-rabbit secondary antibody (1:1000, Jackson ImmunoResearch, West Grove, PA) for 30 min. The antigen was visualized in 0.05% 3,3'-diaminobenzidine, 0.05% ammonium nickel(II) sulfate hexahydrate and 0.015% hydrogen peroxide for 20 min. Sections were then mounted on Frost Plus slides, dried, dehydrated, and coverslipped in preparation for analysis.

For each rat, cFos labeled (cFos⁺) cells were counted manually within each coronal slice throughout the entire RMTg and LHb and within a 2.2 mm² region overlying the dentate gyrus and CA3 region of the hippocampus under 10X magnification using an infinity plan achromatic compound microscope (AmScope, Irvine, CA). Counts were made across four serial sections from one set of a 1-in-6 series (i.e., 40 μm $\times$ 6 = 240 μm apart) spanning the rostrocaudal extent of the region. Thus, counts were made at levels corresponding to approximately AP: -6.36 to -7.08 for the RMTg and AP: -3.36 to -4.08 for the LHb and hippocampus according to the atlas of Paxinos and Watson (2007) (Figure 1C–D). Within each slice, cells were counted in triplicate and these counts were averaged together for a single count per hemisphere per slice. Cell counts from each region from all four slices were subsequently averaged together for each rat. Counts between hemispheres were averaged together to arrive at a single count for each region for each rat.

Statistical analysis

Data were analyzed using multifactorial analysis of variance (ANOVA) where applicable with Paired vs Unpaired conditions, Sex, and Drug group as the between-subject factors and saccharin intake across Conditioning trials and cFos counts as within-subject factors. ANOVA was also used to assess the effect of estrous cycle on fluid consumption and cFos expression across Drug groups and Paired vs Unpaired conditions. Linear regression was used to determine the relationship between cFos expression and CTA magnitude and cFos expression between the RMTg and LHb. Where applicable post-hoc analyses were performed using Tukey's HSD test. All statistical tests and graphs were generated using IBM SPSS Statistics Version 23 and Prism 6 for Mac OS X, respectively.

Results

Baseline saccharin intake is similar across sexes and drug groups

Similar levels of saccharin consumption were observed between groups during the first conditioning trial (Cond 1). A multifactorial ANOVA revealed no significant main effect of Sex [$F(1,67)$ 1.872, $p=0.176$], Paired vs Unpaired conditions [$F(1,67)$ 1.793, $p=0.185$] or Drug group [$F(1,67)$ 1.006, $p=0.371$] on baseline saccharin intake (Figure 2).

LiCl and EtOH induce significant conditioned taste aversion

Analysis of saccharin intake over the course of the CTA procedure revealed a significant main effect of Conditioning trial [$F(3,198) = 9.455$, $p < 0.0001$]. Significant interactions were also observed between Conditioning trial and Sex [$F(3,198) = 4.043$, $p = 0.008$], Conditioning trial and Paired vs Unpaired conditions [$F(3,198) = 55.273$, $p < 0.0001$] and Conditioning trial and Drug group [$F(6,198) = 16.866$, $p < 0.0001$]. A significant three-way interaction between Conditioning trial, Paired vs Unpaired conditions and Drug group was observed [$F(6,198) = 21.821$, $p < 0.0001$], however, no additional significant interactions with Sex as a factor were observed. Therefore, Sex was excluded as a between-subjects factor in subsequent analyses. Post-hoc analyses revealed significant differences in saccharin consumption in rats in the Paired condition only between LiCl and saline, EtOH and saline and EtOH and LiCl on the second and third conditioning trials as well as on test day ($p = 0.01$). In contrast, saccharin intake did not differ significantly between Drug groups in rats in the Unpaired condition. Rats in the Unpaired condition did, however, exhibit a significant increase in saccharin intake across conditioning trials ($p = 0.05$; Figure 3A–D). These data show that, when paired with saccharin exposure, both EtOH and LiCl induced significant CTA compared to saline, whereas no CTA developed in any drug group when the drug was explicitly unpaired with saccharin exposure. Furthermore, saccharin consumption did not differ between sexes in both conditions (Paired vs Unpaired) and drug groups.

CTA enhances cFos expression in the RMTg

The RMTg has been characterized for its role in aversion, but its involvement in signaling the aversive properties of EtOH has not yet been elucidated. To investigate this possibility, cFos expression was measured in the RMTg of male and female rats in both Paired and Unpaired CTA conditions. A multifactorial ANOVA revealed significant main effects of Drug group [$F(2,68) = 4.850$, $p = 0.011$] and Paired vs Unpaired conditions [$F(1,68) = 22.421$, $p < 0.001$] on RMTg cFos expression. No main effect of Sex [$F(1,68) = 0.071$, $p = 0.791$] or interaction between Sex and the other factors were observed. Therefore, Sex was excluded as a between-subjects factor in subsequent analyses. Post-hoc analyses revealed that, within the Paired condition, both LiCl- and EtOH-induced CTA significantly enhanced cFos expression in the RMTg compared to saline ($p = 0.01$; Figure 4A–C). RMTg cFos expression following LiCl- and EtOH-induced CTA was also significantly greater than RMTg cFos expression in all three drug groups in the Unpaired condition ($p = 0.01$). In contrast, RMTg cFos expression was similar between drug groups in the Unpaired condition and did not differ significantly from RMTg cFos expression observed in saline treated rats in the Paired condition. In addition, the magnitude of cFos induction in the RMTg correlated negatively with saccharin consumption on test day [$F(1,78) = 19.79$, $p < 0.0001$] (Figure 4D). Together these data show that expression of LiCl- and EtOH-induced CTA is associated with increased neuronal activity within the RMTg.

CTA enhances cFos expression in the LHb

Previous data has shown that activity within the LHb drives RMTg inhibitory control over dopamine release (Hong et al., 2011). Based on these findings, we also measured CTA-induced cFos expression in the LHb of the same animals. Cell counts were not completed for

one male saline Paired and one male LiCl Unpaired rat due to tissue damage. In addition, one female EtOH Unpaired rat was excluded from analysis after a Grubbs' test identified it as a significant outlier with LHb cFos counts falling outside two standard deviations from the mean. Similar to our results in the RMTg, a multifactorial ANOVA revealed significant main effects of Paired vs Unpaired condition [$F(1,65) = 4.206$, $p=0.044$] and Drug group [$F(2,65) = 9.649$, $p<0.001$] on LHb cFos expression. A significant interaction was also observed between Paired vs Unpaired condition and Drug group [$F(2,65) = 12.636$, $p<0.001$] but no main effect of Sex or interaction between Sex and any other factors was observed. Post-hoc analyses uncovered a significant enhancement of cFos expression following both LiCl- and EtOH-induced CTA compared to saline in the Paired condition ($p<0.05$), whereas, cFos expression in all three drug groups in the Unpaired condition did not differ significantly from that observed in the saline Paired condition (Figure 5A–C). Again, similar to our results in the RMTg, the magnitude of cFos induction in the LHb correlated negatively with saccharin consumption on test day [$F(1,75) = 32.51$, $p<0.0001$] (Figure 5D). In addition, a linear regression analysis demonstrated a significant positive correlation between cFos expression in the LHb and cFos expression in the RMTg [$F(1,75) = 56.23$, $p<0.0001$] (Figure 6). These data indicate that expression of CTA is associated with greater neuronal activity in the LHb and that this activity is significantly correlated with activity within the RMTg.

CTA does not affect cFos expression in the hippocampus

Previous studies have demonstrated a relationship between increased neuronal activity in both the RMTg and LHb and response to aversive stimuli (Jhou et al., 2009a, 2013). In contrast, activity within the hippocampus is not critical for the expression of conditioned taste aversion (Welzl et al., 2001). Consequently, we also measured cFos in the hippocampus of the same rats to serve as a regional control and strengthen our hypothesis that the RMTg and LHb are involved in CTA. A multifactorial ANOVA revealed a main effect of Paired vs Unpaired conditions [$F(1,68) = 6.293$, $p=0.015$] but no effects of Sex or Drug group or significant interactions between any of the three between-subject factors on cFos expression in the hippocampus (Figure 7).

Behavioral and neurobiological measures were unaffected by estrous cycle

A multifactorial ANOVA was used to assess the effect of estrous cycle on saccharin intake across conditioning trials and test day. No main effect of estrous cycle or interactions between estrous cycle and any other between-subject factor was observed (data not shown). Similarly, no main effect of estrous cycle or interaction between estrous cycle and any other between-subjects factor was observed when assessing the effect of estrous cycle on cFos expression in the RMTg, LHb and hippocampus. Although, the present study was not designed to explicitly examine the role of estrous cycle on saccharin intake or cFos expression following CTA, the present data suggest no influence of estrous cycle phase on either measure.

Discussion

The present study demonstrates a significant enhancement of cFos expression in the RMTg and LHb following both EtOH- and LiCl-induced CTA. cFos expression in both regions was inversely related to CTA magnitude indicative of increased neuronal activity within the RMTg and LHb in association with expression of CTA. It is possible that these CTA-induced changes in cFos expression are due to differences in saccharin consumption alone, as opposed to the learned association between saccharin and drug administration, since both drug groups show reduced saccharin consumption compared to saline controls. However, if this were the case, it would mean that low levels of saccharin consumption induce high levels of cFos expression, whereas high levels of consumption suppress cFos expression in the RMTg and LHb. This explanation is unlikely given that previous work has shown similar cFos induction in the RMTg following exposure to other aversive stimuli including shock, shock-predictive cues and food deprivation (Jhou et al., 2009a). In addition, lesions of the RMTg reduce freezing behavior in both conditioned and unconditioned fear paradigms and increases time spent in the open arms of an elevated plus maze (Jhou et al., 2009a). Activity within the RMTg is also involved in the response to pain (Jhou et al., 2012). These data are consistent with the idea that the RMTg serves as a “hub” for aversive signaling (Bourdy and Barrot, 2012). In this theoretical framework, RMTg activity exerts inhibitory control over dopamine release within the VTA and SN to orchestrate emotional and motor responses to aversive stimuli. Evidence for increased RMTg cell firing during shock presentation (Jhou et al., 2009a) and reward omission (Hong et al., 2011) lends additional support to this idea. In agreement with this, the cFos results presented here likely reflect enhanced RMTg and LHb cell firing following presentation of a cue predicting the aversive properties of EtOH or LiCl and suggest that these regions may play a role in signaling the aversive properties drugs including EtOH. Interestingly, recent work has shown increased EtOH intake following muscimol inhibition (Fu et al., 2016b) or dermorphin-saporin ablation of mu-opioid-expressing neurons within the RMTg (Fu et al., 2016a) suggesting that activity within the RMTg plays a key role in the propensity to drink. In light of the present findings, these data suggest that the RMTg may be critical for signaling the aversive properties of EtOH, which may function to limit intake.

In the present study, we observed a significant correlation between cFos expression in the RMTg and LHb. This observation is in agreement with a previous observation of a functional link between the LHb and RMTg where activity within the LHb drives RMTg activity (Hong et al., 2011). Interestingly, recent work investigating the effect of LHb lesions on EtOH-induced CTA reported similar levels of saccharin consumption between lesioned and intact rats following a single pairing of 0.7 g/kg EtOH with saccharin, but increased levels of consumption on subsequent days when saccharin was presented in the absence of EtOH injection (Haack et al., 2014). These data suggest that the LHb is involved in extinction, but not expression, of EtOH-induced CTA. While this observation would appear to be inconsistent with the findings in the present study, lesions of the LHb, unlike lesions of the RMTg, have been shown to have no effect on the expression of conditioned fear or performance in the elevated plus maze (Murphy et al., 1996; Heldt and Ressler, 2006). Similarly, lesions of the fasciculus retroflexus, the white matter tract carrying projections

from the LHb to the RMTg, reduce, but do not eliminate, cocaine-induced cFos expression in the RMTg (Jhou et al., 2013). Thus, the absence of an effect of LHb lesion on CTA expression is generally consistent with previous studies, and suggests that the LHb is neither necessary nor sufficient for behavioral output following exposure to these aversive stimuli. Of note, the RMTg also receives input from several other brain regions known for their involvement in the response to aversive stimuli including the periaqueductal gray and bed nucleus of the stria terminalis (Jhou et al., 2009b). Thus, it is possible that inputs arising from regions other than the LHb facilitate activation of RMTg neurons and the subsequent behavioral response to stimulus exposure. Nevertheless, the present data, together with previous research showing that LHb lesions reduce ethanol intake (Haack et al., 2014) and enhanced LHb cFos expression following exposure to a dose of EtOH that produces conditioned place aversion (Zuo et al., 2015) suggests that this brain region is involved at some level in signaling EtOH's aversive properties.

The cFos data presented here are indicative of heightened neural activity in response to a conditioned stimulus predictive of ethanol exposure in an assay that measures aversive responding. Considering that previous work has shown similar increases in cFos expression in the RMTg following both aversive stimuli and their predictive cues (Jhou et al., 2009a), one might expect enhanced cFos expression in the RMTg following acute ethanol administration. While cocaine and other stimulants have been shown to induce significant cFos expression in the RMTg (Jhou et al., 2009a, 2009b; Kaufling et al., 2010), in a study comparing cFos induction following exposure to a number of drugs of abuse, Kaufling et al. (2010) reported an absence of cFos induction in the RMTg following 1.5 and 5.0 g/kg i.p. EtOH. However, recent work recording RMTg activity *in vivo* demonstrated increased cell firing following acute EtOH (Melis et al., 2014). While dose effects may contribute to these contradictory findings, it should also be noted that Kaufling et al. (2010) obtained all cFos measurements at a single time point three hours following drug administration. Given differences in the rate of drug absorption and metabolism, and the accompanying differences in the onset of euphoric versus dysphoric effects of each drug, it is not surprising that different drugs would produce dissimilar results when measures are taken at the same time point. Indeed, recent work makes clear that responses observed when aversive properties of a drug are at their peak differ greatly from those observed prior this point (i.e., when rewarding properties are more prevalent). Thus, Jhou et al (2013) showed that optogenetic inactivation of the RMTg 15 min following cocaine administration, when aversive symptoms are at their peak, reduced conditioned avoidance to cocaine. In contrast, RMTg inactivation 0–10 min following cocaine had no effect. Similar biphasic activity was also observed in electrophysiological recordings from LHb neurons following cocaine administration (Jhou et al., 2013) suggesting that activity within these regions is tightly correlated with the aversive, but not rewarding, properties of drugs of abuse. These findings are particularly important to consider in the case of ethanol given the well-documented differences in subjective experience during the ascending versus descending limbs of the blood alcohol curve (Morean and Corbin, 2010).

Consistent with previous reports, the present study observed no effect of CTA on cFos expression in the hippocampus (Koh and Bernstein, 2005; Bernstein et al., 2009). There was, however, a significant enhancement of cFos expression in all rats that underwent paired drug

and saccharin exposure. This finding is not completely unexpected given the prominent role the hippocampus plays in learned associations and previous work reporting enhanced cFos expression in this region following other Pavlovian learning procedures (Radulovic et al., 1998; Strekalova et al., 2003). Thus, the cFos induction observed in this region is likely indicative of enhanced neuronal activity in response to a cue that is predictive of drug injection regardless of whether or not that drug retains aversive properties. Importantly, cFos expression in both the RMTg and LHb varied by drug group indicating that, unlike the hippocampus, activity within these regions is responsive to the properties of the drug itself in addition to, or separate from, any response to the injection alone.

No sex differences were observed in either the behavioral or neurobiological measures obtained in the present study. These data support previous work showing little effect of ovariectomy on EtOH-induced CTA acquisition or expression (Morales and Spear, 2013). Nevertheless, given the well-documented inverse relationship between EtOH intake and CTA expression (Green and Grahame, 2008), and frequent reports of greater EtOH intake in female rats compared to males rats (Almeida et al., 1998; Cailhol and Mormède, 2001), the absence of sex differences in the present study was a somewhat unexpected observation. However, not all studies that have examined sex differences are in agreement. For example, while Vetter-O'Hagen et al. (2009) reported greater voluntary EtOH intake in female Sprague-Dawley rats than males, this was not associated with sex differences in EtOH-induced CTA. In contrast, a more recent study reported attenuated EtOH-induced CTA in females compared to males but did not observe differences in EtOH intake between the sexes (Schramm-Sapota et al., 2014). While these data are in agreement with other work reporting similar sex differences in EtOH-induced CTA (Sherrill et al., 2011; Morales et al., 2014), at least one other study reported no significant sex differences in EtOH-induced CTA in adult rats (Morales and Spear, 2013). While strain differences are also known to contribute to contradictory findings (Roma et al., 2007), it should be noted that sex differences in CTA and ethanol intake have not been consistent using the same strain within the same laboratory suggesting that age, housing experience, and differences in experimental procedures can contribute to variability between studies (Vetter-O'Hagen et al., 2009; Morales and Spear, 2013; Morales et al., 2014). Collection of daily vaginal smears throughout the duration of the study allowed us to examine the influence of estrous cycle on CTA and cFos expression. While we did not observe any significant effect of cycle phase on saccharin intake or cFos expression, it should be noted that because cycle phase was not explicitly controlled for, the representation of each phase varied widely between conditioning trials and test day. Consequently, the possibility remains that with greater sample sizes and/or assignment of specific cycle phases to specific experimental groups, a more pronounced effect of cycle phase may be uncovered. Collectively, these data speak to the need for additional, well-controlled studies that are explicitly aimed at investigating sex differences in rodent models of alcohol reward and aversion.

In summary, the data obtained in the present study revealed increased levels of cFos in the RMTg and LHb during LiCl- and EtOH-induced CTA suggesting increased neuronal activity and involvement of these nuclei in signaling the aversive properties of drugs including alcohol. Together with recent work reporting enhanced EtOH intake following RMTg inhibition, these data to suggest that the RMTg may play a critical role in the balance

between reward and aversion and the propensity for alcohol use and misuse. How RMTg activity is affected during the path from recreational drinking to compulsive consumption will be an important avenue for future research.

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References

- Almeida OF, Shoaib M, Deicke J, Fischer D, Darwish MH, Patchev VK. Gender differences in ethanol preference and ingestion in rats. The role of the gonadal steroid environment. *J Clin Invest*. 1998; 101:2677–2685. [PubMed: 9637701]
- Balcita-Pedicino JJ, Omelchenko N, Bell R, Sesack SR. The inhibitory influence of the lateral habenula on midbrain dopamine cells: ultrastructural evidence for indirect mediation via the rostromedial mesopontine tegmental nucleus. *J Comp Neurol*. 2011; 519:1143–1164. [PubMed: 21344406]
- Bernstein, IL.; Wilkins, EE.; Barot, SK. Mapping conditioned taste aversion associations through patterns of c-Fos expression. In: Reilly, S.; Schachtman, T., editors. *Conditioned Taste Aversion: Neural and Behavioral Processes*. New York, NY: Oxford University Press; 2009. p. 328-340.
- Bourdy R, Barrot M. A new control center for dopaminergic systems: pulling the VTA by the tail. *Trends Neurosci*. 2012; 35:681–690. [PubMed: 22824232]
- Cailhol S, Mormède P. Sex and strain differences in ethanol drinking: effects of gonadectomy. *Alcohol Clin Exp Res*. 2001; 25:594–599. [PubMed: 11329501]
- Cailhol S, Mormède P. Conditioned taste aversion and alcohol drinking: strain and gender differences. *J Stud Alcohol*. 2002; 63:91–99. [PubMed: 11925064]
- Cora MC, Kooistra L, Travlos G. Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicol Pathol*. 2015; 43:776–793. [PubMed: 25739587]
- Cunningham CL. Genetic relationship between ethanol-induced conditioned place preference and other ethanol phenotypes in 15 inbred mouse strains. *Behav Neurosci*. 2014; 128:430–445. [PubMed: 24841742]
- Fu R, Chen X, Zuo W, Li J, Kang S, Zhou L-H, Siegel A, Bekker A, Ye J-H. Ablation of μ opioid receptor-expressing GABA neurons in rostromedial tegmental nucleus increases ethanol consumption and regulates ethanol-related behaviors. *Neuropharmacology*. 2016a; 107:58–67. [PubMed: 26921770]
- Fu R, Zuo W, Gregor D, Li J, Grech D, Ye J-H. Pharmacological Manipulation of the Rostromedial Tegmental Nucleus Changes Voluntary and Operant Ethanol Self-Administration in Rats. *Alcohol Clin Exp Res*. 2016b; 40:572–582. [PubMed: 26876382]
- Green AS, Grahame NJ. Ethanol drinking in rodents: is free-choice drinking related to the reinforcing effects of ethanol? *Alcohol*. 2008; 42:1–11. [PubMed: 18164576]
- Haack AK, Sheth C, Schwager AL, Sinclair MS, Tandon S, Taha SA. Lesions of the lateral habenula increase voluntary ethanol consumption and operant self-administration, block yohimbine-induced reinstatement of ethanol seeking, and attenuate ethanol-induced conditioned taste aversion. *PLoS One*. 2014; 9:e92701. [PubMed: 24695107]
- Heldt SA, Ressler KJ. Lesions of the habenula produce stress- and dopamine-dependent alterations in prepulse inhibition and locomotion. *Brain Res*. 2006; 1073–1074:229–239.
- Hikosaka O, Sesack SR, Lecourtier L, Shepard PD. Habenula: Crossroad between the Basal Ganglia and the Limbic System. *J Neurosci*. 2008; 28:11825–11829. [PubMed: 19005047]
- Holdstock L, King AC, de Wit H. Subjective and objective responses to ethanol in moderate/heavy and light social drinkers. *Alcohol Clin Exp Res*. 2000; 24:789–794. [PubMed: 10888066]

- Hong S, Zhou TC, Smith M, Saleem KS, Hikosaka O. Negative reward signals from the lateral habenula to dopamine neurons are mediated by rostromedial tegmental nucleus in primates. *J Neurosci*. 2011; 31:11457–11471. [PubMed: 21832176]
- Zhou TC, Fields HL, Baxter MG, Saper CB, Holland PC. The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. *Neuron*. 2009a; 61:786–800. [PubMed: 19285474]
- Zhou TC, Geisler S, Marinelli M, Degarmo BA, Zahm DS. The mesopontine rostromedial tegmental nucleus: A structure targeted by the lateral habenula that projects to the ventral tegmental area of Tsai and substantia nigra compacta. *J Comp Neurol*. 2009b; 513:566–596. [PubMed: 19235216]
- Zhou TC, Good CH, Rowley C, Xu S, Wang H, Burnham N, Hoffman A, Lupica CR, Ikemoto S. Cocaine Drives Aversive Conditioning via Delayed Activation of Dopamine-Responsive Habenular and Midbrain Pathways. *J Neurosci*. 2013; 33:7501–7512. [PubMed: 23616555]
- Zhou TC, Xu S-P, Lee MR, Gallen CL, Ikemoto S. Mapping of reinforcing and analgesic effects of the mu opioid agonist Endomorphin-1 in the ventral midbrain of the rat. *Psychopharmacology (Berl)*. 2012; 224:303–312. [PubMed: 22669129]
- Kaufling J, Veinante P, Pawlowski SA, Freund-Mercier M-J, Barrot M. Afferents to the GABAergic tail of the ventral tegmental area in the rat. *J Comp Neurol*. 2009; 513:597–621. [PubMed: 19235223]
- Kaufling J, Waltisperger E, Bourdy R, Valera A, Veinante P, Freund-Mercier M-J, Barrot M. Pharmacological recruitment of the GABAergic tail of the ventral tegmental area by acute drug exposure. *Br J Pharmacol*. 2010; 161:1677–1691. [PubMed: 21087442]
- King AC, Houle T, de Wit H, Holdstock L, Schuster A. Biphasic alcohol response differs in heavy versus light drinkers. *Alcohol Clin Exp Res*. 2002; 26:827–835. [PubMed: 12068251]
- King AC, de Wit H, McNamara PJ, Cao D. Rewarding, stimulant, and sedative alcohol responses and relationship to future binge drinking. *Arch Gen Psychiatry*. 2011; 68:389–399. [PubMed: 21464363]
- Koh MT, Bernstein IL. Mapping conditioned taste aversion associations using c-Fos reveals a dynamic role for insular cortex. *Behav Neurosci*. 2005; 119:388–398. [PubMed: 15839785]
- Marcondes FK, Bianchi FJ, Tanno AP. Determination of the estrous cycle phases of rats: some helpful considerations. *Braz J Biol Rev Brasleira Biol*. 2002; 62:609–614.
- Melis M, Sagheddu C, De Felice M, Casti A, Madeddu C, Spiga S, Muntoni AL, Mackie K, Marsicano G, Colombo G, Castelli MP, Pistis M. Enhanced endocannabinoid-mediated modulation of rostromedial tegmental nucleus drive onto dopamine neurons in Sardinian alcohol-preferring rats. *J Neurosci*. 2014; 34:12716–12724. [PubMed: 25232109]
- Morales M, Schatz KC, Anderson RI, Spear LP, Varlinskaya EI. Conditioned taste aversion to ethanol in a social context: Impact of age and sex. *Behav Brain Res*. 2014; 261:323–327. [PubMed: 24406726]
- Morales M, Spear LP. Differences in sensitivity to ethanol-induced conditioned taste aversions emerge after pre- or post-pubertal gonadectomy in male and female rats. *Behav Brain Res*. 2013; 240:69–75. [PubMed: 23195111]
- Morean ME, Corbin WR. Subjective response to alcohol: a critical review of the literature. *Alcohol Clin Exp Res*. 2010; 34:385–395. [PubMed: 20028359]
- Murphy CA, DiCamillo AM, Haun F, Murray M. Lesion of the habenular efferent pathway produces anxiety and locomotor hyperactivity in rats: a comparison of the effects of neonatal and adult lesions. *Behav Brain Res*. 1996; 81:43–52. [PubMed: 8950000]
- National Research Council. Guide for the Care and Use of Laboratory Animals: Eighth Edition. 8. Washington DC: National Academies Press; 2011.
- Paxinos, G.; Watson, C. The Rat Brain in Stereotaxic Coordinates, Sixth Edition: Hard Cover Edition. 6. Amsterdam; Boston: Academic Press; 2007.
- Quinn PD, Fromme K. Subjective response to alcohol challenge: a quantitative review. *Alcohol Clin Exp Res*. 2011; 35:1759–1770. [PubMed: 21777258]
- Radulovic J, Kammermeier J, Spiess J. Relationship between fos production and classical fear conditioning: effects of novelty, latent inhibition, and unconditioned stimulus preexposure. *J Neurosci Off J Soc Neurosci*. 1998; 18:7452–7461.

- Rinker JA, Busse GD, Roma PG, Chen SA, Barr CS, Riley AL. The effects of nicotine on ethanol-induced conditioned taste aversions in Long-Evans rats. *Psychopharmacology (Berl)*. 2008; 197:409–419. [PubMed: 18204997]
- Roma PG, Chen SA, Barr CS, Riley AL. Dissociation between the aversive and pharmacokinetic effects of ethanol in female Fischer and Lewis rats. *Behav Brain Res*. 2007; 182:51–56. [PubMed: 17568694]
- Schramm-Sapota NL, Francis R, MacDonald A, Keistler C, O'Neill L, Kuhn CM. Effect of sex on ethanol consumption and conditioned taste aversion in adolescent and adult rats. *Psychopharmacology (Berl)*. 2014; 231:1831–1839. [PubMed: 24158502]
- Schuckit MA, Smith TL, Anderson KG, Brown SA. Testing the level of response to alcohol: social information processing model of alcoholism risk--a 20-year prospective study. *Alcohol Clin Exp Res*. 2004; 28:1881–1889. [PubMed: 15608605]
- Sherrill LK, Berthold C, Koss WA, Juraska JM, Gulley JM. Sex differences in the effects of ethanol pre-exposure during adolescence on ethanol-induced conditioned taste aversion in adult rats. *Behav Brain Res*. 2011; 225:104–109. [PubMed: 21767576]
- Strekalova T, Zörner B, Zacher C, Sadovska G, Herdegen T, Gass P. Memory retrieval after contextual fear conditioning induces c-Fos and JunB expression in CA1 hippocampus. *Genes Brain Behav*. 2003; 2:3–10. [PubMed: 12882314]
- Trantham-Davidson H, Burnett EJ, Gass JT, Lopez MF, Mulholland PJ, Centanni SW, Floresco SB, Chandler LJ. Chronic alcohol disrupts dopamine receptor activity and the cognitive function of the medial prefrontal cortex. *J Neurosci*. 2014; 34:3706–3718. [PubMed: 24599469]
- Trim RS, Schuckit MA, Smith TL. The relationships of the level of response to alcohol and additional characteristics to alcohol use disorders across adulthood: a discrete-time survival analysis. *Alcohol Clin Exp Res*. 2009; 33:1562–1570. [PubMed: 19485971]
- Verendeev A, Riley AL. Conditioned taste aversion and drugs of abuse: History and interpretation. *Neurosci Biobehav Rev*. 2012; 36:2193–2205. [PubMed: 22921283]
- Vetter-O'Hagen C, Varlinskaya E, Spear L. Sex differences in ethanol intake and sensitivity to aversive effects during adolescence and adulthood. *Alcohol Alcohol*. 2009; 44:547–554. [PubMed: 19767625]
- Welzl H, D'Adamo P, Lipp HP. Conditioned taste aversion as a learning and memory paradigm. *Behav Brain Res*. 2001; 125:205–213. [PubMed: 11682112]
- Zuo W, Fu R, Hopf FW, Xie G, Krnjević K, Li J, Ye J-H. Ethanol drives aversive conditioning through dopamine 1 receptor and glutamate receptor-mediated activation of lateral habenula neurons. *Addict Biol*. 2015

Figure 1. Conditioned taste aversion paradigm and anatomical depiction of regions of cFos assessment

Conditioned taste aversion was produced by restricting fluid access to 30 min each day. For the first seven days of the protocol, rats were provided with daily access to water. Beginning on the eighth day, rats in the Paired condition (**A**) underwent three conditioning trials consisting of access to a novel 0.1% saccharin solution immediately followed by i.p. drug injection (saline, EtOH or LiCl). Each conditioning trial was separated by three days of water recovery. Rats in the Unpaired condition (**B**) underwent the same procedure except that drug exposure occurred on the first water recovery day and was thus, explicitly unpaired with saccharin exposure. On the final day of the experiment, rats in both Paired and

Unpaired groups underwent a fourth saccharin exposure followed by sacrifice 90–105 min later. During sacrifice, brains were harvested and processed for immunohistochemical analysis of cFos expression. cFos⁺ cells were counted within the regions indicated in blue corresponding to the RMTg (**C**), LHb (**D**, blue fill) and hippocampus (**D**, blue box). Cell counts were collected from both hemispheres for each region and are represented here from one hemisphere for presentation purposes only.

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Figure 2. Baseline saccharin consumption does not differ between groups

A multifactorial ANOVA uncovered no significant differences in saccharin consumption during the first conditioning trial between males and females, saline, EtOH or LiCl drug groups or Paired vs Unpaired CTA conditions. n=6–10 rats/group.

Figure 3. Lithium chloride and ethanol induce significant conditioned taste aversion when explicitly paired with saccharin exposure

Within the Paired condition both EtOH and LiCl induced significant taste aversion in male (A) and female (B) rats compared to saline. Significant CTA expression was observed after a single conditioning trial and persisted across subsequent trials and test day. Though both drugs produced significant CTA compared to saline, LiCl-induced CTA was also significantly greater than that induced by EtOH. In contrast, male (C) and female (D) rats in the Unpaired condition did not develop a significant taste aversion to any drug and in fact exhibited a significant increase in saccharin consumption across conditioning trials. No significant sex differences were observed indicating similar levels of saccharin intake between males and females in all three drug groups in both Paired and Unpaired conditions. n=6–10 rats/group; *p 0.05 compared to saline; †p<0.01 compared to EtOH.

Figure 4. Conditioned taste aversion enhances cFos expression in the RMTg

(A) Both EtOH- and LiCl-induced CTA (Paired condition) significantly enhanced cFos expression in the RMTg compared to saline and Unpaired drug groups in a manner similar across sexes. (B) Representative image showing cFos expression in the RMTg (outlined in gray) in a saline control from the Paired condition. (C) Representative image showing cFos expression in the RMTg following EtOH-induced CTA. (D) A significant correlation was observed between RMTg cFos expression and saccharin consumption on test day with greater cFos expression being associated with greater magnitude of CTA expression (lower

saccharin consumption). n=6–10 rats/group; *p 0.01 compared to Paired saline; †p 0.05 compared to Unpaired drug groups; Scale bar = 200 μ m.

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Figure 5. Conditioned taste aversion enhances cFos expression in the LHb

(A) Both EtOH- and LiCl-induced CTA (Paired condition) significantly enhanced cFos expression in the LHb compared to saline in a manner similar across sexes. LHb cFos expression following LiCl-induced CTA was also significantly greater than that observed in Unpaired drug conditions. (B) Representative image showing cFos expression in the LHb (outlined in gray) in saline control from the Paired condition. (C) Representative image showing cFos expression in the LHb following EtOH-induced CTA. (D) A significant correlation was observed between LHb cFos expression and saccharin consumption on test day, with greater cFos expression being associated with greater magnitude of CTA

expression (lower saccharin consumption). n=6–10 rats/group; *p 0.05 compared to Paired saline; †p 0.001 compared to Unpaired counterpart; Scale bar = 200 μ m.

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Figure 6. Positive correlation between cFos expression in the RMTg and LHb
cFos expression in the RMTg correlated significantly with cFos expression in the LHb. Data are presented from rats in all drug groups in both Paired and Unpaired conditions. n=80 rats (6–10 rats/group)

Figure 7. Conditioned taste aversion does not affect cFos expression in the hippocampus
Male and female rats in the Paired CTA condition exhibited significantly greater hippocampal cFos expression compared to rats in the Unpaired CTA condition. However, cFos expression did not differ significantly between drug group including those rats within the Paired condition that exhibited significant EtOH- or LiCl-induced CTA compared to saline. *p 0.05.