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ETHANOL INDUCES CONDITIONED SOCIAL PREFERENCE IN MALE MICE

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Abstract

Background—Affiliative social interactions promote alcohol consumption, and alcohol also promotes affiliative behavior. Furthermore, for most species, moderate doses of ethanol (EtOH) and social affiliation are each rewarding. However, animal studies of drug and EtOH reward typically test individuals in isolation. To address social dimensions of EtOH reward, the present study tested EtOH-induced conditioned social preference in male C57BL/6 mice with (ORCHX +T) and without (ORCHX) testosterone.

Methods—ORCHX+T males received EtOH (0, 1, 2 or 3g/kg) ip and were paired 4x for 30 min each with 1 of 2 stimulus males: with the CS-stimulus male after saline injection, and with the CS + male following EtOH. After pairing, time spent with CS+ and CS– males was measured in a 10 min test.

Results—ORCHX+T test males showed conditioned preference for the CS+ male in response to 3 g/kg EtOH (change in preference: $+71.3 \pm 30.0$ sec/10 min, $p < 0.05$), but not for 0, 1 or 2g/kg. By contrast, ORCHX males did not demonstrate conditioned preference for 3g/kg EtOH ($+16.0 \pm 24.3$ sec/10 min, $p > 0.05$). In separate groups of mice, stimulus males (IS+) received EtOH during pairing to determine if test mice prefer another intoxicated mouse. Both ORCHX+T and ORCHX test mice showed an increase in preference score for the IS+ mouse (ORCHX+T: $+68.1 \pm 24.0$ sec, ORCHX: $+58.9 \pm 19.6$ sec, $p < 0.05$).

Conclusions—These data demonstrate that EtOH promotes social preference in male mice, as it does in females. Testosterone enhances this effect.

Keywords

alcoholic intoxication; association learning; conditioning; classical; testosterone; social behavior

INTRODUCTION

Social context has a substantial impact on ethanol (EtOH) drinking and substance abuse, including binge drinking in social settings (Courtney et al, 2009). Accordingly, current research has emphasized the role of social relationships in initiating and promoting drinking behavior (Ali and Dwyer, 2010). The particular susceptibility of adolescents to social pressure for drinking and drugs further suggests a role for gonadal steroid hormones, which rise at puberty (Sato et al, 2008). In this regard, gonadal steroid hormones are correlated with the onset and quantity of alcohol use in boys (de Water et al, 2013). Likewise, gonadal steroids enhance both social behavior (Choleris et al, 2009) and drug reward in animals (Rajasingh et al, 2007), and thereby provide opportunities to explore interactions of hormones and EtOH reward in a social context.

If social interactions promote EtOH consumption, the opposite is also true. That is, consumption of drugs or EtOH can facilitate the amount and quality of positive social interaction among men (Kirchner et al, 2006). In part, facilitation of social behavior may reflect EtOH-induced loss of social inhibitions (Lithari et al, 2012). This can promote social support (discussion of mental health, showing concern for others) among male friends who drink together (Emslie et al, 2013). Conversely, EtOH enhances aggression in men (Quinn et al, 2013), and agonistic behavior in male mice in a home cage-intruder model, manifest as increased frequency of pursuit, threats and bites (Fish et al, 2002). These contrasting effects of EtOH on male social behavior make it difficult to predict the outcome of social contact in males (increased agonistic vs affiliative behavior) in the presence of EtOH.

We have recently established a model of conditioned social preference (CSP) in female mice to explore EtOH intoxication in a social context (Wood & Rice, 2013). CSP merges conditioned place preference (CPP) with partner preference. CPP has been an important experimental model to study reward in laboratory animals, both natural rewards (food, sex) and drugs of abuse, including EtOH (Tzschenke, 2007). With CPP, mice learn to associate an unconditioned stimulus (US, e.g. food, sex, drugs) associated with a unique environment (conditioned stimulus; CS+). In response to repeated pairing of the CS+ with a reinforcing US, a mouse will prefer the CS+ over an environment with a non-rewarding cue (CS-; e.g. saline), even in the absence of the US. This design allows for behavioral testing in the absence of acute effects of the US (e.g. food-induced satiety, drug-induced immobility).

In CSP, the CS+ and CS- are individual stimulus animals of the same sex, age, and strain. Female mice form a conditioned preference for an unfamiliar female (CS+) with whom they have been paired while intoxicated, over another female (CS-) with whom they have been paired after saline injection. Ovariectomized females without estrogen replacement did not demonstrate this preference. This is similar to the inhibitory effects of ovariectomy on CPP for cocaine (Russo et al, 2003). As estrogen and testosterone are the principal sex steroid hormones in females and males, respectively, we expect that testosterone also interacts with EtOH to enhance social preference in male mice. This was the focus of the present study.

To investigate EtOH-induced CSP in male mice and potential interactions with testosterone, the present study used castrated males with (ORCHX+T) or without (ORCHX) testosterone

replacement. Test mice received EtOH or saline and were paired repeatedly with the CS+ or CS- stimulus male, respectively. The hypothesis was that EtOH intoxication induces CSP in male mice, and that testosterone facilitates this response. We further explored the role of EtOH intoxication in the CS+ stimulus male, and the role of testosterone on individual recognition.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (8–10 weeks) were obtained from Charles River Laboratories (Wilmington, MA). This strain shows high voluntary EtOH intake in a 2-bottle preference test (Yoneyama et al, 2008), and relatively low aversion to EtOH (Broadbent et al, 2002). Mice were group-housed 4 per cage on a reversed 14:10 light-dark photoperiod (lights-on at 10 pm) with access to food and water *ad libitum*, except during pairing and testing. Behavior was evaluated under dim illumination during the first 4h of the dark phase. Experimental procedures were approved by USC's Institutional Animal Care and Use Committee and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals (DHEW Publication 80–23, revised 1985, Office of Sciences and Health Reports, DRR/NIH, Bethesda, MD 20205).

Castration and testosterone replacement

To explore the interaction of EtOH with testosterone on CSP, all test and stimulus males were castrated via a midline scrotal incision under ketamine/xylazine anesthesia (130 and 10 mg/kg). In ORCHX+T males, testosterone was replaced via a Silastic implant sc (o.d. 2.16 mm, i.d. 1.02 mm, Dow Corning, Midland, MI). The 5-mm implant was filled with crystalline testosterone, and the ends were sealed with silicone adhesive. This implant delivers testosterone chronically at physiologic levels as determined by weight of the accessory sex glands (Barkley and Goldman, 1977). ORCHX males received empty Silastic implants. Mice were allowed to recover from surgery up to 2 weeks before testing and pairing.

Conditioned Social Preference

CSP was conducted as described previously (Wood and Rice, 2013). Briefly, pre-testing, pairing and preference testing were conducted in identical plastic arenas (40×50×40 cm; see Figure 1A). During the pre-test, 2 unfamiliar stimulus males were tethered on opposite sides of the apparatus. Stimulus males were of the same age and hormone treatment (ORCHX or ORCHX+T) as the test male, except in Experiment 3 (below). Each stimulus male was further distinguished by a topical odor (coconut or lemon, McCormick Baking Extracts, Sparks MD) rubbed on the fur over the scapular region prior to all testing and pairing sessions. A test male was introduced, and the amount of time spent in proximity to each stimulus male was recorded for 10 minutes by an observer blind to the treatment groups. Proximity was defined when the test male had all 4 paws within reach of the stimulus male (shaded area, Figure 1A).

For the next 8 days, test males were paired individually for 30 min with each stimulus male in the testing arena on alternate days (4 pairings each). 30 minutes before pairing, each test male received an injection ip of 20% EtOH or an equivalent volume of saline, and was temporarily housed individually in a clean cage. EtOH for injection was prepared daily from a commercially-available neutral grain spirit [Everclear, Luxco, St. Louis MO; (Crabbe et al, 2008)], and was injected at a dose of 0.006 to 0.019 ml/g BW.

The lemon-scented stimulus male was designated as the CS-. Following a saline injection, the test male was paired with the CS- male. On alternate days, the test male received an EtOH injection, and was paired with the coconut-scented stimulus male (CS+). During initial pretest sessions, 34% of test mice showed a positive preference score for the coconut-scented stimulus male. The order of pairing (beginning with EtOH or saline) was balanced, and both stimulus males received an injection of saline 30 min before pairing. During pairing, stimulus males were scented, but were unrestrained to facilitate interaction with the test male. The day after the final pairing, CSP was measured in test males under conditions identical to the pre-test.

Experiment 1: Dose-response for EtOH

Separate groups of ORCHX+T males (n=7–10 each) were tested for CSP in response to EtOH at 0, 1, 2 or 3 g/kg. EtOH has been shown previously to facilitate CPP in male mice across this range of doses [1–4 g/kg, (Grobowski et al, 2008)], and stimulates CSP in female mice at 2 and 2.5 g/kg (Wood and Rice, 2013). In male C57Bl/6 mice, injection of 3 g/kg EtOH ip produces peak blood ethanol concentrations of 4.3 ± 0.1 mg/ml, with half-maximal values approximately one hour after injection (Ponomarev and Crabbe, 2002), corresponding to the end of pairing in the present study.

Experiment 2: Effects of testosterone in test males, effects of EtOH in stimulus males

Experiment 2 extended these initial observations to investigate the interactions of testosterone and EtOH on CSP. Based on the results of Experiment 1, CSP was tested in ORCHX and ORCHX+T males (n=10 each) in response to 3 g/kg EtOH. Stimulus males were unfamiliar mice of the same hormonal status (ORCHX or ORCHX+T) as test males.

As shown in Figure 1B and C, we next evaluated the effects of EtOH intoxication in the CS+ stimulus male (Intoxicated Stimulus: IS+) on EtOH-induced CSP in ORCHX and ORCHX+T test males (n=10–11/group). The CS- male received a saline injection, but the test male and IS+ male each received 3 g/kg EtOH 30 minutes before pairing. This comparison determined preference for IS+ vs CS-.

Where test males demonstrated EtOH-induced CSP, we determined if these males would express a generalized preference for unfamiliar males bearing the same scent. Accordingly, CSP was retested on the following day, substituting novel stimulus males scented with lemon and coconut. Generalization of preference for an unfamiliar scented partner has been demonstrated for mating-induced conditioned partner preference in rats [Coria-Avila et al, 2005; Kippin and Pfaus, 2001]. However, female mice do not show a generalized preference for the CS+ scent with EtOH-induced CSP (Wood and Rice, 2013).

To determine if testosterone modifies EtOH sensitivity, we compared ORCHX and ORCHX +T test males on the EtOH-induced loss of righting reflex (LORR) test (Becker et al, 1985). Mice received a hypnotic dose of ethanol (4 g/kg) ip, and were placed on their backs in a V-shaped trough once they were unable to right themselves. Time until the righting reflex was regained was recorded by an experimenter blind to the treatment group.

Experiment 3: Testosterone in stimulus males, olfactory recognition in ORCHX test males

Lastly, we investigated the role of testosterone in the stimulus males on EtOH-induced CSP and individual olfactory discrimination in ORCHX test males. CSP was tested in response to 3 g/kg EtOH in ORCHX males (n=10). Unlike Experiment 2, where stimulus males were of the same hormonal status as test males, CS+ and CS− stimulus males were unfamiliar ORCHX+T males (Fig. 1C). The next day, ORCHX test males were retested for a generalized preference for novel ORCHX+T males bearing the same scents.

Male mice produce urinary pheromones that are testosterone-dependent (Achiraman and Archunan, 2005). Without the presence of these urinary signatures, it may be difficult for test males to identify individual ORCHX stimulus males. Accordingly, we compared the ability of ORCHX males to discriminate urinary odors from pairs of ORCHX+T and ORCHX males in an olfactory habituation/dishabituation test. Per Yang and Crawley (2009), an ORCHX male was introduced into an empty cage (17.5 × 28 × 12 cm). After a 2-min acclimation, the mouse was presented with a series of 3 odorants delivered via cotton swabs. During the 18-min test, each odor was presented on a fresh swab at the top of the cage for three consecutive 2-min trials. Water was presented first, followed by urine (1:10 in water) acquired from 2 unfamiliar males (urine A, urine B). On the first day, test males were exposed to urine from 2 ORCHX males, and to urine from 2 ORCHX+T males on the next day. An observer blind to treatment groups recorded investigation times. Habituation was measured as the decrease in investigation time in seconds from the first to final odor presentation. Dishabituation was measured as an increase in investigation time from the third odor presentation to the first in the next odor series (e.g. from the third water presentation to the first presentation of urine A).

Data Analysis

Preference score (time with CS+ minus CS−, or time with IS+ minus CS−, in seconds) and total interaction (time with CS+ plus CS−, or time with IS+ plus CS−) were determined for each test male during pre-test, CSP, and generalization tests. Except where noted, for all analyses, $p < 0.05$ was considered significant.

For Experiment 1, preference scores and total interaction among the different EtOH doses were evaluated by repeated measures ANOVA (RM-ANOVA) with time (pre-test and CSP) as the repeated measure. Subsequently, preference score and total interaction at each EtOH dose were evaluated during pre-test and CSP by paired t-test.

For Experiment 2, the effects of testosterone (ORCHX+T vs ORCHX) and intoxication of the stimulus males (CS+/CS− vs IS+/CS−) on preference scores and total interaction were evaluated by RM-ANOVA with time (pre-test and CSP) as the repeated measure. Within each experimental group, preference score and total interaction during pre-test, CSP, and

generalization were evaluated by ANOVA, with post-hoc comparison vs pre-test values by Dunnett's test. The LORR test in ORCHX+T and ORCHX males was compared by Student's t-test.

For Experiment 3, preference scores and total interaction in ORCHX test males in response to ORCHX+T stimulus males were evaluated by RM-ANOVA with time (pre-test, CSP and generalization) as the repeated measure. Subsequently, preference scores and total interaction during CSP and generalization were compared with pre-test values by paired t-test, with Bonferroni correction for multiple comparisons.

For the habituation-dishabituation test, habituation and dishabituation to urine A and urine B were evaluated by RM-ANOVA, with urine source (from ORCHX vs ORCHX+T males) as the repeated measure. Habituation compared the decrease in investigation time in response to repeated presentation of the same odor. Dishabituation compared the increase in investigation with the initial presentation of a new odor.

RESULTS

Experiment 1: Dose-response for ethanol

Preference scores and total interaction in ORCHX+T males paired with EtOH at 0, 1, 2, or 3 g/kg are shown in Figure 2. There was a significant interaction of EtOH dose $\times$ time on preference score for the CS+ stimulus male by RM-ANOVA ($F_{3, 34} = 4.235$; $p < 0.05$). However, there was no overall effect of time (pre-test vs CSP) or dose (N.S. $p > 0.05$). For total interaction, there was a significant effect of dose ($F_{3, 34} = 3.884$; $p < 0.05$) and of time ($F_{1, 34} = 4.251$; $p < 0.05$), but no dose $\times$ time interaction (N.S. $p > 0.05$).

Repeated pairing with saline (0 g/kg EtOH) had no effect on preference for the CS+ stimulus male. Preference scores for the CS+ stimulus male decreased by -35.0 ± 29.3 (mean $\pm$ SEM) seconds from pre-test to CSP ($p > 0.05$). After repeated pairing with saline, total interaction with both stimulus males decreased significantly ($p < 0.05$). At 1 and 2 g/kg, EtOH also failed to induce CSP for the CS+ stimulus male ($p > 0.05$), and total interaction was unchanged.

At 3 g/kg, repeated pairing with EtOH caused a significant increase in preference score ($+71.3 \pm 30.0$ seconds from pre-test to CSP, $p < 0.05$). However, total interaction did not change from pre-test to CSP ($p > 0.05$).

Experiment 2: Effects of testosterone in test males, effects of ethanol in stimulus males

Preference scores and total interaction for CS+ and IS+ stimulus males in response to 3 g/kg EtOH in ORCHX and ORCHX+T males are shown in Figure 3. As determined by 2-factor RM-ANOVA, there was a significant effect of time (pretest vs CSP) on preference score ($F_{1, 41} = 18.176$; $p < 0.05$). There was no overall effect of hormonal status (ORCHX+T vs ORCHX; N.S. $p > 0.05$) or intoxication (CS+/CS- vs IS+/CS-; N.S. $p > 0.05$). However, there was a significant interaction of hormonal status with intoxication ($F_{1, 41} = 5.148$; $p < 0.05$). Likewise, for total interaction during pre-test and CSP, there was a interaction of hormonal status and intoxication ($F_{1, 41} = 6.269$; $p < 0.05$).

Among ORCHX+T test males, repeated pairing with 3 g/kg EtOH significantly increased the preference score for the CS+ stimulus male by $+78.6 \pm 32.2$ seconds ($p < 0.05$). However, when retested with novel stimulus males, these ORCHX+T males did not express a significant preference for the coconut-scented male ($+56.9 \pm 23.0$ seconds vs pre-test, $p > 0.05$). Likewise, ORCHX+T test males demonstrated EtOH-induced CSP when paired with the IS+ stimulus male. When tested for CSP, preference scores increased by $+68.1 \pm 24.0$ seconds ($p < 0.05$). During generalization testing, preference score for the unfamiliar coconut-scented male was increased from pre-test by $+51.7 \pm 23.7$ seconds ($p > 0.05$ vs pre-test). For both groups of ORCHX+T test males, total interaction with the 2 stimulus males after pairing with ethanol was unchanged from pre-test values (N.S. $p > 0.05$).

In ORCHX test males, there was no significant change in preference score for the CS+ stimulus male when tested for CSP ($+16.0 \pm 24.3$ seconds, $p > 0.05$). Accordingly, these males were not retested for generalization. However, ORCHX test males did express a CSP when EtOH was paired with the IS+ stimulus male. The mean preference score increased by $+58.9 \pm 19.6$ seconds during CSP testing ($p < 0.05$). Notably, these ORCHX males also expressed a generalized preference for an unfamiliar coconut-scented male ($+54.3 \pm 20.0$ seconds, $p < 0.05$). Total interaction with the two stimulus males was unchanged for both CS +/CS- and IS+/CS- pairs (N.S. $p > 0.05$).

For the LORR test in response to 4 g/kg EtOH, ORCHX+T males righted themselves 35.2 ± 5.1 min after EtOH injection. By contrast, ORCHX males required 63.3 ± 8.2 min to right themselves ($p < 0.05$, data not shown).

Experiment 3: Testosterone in stimulus males, olfactory recognition in ORCHX test males

Preference scores and total interaction in response to 3 g/kg EtOH in ORCHX males paired with ORCHX+T stimulus males are shown in Figure 4. As determined by RM-ANOVA, there was a significant effect of time (pre-test, CSP and generalization) on preference scores for the CS+ stimulus male ($F_{2, 18} = 5.2373$; $p < 0.05$). Repeated pairing with EtOH significantly increased the preference score during CSP by $+81.9 \pm 28.2$ seconds ($p < 0.025$). However, ORCHX test males did not demonstrate a generalized preference for an unfamiliar ORCHX+T male scented with coconut. Preference scores during generalization testing decreased by -0.3 ± 42.3 seconds vs pre-test (N.S.; $p > 0.025$). Total interaction with the two stimulus males was unaffected (N.S. $p > 0.05$).

Figure 5A presents investigation time for a representative ORCHX test male in response to repeated presentation of urine from pairs of unfamiliar ORCHX and ORCHX+T males; Figure 5B illustrates the change in investigation for the entire group during habituation and dishabituation to urine A and urine B. By RM-ANOVA, there was a significant effect of order (urine A vs urine B; $F_{1, 32} = 5.7696$; $p < 0.05$) and urine source (ORCHX vs ORCHX +T; $F_{1, 32} = 7.1165$; $p < 0.05$) on investigation. With the first presentation of urine A from an ORCHX+T male, test males investigated for 6.8 ± 1.8 sec/2-min test. With habituation on the third presentation, investigation declined to 1.3 ± 0.5 sec (a -5.4 ± 1.9 sec decrease). Compared with the response to urine A, investigation of urine B was significantly reduced. Test males investigated urine B from an ORCHX+T male for 4.8 ± 1.3 seconds at first presentation, followed by habituation to 0.9 ± 0.3 seconds at the third presentation. Response

to urine from ORCHX males was significantly lower: 4.4 ± 0.7 sec at initial presentation of urine A, 1.9 ± 0.7 sec for the first exposure to urine B.

DISCUSSION

The present study demonstrates that male mice form a CSP for an unfamiliar male with whom they have repeatedly been paired following EtOH intoxication. Testosterone enhances this effect, as castrated males fail to show CSP for the CS+ stimulus male. However, when both the test and stimulus male (IS+) are intoxicated, EtOH induces CSP in ORCHX and ORCHX+T males. Castration increases recovery time in the LORR test. Furthermore, test males show reduced response to individual odors from ORCHX stimulus males. The mostly stimulatory interactions among EtOH, testosterone and social behavior have implications for drinking and social bonding.

Initial studies using CSP were limited to sexual reward. Male rats form a conditioned preference for scented females with whom they have mated (Kippin and Pfau, 2001). This preference generalizes to unfamiliar females bearing the same scent. Female rats form a conditioned preference for males with whom they have engaged in paced mating (Coria-Avila et al, 2005). This preference extends to unfamiliar males of the same strain. Our recent study of EtOH-induced CSP in female mice (Wood and Rice, 2013) broadens the CSP model from social reward (sex) to drug reward. Similar to the present study, ovariectomized female mice with systemic estrogen replacement demonstrate CSP in response to EtOH, either when the CS+ stimulus female is also intoxicated (IS+) or not (CS+). Ovariectomized females without estrogen show CSP for the IS+ stimulus female, but not for the CS+ female. As with males in the present study, EtOH-induced CSP does not generalize to unfamiliar stimulus females with the same scent.

It was somewhat more difficult to predict the outcome of EtOH on social preference in males. Unfamiliar male mice often fight (Pinna et al, 2004), and EtOH can intensify aggression (Fish et al, 2002). Nonetheless, in the present study we did not observe fighting between test and stimulus males during pairing. In part, this may reflect that males in the present study were group-housed, and pairing took place in a neutral arena. By contrast, inter-male aggression is especially pronounced when an intruder is introduced into the home-cage of a singly-housed male (Van Loo et al, 2003). Furthermore, EtOH-enhanced aggression is observed most often with low doses of EtOH, and is not evident in all males (Chiavegatto et al, 2010). In this regard, 3g/kg EtOH induces substantial motor deficits. This dose produces intoxication greatly exceeding social drinking in humans (Stowell and Stowell, 1998). Even so, if test males had fought with stimulus males during pairing, this would not have precluded formation of CSP, since aggression is also rewarding (Couppis and Kennedy, 2008; Fish et al, 2005; Meisel and Joppa, 1994).

Comparing EtOH-induced CSP in male and female mice, the responses were overwhelmingly similar, with one exception. Ovariectomized females with estrogen replacement demonstrated CSP in response to EtOH at 2 g/kg, while this dose was ineffective in ORCHX+T males in the present study. On the other hand, it might be that CSP would emerge in males at lower EtOH doses with additional pairings, as reported previously

(Nocjar et al, 1999). Most previous studies of male and female mice have not reported sex differences in CPP across a range of EtOH doses [1–3 g/kg (Nocjar et al, 1999; Song et al, 2007; Itzhak et al, 2009)]. It is also important to note that none of the studies of EtOH-induced CPP have controlled for estrous cyclicity in females. However, a single study of OF1 mice reported CPP in late adolescent females in response to 2.5 g/kg EtOH, a dose that was ineffective in males (Roger-Sanchez et al, 2012). Conversely, early adolescent males also demonstrated CPP in response to EtOH at 1.25 g/kg, a dose that was ineffective in older males, and in females at either age. Thus, the relationship between age, sex, and gonadal steroids on response to EtOH is complex. This is true not only for CPP, but also for EtOH self-administration. Previous studies have shown that females will make more operant responses for access to EtOH (Ferraro et al, 2006) and will voluntarily consume more EtOH than males (Meliska et al, 1995). Furthermore, with prolonged access, EtOH intake increases in females, but not in males (Middaugh et al, 1999). However, these effects depend on both response requirements and response access time: females respond more than males when response requirements are low and response access is prolonged (Middaugh and Kelley, 1999).

Gonadectomy had similar effects on EtOH-induced CSP in males and females. That is, gonadectomized males and females failed to demonstrate CSP for a CS+ stimulus mouse, although the same dose of EtOH was effective in ORCHX+T males or estrogen-treated ovariectomized females. Gonadal steroids promote social behavior (Choleris et al, 2009), and testosterone increases behavioral sensitivity to EtOH, at least with regard to aggressive behavior (DeBold and Miczek, 1985). Importantly, the present study in males significantly extends our understanding of how testosterone may modify EtOH-induced CSP, particularly with regard to testosterone's modulatory effects on EtOH intoxication and testosterone's stimulation of a unique olfactory signature.

In the LORR test, ORCHX males took longer than ORCHX+T males to regain ventral recumbency in response to 4 g/kg EtOH. This could reflect increased sensitivity to EtOH's hypnotic effects and/or reduced EtOH metabolism in ORCHX males. The implication is that intoxicated ORCHX test males may have difficulty interacting with a CS+ stimulus male during pairing, due to impaired locomotor function. An intoxicated ORCHX stimulus male (IS+) would have a similar impairment. Somewhat surprisingly, there is only a limited literature exploring hormonal effects on the LORR test. Specifically, ovariectomy had no effect on sleep time during the LORR test in female mice (Becker et al, 1985). However, there is potential for castration to reduce activity of the hepatic alcohol dehydrogenase enzyme, resulting in increased blood acetaldehyde levels and reduced EtOH self-administration (Quintanilla et al, 2007; but see also Aasmoe and Aarbakke, 1999; Simon et al, 2002). If so, impaired EtOH metabolism could contribute to longer sleep time among ORCHX males in the LORR test. Based on the results of EtOH-induced CSP, it is tempting to speculate that ORCHX males might also be more sensitive than ORCHX+T males to EtOH in a CPP test. However, we are unaware of any published reports comparing EtOH-induced CPP in ORCHX+T vs ORCHX males.

In addition, castration of the two stimulus males also appears to limit individual discrimination. ORCHX test males paired with ORCHX stimulus males failed to

demonstrate CSP. However, in the follow-up to our initial test, ORCHX test males did form a CSP for the CS+ male when both stimulus males were ORCHX+T. Furthermore, when ORCHX males were tested for recognition of urinary odors from ORCHX or ORCHX+T males in the habituation-dishabituation test, the response to urine from ORCHX males was reduced. We note that overall investigation time during the 2-minute exposure to urine from unfamiliar males was limited (<7 seconds). This is due to both the presentation (mouse vs urine) and sex (male vs female) of the odor cue. Male mice spend more time investigating novel conspecifics vs urine alone, and they show greater interest in female urine compared with urine from other males (Pankevich et al, 2004, Tejada and Rissman, 2012). Nonetheless, the results of the habituation-dishabituation test are consistent with impaired discrimination of individual ORCHX odor cues. From these findings, we infer that test males recognize the two stimulus males based on unique androgen-dependent odor signatures, and not on the artificial scents (coconut, lemon) applied to them. This is consistent with our observation that test males do not express a generalized preference for unfamiliar ORCHX+T males with the same scent. Accordingly, we predict that male mice would express EtOH-induced CSP even if tested and paired without artificial scents. However, this remains to be tested. Finally, our conclusion is supported by previous studies showing that male mouse urine has androgen-dependent pheromones (Achiraman and Archunan, 2005).

Among EtOH, testosterone and social behavior, there is considerable positive interaction. Testosterone promotes social behavior (Choleris et al, 2009), while social interactions stimulate testosterone secretion (Gleason et al, 2009). The same is true of EtOH. At moderate doses, EtOH increases social behavior in humans (Källmén and Gustafson, 1998) and aggression in mice (DeBold and Miczek, 1985), while social interaction can modulate EtOH consumption. In this regard, rats paired with an intoxicated cage-mate will voluntarily consume more ethanol than rats paired with a sober companion (Fernandez-Vidal and Molina, 2004). Conversely, voles with high voluntary EtOH intake will reduce their EtOH consumption when paired with a low-intake cage-mate (Anaker et al, 2011). And, although the present study controlled for potential EtOH-induced fluctuations in endogenous testosterone by using an ORCHX+T model, there is a further potential for EtOH to interact with testosterone. Higher androgen levels in pubertal boys correlate with greater probability of lifetime EtOH use (de Water et al, 2013), yet androgens also appear to protect the central nervous system and liver from EtOH toxicity (Celec et al, 2003; Jaya et al, 1995). On the other hand, chronic EtOH exposure suppresses testosterone production and increases estradiol levels due to impaired liver metabolism of estrogen (Colantoni et al, 2000). This can lead to gynecomastia in alcoholics as a result of increased circulating estrogens (Martínez-Riera et al, 2005). Thus, while the acute interactions of EtOH, social behavior and testosterone are generally stimulatory, this effect may shift under chronic EtOH exposure due to hypogonadism.

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Figure 1. Experimental design. A: Testing conditioned social preference (CSP) for a stimulus male mouse (CS+) paired with ethanol vs another stimulus male (CS-) paired with saline. See Methods for details. B: In separate groups of mice (IS+ vs CS-), the conditioned stimulus male (IS+) also receives ethanol before pairing. C: Summary of pairings (CS+ or IS+) in gonadectomized male mice with (ORCHX+T) or without (ORCHX) testosterone replacement across the three experiments.

Figure 2.

A: Preference score (mean±SEM) for the CS+ stimulus male mouse paired with 0, 1, 2 or 3 g/kg ethanol vs the CS- stimulus male during pre-test (open bars) and conditioned social preference (CSP, closed bars) in castrated male mice with testosterone replacement (ORCHX+T; n=7-10 each). B: Total interaction (seconds/10 min) with both stimulus males during pre-test and ethanol-induced CSP. Asterisks indicate significant difference by paired t-test vs pre-test values at the same ethanol dose.

Figure 3.

A: Preference score (mean±SEM) for the conditioned stimulus male mouse (CS+ or IS+) paired with 3 g/kg ethanol vs the CS- stimulus male during pre-test (open bars), conditioned social preference (CSP, closed bars), and generalization (gray bars) in gonadectomized male mice with (ORCHX+T) or without (ORCHX) testosterone replacement (n=10–11 each). B: Total interaction (seconds/10 min) with both stimulus male mice during pre-test, ethanol-induced CSP and generalization tests. Asterisks indicate significant difference by Dunnett's test vs pre-test values in the same experimental group.

Figure 4.

A: Preference score (mean $\pm$ SEM) for the CS+ stimulus male mouse paired with 3 g/kg ethanol vs the CS- stimulus male during pre-test (open bars), conditioned social preference (CSP, closed bars), and generalization (gray bars) in castrated (ORCHX) male mice (n=10). CS+ and CS- stimulus males were castrates with testosterone replacement (ORCHX+T). B: Total interaction (seconds/10 min) with both stimulus male mice during pre-test, ethanol-induced CSP and generalization tests. Asterisk indicates significant difference by Dunnett's test vs pre-test values.

Figure 5.

A: Olfactory investigation in seconds by a representative castrated (ORCHX) male mouse in response to repeated exposure to water and urine from 2 unfamiliar males (urine A and urine B). Urine was collected from castrates with (ORCHX+T, closed symbols) and without (ORCHX, open symbols) testosterone replacement. B: Change in investigation time (mean $\pm$ SEM) during habituation and dishabituation ($n=10$). Asterisks indicate significant effects of urine order and source by RM-ANOVA.