Drinking alcohol has sex-dependent effects on pair bond formation in prairie voles

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Alcohol use and abuse profoundly influences a variety of behaviors, including social interactions. In some cases, it erodes social relationships; in others, it facilitates sociality. Here, we show that voluntary alcohol consumption can inhibit male partner preference (PP) formation (a laboratory proxy for pair bonding) in socially monogamous prairie voles (*Microtus ochrogaster*). Conversely, female PP is not inhibited, and may be facilitated by alcohol. Behavior and neurochemical analysis suggests that the effects of alcohol on social bonding are mediated by neural mechanisms regulating pair bond formation and not alcohol's effects on mating, locomotor, or aggressive behaviors. Several neuropeptide systems involved in the regulation of social behavior (especially neuropeptide Y and corticotropin-releasing factor) are modulated by alcohol drinking during cohabitation. These findings provide the first evidence to our knowledge that alcohol has a direct impact on the neural systems involved in social bonding in a sex-specific manner, providing an opportunity to explore the mechanisms by which alcohol affects social relationships.

anxiety | ethanol | substance use | oxytocin | vasopressin

Prairie voles are a valuable animal model of social monogamy. Males and female mates form durable bonds in the wild and in the laboratory (1, 2), and the neural mechanisms of social bonding delineated in this model species have translated with high predictive validity to humans (3, 4). In both species, social reward and drug reward show striking parallels at the behavioral and neurobiological levels (5–9). Prairie voles are now being used to explore the interactions between social relationships and drug abuse (10–19).

We previously demonstrated that prairie voles voluntarily self-administer substantial amounts of alcohol (ethanol) and can influence the drinking patterns of a social partner (16–19), similar to social drinking in humans (20). Because alcohol is known to influence social bonds in humans (21–24), we asked here whether alcohol consumption can affect the formation of adult social attachments in prairie voles. Adult male and female prairie voles were paired for 24 h and simultaneously given access to alcohol (10% ethanol by volume in water) and water or only water. They were then tested in the 3-h partner preference (PP) test (PPT), which has proved to be a remarkably sensitive assay for assessing the effects of genetics (25, 26), early social environment (27), and a range of pharmacological agents on social bond formation (28, 29).

Results

PP was first measured in female prairie voles that drank alcohol during cohabitation without mating. Animals consumed 12.48 ± 1.03 (mean ± SE) grams of alcohol per kilogram of body weight (g/kg) in the 24-h drinking period and showed a 58 ± 5.7% preference for alcohol. Analysis of behavior in the PPT revealed a significant effect of stimulus animal (partner or stranger) on huddling time [$F(1,56) = 26.86$, $P < 0.0001$]; no main effect of alcohol on total huddling time [$F(1,56) = 0.02$, $P = 0.89$]; and, most importantly, a significant interaction between alcohol and stimulus animal [$F(1,56) = 4.25$, $P = 0.044$]. Control females did not display a significant PP [$t(28) = 1.90$, $P = 0.068$], whereas females drinking alcohol during cohabitation exhibited a robust and statistically significant preference for the partner over the stranger [$t(28) = 6.34$, $P < 0.0001$] (Fig. 1*A*). These findings indicate that alcohol facilitated PP in females.

Next, we tested effects of alcohol on PP in male prairie voles. In contrast to females, males that drank alcohol during cohabitation with sexually unreceptive (ovariectomized) stimulus females (average consumption: 11.2 ± 0.81 g/kg, average preference: 74 ± 2.8%) exhibited no PP (Fig. 1*B*). There were no significant effects of stimulus animal [$F(1,58) = 2.57$, $P = 0.11$] or alcohol [$F(1,58) = 0.37$, $P = 0.54$] on huddling time and no significant interaction between alcohol and stimulus animal [$F(1,58) = 0.13$, $P = 0.72$]. These findings indicate that males, unlike females, did not experience facilitation of the PP by alcohol.

To determine whether alcohol can have a negative impact on PP in males, we altered the experimental conditions by priming ovariectomized stimulus females with estradiol benzoate (EB) to make them sexually receptive. Voles drank, on average, 14.3 ± 1.87 g/kg and showed a 73 ± 7.2% alcohol preference. Analysis of the PPT revealed a significant effect of stimulus animal on huddling time [$F(1,26) = 11.23$, $P = 0.0025$]; no main effect of alcohol on total huddling time [$F(1,26) = 1.24$, $P = 0.28$]; and, most importantly, a significant interaction between alcohol and stimulus animal [$F(1,26) = 9.88$, $P = 0.0041$]. Planned *t* tests for each group revealed that, in contrast to the females, there was

Significance

This study provides the first evidence to our knowledge that the effects of alcohol on social bonding can be mediated by biological mechanisms. The observed effects differed between males and females, such that alcohol inhibited social bonding in males and facilitated the partner preference in females. In addition to affecting behavior, alcohol affected neuropeptide systems known to be involved in social and stress/anxiety-like behaviors. These findings allow us to understand the factors involved in regulation of social behaviors, and effects of alcohol on them, better. Identification of these factors can help develop ways to prevent or treat the devastating effects of alcohol abuse on social relationships.

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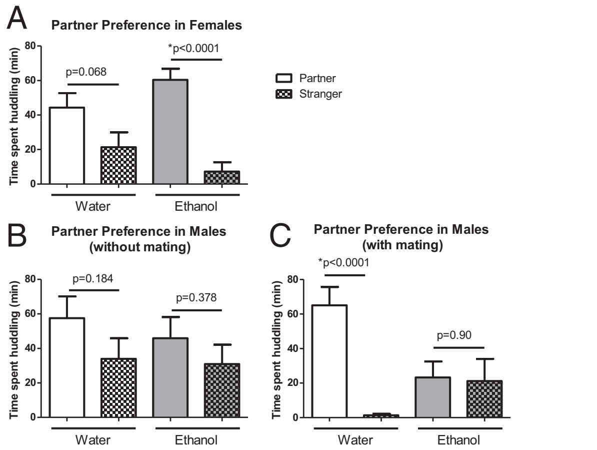

Fig. 1. Results of the PPT in voles drinking water or alcohol during cohabitation. (*A*) Females showed a trend for a PP under control conditions (n = 15), and this trend was enhanced in pairs exposed to alcohol (n = 15). (*B*) Males paired with sexually nonreceptive females did not show a significant PP with (n = 16) or without (n = 15) alcohol. (*C*) Males that mated with estrogen-primed females during cohabitation showed a significant PP (n = 7), whereas males that mated and had access to alcohol did not (n = 8). Values represent mean minutes huddling + SEM. *P < 0.0001.

a significant PP in the water control group [$t(12)$ = 5.98, P < 0.0001] but there was no significant PP in males that had access to alcohol during cohabitation [$t(14)$ = 0.13, P = 0.90] (Fig. 1*C*). Alcohol inhibited PP in male prairie voles.

To examine the behavioral effects of alcohol on individual PP formation further, we analyzed the number of subjects within each group expressing a PP, a stranger preference, or no preference according to established criteria (30), where an individual PP is demonstrated by the subject spending more than twice as much time with the partner as with the stranger. In the female experiment, two-thirds of the subjects drinking water (10 of 15 subjects) exhibited a PP, whereas the remaining subjects had no preference or a stranger preference; in the alcohol group, 14 of 15 subjects showed a PP (Table 1). A comparison of the number expressing a PP vs. no PP showed no effect of alcohol [P = 0.17, Fisher's exact test (FET)]. In the male experiment that showed a difference between the control and alcohol groups, all of the subjects in the water-drinking group exhibited a PP, whereas those in the alcohol group were approximately evenly split between exhibiting a PP, no preference, or a stranger preference (Table 1). A comparison of the number expressing a PP vs. no PP showed a significant effect of alcohol (P = 0.026, FET).

To determine whether the effects of alcohol on PP could be attributed to effects on mating, we quantified mating bouts. As expected, no mating was observed in either the first or last 2 h of cohabitation with unprimed female test subjects, and mating during the PPT occurred infrequently, with no effect of alcohol [$F(1,56)$ = 1.70, P = 0.20], no effect of stimulus animal on mating [$F(1,56)$ = 1.47, P = 0.23], and no interaction between these factors [$F(1,56)$ = 1.62, P = 0.21] (Fig. S1).

For males cohabiting with sexually receptive females, mating was observed in all subjects. There was no effect of alcohol on the number of mating bouts observed in the first or last 2 h of the 24-h cohabitation period [$F(1,12)$ = 0.15, P = 0.71] (Fig. 2*A*). The number of mating bouts during the PPT was not significantly different between treatment groups [$F(1,28)$ = 0.24, P = 0.57], there was no main effect of stimulus animal on number of mating bouts [$F(1,28)$ = 0.035, P = 0.85], and there was no interaction [$F(1,28)$ = 0.48, P = 0.50], indicating that both water and ethanol treatment groups mated equally with partner and stranger stimulus animals (Fig. S1). These findings indicate that the differences in PP between alcohol-exposed voles and water-drinking controls were not due to differences in mating behavior.

Alcohol also has well-known effects on locomotor activity and sedation (31), as well as on aggression (32), which are factors that might influence the expression of a PP. We found no evidence that any of these factors played a role in the PPT results: There was no difference in the number of chamber crossings (a standard quantitative measure of locomotor activity in the PPT) [$F(3,30)$ = 0.54, P = 0.66] (Fig. 2*B*) or in the number of aggressive bouts made between treatment groups by males during the first or last 2 h of cohabitation with primed females [$F(1,14)$ = 0.46, P = 0.51] (Fig. S1) or during the PPT [$F(1,26)$ = 2.79, P = 0.11] (Fig. 2*C*). Female aggression was very low, and there was no difference in the number of aggressive bouts between treatment groups during the first or last 2 h of cohabitation [$F(1,28)$ = 0.13, P = 0.72] (Fig. S1). In the PPT, females exhibited a trend for an effect of alcohol increasing aggression [$F(1,56)$ = 3.78, P = 0.057] and for an effect of stimulus animal [$F(1,56)$ = 2.92, P = 0.093], but there was no interaction between treatment and stimulus animal [$F(1,56)$ = 0.04, P = 0.85] (Fig. 2*D*). There was a larger number of aggressive bouts exhibited between male test subjects and primed stranger stimulus females compared with the primed partners [$F(1,26)$ = 13.37, P = 0.001] but no interaction between treatment and stimulus animal [$F(1,26)$ = 2.49, P = 0.13] (Fig. 2*C*). This finding is in accordance with other studies showing selective aggression in prairie voles (12, 33–35).

To explore the effect of alcohol on the neural systems involved in social bonding, we quantified immediate early gene product (Fos) immunoreactivity and the neuropeptides arginine vasopressin (AVP), oxytocin, corticotropin-releasing factor (CRF), neuropeptide Y (NPY), and urocortin 1 (Ucn1) in immunopositive cells and/or fibers in neural systems that have been associated with social bonding and alcohol abuse in a separate group of paired prairie voles after 24 h of cohabitation with (n = 9 pairs) or without (n = 9 pairs) access to alcohol. Immediately following 24 h of cohabitation and alcohol self-administration, animals were euthanized and brains were preserved for immunohistochemistry (IHC). The brain regions examined are listed in Table S1, along with the mean and SEM for the number of cells and fibers containing each neuropeptide, the number of cells expressing Fos, and corresponding probability values of ANOVA. All regions examined for neuropeptide expression were also examined in the Fos-stained tissue; those regions without data (–) had negligible numbers of neuropeptide- or Fos-positive cells, and therefore were not quantified. The findings suggesting differential effects of alcohol in male vs. female animals are shown in Fig. 3, with representative photomicrographs shown in Fig. S2, and described below.

We found significant interactions between sex and alcohol drinking affecting several markers traditionally associated with

Table 1. Number of subjects expressing preference for the partner, stranger, or neither

	Females			Males		
Treatment	PP	No preference	Stranger preference	PP	No preference	Stranger preference
Water	10	2	3	7	0	0
Ethanol	14	0	1	3	2	3

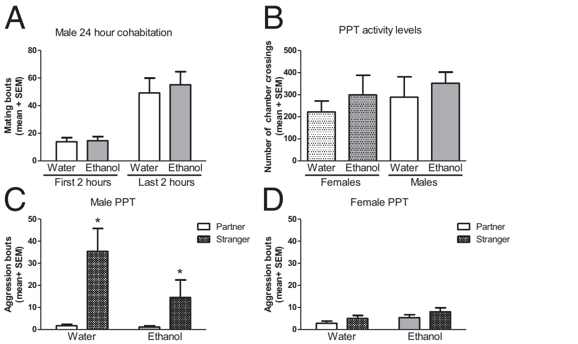

Fig. 2. Mating, activity, and aggression during 24-h cohabitation period and the PPT did not differ with alcohol access. (*A*) Number of mating bouts exhibited by male test subjects ($n = 8$ per group) in the first or last 2 h of the 24-h cohabitation period was not different between water and alcohol groups. (*B*) Activity levels in males ($n = 7$ drinking water, $n = 8$ drinking ethanol) and females ($n = 15$ per group) in the PPT did not differ between treatment groups. (*C*) Males ($n = 7$ drinking water, $n = 8$ drinking ethanol) exhibited no effect of alcohol on the number of bouts of aggression during the PPT (*$P = 0.001$; effect of stimulus animal on the number of aggressive bouts). (*D*) Females ($n = 15$ per group) exhibited a trend for an effect of alcohol increasing aggression in the PPT and for an effect of stimulus animal. Values represent mean + SEM.

measures of anxiety but not oxytocin- or AVP-immunoreactive cells or fibers. Specifically, there was a significant interaction between sex and alcohol on NPY fiber density in the medial amygdala [$F(1,32) = 5.68$, $P = 0.028$], with alcohol-exposed males having greater levels than water-drinking controls ($P = 0.039$). There was also a significant effect of alcohol on CRF fiber density in the ventral bed nucleus of the stria terminalis [BNST; $F(1,32) = 6.93$, $P = 0.013$]. In addition to the effects on neuropeptides, there was a significant interaction between the effects of sex and alcohol on the number of cells expressing the transcription factor protein Fos in the arcuate nucleus of the hypothalamus [$F(1,21) = 4.45$, $P = 0.047$], where females drinking alcohol had more Fos-immunoreactive (ir) cells than females drinking water ($P = 0.045$). There was also a significant interaction between sex and alcohol on Fos-ir cells in the centrally projecting Edinger–Westphal nucleus [EWcp; $F(1,32) = 5.88$, $P = 0.021$], which produces Ucn1, although post hoc tests revealed no significant differences to explain the interaction. There was a trend for a difference in males with access to alcohol compared with males without access to alcohol, and levels were significantly higher in males overall (Fig. 3, Fig. S2, and Table S1). Ucn1-labeled cells were largely colocalized (83.5%) with Fos in the EWcp of alcohol-drinking males (Fig. S3). Combined with the above data, this finding indicates that Ucn1 cells were activated in response to alcohol, particularly in male voles.

To test the hypothesis that alcohol decreases anxiety-like behaviors in prairie voles as has been demonstrated for other animals, we tested the effect of an injection of a low dose of alcohol (1.06 g/kg), comparable to what is consumed in a drinking bout (16, 18), on anxiety-like behavior in the elevated plus maze (EPM). As expected, the alcohol-treated voles spent more time in the open arms [$t(13) = 2.02$, $P = 0.032$] and less time in the closed arms [$t(13) = 1.87$, $P = 0.042$]; accordingly, they had a greater proportion of time in the open arms [$t(13) = 1.97$, $P = 0.035$] than did saline-treated subjects. They exhibited a trend toward a shorter latency to enter the open arms [$t(13) = 1.52$, $P = 0.076$] and more crossings through the center of the maze [$t(13) = 2.36$, $P = 0.017$], consistent with the stimulatory effects of low-dose alcohol on locomotor activity (Fig. S4). We also examined whether alcohol decreases stress hormone levels in prairie voles by assessing levels of corticosterone following 24 h of cohabitation of intact male and female voles. We found no differences in plasma corticosterone levels between alcohol-drinking prairie voles and water-drinking controls (Fig. S5).

Discussion

The main finding of this study is that alcohol can affect social bonds through biological mechanisms. Specifically, our animal model shows that alcohol drinking disrupts PP in males but not in females. Further, female prairie voles may show moderate enhancement of PP. These findings add to a limited body of literature demonstrating that drugs of abuse can affect social bonding and affiliative behavior in socially monogamous species. Specifically, administration of morphine has been shown to decrease huddling behavior in male-female pairs of prairie voles but not montane voles (36) and administration of amphetamine has been shown to disrupt PP in male prairie voles (13). Importantly, in the present study, the effects on social bonds are observed not after administration of the drug by an experimenter, which could produce stress, but following voluntary self-administration by the voles.

Interestingly, the selective aggression toward stranger voles following cohabitation regardless of alcohol intake indicates that alcohol acts on a specific and dissociable part of the pair bond formation neural processes to affect PP but not aggressive behaviors. This finding is in accordance with studies showing that PP formation and selective aggression require similar but not entirely overlapping neural substrates: AVP and activation of the AVP receptor 1a are required for both, but the receptors are activated in the anterior hypothalamus for aggression and in the ventral pallidum and lateral septum for PP (12, 35, 37, 38). It is possible that alcohol acts on mechanisms responsible for the development of PP but not on those responsible for selective aggression. In addition, the lack of alcohol's effects on selective aggression indicates that the inhibition of PP in male prairie voles is not due to attenuation of social learning or recognition of the partner vs. stranger. The lack of effects of alcohol on behaviors other than PP that could have potentially interfered with bond formation supports our hypothesis that alcohol acts directly on neurobiological substrates involved in aspects of bond expression. In this regard, our study also differs from results with other drugs of abuse. Specifically, morphine disrupted huddling at doses that were inhibiting locomotor behavior (36), and amphetamine disrupted PP at a dose nonspecifically enhancing aggression (12, 13). This finding indicates that drugs of abuse can affect pair bonding through different neurobiological mechanisms.

The self-administration paradigm used in the present study is advantageous in that it does not involve stress of experimental handling; however, its disadvantage is in the difficulty of testing dose effects. In this study, we did not measure blood ethanol concentration (BEC) due to the potential effect of stress of blood sampling on behavioral outcomes, as well as to the pattern of alcohol drinking exhibited by prairie voles, which drink moderate amounts of alcohol throughout the day and night, making it impossible to sample at a time of peak BEC. However, we have previously demonstrated that prairie voles exhibit a peak BEC similar to that of C57BL/6 mice following an injection of ethanol and that the rates of elimination are not different between sexes (16). Moreover, the subjects in the present experiments consumed doses of alcohol similar to what we have observed in other studies in voles and C57BL/6 mice, and which are likely to produce an intoxicating BEC (16, 17, 19). One indication of the intoxication is the anxiolytic effect of alcohol injection observed here. A limitation to the self-administration procedure used in these experiments was the inability to distinguish the subject's fluid intake from the partner's. Nevertheless, previous experiments have not demonstrated any consistent sex-dependent differences in alcohol consumption (16, 17, 19); thus, a sex-

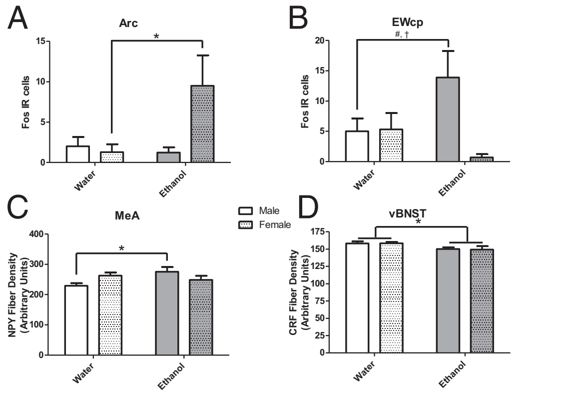

Fig. 3. Immunoreactivity for Fos or neuropeptides. Regions shown exhibit a significant interaction between sex and alcohol or an effect of alcohol in a region with known sex-related differences in receptor levels. (*A*) Number of Fos cells is significantly higher in the arcuate nucleus (Arc) of females with access to alcohol. (*B*) Number of Fos cells in the EWcp exhibits a trend for a significant difference in males with access to alcohol compared with males without access to alcohol, and is significantly higher in males overall. (*C*) Density of NPY immunoreactivity in the medial amygdala (MeA) is higher in males with access to alcohol than in males without access to alcohol. (*D*) Density of CRF fibers in the ventral BNST (vBNST) is significantly lower in voles with access to alcohol (n = 9 per sex per treatment). Values represent mean + SEM. *$P < 0.05$; $^{\#}P < 0.1$; †main effect of sex, $P < 0.05$.

specific difference in alcohol dose is unlikely to be responsible for the observed behavioral effects of alcohol on PP.

Our findings indicate that alcohol drinking during cohabitation affects a number of brain regions and neuropeptide systems that could regulate social behaviors through several potentially independent mechanisms. Extensive literature implicates oxytocin and AVP in regulation of PP (28, 39, 40), and although we found some effects of alcohol or sex on these neuropeptide levels in a few brain regions (Table S1), there were no significant interactions to indicate a role for either of these neuropeptides in the sex-specific effects of alcohol on behavior. Because the roles of oxytocin and vasopressin are so essential to pair bonding, and because the effects of alcohol on these neuropeptide systems have been described previously (41–50), it would be worth pursuing further experiments testing whether these systems do play a role in the effect of alcohol on PP that is simply not detected in changes in cellular or fiber peptide levels at 24 h of cohabitation.

We also examined neural substrates involved in stress and anxiety, because alcohol is known to act as an anxiolytic and stress and corticosterone have been shown to stimulate PP in male prairie voles but to inhibit PP in female prairie voles (51). In agreement with alcohol's effect on decreasing anxiety, we find a decrease in the levels of CRF within the BNST after alcohol exposure. This finding may indicate decreased activation of the stress pathway in response to alcohol, leading to facilitation of PP for females and inhibition for males (30, 52). This region is of particular interest because there is a sex-dependent difference in the number of CRF2 receptors in the BNST, with male prairie voles having greater receptor binding than females (53). More recent studies in rats have also demonstrated sex-dependent differences in CRF receptor binding levels; similar to prairie voles, male rats have more CRF2 receptors than female rats in the BNST. There are additional sex-dependent differences in CRF1 and CRF2 receptor levels in several other brain regions, but the differences are dependent on age relative to puberty (54). We also find that alcohol-exposed males exhibit an increase in amygdalar fiber density of NPY, which is known to act as an anxiolytic (49), and may thereby inhibit pair bond formation. This finding may parallel sex-dependent differences in rats in response to a stressor, where males release more NPY in the circulating plasma in response to prolonged acute stress, whereas females release NPY and then return to baseline even throughout prolonged stress (55). In addition, there are known sex-dependent differences in the region-specific production of NPY in rats, where males express more than females in the caudal Arc (56), and in the present study, there was an interaction between the effects of alcohol and sex on Fos-ir neurons in the Arc. Similarly, we observed an increase in the number of cells expressing Fos in the EWcp of males, the main central source of the neuropeptide Ucn1, which is also thought to be involved in stress-coping mechanisms (57). Importantly, the majority of the cells expressing Ucn1 were activated (as seen by coexpression of Fos in alcohol-drinking male voles), and there was no difference in the number of Ucn1-positive cells between treatments, indicating that the greater levels of Fos in the EWcp were due to activation by alcohol and not simply to a greater number of cells in this region.

To date, stress is the only mechanism that has shown opposite effects on PP in male and female prairie voles: A short swim stress before cohabitation, or administration of corticosterone, facilitated PP in males but inhibited it in females, whereas adrenalectomy reversed these effects (51). Combined with results from the present study indicating the sex-dependent effects of alcohol on neuropeptides and brain regions relevant to stress and anxiety, this evidence leads us to hypothesize that alcohol self-administration during cohabitation acts as an anxiolytic, thereby facilitating PP in females but inhibiting it in males. This hypothesis is supported by behavioral data from the EPM test demonstrating decreased anxiety-like behavior following a low dose of alcohol but not by data from analysis of corticosterone levels following alcohol intake. Although the lack of observed effects of alcohol on levels of corticosterone could suggest that effects of alcohol on PP are mediated through central mechanisms, another potential explanation could be the short peak of corticosterone following a stimulus (58); similar to BECs, changes in corticosterone levels may be impossible to detect when voles drink at staggered intervals throughout the 24-h access period.

In addition to testing the hypothesis that alcohol decreases stress and anxiety, leading to the sex-dependent difference in the effect on PP, future studies should examine the levels of neuropeptide release in the relevant brain regions indicated to determine a more precise mechanistic role. The role of dopamine, serotonin, and opioid systems should also be investigated because these neurotransmitters are involved in the rewarding properties of alcohol and other drugs, and play a role in vole social behavior as well, including through interactions with the oxytocin system (59–61).

The enhancement of attachment in female prairie voles parallels the prosocial effects of alcohol in humans (24, 62, 63). The inhibition of bond formation in males is reminiscent of the negative effects of alcohol on long-term attachments and marital happiness, which occur for both men and women (23, 64–66). It appears that both sexes of prairie voles can model a different aspect of the effects of alcohol observed in both male and female humans. Our findings do not argue against the importance of uniquely human cultural and socioeconomic factors that contribute to alcohol's effects on human bonding (20, 21, 62) but indicate that biological effects of alcohol on social bonds should be considered. Moreover, we also acknowledge that the biological effects of alcohol on social attachments are most likely more complex than those modeled in our study, and alcohol could exhibit differential effects on human bonding depending on the status of alcohol use disorders or the stage of the human relationships (e.g., formation vs. maintenance of pair bonds). Importantly, the paradigm established here allows us to model significant aspects of human alcohol abuse and can ultimately lead to understanding of neural and behavioral factors contributing to the abnormal social behavior that occurs during alcohol use and abuse, as well as development of effective therapies that can improve affiliations and alcohol drinking in problem drinkers.

Methods

Animals. Adult male and female prairie voles (67–115 d old) from our breeding colony at the Portland Veterans Affairs Medical Center Veterinary Medical Unit were used in these experiments. All experiments were approved by the Institutional Animal Care and Use Committee. Voles were weaned at 21 d of age and housed with same-sex siblings under a 14:10-h light/dark cycle. Females were housed separately to suppress ovulation, because prairie voles are induced ovulators. The animals were given ad libitum access to food and water throughout experiments except where noted.

Effects of Ethanol Self-Administration on PP in Females. Female test subjects (n = 15 per group) were weighed and placed in clean home cages, followed within minutes by a "partner" male. Once paired, both animals were given access to tubes containing ethanol and water or only water for control pairs. The volume of each fluid was recorded 0, 22, and 24 h from the beginning of cohabitation. The total volume of alcohol or water consumed per cage for each time period was divided by 2 and assigned to each member of the pair. These measured volumes were then used to calculate alcohol preference (volume of alcohol divided by total volume of fluid consumed) and the dose of alcohol consumed (grams of alcohol per kilogram of body weight).

"Stranger" stimulus males were also placed in clean home cages, but in isolation. Strangers received ethanol and water or water only to match the condition of the subject with which they would later be tested.

Female prairie voles are induced ovulators and are not usually sexually receptive until after being exposed to a male for 24 h (67). Thus, mating was not expected during the 24-h cohabitation period for these pairs. However, to detect any potential differences in mating behavior, the last 2 h of cohabitation were digitally video-recorded.

Following 24 h of cohabitation, the PP formation was assessed with a 3-h PPT. Although other laboratories have demonstrated a strong PP in females following 24 h of cohabitation, this amount of time did not lead to a significant PP in our laboratory. This effect is likely due to differences between laboratories and across time, although some experiments from the same laboratory can vary in the time necessary to form a PP in control subjects, ranging from 3 to 24 h (58, 68). The PPT occurred in a three-chambered testing box with the partner stimulus animal tethered to one end of the cage, the stranger stimulus animal tethered to the other end, and the test subject placed in the center and allowed to move freely throughout the cage (40, 69). The PPT lasted 3 h, during which time the voles did not have access to food, water, or alcohol. The entire 3-h test was digitally video-recorded for later analysis.

Effects of Ethanol Self-Administration on PP in Males. The test for PP in males was conducted in two experiments, with or without mating. The tests were conducted in the same way as described above, but with a male test subject and female stimulus animals and with the following exceptions.

In the first male experiment, female stimulus animals were ovariectomized (*SI Methods*) so that they were not sexually receptive. For this experiment, we used 16 subjects in the alcohol group and 15 subjects in the water control group.

In the second male experiment, female stimulus animals were ovariectomized and given priming doses of estradiol to induce sexual receptivity (*SI Methods*). Because mating was expected under these conditions, the first 2 h of cohabitation were recorded, in addition to the last 2 h, to detect whether mating occurred in all pairs and to assess whether there were differences in mating behaviors between groups. For this experiment, we used eight test subjects per group. One subject from the water control group was removed from analysis for fighting with the partner.

IHC. To assess potential neuropeptide involvement in the effects of alcohol on PP, and to rule out any effects of the PPT itself, a separate group of animals (18 male-female pairs) were given similar access to 10% (vol/vol) ethanol and water or only water during a 24-h cohabitation period. Immediately following cohabitation, they were euthanized by CO_2 inhalation. Brains were extracted, fixed in paraformaldehyde (2% in 10 mM PBS) for 24 h, and cryoprotected by 30% sucrose for immunohistochemical staining.

Brain tissue was sliced in 40-μm coronal floating sections and preserved until IHC in PBS with sodium azide. Each brain region was chosen based on involvement in PP behaviors and response to alcohol or on known differences in receptor levels between sexes or species with different social systems, and two slices from each region were selected and assayed for each subject. The IHC protocol was based on previous publications (70–73). Antibodies were diluted as follows: antioxytocin (Peninsula Laboratories), 1:20,000; anti-AVP (Peninsula Laboratories), 1:50,000; anti-CRF (Peninsula Laboratories), 1:10,000; anti-NPY (Sigma–Aldrich), 1:50,000; anti-Ucn1 (Santa Cruz Biotechnology), 1:5,000; and anti–c-Fos (Santa Cruz Biotechnology), 1:2,000. All primary antibodies were polyclonal, made in rabbit against mouse, with the exception of the Ucn1 antibodies made in goat. Anti-rabbit secondary antibodies made in goat or anti-goat antibodies made in rabbit (Vector Laboratory, Inc.) were applied, and the antibody signal was amplified with a Vectastain ABC kit (Vector Laboratory, Inc.). The tissue was processed for visualization using metal-enhanced diaminobenzidine (Pierce).

Immunoreactive cells and fibers were visualized using a Leica DM4000 bright-field microscope (Bartels and Stout, Inc.). All cells stained above background in each region were manually counted by an experimenter blinded to the condition of the subjects. Fibers in some regions were manually counted, and fiber density was determined in other regions using ImageJ software (National Institutes of Health) by an experimenter blinded to the condition of the subjects. For density analysis in ImageJ, each image was converted to eight-bit and calibrated to a straight-line function using the darkest and lightest spots in the background tissue to control for differences in background staining. A polygon was drawn around the borders of the region of interest, capturing a consistent area between images, and the density of staining was measured for each region. Thus, the density reported was based on the calibration curve and is expressed in arbitrary units, with greater values corresponding to greater staining.

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