INTERACTIONS BETWEEN SOCIAL RELATIONSHIPS AND ALCOHOL DRINKING IN PRAIRIE VOLES

By

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LIST OF ABBREVIATIONS

ABC – avidin-biotin complex

ACF – auto-correlation function

AHA – anterior hypothalamic area

Amyg - amygdala

ANOVA – analysis of variance

Arc – arcuate nucleus of the hypothalamus

AVP – arginine vasopressin

BEC – blood ethanol concentration, blood alcohol concentration

BLA – basolateral amygdala

BNST – bed nucleus of the stria terminalis

C57 – C57BL/6J mice

CeA – central nucleus of the amygdala

CO2 – carbon dioxide

CORT - corticosterone

CP – caudate-putamen

CRF – corticotropin releasing factor

CRFR1 – corticotropin releasing factor receptor 1

CRFR2 – corticotropin releasing factor receptor 2

CTX – cortex

D2R – dopamine receptor 2

DA – dopamine

DBA – dilute brown non-agouti mice

EB – estradiol benzoate

ELISA – enzyme-linked immunosorbent assay

EtOH – ethanol; alcohol

EW; EWcp – centrally-projecting Edinger-Westphal nucleus

GR – glucocorticoid receptors

Hipp - hippocampus

HPA – hypothalamic-pituitary-adrenal

IHC – immunohistochemistry

i.p. – intraperitoneal

IR – immunoreactivity

LDT – lateral dorsal tegmental area

LS – lateral septum

MeA – medial amygdala

MGN – medial geniculate nucleus

MOR - μ opioid receptor

MPA – medial preoptic area

MR – median raphe

MR – mineralocorticoid receptors

MS – microsatellite

NAc – nucleus accumbens

NaN_3 – sodium azide

NPY – neuropeptide Y

OB – olfactory bulb

OT – oxytocin

OTR – oxytocin receptor

PBS – phosphate-buffered saline

$pIII_U$ – perioculomotor urocortin-containing neurons (see EW above)

PP – partner preference

PPT – partner preference test

PVN – paraventricular nucleus of the hypothalamus

RCF – relative centrifugal force

SEM – standard error of the mean

SNP – single nucleotide polymorphism

SON – supraoptic nucleus

V/V – volume per volume

V1aR – vasopressin 1a receptor

VMH – ventral medial hypothalamus

VP – ventral pallidum

VTA – ventral tegmental area

ZT – zeitbeger time

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ABSTRACT

Alcohol use disorders are a prevalent problem that can lead to harmful effects on an individual's health, functioning, and social relationships. Social interactions can in turn affect alcohol intake. It is important to study these behavioral interactions, and in particular the biological underpinnings of these behaviors, in order to understand how they influence alcohol use disorders, and how these disorders may be prevented or treated.

Animal models of human disease can be particularly useful for studying the biological bases of related mechanisms and behaviors, and for testing potential treatments. However, until now, an adequate laboratory animal model of the interactions between alcohol drinking and specific adult social attachments did not exist. Thus, the aim of this dissertation was to establish a novel animal model of the interactions between social relationships and alcohol drinking.

To accomplish this goal, prairie voles (*Microtus ochrogaster*) were used to model these behavioral interactions. Prairie voles are socially monogamous and readily form strong, lasting bonds between specific individuals, unlike traditional laboratory rodents. Also unlike most other animals, prairie voles show a natural high preference for unsweetened alcohol, as demonstrated here. These features combined make prairie voles an ideal model for examining the interactions between social bonds and alcohol drinking.

Here we find that prairie voles show a higher preference for alcohol when they are housed in pairs with siblings or peers, rather than housed in isolation, and they match each other's alcohol intake. This models the social facilitation of alcohol drinking often observed in humans. Furthermore, when a high-drinking prairie vole is paired with a low

drinker, the high drinker usually decreases its alcohol intake, demonstrating a direct peer influence to decrease drinking. Susceptibility to peer influence may be predicted by individual variation in features of drinking behavior, but not by voles changing drinking patterns to drink together. A genetic polymorphism known to play a role in prairie vole social behaviors did not predict the susceptibility to alter drinking when influenced by a peer. The procedures established in the experiments described here allow us to model the important role of social influences, particularly to decrease problem drinking.

In another set of experiments we tested the effects of alcohol on pair bond formation in adult prairie voles. We found that the effects were opposite for males and females: alcohol drinking during the bond formation period facilitated a preference for the partner in female prairie voles but inhibited it in males. We explored a number of possible neurobiological mechanisms behind these effects and found support for the role of several neuropeptides known to be involved in stress, anxiety, and response to alcohol.

With these experiments, we establish that prairie voles can model a number of human behaviors relevant to interactions between social relationships and alcohol drinking: social facilitation of alcohol drinking, peer influence to decrease drinking, and alcohol acting to facilitate or inhibit formation of social bonds. Future studies of these behaviors and underlying neurobiological mechanisms will allow us to gain a better understanding of causes, influences, and treatment strategies that can help people with alcohol use disorders.

GENERAL INTRODUCTION

Alcohol use and social relationships in humans

Alcohol use disorders are extremely prevalent, with approximately 30% of the population of the United States meeting diagnostic criteria for alcohol abuse or dependence at some point in their lifetimes (Chapter 2, Hasin, et al., 2007). This often leads to problems on an individual level such as straining relationships between family or friends, job loss or economic hardship, and severe health problems (Hasin, et al., 2007). There are similar problems at the level of society as well. Alcohol is the third greatest cause of preventable mortality behind diet and tobacco, not only due to direct effects of alcohol on health, but also to alcohol-related car crashes; furthermore, morbidity and mortality due to alcohol use contribute to a substantial loss of economic productivity (Mokdad, et al., 2004).

The tendency to develop an alcohol use disorder is approximately half due to heritable genetic factors and half due to environmental factors such as culture, socio-economic status, and social influences, and there is considerable interaction between these factors (Enoch, et al., 2003; Goldman, Oroszi, & Ducci, 2005; Stacey, Clarke, & Schumann, 2009). The interactions between social relationships and alcohol drinking are important at every stage in the progression of alcohol use disorders and recovery. For example, peer influences are an important factor in the initiation of alcohol drinking (Windle, et al., 2008), and the level of use in adolescence, college, and beyond (Fisher, et al., 2007; Homish & Leonard, 2008; Leonard & Mudar, 2000; Park, Sher, & Krull, 2008). Alcohol abuse is often linked with aggression, particularly intimate partner violence (Foran & O'Leary, 2008; Hoaken & Stewart, 2003; Leonard, et al., 1985; Testa, et al., 2012), and plays a large role in marital dissatisfaction and divorce (Leonard & Eiden,

2007; Leonard & Rothbard, 1999; Levinger, 1966; Marshal, 2003). Further, a strong social support network is vital for recovering alcoholics working to maintain abstinence (Bond, Kaskutas, & Weisner, 2003; Dawson, et al., 2006; Gordon & Zrull, 1991; Kaskutas, Bond, & Humphreys, 2002; Kelly, et al., 2011; Longabaugh, 2003; Wu & Witkiewitz, 2008).

The role of social relationships in alcohol-related behavior is in some cases different for men and women. While the number of 'drinking buddies' in a person's social network is a predictor of alcohol drinking levels for both sexes, men often have more drinking buddies than women, even with similarly-sized social networks, and correspondingly consume more alcohol than women (Homish & Leonard, 2008). Women are less likely than men to seek treatment for alcoholism, and when they do, they report less support from their families, although women also have more social resources in long-term follow-up reports (Timko, Finney, & Moos, 2005). On the other hand, while there are often sex biases in expectations of the effects of alcohol on behavior, these expectations do not necessarily hold true (Critchlow, 1986). For example, increases in alcohol use correlate with increases in the proportion of young adults reporting multiple sexual partners at a similar rate in both men and women (M. L. Cooper, 2002; Graves, 1995; Santelli, et al., 1998). All together, this and other examples illustrate that there are several differences between men and women experiencing the effects of alcohol and social influences, but that many perceptions of differences may not be based in truth. The cultural influences on these differences and perceptions are important to keep in mind.

Animal models of human behaviors

It is very difficult to tease apart the effects of innate biological factors, environmental elements, cultural influence, and specific social experiences that can all

contribute to alcohol use disorders or recovery in a human population. Furthermore, it is impossible to determine precise neurobiological mechanisms involved in predisposition or escalation of alcohol drinking to alcohol abuse in humans using methods currently available. Therefore, it is useful to examine alcohol drinking behaviors and underlying biological factors in animal models. This allows determination of causal rather than correlational effects and examination of specific neurobiological mechanisms on the molecular level. While there are important differences between humans and various other animals, laboratory animals can be used to model specific aspects of human behavior. Further, it can be advantageous to eliminate effects of culture or socioeconomic status in order to focus on the strict biological and behavioral factors that influence alcohol drinking. We can then use findings from studies with animals to inform future studies in humans, in order to confirm similarities and potentially test new therapeutic strategies.

Interactions between social behaviors and alcohol drinking have been assessed in a number of other animal models (reviewed in Anacker & Ryabinin, 2010). For example, the role of the mother-infant bond has been studied in non-human primates. Rhesus macaques reared with peers instead of by a mother show increased alcohol drinking in adulthood (Fahlke, et al., 2000; Higley, et al., 1991; Higley, Suomi, & Linnoila, 1996). These studies are thought to model the impact of unfavorable early life experiences on alcohol intake later in life (Foxcroft & Lowe, 1991).

The converse, effects of alcohol on social behaviors, has been explored in laboratory rodents. Alcohol increases aggressive behavior in mice (Chiavegatto, et al., 2010; Cruz, et al., 2008; Faccidomo, Bannai, & Miczek, 2008; Fish, et al., 2008; Takahashi, Kwa, et al., 2010; Takahashi, Shimamoto, et al., 2010), modeling the same

effects problematic in humans (Foran & O'Leary, 2008). Alcohol also decreases social inhibition and facilitates social play behaviors in rats (Varlinskaya & Spear, 2002, 2006, 2009), which models the similar facilitation of positive social experiences and bonding reported by humans (Sayette, et al., 2012).

Many studies have examined the effects of social housing and isolation on alcohol intake in mice and rats, and most results indicate that these rodents drink more when housed in isolation (Ehlers, et al., 2007; Hall, et al., 1998; McCool & Chappell, 2009; Rockman, Gibson, & Benarroch, 1989; Wolffgramm & Heyne, 1991). These effects model particular aspects of human drinking. Effects of social isolation stress, for example due to divorce, can increase alcohol drinking (Jose, et al., 2000; Leonard & Rothbard, 1999). Heavy drinking alone, while not necessarily an indication of alcohol abuse in itself, can be a sign of other alcohol-related problems (Chalder, Elgar, & Bennett, 2006; Swahn, et al., 2004).

These studies of interactions between alcohol drinking and social behaviors have led to important findings, but they have one major shortcoming that prevents these animal models of social behaviors and alcohol drinking interactions from modeling key aspects of human alcohol intake: the rodents studied do not demonstrate social affiliations with a *specific* peer, sibling or mate, and these types of relationships have not been examined in the context of alcohol drinking in non-human primates. These types of peers can exert the greatest social influences on drinking in people, and they typically experience the greatest effects of an individual's alcohol abuse.

Modeling social behaviors in prairie voles

Prairie voles (*Microtus ochrogaster*) are a valuable model of adult social attachments. These rodents have been well-studied due to their relatively rare

monogamous social structure; only an estimated 3% of mammalian species exhibit monogamy (Kleiman, 1977). In the wild, male and female prairie voles form an attachment called a pair bond, and they nest together and continue to spend time together even outside of the breeding season. Prairie vole pairs exhibit other features of monogamy such as both parents participating in rearing of offspring, and aggression toward strangers of either sex (selective aggression or mate guarding) (Carter & Getz, 1993; Getz, Carter, & Gavish, 1981; Getz, et al., 1993; Gruder-Adams & Getz, 1985).

Some prairie voles live in typical single-family units comprised of a bonded male-female pair and their offspring, while others live in communal nests having at least two adults of the same sex (Getz & McGuire, 1997; McGuire & Getz, 1995). A majority of the time in the wild, prairie vole young will not disperse after weaning and instead will remain in the natal nest, where they may help rear subsequent litters of offspring (McGuire, et al., 1993; Solomon, 1991). Under these conditions, offspring typically remain sexually inactive, while one or more unrelated adults of each sex are reproductively active (Getz & McGuire, 1997). Dispersal is more common during the breeding season and when the population density is low, and if offspring do become reproductively active, but is not dependent on competition for mates or resources (McGuire, et al., 1993). Taken together, this evidence from the field indicates that male-female, parent-offspring, and sibling affiliations are all important for prairie voles.

All of these relationships have been studied in the laboratory in addition to the reports of wild populations. The formation of the pair bond between a male and female has been the most extensively characterized. To assess this bond in the lab, an adult male and female are paired and allowed to cohabitate, and in some cases mate, which can

facilitate bond formation (Insel, Preston, & Winslow, 1995). Following cohabitation, the presence of the pair bond can be assessed with the partner preference test. In this test, the subject is presented with two stimulus animals, the partner and a matched stranger, tethered on opposite ends of a three-chambered cage. The amount of time the subject spends huddling in side-by-side, motionless contact with each stimulus animal is measured. After a sufficient cohabitation period (usually 24 hours), prairie voles will exhibit a significant preference for huddling with the partner over the stranger, which is called the partner preference (Carter & Getz, 1993; Getz, Carter, & Gavish, 1981; K. A. Young, Liu, & Wang, 2008). This test has also been used to assess same-sex pairs with similar results (DeVries, Johnson, & Carter, 1997).

The formation of the pair bond can be manipulated experimentally by exposing subjects to experiences or pharmacological agents before or during cohabitation. Using these techniques, several mechanisms of pair bond formation have been elucidated. In particular, the roles of neuropeptides arginine vasopressin, oxytocin, and corticotropin releasing factor (CRF), along with specific receptors, have been established. Activation of the vasopressin 1a receptor in the lateral septum and ventral pallidum is necessary and sufficient for pair bond formation and expression in male prairie voles (Donaldson, Spiegel, & Young, 2010; Lim & Young, 2004; Liu, Curtis, & Wang, 2001; Pitkow, et al., 2001; Winslow, et al., 1993). Oxytocin receptor, along with dopamine D2 receptor activation is required for bond formation in female prairie voles (Liu & Wang, 2003; Williams, Carter, & Insel, 1992; Williams, et al., 1994). Further, activation of CRF receptors in the nucleus accumbens is necessary for pair bonding in males (DeVries, et al., 2002; Lim, et al., 2007). Interestingly, stressors (or manipulations of corticosterone

levels) have opposite effects on bond formation in male and female prairie voles (DeVries, et al., 1996). When a male and female prairie vole meet, corticosterone levels decrease, which facilitates partner preference formation in females, but it is the subsequent return to baseline corticosterone levels that is necessary for bond formation in males (Carter, et al., 1995; DeVries, et al., 1995; DeVries, et al., 1996; DeVries, Taymans, & Carter, 1997).

In addition to discovering molecules and receptor systems involved in pair bonding, the role of specific genetic elements has been determined using transgenic techniques coupled with the partner preference test. One specific genetic polymorphism in the regulatory region of the prairie vole gene encoding the vasopressin 1a receptor has been of particular interest. Within this regulatory region, upstream of the 5' end of the promoter sequence, there lies a microsatellite, a sequence of several hundred nucleotide base pairs that includes many di- and tri-nucleotide repeats. The length of the microsatellite has been linked to differential V1aR expression levels between prairie voles (Hammock, et al., 2005; Ophir, Campbell, et al., 2008; Phelps & Young, 2003), and different patterns of expression between these monogamous voles and non-monogamous meadow voles or mice (Hammock & Young, 2004). Further, introduction of the prairie vole regulatory region to non-monogamous rats or meadow voles via viral-mediated gene transfer induced more prairie vole-like social affiliations (Landgraf, et al., 2003; Lim, et al., 2004). Interestingly, natural individual differences in the receptor expression levels between prairie voles (and corresponding microsatellite lengths) were correlated with differences in partner preference expression and other social behaviors (Hammock, et al., 2005; Solomon, et al., 2009). There are similar microsatellite regions and single

nucleotide polymorphisms in the vasopressin 1a receptor regulatory regions of the human gene, which have been associated with social behaviors such as pair-bonding in men, as well (Walum, et al., 2008).

Interactions between social relationships and alcohol drinking in prairie voles

All of these systems found to be involved in social bonds in prairie voles have also been shown to play an important role in alcohol (and other drug) use. For example, vasopressin and oxytocin levels in the plasma and brain change following alcohol intake (Guillaume, Gutkowska, & Gianoulakis, 1994; Harding, et al., 1996; Inder, et al., 1995; Linkola, et al., 1978; Madeira & Paula-Barbosa, 1999; Madeira, et al., 1993; Mennella & Pepino, 2006; K. M. Ogilvie, S. Lee, & C. Rivier, 1997; Rivier & Lee, 1996; Silva, et al., 2002). Vasopressin is also necessary for the development and maintenance of functional tolerance to alcohol (Hoffman, et al., 1990; Hoffman, et al., 1978). As with other rewarding experiences, alcohol is associated with an increase in dopamine in the nucleus accumbens (Di Chiara & Imperato, 1985; Everitt, et al., 2008; Gessa, et al., 1985; Koob, et al., 1998; Robinson & Berridge, 1993, 2008; Wise & Bozarth, 1981). Alcohol can act as a stressor, increasing CRF and corticosterone (Rivier, Bruhn, & Vale, 1984; Rivier & Lee, 1996), although it is often reported to be anxiolytic (Book & Randall, 2002; Carrigan & Randall, 2003; Sutker, et al., 1982).

Based in part on the similar neural mechanisms behind both alcohol drinking and social behaviors in prairie voles, and in part on the observed interactions between these behaviors in humans, it follows that there may be biological factors underlying the interactions between social relationships and alcohol drinking, and that we may be able to detect them by modeling these behaviors in prairie voles.

The first goal of this dissertation was to establish prairie voles as a novel animal model of human behaviors that had not previously been modeled in other animals. Toward this goal, the interactions between social circumstances and alcohol drinking were explored to identify specific behaviors exhibited by voles that could model human behaviors. In Chapter 1 we examine the effects of the social environment on alcohol drinking and explore similarities and differences between these effects and those seen in other laboratory rodents. In Chapter 2 we investigate whether a peer can directly influence alcohol drinking levels, and Chapter 3 further examines the behavioral mechanisms behind socially-influenced changes in drinking. In Chapter 4, we explore the effects of alcohol drinking on the formation of social attachments, measured by the partner preference in male and female prairie voles.

The second goal was to examine the genetic and neurobiological factors underlying the interactions between social relationships and alcohol drinking. The role of the vasopressin 1a receptor in these behaviors was investigated in Chapters 3 and 4. Additionally, a panel of key neuropeptides was assessed for their potential roles in alcohol's effects on pair bonding in Chapter 4.

A third goal was to begin to manipulate the social environment to determine whether these changes could exert a positive influence on drinking, toward development of methods that could treat alcohol use disorders or augment existing drug or behavioral therapies. Chapters 2 and 3 demonstrate the effects of social circumstances on changing alcohol drinking. Together, these studies show that prairie voles can model many specific aspects of the interactions between social relationships and alcohol drinking in humans,

and provide insight into the biological mechanisms mediating these interactions and potential behavioral and pharmacological therapies for alcohol abuse.

CHAPTER 1: Prairie Voles as a Novel Model of Socially Facilitated Excessive Drinking

(This chapter has been reformatted and minimally edited for inclusion in this dissertation from: Anacker, A. M., Loftis, J. M., Kaur, S., & Ryabinin, A. E. (2011). Prairie voles as a novel model of socially facilitated excessive drinking. *Addict Biol, 16*(1), 92-107.)

Introduction

Social relationships and alcohol (ethanol) drinking have complex effects on each other. In one direction of these effects, social situations can affect alcohol intake patterns and quantities. For example, social stress or separation from a loved one through death or divorce can lead to increased alcohol intake (Hajema & Knibbe, 1998; Jose, et al., 2000; Temple, et al., 1991), while a supportive social network is a major aide for abstinent alcoholics (Groh, Jason, & Keys, 2008; Kelly, et al., 2008). There are many examples of effects in the opposite direction, where alcohol intake impacts social relationships. Male alcohol abuse is considered a causal risk factor for intimate partner violence (Heise, 1998; Leonard, et al., 1985), and alcohol use is a commonly accepted cause of marital dissatisfaction and dissolution (for discussion of these findings, see Leonard & Eiden, 2009).

While adverse effects of social relationships can lead to increased drinking, many enjoyable relationships and circumstances may also lead to increased drinking. Alcohol is often considered a 'social lubricant,' such that there is a reciprocal relationship between the social network and individual drinking patterns (Park, Sher, & Krull, 2008), and a person's social network is a primary factor in modulation of his or her alcohol use (Homish & Leonard, 2008).

Previously, there has not been an adequate laboratory rodent model to investigate socially facilitated drinking, or the effect of social relationships on alcohol consumption. It is difficult or impossible at this time to study the neural mechanisms in humans that are involved in influencing one another's drinking or peer pressure. A rodent model that exhibited behaviors relevant to elevated drinking in social situations would be invaluable.

The primary reason for the absence of an adequate rodent model is the lack of strong specific bonds in traditional laboratory rodents. While mice and rats do prefer social environments, particularly in adolescence (Douglas, Varlinskaya, & Spear, 2004; Panksepp & Lahvis, 2007), can show signs of anxiety- and depression-like symptoms when they are socially isolated (Yates, et al., 1991), and can show differences in alcohol intake dependent on their social housing conditions (Deehan, Cain, & Kiefer, 2007; Doremus, et al., 2005; Ehlers, et al., 2007; Schenk, Gorman, & Amit, 1990), there is no evidence that they show strong pair bonds with, or prefer to spend time with, a particular individual.

In contrast, prairie voles (*Microtus ochrogaster*) are socially monogamous rodents that have been extensively studied because they form specific pair bonds. In the wild, mated pairs nest together, both parents participate in caring for offspring, and they typically mate for life (Getz, Carter, & Gavish, 1981). The pair bond can be demonstrated in the laboratory by more time spent with a partner than with a stranger in the partner preference test (Williams, Carter, & Insel, 1992; for a review, see K. A. Young, Liu, & Wang, 2008). While traditional laboratory animals can model altered behavior in response to social isolation, the prairie vole can be used to model more fully the formation, maintenance, and effects of a specific pair bond relationship.

While male-female pair bonds have been widely studied in prairie voles, the sibling bond is another important relationship. Under certain circumstances in the wild, a high percentage of juvenile prairie voles remain in the natal nest with their parents and siblings instead of dispersing (Carter & Roberts, 1997). In the lab, prairie voles exhibit signs of depression- and anxiety-like behaviors when separated from a partner or a same-

sex sibling, such as in the elevated plus maze, sucrose consumption, and resident-intruder tests (Bosch, et al., 2009; Grippo, Cushing, & Carter, 2007; Grippo, et al., 2007; Pan, et al., 2009). These bonds and the effects of separation can be used to examine the influences of specific social relationships on alcohol drinking. Here the sibling bond is explored, in order to avoid issues of pregnancy (or gonadectomy) that could independently affect alcohol consumption.

In our pilot experiments we observed a preference for alcohol intake in prairie voles (unpublished observations: Loftis, et al., 2006), suggesting that voles can be useful for alcohol self-administration studies. Most other laboratory rodents that exhibit a high preference for alcohol are inbred or selectively bred lines of mice and rats. Therefore, the prairie vole, which remains outbred and exhibits a high degree of genetic diversity relative to these strains of mice and rats, might be valuable for examining genetic underpinnings of alcohol preference and intake, and for observing individual variation.

Interestingly, there is considerable overlap in a number of neural systems that have been implicated in pair bond formation in prairie voles and in alcohol intake. For example, the role of the dopamine system in pair bonding has been established in prairie voles (Aragona, et al., 2003; Aragona, et al., 2006; Wang, et al., 1999), and it has been well-established that dopamine plays a role in response to alcohol and other addictive substances (Everitt, et al., 2008; Robinson & Berridge, 1993, 2008; Wise & Bozarth, 1981). The commonalities and potential interplay of the dopamine system involved in these behaviors have been reviewed and discussed (Curtis, et al., 2006). Additionally, vasopressin, a neuropeptide necessary for pair bond formation in male prairie voles (Winslow, et al., 1993), has long been known to influence alcohol intake (Finkelberg,

Kalant, & Blanc, 1978). Similarly, the corticotropin-releasing factor (CRF) system plays a role in partner preference formation in male prairie voles (DeVries, et al., 2002; Lim, et al., 2007), as well as in coping with isolation or loss of a partner (Bosch, et al., 2009; Grippo, et al., 2007), and has been shown in a large body of literature to regulate alcohol intake (for reviews, see Heilig & Koob, 2007; Ryabinin, et al., 2002; Valdez & Koob, 2004). The overlap of the neural mechanisms for social bonding and alcohol intake implies that there could be some common regulation of these behaviors, and that they may affect one another.

The goal of these studies was to establish the prairie vole as a novel model to investigate the interrelation of social affiliations and alcohol drinking. The preliminary hypothesis was that the stress of separation from a partner would lead to increased drinking relative to voles that remained with their partner. The results described here show an effect in the opposite direction, implying that prairie voles prefer to drink alcohol more when they are with a partner, and that they may be a model for socially-facilitated excessive drinking. Subsequent experiments were performed to establish a procedure for limited access to alcohol useful for studies of pharmacological manipulations that could influence drinking behavior.

Materials and Methods

Animals

The prairie vole colony was originally established from eight pairs generously provided by Dr. Joseph Lonstein at Michigan State University in March 2005. These voles originated from a colony at Emory University, which was derived from field-caught prairie voles in Illinois. Diversity in our colony was maintained by the generous donations of prairie voles from Dr. Phillip Smith at Texas Tech University in November

2007, and from Dr. Karen Bales at the University of California at Davis in February 2008.

Prior to any experimentation, prairie voles were housed in same-sex sibling groups after weaning at around 21 days, and maintained on a diet of mixed rabbit chow (LabDiet Hi-Fiber Rabbit), corn (Nutrena Cleaned Grains), and oats (Grainland Select Grains). All experiments took place in the vole colony room at the Portland Veterans Affairs Medical Center (VAMC). All animals were kept on a 12:12 hour light-dark cycle. All animals had *ad libitum* access to food and water throughout each experiment. Animals were alcohol- and experimentally-naïve, except where noted in Experiments 1 and 4.

In Experiment 1, we also used alcohol-naïve mice of C57BL/6J (C57) background bred in our animal colony at OHSU that were housed at four to five per cage with water and food constantly available, and C57 mice purchased from Jackson Laboratories (Sacramento, CA, USA) and housed five per cage for one week prior to testing.

All animal care, breeding, and testing procedures were in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and approved by the local Institutional Animal Care and Use Committees at the VAMC and OHSU, Portland, OR, USA.

Drinking solutions and drugs

Fluids were available from 25 ml glass tubes with metal sipper tubes attached with a rubber stopper. For 24-hour access experiments, the bottles were filled to 25 ml, and for the limited access experiment the bottles were filled to 10 ml. After the period of

consumption, the bottles were carefully removed to avoid spillage, and remaining volumes were read to the nearest 0.2 ml.

The animals in each alcohol drinking experiment were given a two-bottle choice test, always with one bottle of tap water, and the other bottle containing one of the following solutions. Alcohol solutions (3%, 6%, or 10%) were made as volume/volume (v/v) concentrations from 95% ethanol and tap water. Saccharin and quinine concentrations were 0.05% and 0.0025%, respectively, weight/volume (w/v) in tap water. Each solution was made fresh every other day and stored in an airtight container, and the solutions were replaced in the drinking tube at the start of each consumption period.

Experiment 1 – Assessment of alcohol elimination rates in voles and mice

In order to test whether high alcohol intake in voles is not due to unusually high ethanol elimination rates, we compared blood ethanol concentration (BEC) in voles and C57 mice after an intraperitoneal (i.p.) injection of 2.5 g/kg of ethanol (20%, v/v). Twenty ethanol-naïve prairie voles (10 females and 10 males; weights 36.2 ± 1.4 g) with ages ranging from 83 to 109 days and 20 ethanol-naïve mice (9 females and 11 males; weights 22.8 ± 0.7 g; 72-103 days old) were euthanized either 30, 60, 120, or 180 minutes after the injection by an overdose of CO_2, and trunk blood was collected.

In addition, a separate study of alcohol elimination rate was conducted in 35 voles (17 females and 18 males; weights 42.1 ± 1.0 g; 106-134 days old) and 40 mice (20 females and 20 males; weights 23.5 ± 0.6 g; 86 days old) that were not naïve to alcohol. Briefly, these animals were given continuous access to increasing concentrations of alcohol (3%, 6%, 10%) over 12 days in a two-bottle choice test with water as described in subsequent experiments, while half the animals were housed in pairs and the others were

housed in isolation. Ethanol injections (2.5 g/kg) were given eleven days following the final access to drinking alcohol, and the animals were euthanized at the same relative times that the naïve animals were.

Blood samples were centrifuged at 5223 relative centrifugal force (RCF) for 10 minutes, after which serum was removed and stored at -20°C before processing. BEC was determined using an Analox Analyzer (Analox Instruments, Luneburg, MO, USA) and is reported in milligrams per deciliter (mg/dl).

Experiment 2 – Investigation of the effects of social separation on alcohol intake

The effect of social housing on alcohol intake was tested in 30 female (44.2 ± 1.2 g) and 32 male (44.3 ± 1.3 g) adult prairie voles, ranging from 68 to 85 days old on the first day of the experiment. First they were moved from their home cage into a new cage with one of the same-sex littermates, where they remained for five days, with water available from the drinking tubes. On the sixth day, the pairs were moved to new cages, where half of the pairs were kept together, and the other half of the pairs were separated into individual cages. In order to monitor drinking behavior of each subject, we created a cage that would house a pair of prairie voles in a manner that would allow each exclusive access to drinking solutions. A wire mesh divider down the center of the cage kept each of the paired voles in one half of the cage, where it had access to its own drinking tubes, but could still see, hear, smell and interact with the other vole through the mesh. The cage was approximately 26.7 x 26.7 x 13.3 cm, the mesh wire was less than 2 mm thick, and the distance between wires was 1.3 cm in the length dimension of the cage and 2.6 cm in the height dimension. The individual cages were approximately equal in size to one side of the mesh-divided cage (26.7 x 16.5 x 13.3 cm).

On the day the animals were moved to new cages, they began a continuous access two-bottle choice test with water and 3% ethanol. The position of the alcohol bottle relative to the water bottle was switched daily to avoid the potential effects of side preference. The choice test consisted of four days at each concentration (3%, 6%, 10%), given in increasing order to all animals. For all choice tests, consumption from both tubes was monitored every 24 hours, and preference for the solution relative to total fluid intake was calculated, in addition to dose of ethanol consumed per body weight (g/kg). Voles were weighed on the first day of the experiment, and every third day throughout. Following the last day of the choice test with ethanol, the bottles were all switched to water for 24 hours, and then a two-bottle choice tastant test began with 0.05% saccharin and water for two days, followed by 0.0025% quinine and water for two days.

Experiment 3 – Investigation of a circadian pattern of fluid consumption using a lickometer system

In order to designate the best time of day for a procedure for limited access to alcohol, fluid consumption was monitored throughout the circadian cycle to determine whether there existed a peak period of consumption in prairie voles, as has been observed in mice and rats (Aalto, 1986; Freund, 1970), and utilized to achieve high alcohol intake (Rhodes, et al., 2005; Ryabinin, et al., 2003; Sharpe, Tsivkovskaia, & Ryabinin, 2005). To examine drinking at regular short intervals without having to disturb the animals, we utilized a “lickometer” apparatus that would record the precise time of each lick on a drinking spout. The apparatus has been described previously (Ford et al., 2005). Briefly, it consisted of a raised, stainless steel rod floor beneath a four-sided Plexiglas box with a perforated lid for ventilation, nested inside a shoebox cage with bedding beneath the rod

floor. Each animal had access to two drinking tubes, one containing water, and the other containing either saccharin or 10% ethanol, available through two holes in one side of the box. Modifications to the apparatus used by Ford et al. included the use of 50 ml conical polypropylene tubes fitted with a rubber stopper with a metal sipper tube, and the addition of a small Petri dish secured to the rod floor opposite the drinking tubes to contain food. The metal floor and sipper tubes created a circuit that was completed when the animal made contact with the sipper, which was recorded by a lickometer device (MED Associates, Inc., St. Albans, VT) interfaced to a computer with MED-PC IV software (MED Associates, Inc.) for collection of cumulative lick records. The tubes were filled at the start of each day, weighed, and secured to the cage. At the end of each session, the tubes were weighed again before refilling, to determine the amount of fluid that was consumed. Food was replenished at the start of each session. To avoid a potential entrainment of activity to the time of new food and fluid delivery, and to record a full 24 hours without interruption, each session started at a slightly different time each day (noted in Fig. 4).

In this experiment, 24 adult prairie voles (12 male, 12 female; 30.7 ± 0.9 g; 95-137 days old on the first day) were housed individually in the apparatus described. During the first four days, they had access to water and saccharin, followed by three days with only water available, and then four days with water and 10% ethanol available. The voles were weighed immediately before commencing the saccharin and ethanol experiments.

Preference for alcohol over water was calculated based on fluid consumption and also on recorded licks from each fluid, and alcohol dose consumed was calculated based on the weight of ethanol solution consumed and the body weight of the subject.

Experiment 4 – Establishing a limited access two-bottle choice procedure

To determine whether prairie voles could voluntarily self-administer alcohol in quantities sufficient to produce substantial BECs and changes in neural activity as indicated by increased Fos immunoreactivity (IR) we established a two-hour limited-access procedure. The animals used here were 26 of the same male and female animals used in Experiment 2, and so were not naïve to alcohol, and continued to be pair- or singly-housed. In this study, begun 26 days after the last alcohol consumption, the voles were given a two-bottle choice test with 10% ethanol and water for two hours, starting at the onset of the light cycle (zeitgeber time [ZT] 0, based on the results of Experiment 3), and repeated over four consecutive days. Preference for alcohol and dose consumed were calculated as described above, also subtracting the average volume missing from four control tubes in empty cages from the volume of fluid consumption for each solution.

Immediately after the end of the two-hour drinking session on the last day, animals were euthanized by CO_2 inhalation, followed by decapitation. An additional 12 age-matched voles that were alcohol- and experimentally-naïve were euthanized at the same time. Trunk blood samples were collected for analysis of BEC, as described in Experiment 1. Brains were removed and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours. Subsequently, brains were transferred to 20% sucrose in PBS with 0.1% sodium azide (NaN_3) overnight, followed by 30% sucrose (in PBS with 0.1% NaN_3) until slicing.

Brains were sliced into 30 µm floating sections in 0.1% NaN_3 in PBS. Slices containing the nucleus accumbens (NAc), the lateral septum (LS), the central nucleus of the amygdala (CeA), and the perioculomotor urocortin-containing neurons (pIIIu) were chosen for immunohistochemistry (IHC) to detect levels of the transcription factor protein Fos. These brain regions were selected for analysis because they most frequently showed changes in Fos IR following alcohol administration in previous rodent studies (Bachtell, et al., 1999; Sharpe, Tsivkovskaia, & Ryabinin, 2005; Vilpoux, et al., 2009).

The Fos IHC protocol used here was based on previously published procedures in mice and rats (Ryabinin, et al., 1999). Endogenous peroxidase activity was quenched with 0.3% peroxide in PBS, followed by blocking with goat serum in PBS/Triton-X 100. The slices were incubated overnight with a primary rabbit polyclonal antibody to c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA, 1:2000). Slices were subsequently incubated in biotinylated anti-rabbit antibody, made in goat (Vector Laboratory Inc., Burlingame, CA), ABC solution (Vector Laboratory Inc.), and diaminobenzidine (Thermo Scientific, Rockford, IL) to visualize the stain.

In the NAc, CeA, and pIIIu, Fos IR was quantified by counting the number of cells stained above background. Counting was performed manually by a trained experimenter blind to the identification of the samples. In the LS, little or no staining was observed in tissues, and so this area was not quantified.

Statistical analyses

For each day of drinking, preference for alcohol over water was calculated by dividing the volume of alcohol consumed by the total volume of fluid consumed. Additionally, g/kg consumed was calculated for each session by dividing the grams of

alcohol consumed (the density of alcohol multiplied by the v/v concentration multiplied by the volume consumed) by the weight of the animal in kilograms.

Several of the voles occasionally chewed through the rubber stopper of the drinking tubes, leading to spillage of the fluids. This behavior has not been observed in mice using the same equipment in our lab. On these occasions, animals that chewed through the stopper were removed from analysis for that day, and will be referred to as outliers. In addition, statistical outliers defined as animals having intake of at least one fluid more than two standard deviations from the mean intake were removed from analysis for that day (this information is included in the Results section). However, these individuals were included in analysis of Fos and BECs, where consumption was not used as a dependent variable, and outliers for measures of Fos IR and BEC were not removed, based on the intra-experiment reliability of these measures. The statistical results obtained with exclusion of outliers never contradicted the results obtained with them in the analyses.

In Experiment 1, alcohol elimination rates were determined by use of a regression line, and the slopes and intercepts of prairie voles and mice were compared with an F test. Effects of sex, housing, and age were assessed as appropriate using a two-factor ANOVA with sex, housing, or age (old or young) as one factor, time of BEC assessment as the other between-subjects factor, and BEC as the dependent variable.

For Experiment 2, group differences in preference and g/kg were determined by two-factor repeated measures ANOVA, with sex and housing condition as between-subject factors, and alcohol concentration as the repeated measure. Preference and g/kg were each averaged across the four days of drinking at each concentration of alcohol and

used for the repeated measures analyses. A two-factor repeated measures ANOVA, with sex and housing condition as between-subject factors, and alcohol concentration as the repeated measure, was also used to analyze water intake (g/kg). Body weights were monitored throughout the experiments, but were not affected by the experimental manipulations, as was expected, and are therefore not described here. The tastant tests for saccharin and quinine were analyzed by two-way ANOVA with sex and housing condition as independent variables, and saccharin and quinine preference as dependent variables. Where appropriate, Fisher's PLSD was used for post-hoc comparisons, and tests of simple effects were used to discover the basis of interaction effects.

The correlation of alcohol consumption between sibling partners was analyzed in Experiment 2 to determine whether individual members of a pair drank similar amounts. Separate correlations were performed for pair-housed animals and separated partners, using the average g/kg consumed from the 10% ethanol solution by one member of a pair as the X variable and g/kg consumed by the partner as the Y variable. Pearson's r was computed, and a threshold $\alpha = 0.05$ level of significance was applied to the correlation. The same test was applied to consumption of saccharin and quinine.

In Experiment 3, the number of licks was determined for each solution during each hour using SoftCR for Windows (MED Associates, Inc.). A software error caused nearly all of the data from the second day of the saccharin consumption study to not be recorded. As a result of this and the irregular drinking pattern observed on the first day of the experiment, which was likely due to the novelty of the cage and the saccharin, only the third and fourth days of saccharin consumption were examined. All four days of

ethanol consumption were analyzed, excepting only the first hour of the first day, where unusually high numbers of licks for both fluids were recorded.

A repeated measures ANOVA was used to analyze consumption separately for each fluid (saccharin, ethanol, or water), and for the total amount of fluid (saccharin or ethanol plus water) consumed during each period. First the statistical test was applied to each day separately, with each hour as the repeated measure. Then the data were collapsed across all days of consumption, and again analyzed with each hour as the repeated measure. The timing of fluid replacement changed each day, and so data from the first four hours were not present for all days, and could not be analyzed by repeated measures, so they were eliminated from analysis although data from the remaining days is shown with in Fig. 4. Fisher's PLSD was used for post hoc comparisons. The preference ratios calculated from lick data and fluid data were compared by a Pearson correlation.

In Experiment 4, induction of Fos by alcohol consumption was compared to that of naïve animals for each brain area investigated, using the Mann-Whitney test, since the measures were not normally distributed. For pIIIu, which showed significant Fos induction, correlational analyses were performed to examine the relationship between Fos IR and preference or alcohol consumption (g/kg), using the Spearman rank r test. This nonparametric analysis was also used to examine the relationship between BEC and preference or alcohol consumption since neither Fos nor BEC data were normally distributed. Naïve animals were not included in the correlational analyses. An α level of 0.05 was used for all tests.

Results

Experiment 1 – Assessment of alcohol elimination rates in voles and mice

Our pilot experiments indicated that prairie voles exhibited high intakes of ethanol (unpublished observations: Loftis, et al., 2006). To see whether these intakes could be due to unusually high ethanol elimination rates, we compared BECs in prairie voles and C57 mice following an i.p. injection of 2.5 g/kg ethanol. We observed similar levels of behavioral responses (i.e. abnormal gait, loss of righting reflex, increased activity followed by sedation) in voles and mice after ethanol injections. Naïve C57 mice exhibited BECs and a rate of ethanol elimination in the expected range, based on previous reports (Grisel, et al., 2002) (Fig. 1A). While the blood ethanol levels of prairie voles were near those of the mice, the rate of alcohol elimination of naïve C57s (slope of regression line: -0.94 ± 0.12) was slower than that of naïve prairie voles (slope of regression line: -1.65 ± 0.036) [$F(1,4) = 29.90$; $p < 0.01$] (Fig. 1A). However, in the alcohol-experienced animals, the elimination rates (slopes: -1.11 ± 0.14 for C57s, -1.40 ± 0.089 for prairie voles) were not significantly different [$F(1,4) = 3.88$; $p = 0.12$], while the intercepts were different [$F(1,5) = 15.43$; $p < 0.05$] (Fig. 1B). There was no difference in elimination rate between sexes or age groups in mice or voles in either study, or between housing conditions in the alcohol-experienced study, so the groups were combined.

Experiment 2 – Investigation of the effects of social separation on alcohol intake

Prairie voles housed together with a mesh divider showed a higher preference for alcohol than voles that had been separated from a sibling partner (Fig. 2A). Accordingly, there was a significant main effect of housing on preference [$F(1,55) = 15.2$; $p = 0.0003$], and no significant effect of sex on preference. The housing effect was significant at each alcohol concentration tested [$F(1,57) = 12.66, 13.16, 12.43$ at 3%, 6%, 10%, respectively;

$p < 0.001$]. There was a significant effect of concentration on preference [$F(2,110) = 23.52$; $p < 0.0001$], where the preference for 10% ethanol was significantly lower than the preference for 3% or 6% ethanol solutions. In contrast, there was no significant effect of housing condition on the dose of alcohol consumed, but there was a significant effect of concentration [$F(2,112) = 92.84$; $p < 0.0001$] such that a higher dose of alcohol was consumed at each increase in concentration ($p < 0.0001$ for each pairwise comparison). There was a significant interaction of sex and concentration [$F(2,112) = 3.12$; $p = 0.048$], and a test of simple effects revealed that the males consumed more alcohol than females, but only of 10% ethanol solution [$F(1,58) = 4.12$; $p = 0.047$]. There was a significant three-way interaction between housing condition, sex, and concentration [$F(2,112) = 3.62$; $p = 0.030$]; intake of 3% and 6% solutions reflects the higher alcohol preference demonstrated in pair-housed voles of both sexes, compared to isolated voles, but the pattern persists only in females for the 10% solution, while males show no effect of housing at this concentration.

Analysis of water intake revealed results complementary to alcohol intake. There was a significant effect of housing on amount of water consumed [$F(1,110) = 12.97$; $p = 0.0007$], such that the isolated animals drank more water than the pair-housed voles.

In the tastant test, there was no significant effect of sex or housing on preference for saccharin, though there was a significant effect of housing on preference for quinine [$F(1,54) = 6.28$; $p = 0.015$] (Fig. 2C). In this case, pair-housed voles showed less preference for (actually an avoidance of) quinine when compared to isolated voles, which exhibited an indifference to quinine, as the mean preference was near 50%. Several

outliers in each group were removed from analysis on various days of testing, and so the final number in each group is indicated at each concentration in Fig. 2.

A strong correlation between alcohol consumption by one member of a pair and consumption by the other member of the pair was revealed between siblings housed together ($r = 0.79$; $p = 0.0003$; $n = 16$), but there was no correlation between consumption of siblings that were separated ($r = 0.20$; $p = 0.46$; $n = 15$) (Fig. 3A). The goodness of fit of the regression line (r^2) of the relationship between alcohol consumption in the mesh-paired voles was 0.62, indicating that 62% of the variability in alcohol intake in partner 'A' could be could be accounted for by the variation in intake in partner 'B'. However, there was no correlation of saccharin (Fig. 3B) or quinine (Fig. 3C) intake between partners.

Experiment 3 – Investigation of a circadian rhythm of fluid consumption

Prairie voles consumed fluids in the lickometer apparatus, and the number of licks was successfully recorded in this procedure. During alcohol consumption, a number of voles showed behavioral evidence of intoxication (i.e. abnormal gait, hind footslips, difficulty balancing while drinking). The general pattern of drinking for an individual vole consisted of discrete bouts of licks separated by long periods of time (often over an hour) without licking.

The graphical representation of the lickometer data combined for all animals shows that prairie voles generally drank more fluid during the light period than in the dark (Fig. 4). Time had a significant effect on saccharin consumption on day 3 [$F(21,483) = 2.84$; $p < 0.0001$], as well as water [$F(21,483) = 1.78$; $p = 0.018$] and total volume [$F(21,483) = 2.84$; $p < 0.0001$]. On day 4, time had a significant effect on saccharin licks

[F(21,483) = 2.40; p = 0.005]. In the four-day test with ethanol, only the fourth day alone had significant differences over time, in water [F(23,506) = 1.58; p = 0.043] and total fluid [F(23,506) = 1.84; p = 0.010] licks.

When the data were collapsed across days, time had a significant effect on saccharin [F(21, 987) = 4.50; p < 0.0001] and total fluid [F(21, 987) = 2.52; p = 0.0002] licks (but not water) on the third and fourth days of saccharin consumption (Fig. 4A). There was a significant effect of time on ethanol [F(19,1805) = 1.93; p = 0.0094], water [F(19,1691) = 2.87; p < 0.0001], and total fluid [F(19,1691) = 3.28; p < 0.0001] licks during the four days of alcohol consumption when the data were collapsed across days (Fig. 4B). Post hoc tests revealed a number of hours with significantly elevated intakes. Peak drinking of saccharin occurred at ZT 10 and 11 with slightly smaller peaks at ZT12, 0, and 6. Total drinking (saccharin and water combined) reflected the same peaks in the hours preceding 'lights off' (ZT 10 and 11and a lower peak at ZT 12), but had a different peak following the dark phase at ZT 3.

Peak drinking of ethanol occurred at ZT 0 ('lights on'), with slightly lower peaks at ZT 23 and 9. In contrast, water intake during ethanol exposure was highest at ZT 9, with a slightly lower peak at ZT 10. As a result, the highest peaks for total fluid intake during these four days were at ZT 9 and ZT 0, with lower peaks at ZT 10 and 12.

The preference ratio for saccharin or alcohol compared to water was determined each day for each animal by the number of licks on each drinking tube (licks on alcohol or saccharin divided by total number of licks each day), and also by the mass of each fluid consumed (mass of alcohol or saccharin consumed divided by total mass of fluid consumed each day). The average saccharin preference by fluid mass was 0.71 ± 0.028

(mean ± SEM), and the average ethanol preference was 0.45 ± 0.023. The correlation between preference calculated by fluid and by number of licks was significant for both saccharin ($r = 0.85$; $p < 0.0001$; Fig. 5A) and ethanol ($r = 0.56$; $p < 0.0001$; Fig 5B).

Experiment 4 – BEC and Fos immunoreactivity in the brain after limited access to alcohol

A limited access procedure was designed to determine whether voles consume pharmacologically relevant doses of alcohol, and whether BEC and Fos immunoreactivity indicating activation of particular brain regions would reflect alcohol intake. The 2-hour limited access was started immediately at the onset of the light cycle corresponding to the peak of ethanol consumption according to Experiment 3. On the final day of two-hour access to alcohol, voles showed a moderate preference averaging 0.60 ± 0.06 (range 0.048 – 0.996). They drank on average 1.99 ± 0.24 g/kg ethanol (range 0.015 – 3.933 g/kg), and exhibited an average BEC of 34.65 ± 7.01 mg/dl (range 13.1 – 133.3 mg/dl). There was no significant effect of sex or housing (mesh-housed or isolated) on alcohol preference, intake, or BEC, and so these animals were collapsed into one group for analysis.

Representative micrographs of Fos staining are shown in Fig. 6. The presence of Fos staining was not observed in the lateral septum of any animals, and thus was not counted and is not shown here. Alcohol did not induce Fos above the level of naïve controls in NAc (Fig. 7A) or CeA (Fig. 7B). Alcohol did significantly induce Fos in the pIIIu, compared to naïve controls ($p = 0.0065$; Fig. 7C). There was no effect of sex or housing condition on Fos IR in any of the brain regions analyzed, so the data are collapsed across these conditions.

A Spearman correlation showed that variability in Fos IR in the pIIIu did not account for a significant portion of the variability observed in alcohol preference, but did account for 44% of the variation of alcohol consumption ($r_s = 0.66$; $p = 0.005$). Similarly, the variation in BEC did not significantly account for the variation seen in preference, but there was a trend for a correlation with alcohol consumption ($r_s = 0.39$; $p = 0.058$).

Discussion

Alcohol intake in pair-housed or socially isolated siblings

The results of the experiments described here show that prairie voles exhibit a high intake of alcohol. During continuous access to the 10% ethanol solution, most groups consumed over 15 g/kg ethanol in 24 hours, and a number of individuals consumed over 20 g/kg (see Fig. 2B). In comparison, only C57 mice and C57 x FVB/NJ hybrids have been shown to consume comparable amounts of roughly 16-20 g/kg/day of 10% unsweetened ethanol, which is more than any other strains of mice (Blednov, et al., 2005; Yoneyama, et al., 2008). Our experiments show that the elimination rate of alcohol appears to be slightly higher in voles than in C57 mice; however, the voles do experience high levels of BECs in the same range as mice, and they also exhibit noticeable behavioral effects of intoxication. Moreover, although C57 mice show higher levels of alcohol consumption compared to other strains, a substantial number of recombinant inbred strains are known to have significantly lower or higher rates of alcohol elimination (Grisel, et al., 2002; Phillips, et al., 1994). Thus, while voles may metabolize ethanol faster than mice, this slightly faster metabolism alone cannot explain the high intake of alcohol observed in prairie voles, and should not serve as an obstacle in future studies. This idea is in agreement with high BECs observed in animals voluntarily consuming alcohol in our experiments. Therefore, prairie voles make a novel animal model for high

alcohol preference and intake, which differs from traditional rodent models of high intake in that they are not inbred or selectively bred, and maintain a high degree of genetic variability.

Moreover, experiments examining the effects of social pairing or isolation on alcohol drinking behavior showed that prairie voles exhibit higher alcohol preference when they are housed together than when they have been separated from a sibling and are housed alone. The novelty of this surprising finding compared to other rodents that tend to increase drinking when in isolation (Advani, Hensler, & Koek, 2007; Daoust, et al., 1985; Ehlers, et al., 2007; Hall, et al., 1998; Juarez & Vazquez-Cortes, 2003; McCool & Chappell, 2009; Nunez, et al., 2002; Nunez, et al., 1999; Rockman, Gibson, & Benarroch, 1989; Schenk, Gorman, & Amit, 1990; Wolffgramm & Heyne, 1991; Yanai & Ginsburg, 1976) indicates that the prairie vole is unique in the way its social circumstances affect alcohol intake. As such, this could be a valuable species to model socially-facilitated excessive or binge drinking. Future studies will attempt to elucidate whether the difference in alcohol intake is due to an increase in socially-housed voles, a decrease in isolated voles, or both, and to determine whether introduction of alcohol at a later time relative to separation from a partner will show the same effect of social circumstance.

The use of the mesh-divided cage that allowed observation of each vole's drinking behavior in Experiment 2 also allowed the animals to interact. One could theorize that the increased drinking is due to the stress of separating the voles by a mesh. However, this possibility is highly unlikely because in our pilot experiments we observed that voles do not show behavioral signs of ever having been separated when they are reunited after being mesh-separated, and, importantly, that vole siblings housed together

without a mesh divider also have higher intakes than singly-housed voles (Anacker, Loftis and Ryabinin, unpublished results).

One of the most interesting findings of these experiments is the strong correlation of intake between siblings housed together, compared to the lack of correlation between siblings housed apart. This finding indicates that prairie voles housed together influence each other's alcohol intake. Future studies should investigate behavioral mechanisms of this phenomenon to see whether the paired voles drink alcohol at the same time, and whether one individual drives drinking of the pair, which can be assessed with an adaptation of the lickometer system utilized in Experiment 3 that would allow voles to be housed in pairs in the apparatus. These studies should not only address the mechanisms by which voles influence each other's drinking, but also whether this behavior has evolutionary origins. Prairie voles are a highly social species that choose to spend most of their time with another animal, rather than alone. This is exemplified by original field results that found pairs captured together in the same cages repeatedly (Getz, Carter, & Gavish, 1981). If the natural history of the species is based upon partners spending much of their time engaged in the same activities, then the traits that facilitate that behavior must be conserved in the species, and here extend to drinking alcohol together.

The facilitating effect of pair-housing on alcohol preference was specific, as it did not extend to saccharin, a rewarding, sweet-tasting solution. However, there was an effect of housing condition on quinine preference, where mesh-housed voles showed less preference for (in fact, an avoidance of) the bitter solution compared to isolated voles. While the reason for this remains unclear, this shows that pair-housed voles do not necessarily exhibit a higher preference for any substance over water, and that this was

specific to alcohol in the current study. Importantly, only consumption of alcohol but not saccharin or quinine was correlated between pair-housed voles.

The variation in alcohol preference observed between animals is quite large, certainly due in large part to the high degree of genetic variation in the outbred prairie vole colony. In future studies, relatively large sample sizes may still be needed to observe significant effects in this population. The method of reading fluid volumes used here could also potentially have introduced error into the data. However, with care taken in handling, the amount of error due to the method of reading was minimized.

The effect of housing on alcohol intake reported in grams per kilogram body weight is similar in direction to the effect on alcohol preference, but of smaller magnitude. Meanwhile, the effect of housing on water intake was also significant, and in the opposite direction, with isolated animals drinking more water than pair-housed voles. The explanation of these combined results is that voles housed alone moderate their alcohol intake with more water, yielding a lower preference for alcohol in overall fluid intake, while not appreciably changing the dose they consume relative to pair-housed voles.

Taken together, the results of Experiment 2, and especially the correlation of drinking, show that prairie voles can serve as a unique animal model for the examination of the effects of social bonds on alcohol drinking behavior.

Circadian pattern of fluid consumption in voles

In natural environments, voles exhibit 2-6 hour ultradian or polyphasic activity cycles with bouts of activity followed by periods of rest, and with slightly higher diurnal activity during winter and slightly higher nocturnal activity during summer (Halle &

Lehmann, 1987; Tamarin, 1985). Our study demonstrates that under a 12:12 hour light cycle, the circadian rhythms of fluid consumption in laboratory prairie voles are relatively flat with a tendency for higher diurnal activity. This pattern is quite different from the high intake of food, water, or alcohol observed in laboratory mice and rats during the dark period (Aalto, 1986; Agabio, et al., 1996; Freund, 1970), but is consistent with other observations of increased diurnal activity and a shallower circadian rhythm in laboratory prairie voles relative to rats and other nocturnal rodents (Dewsbury, 1980; Taymans, et al., 1997).

Analysis of number of licks and fluid intake indicated that voles exhibited no preference for ethanol. This was expected for single-housed animals based on our previous experiments. However, both analysis of licks and fluid consumption detected a clear preference for saccharin indicating the lickometer system reliably detects differences in fluid intake. Importantly, the lickometer system detected that peaks in fluid intake occurred at the same times each day, even though the tubes were removed at different times each day, and peaks occurred at least several hours after the fluids and food had been replenished (see Fig. 4B), indicating that this potential disruption did not disturb normal cycles of fluid intake. Moreover, the peak following the end of the dark phase is unlikely to have been influenced by any manipulations occurring during the light phase over 12 hours prior. The subsequent limited access procedure used in Experiment 4 was conducted during the two-hour period just after 'lights on' time, ZT 0-2, which would take advantage of relatively elevated levels of drinking, including the largest peak for alcohol intake.

BECs and Fos induction after limited access to alcohol

A portion of tested prairie voles (6/26) showed pharmacologically-relevant high BECs in the limited access procedure, ranging from 74 – 133 mg/dl, while the rest had negligible BECs (total range 13.1 – 133.3 mg/dl). The BECs showed a trend toward a significant correlation with alcohol dose consumed, although this correlation was weakened by the number of voles that exhibited low BECs even after consuming large quantities of alcohol in the two-hour session. However, the timing of each animal's drinking can influence the BEC that is determined at the end of the drinking period. For example, BECs may not show a strong correlation with consumption when some individuals drink at the beginning of the access period. If that is the case, then moderate amounts of alcohol consumed within the first part of the session would not be expected to induce significant BECs at the time the blood samples were taken (Livy, Parnell, & West, 2003).

Since BEC was not strongly correlated with alcohol consumption in the present experiment, the possibility that Fos activation could add another measure of alcohol consumption was investigated. Previous findings showed that various alcohol self-administration procedures in mice and rats lead to consistent induction of Fos in the perioculomotor urocortin containing neurons (pIIIu), and less consistent Fos induction in the nucleus accumbens (NAc), central nucleus of the amygdala (CeA), or reduction of Fos in the lateral septum (LS) (Bachtell, et al., 1999; Sharpe, Tsivkovskaia, & Ryabinin, 2005; Topple, Hunt, & McGregor, 1998; Vilpoux, et al., 2009). In agreement with results of previous studies, pIIIu exhibited a significant Fos response in alcohol-drinking voles. Neither the NAc nor CeA showed significant induction in alcohol-exposed animals following a two-hour two-bottle choice test, compared to naïve controls. It should be

noted that there were procedural differences between this and previous studies, since the voles were killed following two-hour access to ethanol, as opposed to 90 minutes after 30-minute access to ethanol, where animals consumed larger doses of ethanol in a shorter period. It could be also theorized that since Fos immunoreactivity in alcohol-drinking voles was compared to naïve control voles and not similarly-treated matched voles, this could have obscured our ability to detect differences in some of the brain regions. However, our naïve controls had virtually no Fos immunoreactivity in NAc and CeA. Therefore, this lack of immunoreactivity should actually improve our ability to see ethanol-induced Fos activation and does not explain lack of activation in NAc and CeA. With larger sample sizes or the ability to use a parametric test, we may have been able to reveal a significant difference between naïve and alcohol-exposed animals in these brain regions, but the magnitude of this response would remain minimal (Fig. 7), and the physiological relevance of such a small level of activation is questionable.

In contrast, the pIIIu exhibited strong induction of Fos, which was correlated with alcohol intake in individual voles. As with BEC, the fact that the predictive ability of Fos for alcohol intake levels was not stronger can be explained in part by differential timing of onset and peak drinking between individuals. Fos IR is increased starting at 60 minutes after alcohol exposure (Chang, Patel, & Romero, 1995). Thus, since the animals were killed at 120 minutes after the alcohol was introduced to the cages, Fos IR may only have been evident in individuals that drank physiologically significant quantities of alcohol within the first hour of the session, but not in animals that tended to drink majority of alcohol at the end of the drinking session. Future studies on the microstructure of drinking behavior within a session may help elucidate details of the relationships of Fos

and BEC with alcohol consumption over time. Since ethanol effects on c-Fos expression in pIIIu neurons are mediated by several mostly unknown signal transduction mechanisms (Bachtell, Tsivkovskaia, & Ryabinin, 2002), BEC remains a much more direct measure of alcohol consumption than Fos IR. However, the significant increase in Fos IR in this brain region after voluntary alcohol intake indicates that prairie voles consume alcohol in quantities sufficient to produce central effects.

The fact that there was no effect of housing condition on alcohol preference, or any of the other measures observed here, indicates that this limited access procedure may not be sufficient for showing differences due to social circumstances. One possible reason for this may be that the voles housed together influence each other to increase alcohol preference and intake in an ongoing process throughout the day, and that two hours is not enough time for them to coordinate or influence each other's drinking.

Importantly, the limited access procedure described here can be used to test effects of pharmacological manipulations (which often have transient effects that are difficult to demonstrate in a continuous access procedure), in order to elucidate the roles of neurotransmitter systems involved in alcohol drinking in voles, and to test potential drug treatments for alcoholism. In fact, our lab has demonstrated that naltrexone, an opioid receptor antagonist and approved treatment for human alcoholics, decreases alcohol intake in this limited access procedure (Anacker & Ryabinin, 2010). Future studies will utilize this procedure in elucidating the neurobiological factors involved in socially-facilitated excessive drinking, as well as determining potential therapeutic targets.

General conclusions

The experiments described here delineate two different procedures for studying alcohol consumption in prairie voles. The first is a 24-hour access procedure (Experiment 2), and the second is a two-hour limited access procedure that can be useful for testing effects of drugs, or involvement of certain neural substrates on alcohol drinking (Experiment 4). Notably, prairie voles prefer alcohol more when they are housed together in the 24-hour procedure, so they may be useful to model socially-facilitated excessive drinking behavior.

It is tempting to speculate on the neural substrates that underlie the behaviors demonstrated here, although they were not investigated in these experiments. For example, it was recently demonstrated that female (but not male) voles separated from a sibling showed elevated HPA axis reactivity, in increased levels of CRF IR in the paraventricular nucleus of the hypothalamus, and increased ACTH and corticosterone following a stressor (Grippo, et al., 2007). Also relevant to HPA axis activity, alcohol consumption elevates mRNA expression levels of CRF receptors (CRFR1 and CRFR2) and AVP in the hypothalamus, and these are speculated to contribute to a 'stop signal' (Pickering, et al., 2007) for drinking via negative feedback. Altogether, a hyper-reactive HPA axis in isolated voles could more easily activate the stop signal pathway, leading to decreased drinking in separated compared to paired animals.

Additionally, the anhedonia described in both sexes of the isolated voles (Grippo, et al., 2007) may be extended to include lack of perceived reward from alcohol, leading to decreased alcohol consumption as seen in the current experiments. In contrast, paired voles may have greater NAc dopamine D1-like receptor levels (Aragona, et al., 2006) than isolated voles, and may experience a greater reward due to dopamine release in the

NAc when drinking alcohol under these social circumstances, ultimately leading to greater drinking after learning the reward value of alcohol. Interestingly, the strong correlation in alcohol intake of voles housed together relates to a feature of human relationships. A study by Homish and Leonard (2005) found that married couples who drank not only at similar levels, but also drank together at the same time, reported higher levels of marital satisfaction than those that did not. This "congruent drinking" is also a striking finding from the current study in prairie voles, and suggests further studies.

In conclusion, the experiments described here suggest that the prairie vole could be a valuable animal for investigating the interplay between social relationships and alcohol intake. In particular, this species could be used to model socially-facilitated excessive drinking. Several procedures and measures developed in the described studies will allow us to comprehensively examine the behavioral and neural mechanisms underlying the phenomenon of socially-facilitated excessive drinking in the future.

Figure 1. Alcohol elimination rate in prairie voles and C57BL/6J mice

A) Blood ethanol concentration (BEC) following 2.5 g/kg i.p. injection of ethanol, in naïve prairie voles and mice (n = 4-5 per group per time point). B) BEC following 2.5 g/kg i.p. injection of ethanol in prairie voles and mice that had previously self-administered alcohol orally (n = 7-10 per group per time point). Values represent mean ± SEM; lines indicate linear regression, and rate of ethanol elimination; 'X' indicates X-intercept. * Slopes are significantly different ($p < 0.01$). # Intercepts are significantly different ($p < 0.05$).

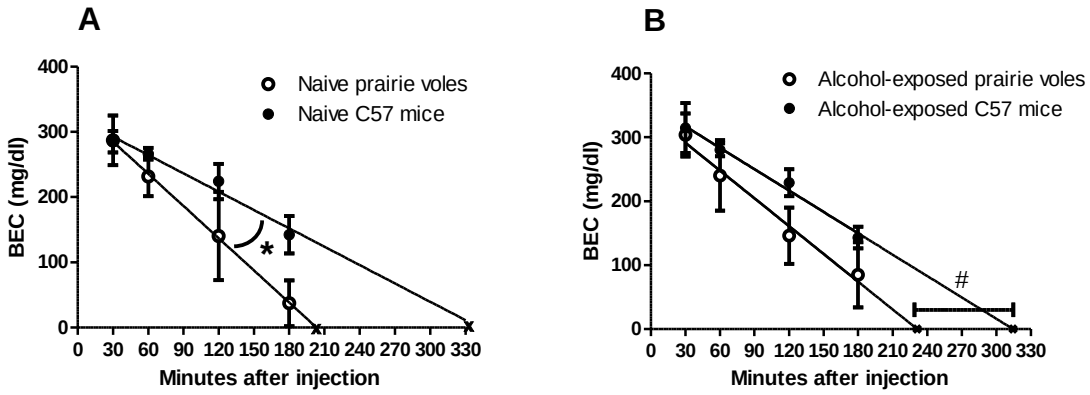


Figure 2. Alcohol drinking in same-sex sibling pairs, housed together with a mesh divider, or separated from sibling

A) Alcohol preference for females and males housed with a sibling (checkered bars) or separated from a sibling (solid bars), at increasing concentrations of ethanol (3%, 6%, 10%). B) Alcohol consumption (g/kg) for females and males housed with a sibling (checkered bars) or separated from a sibling (solid bars), at increasing concentrations of ethanol (3%, 6%, 10%). C) Saccharin and quinine preference for females and males housed with a sibling (checkered bars) or separated from a sibling (solid bars). Values represent mean + SEM; number of animals per group per concentration is indicated. * Effect of housing ($p < 0.01$). # Effect of housing ($p < 0.05$). + Effect of sex ($p < 0.05$).

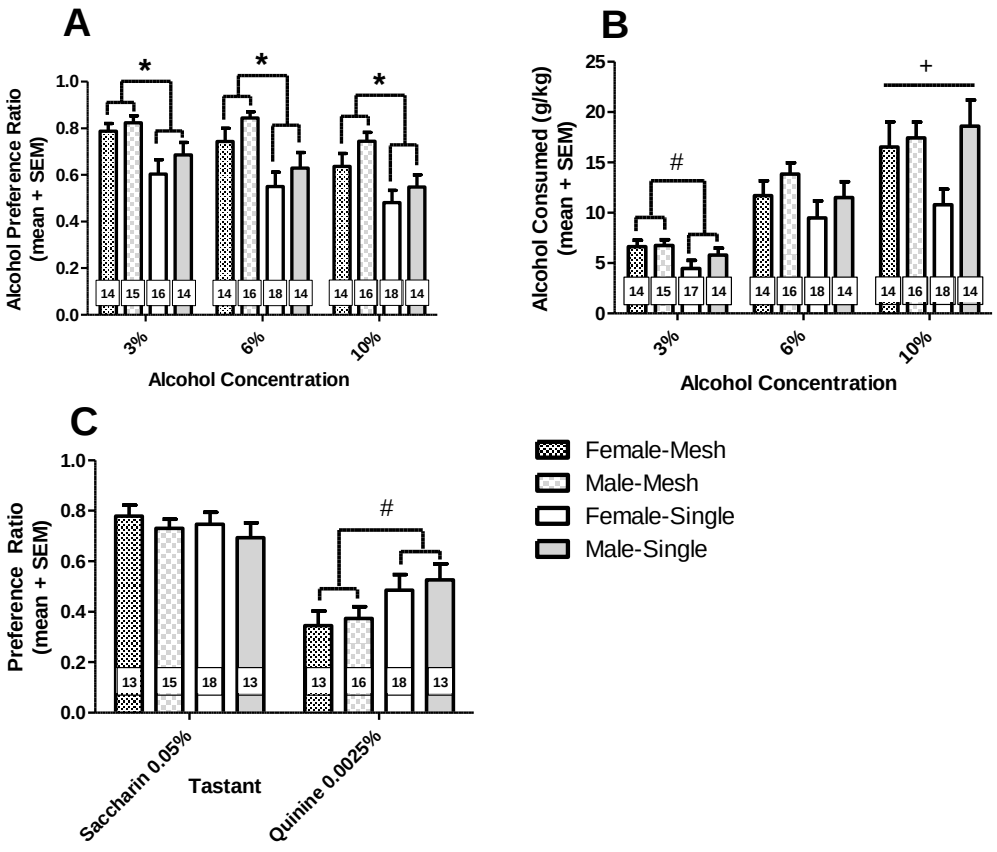


Figure 3. Fluid consumption of prairie vole partners

Correlation of consumption of A) alcohol (10% ethanol), B) saccharin, and C) quinine between siblings in each housing condition. Values represent the average dose of each substance consumed by an individual vole per day, during four days for alcohol and two days for saccharin and quinine.

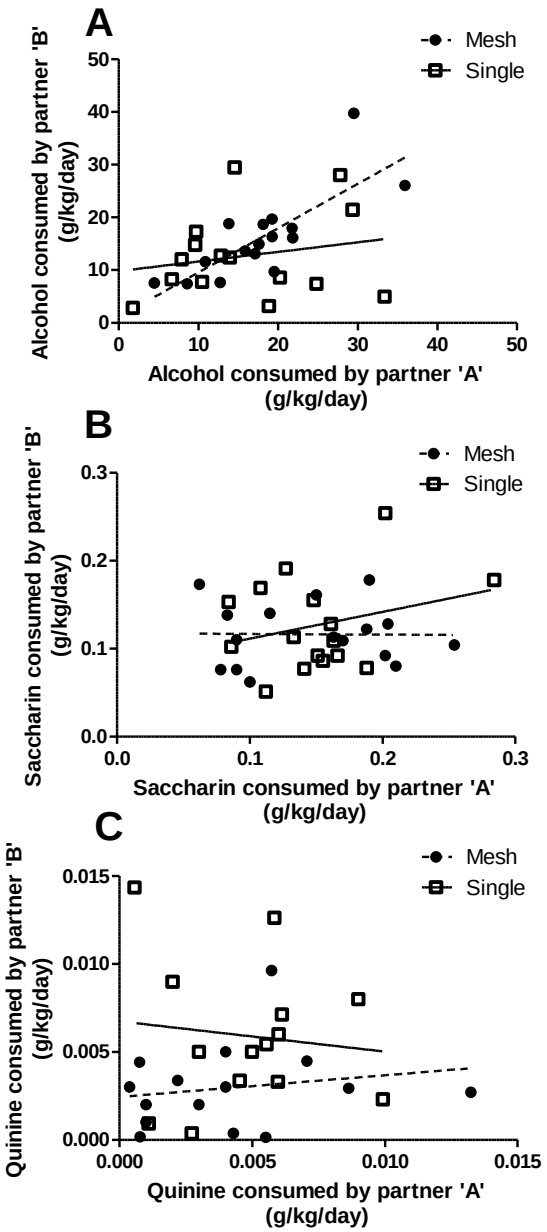
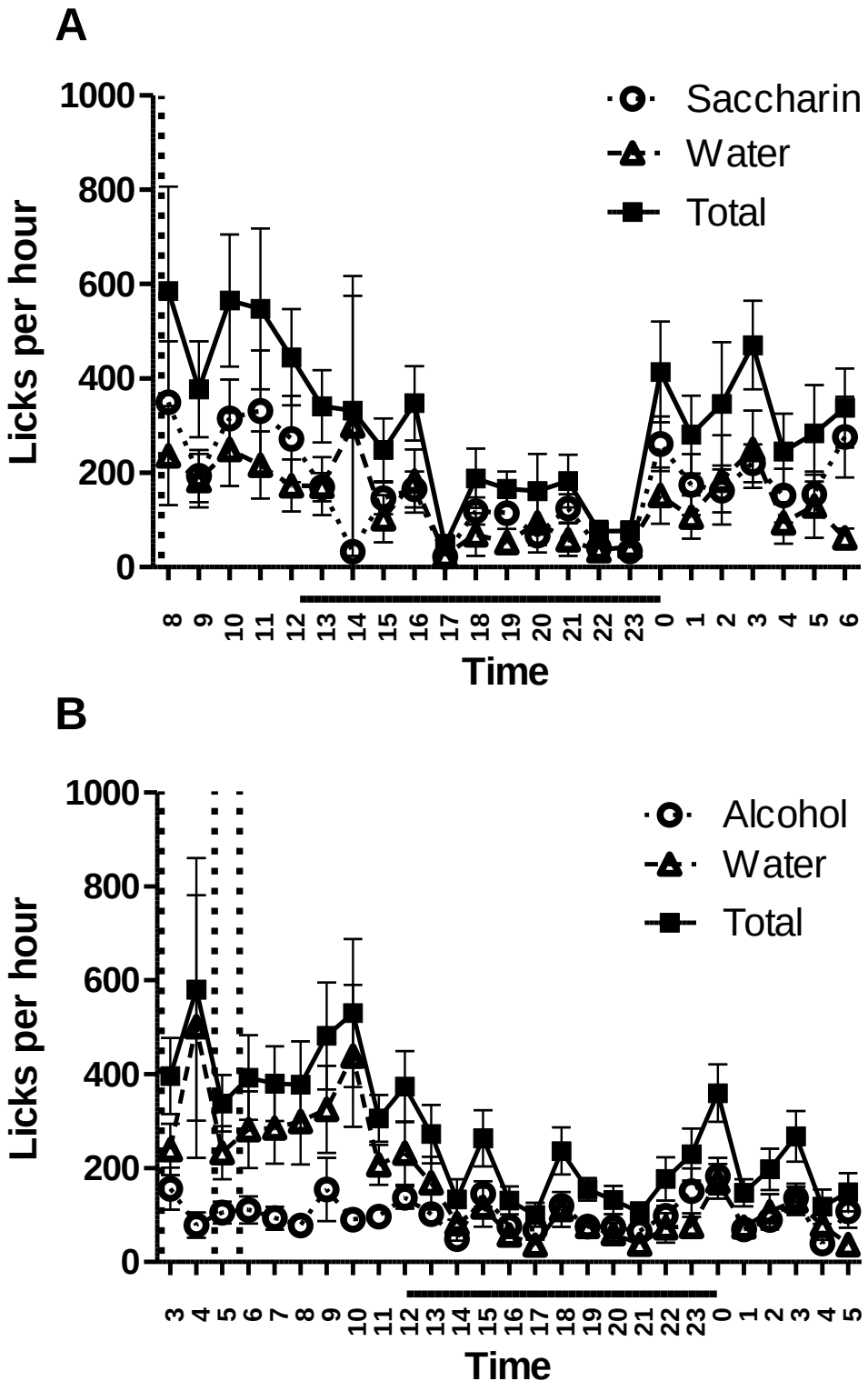


Figure 4. Fluid intake and the circadian cycle

A) Average intake of saccharin, water, and total fluid, collapsed across days 3-4 of testing with saccharin. B) Average intake of ethanol, water, and total fluid, collapsed across days 1-4 of testing with ethanol. Values represent number of licks per hour for all animals, indicating mean ± SEM; n = 24. X-axis values represent zeitbeger time, where ZT 0 = 'lights on'. Horizontal black bars indicate the dark phase of the circadian cycle. Vertical dotted lines indicate the time at which fluids were replaced between successive test days, and hours before these changes were not included in the repeated measures analysis.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Licks per hour
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3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 0 1 2 3 4 5
Time

Figure 5. Solution preference ratios by fluid consumption and recorded licks

Correlation of preference ratios for A) saccharin and B) 10% ethanol over water, calculated from fluid consumption by number of licks recorded (X-axis) and by weight (Y-axis) for each solution. Values represent individual animal preference for each day.

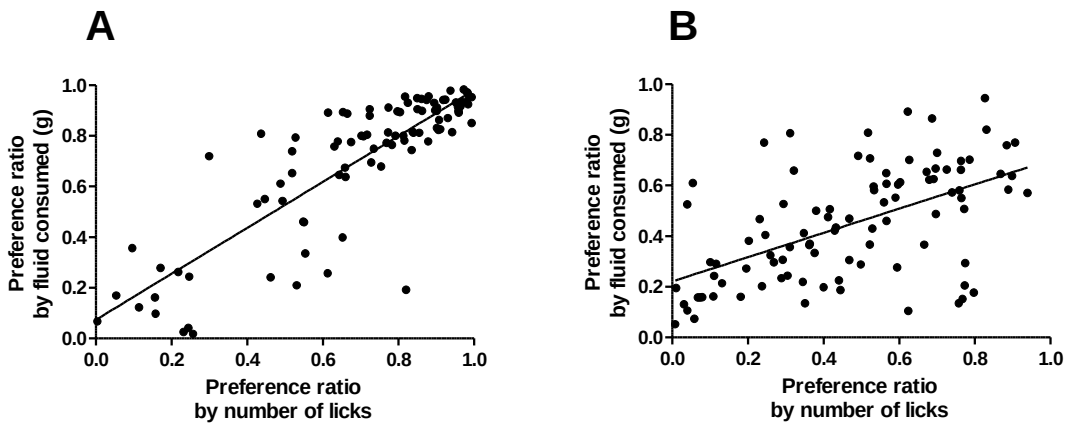

Figure 6. Fos immunoreactivity after limited access to alcohol

Induction of Fos by alcohol self-administration was inspected by comparing immunohistochemical staining in brains of alcohol-naïve voles (A,C,E) to that of voles after two-hour access to alcohol (B,D,F) at 20X magnification. Very little induction was observed in the nucleus accumbens (A,B) or central nucleus of the amygdala (C,D), but there was apparent induction of Fos in the perioculomotor urocortin containing neurons (E,F). Arrows indicate examples of Fos positive nuclei.

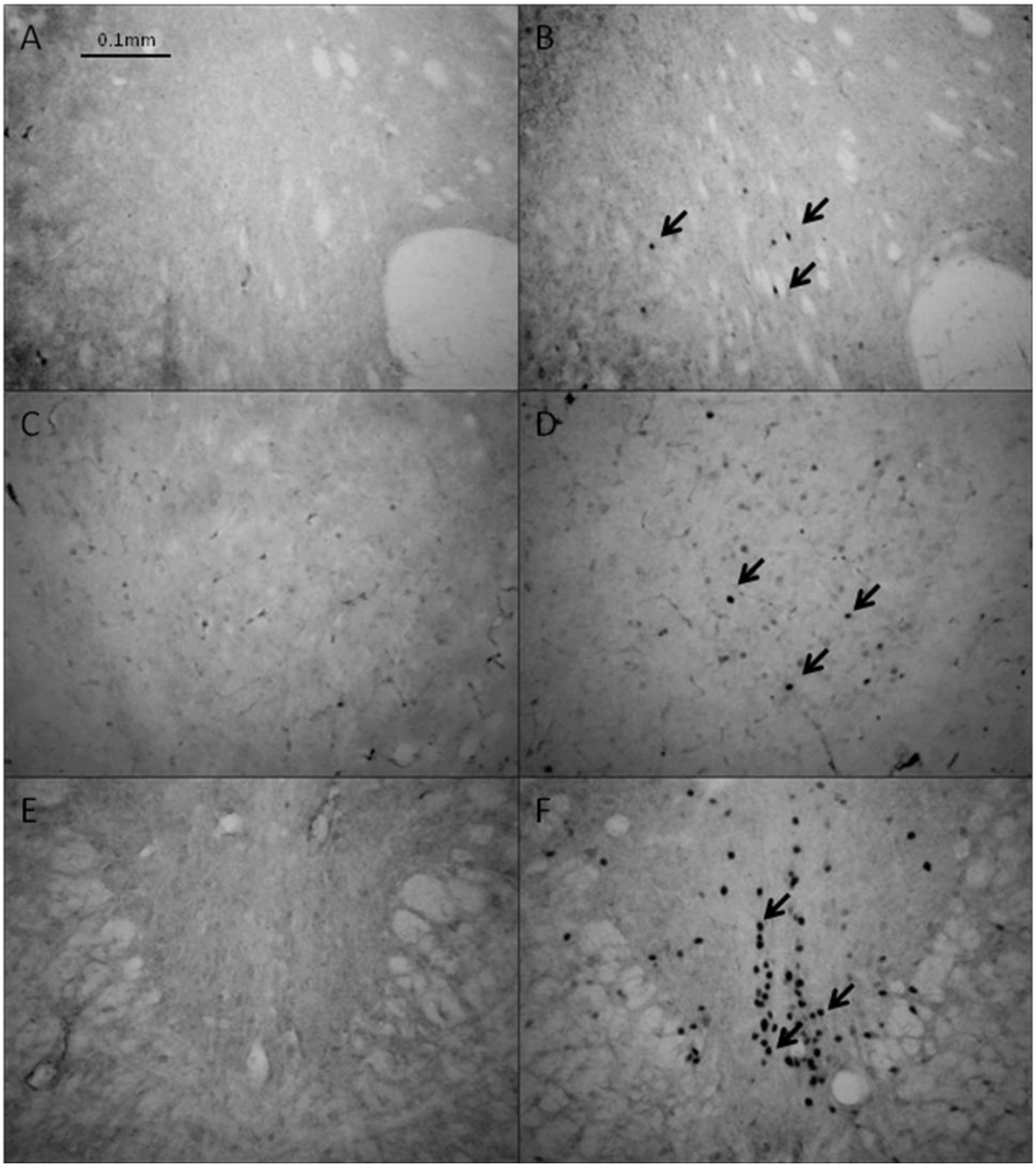
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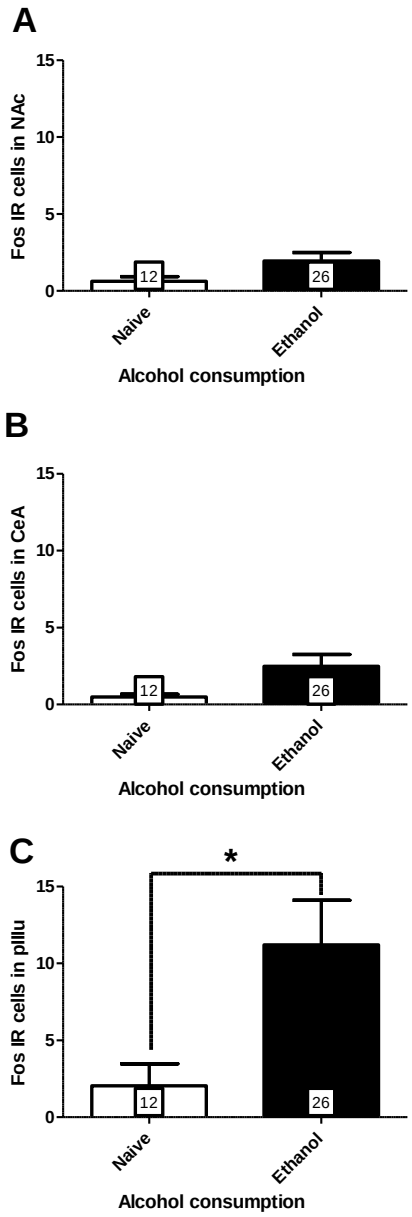
Figure 7. Fos IR as a measure of alcohol consumption

Fos immunoreactivity in limited-access alcohol drinking voles compared to naïve animals, in A) nucleus accumbens B) central nucleus of the amygdala and C) perioculomotor urocortin containing neurons. Values represent mean number of Fos-positive cells + SEM; number of animals per group is indicated. * Difference between groups ($p < 0.01$).



CHAPTER 2: Alcohol Intake in Prairie Voles is Influenced by the Drinking Level of a Peer

(This chapter has been reformatted and minimally edited for inclusion in this dissertation from: Anacker, A. M., Loftis, J. M., & Ryabinin, A. E. (2011). Alcohol intake in prairie voles is influenced by the drinking level of a peer. *Alcohol Clin Exp Res, 35*(10), 1884-1890.)

Introduction

Social influences are an important factor in the onset and development of alcohol (ethanol) drinking patterns. An older sibling's drinking is a significant predictor of an early adolescent's drinking (Needle, et al., 1986), and the number of alcohol-using friends is the greatest predictor of this behavior (Windle, et al., 2008). While selection of friends is often a factor for substance abuse, socialization within a peer group and adoption of norms (real or perceived) are also important influential factors in alcohol use, even more than in use of other drugs (Bot, et al., 2005; Kiuru, et al., 2010; Knecht, et al., 2011; Park, Sher, & Krull, 2009). Two of the greatest factors in predicting heavy drinking in social circumstances among adolescents and young adults are the alcohol-related norms of an individual's family and friends, and modeling behavior after observed drinking of family or peers (Oostveen, Knibbe, & de Vries, 1996).

Modeling the effects of social influences on alcohol intake in laboratory animals is a challenge, due to species differences in the nature and complexities of relationships. We have previously reviewed this literature (Anacker & Ryabinin, 2010) and found that while mice and rats do exhibit effects of social housing on alcohol intake (e.g., Advani, Hensler, & Koek, 2007; Schenk, Gorman, & Amit, 1990; Thorsell, et al., 2005; Yanai & Ginsburg, 1976), there is no evidence of effects on changes in alcohol consumption due to influences from a specific animal.

To address this issue, we have previously established a novel animal model to examine the effects of social behaviors on alcohol drinking, using the prairie vole (*Microtus ochrogaster*). Prairie voles, like as few as three percent of mammalian species (Kleiman, 1977), are socially monogamous, highly affiliative animals that form lasting bonds with specific animals (Carter, et al., 2008; Getz, Carter, & Gavish, 1981; K. A.

Young, et al., 2011; L. J. Young, Murphy Young, & Hammock, 2005), and are also unusual in that they will voluntarily self-administer large doses of alcohol (Chapter 1, Anacker, et al., 2011). In that study, we determined that prairie voles will reach intoxicating levels of blood ethanol concentration similar to those of mice when given the same dose of alcohol. We also showed that prairie voles can demonstrate socially-facilitated alcohol intake, exhibiting a higher preference for alcohol over water when gradually introduced to alcohol housed with a sibling, compared to animals introduced to alcohol in isolation. Interestingly, we observed a correlation of average intake of 10% alcohol over four days between voles housed in sibling pairs, which was not present between siblings housed apart. This correlation was only observed in animals consuming alcohol, but not in animals consuming water or saccharin.

Since individually-housed voles exhibit a substantial variability in their alcohol preference and intake, we hypothesized that the observed correlation in intake between individual members of pairs of animals results from one of the voles altering its level of intake to match the intake of its partner. In the present study we addressed this hypothesis by first measuring alcohol intake in individually-housed voles introduced to a choice between 10% alcohol and water, and then housing animals in pairs based on robust differences in the basal alcohol drinking. After observing significant changes in the drinking levels in pair-housed animals, we tested whether these changes persist when animals are isolated again, and performed control experiments addressing whether these effects are indeed due to social influences and whether they are specific to ethanol.

Materials and Methods

Prairie voles were housed and tested in our breeding colony room at the Portland Veterans Affairs Medical Center Veterinary Medical Unit. The animals were housed

under a 12:12 light-dark cycle, weaned at 21 days and housed with the entire litter until sex was determined at approximately 40 days of age and animals were housed with same-sex siblings. While the 12:12 cycle is not standard for vole studies which commonly use the 14:10 cycle, the 12:12 matches cycles that are typically used in other rodents and that we used in our previous work (Chapter 1, Anacker, et al., 2011; Anacker & Ryabinin, 2010). Only animals that had been housed with 1-3 siblings prior to testing were used in these experiments. Animals were given *ad libitum* access to water and a diet of mixed rabbit chow (LabDiet Hi-Fiber Rabbit), corn (Nutrena Cleaned Grains) and oats (Grainland Select Grains) throughout the experiments. All animals were experimentally naïve.

In Experiment 1, we tested the hypothesis that one vole alters its alcohol intake to match that of its drinking partner when their drinking levels differ. For this, 81 prairie voles aged 69-100 days at the start of testing, weighing 39.8 ± 0.8 g (mean ± SEM), were tested in the first phase of the experiment. In this first phase, voles were weighed and then moved into individual housing, where they were given continuous access to two 25 mL glass cylinders fitted with a metal sipper tube and rubber stopper. One bottle contained tap water, and the other contained 10% ethanol (diluted volume/volume with tap water from 95% ethanol) for four days. During this time, the volume of fluid was monitored and refilled every 24 hours, and the position of the bottles was rotated daily to avoid a bias due to side preference.

Each animal's preference for alcohol (volume of alcohol divided by total fluid volume consumed) and dose of alcohol consumed (g/kg body weight) was assessed and categorized as high, medium, or low each day, based on the criteria presented in Table 1.

Both preference and dose were normally distributed. The specific criteria were chosen because in preliminary tests they yielded approximately equal numbers of high, medium, and low drinkers. After four days of baseline drinking in isolation, each animal was then categorized by subtracting the number of 'low' scores for preference and dose from the number of 'high' scores. Animals receiving a positive number were labeled 'high drinkers' while those receiving negative numbers were labeled 'low drinkers.' The categorization was done in this manner to allow the stability of the drinking category over the four days to be taken into consideration along with the overall mean intake and preference for alcohol. Following categorization from baseline intake, 60 high- or low-drinking prairie voles were used for the study (30 female, 30 male), while the remaining 'medium drinkers' were not tested further.

For the second phase of the experiment, high drinkers were paired with low drinkers of the same sex, which were strangers to each other. Pairs were determined as follows, in order to achieve similar relative differences in alcohol intake between the high drinker and low drinker when compared between pairs. The highest drinker of the high group was paired with the highest drinker of the low group, the second-highest high with the second-highest low, and so on, until the lowest of the high drinkers was paired with the lowest of the low drinkers. The exceptions to this rule were when the pairing would lead to siblings being paired, and in such cases, the closest ranking animals would be switched. Using this method for pairing, there was a relatively similar difference in the drinking levels of the paired animals for each pair.

The paired animals were each housed on one side of a cage with a wire mesh divider that allowed the voles to interact but gave each exclusive access to its own

drinking tubes. This housing was identical to our previously validated procedure showing that the voles' alcohol drinking behavior is similar when they are housed in non-divided cages and in cages divided by a mesh (Chapter 1, Anacker, et al., 2011). The pairs were housed together for four days, with the relative positions of the high and low drinkers within the cage determined randomly. During this period, each animal again had continuous access to 10% alcohol and water. The position of the bottles for each member of a pair was identical (for example, ethanol bottles on the left for both animals on day 1 of pair-housing, and ethanol bottles on the right on day 2). Animals were monitored daily for preference and intake, in order to determine whether any changes in drinking level occurred for each animal.

In the third phase of the experiment, the voles were placed in isolation and drinking was monitored with the same two-bottle choice test for four more days, in order to determine whether any changes in drinking level that occurred during the paired period persist in a subsequent isolation.

In Experiment 2, we tested whether any changes in drinking would occur when paired animals started with similar drinking levels, in order to show that the results of Experiment 1 were due specifically to the presence of the partner drinking at a different level, and not due to the development of an aversion to alcohol that could occur independent of the partner's drinking. The same procedure was performed for the first phase of the experiment, using 51 animals, 71-112 days old, weighing 38.4 ± 0.7 g. Of these, 42 voles (20 female and 22 male) were classified as high and low drinkers and used in the second phase. In this experiment, high drinkers were paired with high

drinkers, and low with low, and otherwise the second and third phases of the experiment were performed with the same methods as described for Experiment 1.

Experiment 3 was conducted just as Experiment 1, except that a saccharin solution (0.05%, weight/volume of tap water) was self-administered instead of alcohol, in order to test whether the effects of Experiment 1 were specific to alcohol or would extend to another rewarding substance. We have previously tested saccharin consumption in prairie voles and found that they show a high preference for this substance at the same concentration used here, and that consumption of saccharin is not correlated between partners as was seen for alcohol consumption (Chapter 1, Anacker, et al., 2011). In this experiment, 39 animals from 67-101 days old, weighing 34.6 ± 1.2 g were used for the first phase, and 34 (18 female and 16 male) were labeled high or low for saccharin consumption and went on to the second phase. The criteria for categorization are presented in Table 2.

Since daily alcohol intake for individual animals differ due to common variability as well as the position of the bottle (some voles show a notable side preference), average intake was calculated across the four days at each phase of the experiments. Then, a repeated measures ANOVA was applied to the data from each experiment, using sex and baseline drinking category (high or low) as between subjects factors, the average drinking throughout each of the three four-day sessions (housing type: isolation 1, paired, or isolation 2) as the repeated measure, with dose of alcohol (g/kg) or alcohol preference as the dependent measures. When warranted by a significant main effect or interaction, the data were further assessed by Fisher's PLSD or tests for simple effects. A few animals were eliminated from statistical analysis of alcohol preference, or both preference and

dose, due to faulty (leaky) tubes; corresponding degrees of freedom are reported in the results for each test.

Results

Experiment 1 – High and low alcohol drinkers paired

As expected based on categorization, high drinkers exhibited a higher preference for alcohol and a higher intake of alcohol than low drinkers (main effect of drinking category on preference [$F_{(1,46)}$=30.73 p<0.0001] and intake [$F_{(1,51)}$=42.57; p<0.0001]). Females showed a higher preference for alcohol and a higher intake of alcohol than males (main effect of sex on preference [$F_{(1,46)}$=5.17; p=0.0277] and intake [$F_{(1,51)}$=8.68; p=0.0048]), but there were no interactions with other variables, and so data are presented in the figures by collapsing across sexes.

There was a significant effect of the repeated measure of housing on preference ($F_{(2,92)}$=6.75; p=0.0018) and intake ($F_{(2,102)}$=6.75; p=0.0018) of alcohol. Post hoc tests revealed that overall preference and intake were lower in the second isolation period in comparison to the first isolation (preference: p=0.0003; intake: p=0.0005) or the paired period (preference: p=0.0241; intake: p=0.0190).

Importantly, there was a significant interaction between drinking category and the repeated measure of housing type for preference ($F_{(2,92)}$=5.92; p=0.0038) and intake ($F_{(2,102)}$=6.22; p=0.0028) of alcohol. A test of simple main effects revealed an effect of housing type on preference and intake in high drinkers but not low drinkers (Fig. 1). In high drinkers, drinking during the paired period was lower than in the first isolation (preference: p=0.0061; intake: p<0.0001), and drinking during the second isolation was also lower in comparison with the first isolation (preference: p=0.0088; intake: p<0.0001). Thus, our data indicated that animals categorized initially as high drinkers

decreased their drinking when housed with animals initially categorized as low drinkers. Moreover, this decrease persisted when animals were again placed in isolation. Although the statistical analysis clearly indicated this change in drinking, there was noticeable variability with some low drinking individuals increasing their alcohol drinking when paired with high drinkers (Fig. 2).

Experiment 2 – Matched alcohol drinkers paired

Initial alcohol intakes and preference in this experiment were very similar to Experiment 1. As in Experiment 1, high drinkers exhibited a higher preference for alcohol and a higher intake of alcohol than low drinkers (main effect of drinking category on preference [$F_{(1,26)}$=96.69; p<0.0001] and intake [$F_{(1,27)}$=33.65; p<0.0001]). In contrast to Experiment 1, the effect of sex on either preference or intake of alcohol was not significant ($F_{(1,26)}$=0.01; p=0.93). There were no interactions of sex with other variables, and so data are presented in figures by collapsing across sexes.

Importantly, in contrast to Experiment 1, there was no effect of the repeated measure of housing type (preference: [$F_{(2,52)}$=1.94; p=0.15]; intake: [$F_{(2,54)}$=1.05; p=0.36]), or any interaction with drinking category (preference: [$F_{(2,52)}$=0.18; p=0.84]; intake: [$F_{(2,54)}$=0.06; p=0.94]), indicating that neither the high nor low drinkers significantly altered their alcohol drinking behavior when paired with an animal with similar intake, or in subsequent isolation (Fig. 3).

Experiment 3 – High and low saccharin drinkers paired

As in Experiments 1 and 2 with alcohol, high saccharin drinkers exhibited a higher preference for saccharin and a higher intake than low drinkers (main effect of drinking category on preference [$F_{(1,27)}$=20.49; p=0.0001] and intake [$F_{(1,27)}$=22.45;

p<0.0001]). There was no effect of sex on either preference or intake of saccharin, and there were no interactions of sex with other variables, and so data are presented in figures by collapsing across sexes.

In saccharin drinkers, there was a significant effect of the repeated measure of housing on preference ($F_{(2,54)}$=6.55; p=0.0029) and intake ($F_{(2,54)}$=5.01; p=0.0101) of saccharin. Post hoc tests revealed that preference and intake were higher in the paired period in comparison to the first isolation (preference: p=0.0006; intake: p=0.0018), and preference only was lower in the second isolation in comparison to the paired period (p=0.0277).

There was no interaction between the repeated measure of housing type and drinking category (preference: [$F_{(2,54)}$=2.01; p=0.14]; intake: [$F_{(2,54)}$=0.42; p=0.66]), indicating that neither the high nor low drinkers significantly altered their saccharin drinking behavior when paired with an animal with opposite intake, or in subsequent isolation (Fig. 4).

Discussion

In the present studies, we confirm our hypothesis that the alcohol drinking behavior of one prairie vole changes to better match the drinking of the other animal it is housed with. Thus, animals initially categorized as high drinkers decreased their intake and preference when housed with animals categorized as low drinkers. This evidence provides some explanation for the previously observed correlation of alcohol intake in voles housed together (Chapter 1, Anacker, et al., 2011). To the best of our knowledge, this is the first demonstration of a direct peer influence on alcohol consumption in a laboratory animal model, further demonstrating the utility of the prairie vole model for examining effects of specific social relationships on alcohol drinking.

Experiment 1 demonstrates the main finding of this study, showing that high alcohol drinkers paired with low drinkers decrease their drinking, and that this decrease persists even in the absence of a direct social influence. High drinking animals demonstrated average intakes over 12 g/kg/day. This is in agreement with our earlier observations that prairie voles in general drink high levels of alcohol, on average comparable to intake of C57BL/6J mice (Chapter 1, Anacker, et al., 2011). Interestingly, even the low drinkers consumed approximately 5 g/kg/day, which is still high enough to observe a reduction in drinking if one were to occur, making it clear that the only group change occurred in the high drinkers.

Although these intakes are extremely high, they are lower than average intakes observed in our previous published study (Chapter 1, Anacker, et al., 2011) at the same concentration of ethanol (about 15 g/kg). This difference is most likely due to the fact that voles here were exposed to 10% ethanol from the first day, while in the previous study they were introduced to gradually increasing concentrations of ethanol (3%, 6%, and only then 10%). Therefore, voles in the present experiment could have experienced aversive effects of ethanol if they consumed excessive doses of this drug, and so it was important to test whether the decrease in intake in the high drinkers in the present experiment could be due to a general tendency to decrease ethanol intake because of exposure to extremely high doses of ethanol. This possibility was addressed in Experiment 2. Should the high drinking animals have decreased alcohol intake due to potential general aversive effects of ethanol, we would have expected to observe a decrease in intake in high drinkers paired with high drinkers in this experiment. This effect was not observed. This finding is in agreement with the idea that the change in

intake and preference in high drinkers in Experiment 1 is due to the social influence of their partners.

The results of Experiment 3 suggest that the peer-mediated change in drinking level to match a partner is specific to alcohol, in that the same behavioral change is not observed for consumption of saccharin, a rewarding sweet substance. Here, as in Experiment 2, the small difference between intakes in high saccharin drinkers in the paired versus isolated phase is in the direction of higher intakes in the paired condition, indicating that no decrease in intake occurs when animals are paired. This result agrees with our previous finding that only ethanol but not saccharin or water intake is correlated between members of vole pairs (Chapter 1, Anacker, et al., 2011). This finding is a further indication that access to any rewarding substance is insufficient to lead to coordinated drinking behavior.

In Experiment 1, we show that on average voles that start out as high drinkers in isolation decrease their alcohol preference and intake when they are paired with low drinkers, while the low drinkers do not change their alcohol preference or intake in the presence of high drinkers. However, we also observed a substantial degree of variability in individual behavioral changes. There was a notable amount of individual variability within groups, where some low drinkers increased their drinking while high drinkers remained high, in contrast to the opposite overall group effects. While this was not frequent enough to lead to a bimodal distribution in each group, it is still important to consider this phenomenon. The reason is that if all high drinkers decreased their drinking, one would presume that the drinking level itself was the predictive factor for which animals would alter their drinking. Since that is not the case in all pairs, there must be

another explanation for which animal alters its drinking. Our preliminary experiments suggest that prairie voles exhibit dominant-subordinate relationships within their pairs. Future studies will examine whether social dominance plays a role in this behavior. One would hypothesize that, within a pair, the dominant animal tends to maintain its original drinking level while the subordinate animal changes its behavior to more closely match the dominant animal. The relation of this hypothesis to the present study is consistent with a number of reports of an inverse correlation between dominance rank and alcohol intake level in non-human primates and rodents (reviewed by Anacker & Ryabinin, 2010; Blanchard, et al., 1987; Blanchard, Yudko, & Blanchard, 1993; Kaur, et al., 2008; N. Kudryavtseva, et al., 2006; N. N. Kudryavtseva, Madorskaya, & Bakshtanovskaya, 1991; McKenzie-Quirk & Miczek, 2008; Wolffgramm & Heyne, 1991); if the same relationship holds for prairie voles, then one would expect that the low drinkers in the present study were more likely to be dominant, and the high drinkers subordinate, and therefore the high drinkers may be more likely to decrease their drinking to match the dominant low drinkers.

The main finding of this study is relevant to the understanding of establishment of alcohol drinking levels or patterns in social circumstances, as well as the effect that a social influence can have on changing alcohol intake. There is considerable literature examining peer influences on alcohol drinking in humans, including both social influences that increase drinking as well as those that are protective against high drinking or relapse (e.g., Andrews, et al., 2002; Henry, Oetting, & Slater, 2009; Homish & Leonard, 2008; Litt, Kadden, & Kabela-Cormier, 2009), but the literature in animal models is relatively sparse. However, there are several studies showing effects of a social

influence, or social learning, on alcohol intake levels. In modifications of Galef's demonstrator-observer paradigm (1985), young rats exposed to either an intoxicated sibling (Hunt, Holloway, & Scordalakes, 2001) or an alcohol-drinking adult female (Honey, Varley, & Galef, 2004) increased subsequent alcohol intake. In these studies, the rats increased their alcohol intake after observing olfactory cues from the demonstrator animal, as has been described for a number of other substances. However, if the presence of the alcohol odor on one animal is a cue for another that it is acceptable to drink the same substance, then the expected result in our study would be an increase in the low drinkers, or even in both groups. Since the opposite effect is observed here, another mechanism must be responsible for the changes in drinking observed in the present study.

We are aware of only one other series of studies in mice showing bidirectional effects relevant to the present investigation. Juvenile mice of a genetic background that normally do not prefer alcohol (DBA) will consume significantly more if they are raised to adulthood with adult C57BL mice, while C57BL mice decrease their drinking when housed from weaning with adult DBA mice (Randall & Lester, 1975). This study and the current study both show that not only can the social environment influence alcohol intake, but the direction of the effect may be dependent on individual predisposition as well as the specific social influences. Randall and Lester concluded that the most likely explanations for the alterations in drinking behavior were peer pressure and setting examples of behavior.

These studies in other animal models show that social influences can have important effects. However, they do not model human peer interactions in which people drink together at the same time. Although in Randall and Lester's (1975) study the mice

were exposed to alcohol in groups, their drinking was only monitored during an eight-day isolation period, and so the proximal effects of peer drinking are not known. The prairie vole model used here is able to address this issue by examining drinking in each individual while they are together, drinking at the same time.

Interestingly, the observed lowered drinking level of high drinkers persists in a subsequent isolation period, even when the presence of the low drinker is no longer a direct influence. This finding suggests that social interactions during pair housing may lead to long-term modifications in drinking behaviors that could involve learning mechanisms. This phenomenon could be of great importance for the understanding of how establishment of drinking patterns with peers can extend into drinking alone or under other circumstances. Future studies in this animal model could examine the neural changes that occur and persist following drinking with a peer, in order to better understand these mechanisms and potential therapeutic targets.

In summary, these studies provide the basis for a novel animal model for examination of direct peer influences on alcohol intake, including their prolonged effect. Understanding specific behavioral mechanisms leading to social influences on alcohol intake akin to peer pressure could be very informative for psychotherapy of alcohol use disorders. The prairie vole animal model has led to fruitful mechanistic discoveries in the field of social neuroscience, and appears likely to provide important insights into the social neurobiology of alcoholism and addiction.

Table 1. Criteria for alcohol drinking level group assignment

Group	Alcohol Preference Ratio	Alcohol Dose (g/kg/day)
Low	< 0.5	< 5
Medium	0.5 – 0.749	5 – 8.999
High	≥ 0.75	≥ 9

Table 2. Criteria for saccharin drinking level group assignment

Group	Saccharin Preference Ratio	Saccharin Dose (g/kg/day)
Low	< 0.65	< 0.075
Medium	0.65 – 0.85	0.075 – 0.125
High	> 0.85	> 0.125

Figure 1. High drinkers paired with low drinkers decrease alcohol drinking

Compared to baseline drinking in isolation, high drinkers (black bars) decrease A) preference for alcohol, and B) intake of alcohol, when paired with low drinkers (white bars), and the decrease in drinking persists during a subsequent isolation. n=30 per drinking category. Values indicate mean + SEM. *Statistically significant post-hoc differences ($p<0.01$).

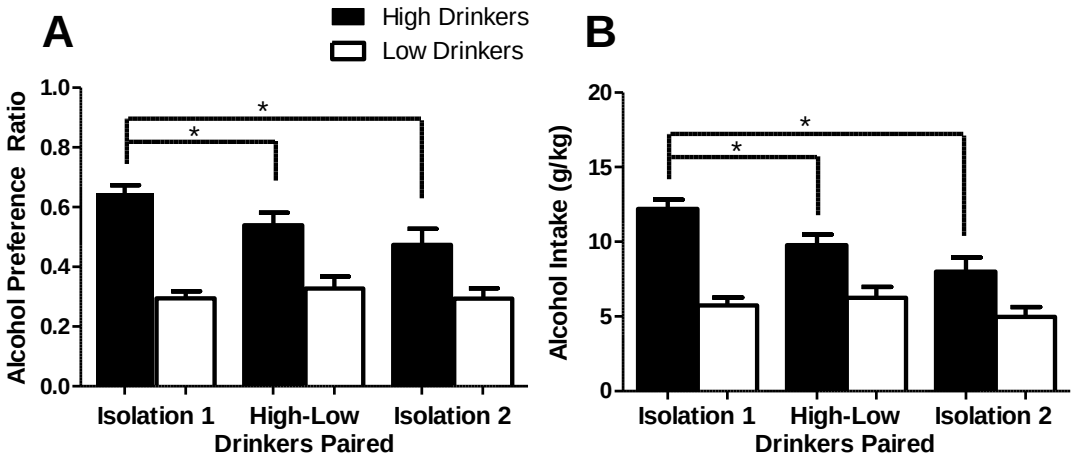


Figure 2. Example of variation between pairs in intake level changes

Average alcohol intake (g/kg/day) across each four-day period is shown for individuals from two pairs. Pair 1 (open triangles) exhibits the alterations in alcohol drinking level corresponding to the group effects: the high drinker (upright triangle) decreases alcohol intake when paired with the low drinker, and the decrease persists in the subsequent isolation, while the low drinker (upside-down triangle) maintains its low drinking level. In contrast, Pair 2 (filled triangles) exhibits the opposite effect: the high drinker (upright triangle) maintains its high level of alcohol intake, even increasing when paired with the low drinker, while the low drinker (upside-down triangle) increases its intake to the level of the high drinker, and the increase persists in the subsequent isolation.

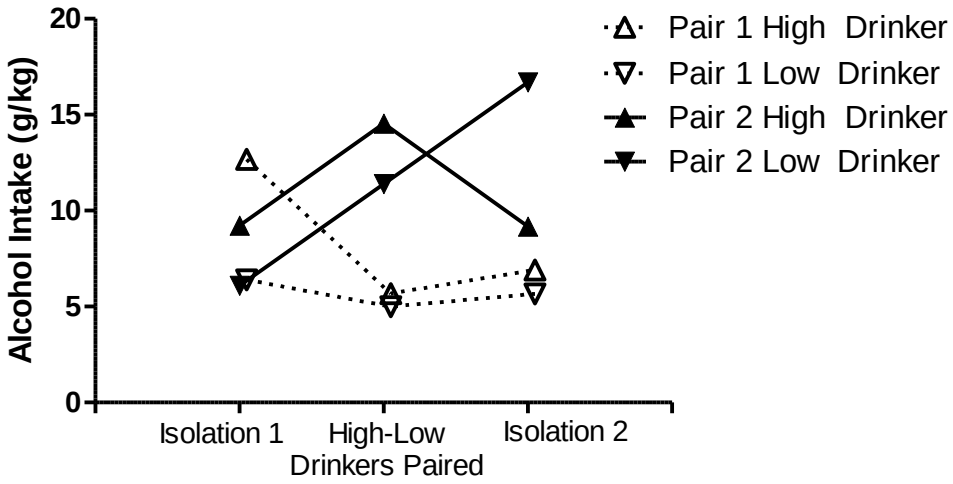


Figure 3. Matched drinkers exhibit no change in alcohol drinking

High drinkers (black bars) and low drinkers (white bars) do not alter their alcohol A) preference, or B) intake, when paired with same-level drinkers, or in a subsequent isolation. n=14 high drinkers, n=18 low drinkers. Values indicate mean + SEM.

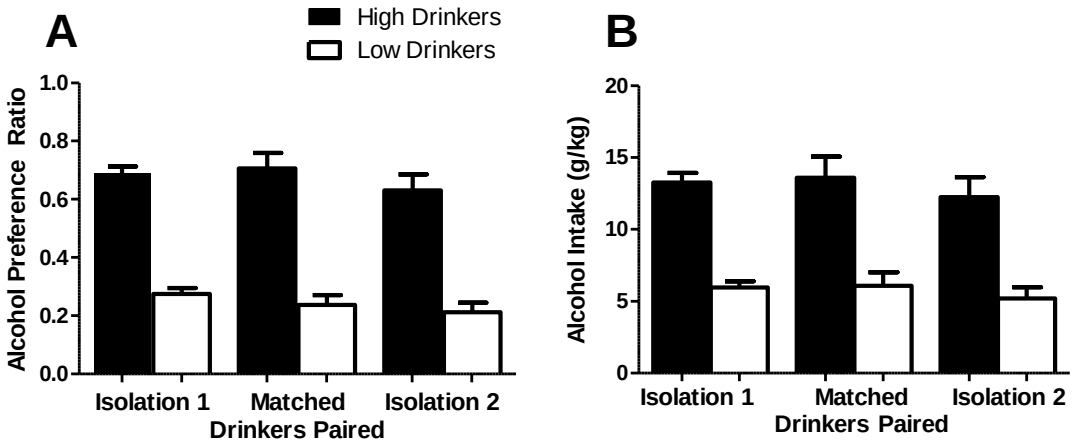
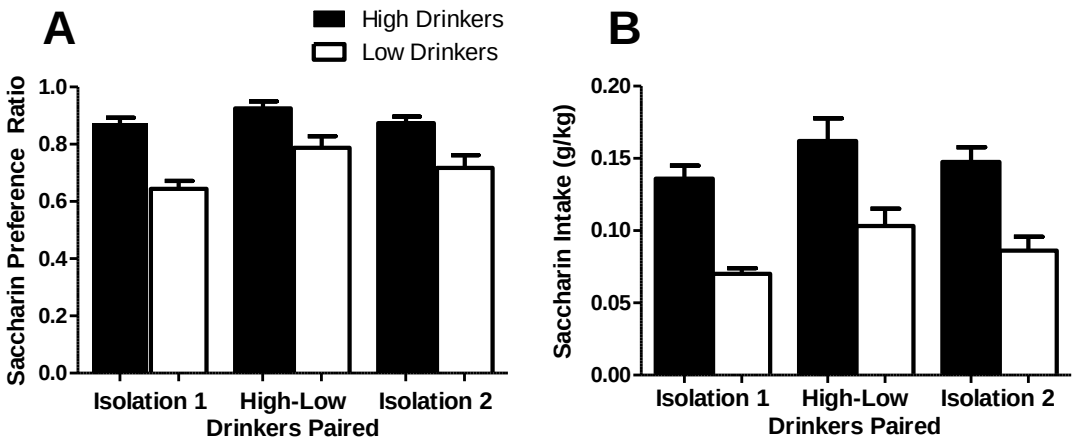


Figure 4. Saccharin drinkers exhibit no decrease in drinking compared with initial levels High drinkers (black bars) and low drinkers (white bars) do not decrease their saccharin A) preference, or B) intake, when paired with opposite-level drinkers, or in a subsequent isolation. n=16 per drinking category. Values indicate mean + SEM.



CHAPTER 3: Susceptibility to Peer Influence on Alcohol Drinking is Predicted by Flexibility in Features of Drinking Bouts

(A modified version of this chapter has been submitted for publication.)

Introduction

Peer influences on alcohol drinking can lead to increases in intake in some cases, and to decreases in others. Both types of influence can be crucial on the path to either alcohol abuse (Fisher, et al., 2007; Park, Sher, & Krull, 2008), or reduced problem drinking or abstinence (Bond, Kaskutas, & Weisner, 2003; Gordon & Zrull, 1991; Kelly, et al., 2011; Wu & Witkiewitz, 2008). Understanding the processes by which peer influences take effect will help inform and improve prevention and treatment strategies for alcoholism.

Biological mechanisms underlying peer influence are underexplored. We have previously modeled social influences of alcohol drinking in socially monogamous prairie voles. Specifically, we have shown that depending on the experimental set-up, housing with siblings or peers can facilitate (Chapter 1, Anacker, et al., 2011) or inhibit alcohol drinking in these animals (Chapter 2, Anacker, Loftis, & Ryabinin, 2011). Moreover, such influence on alcohol drinking is specific to same-sex peers, and not male-female pairs (Hostetler, Anacker, et al., 2012).

The positive (inhibitory) influence of voles drinking low doses of alcohol on voles drinking high doses of alcohol was specific to alcohol, and was not observed with other palatable fluids (Chapter 1, Anacker, Loftis, & Ryabinin, 2011). Moreover, the change in intake due to peer influence was long-lasting and maintained even after the voles were separated. However, we also observed that while some of the voles changed their drinking behaviors due to influence of a peer, others did not. It is important to understand what makes a specific individual susceptible or resistant to peer influence, in order to target prevention or treatment accordingly. We hypothesized that high drinkers' decrease in alcohol intake would be due to the development of a drinking pattern that was linked to

that of a low-drinking peer when they were housed together, since alcohol drinking levels are correlated in pairs housed together (Chapter 1, Anacker, et al., 2011) and since prairie vole pairs exhibit strong coordination of other behaviors such as parenting (Ahern, Hammock, & Young, 2011). To address this hypothesis here, we investigated features of prairie voles' drinking patterns using a lickometer system.

Reports from other laboratories have demonstrated that the establishment of social bonds in prairie voles is dependent on the neuropeptide arginine vasopressin, acting via the vasopressin 1a receptor (V1aR) (Carter, DeVries, & Getz, 1995; Donaldson, Spiegel, & Young, 2010; Liu, Curtis, & Wang, 2001; Nair & Young, 2006; Winslow, et al., 1993). The gene for this receptor in prairie voles contains a polymorphic microsatellite region upstream of the transcription start site (Hammock, et al., 2005; Hammock & Young, 2002, 2004; Ophir, Campbell, et al., 2008; Solomon, et al., 2009; L. J. Young, et al., 1997). Studies have demonstrated that the length of the microsatellite influences gene expression and receptor levels in many brain regions, and the expression in turn affects behavior (Hammock, et al., 2005; Solomon, et al., 2009). Specifically, several types of social behaviors including partner preference have been correlated with microsatellite length. In addition to vasopressin's involvement in social behaviors, the neuropeptide levels are also affected by alcohol drinking and thought to play a role in the development of tolerance (Harding, et al., 1996; Hoffman, et al., 1990; Inder, et al., 1995; Linkola, et al., 1978; Madeira & Paula-Barbosa, 1999; Madeira, et al., 1993; K. M. Ogilvie, S. Lee, & C. Rivier, 1997; Resstel, et al., 2008; Rivier & Lee, 1996; Silva, et al., 2002; Taivainen, et al., 1995). Therefore, we further hypothesized that the length of the V1aR

microsatellite could be correlated with the degree of social influence on alcohol drinking behavior, and addressed this hypothesis in this study.

Materials and Methods

Animals

Prairie voles were bred in our colony at the Portland Veterans Affairs Medical Center Veterinary Medical Unit. All procedures were approved by the Institutional Animal Care and Use Committee. Voles were weaned around 21 days of age and housed in same-sex sibling pairs, with females and males housed in different rooms, until beginning the experiment. Voles were housed under 14L:10D lighting conditions analogous to the breeding season, and had continuous *ad libitum* access to water and food (corn, oats, and rabbit chow). Adult voles of both sexes (n=95) were used in these studies, ranging from 58-95 days of age at the start of the experiment.

Apparatus

The 'lickometer' apparatus used in these experiments was a variation of that described previously (Chapter 1, Anacker, et al., 2011; Ford, et al., 2005). As before, the apparatus consisted of a metal floor (10 x 20cm and 30mm high; VWR, Tualatin, USA), connected by electrical wires to metal spouts of the drinking tubes to create an open circuit through a dual lickometer device (MED Associates, Inc., St. Albans, USA), which was connected to a PC. The wire bottom was positioned underneath the sipper tubes so that the animals were required to have at least one paw on the metal rack to touch the drinking spout, thereby completing the electrical circuit. Each lick exhibited by a subject was recorded by MED-PC IV software (MED Associates, Inc.) and stored for later analysis. The cage containing each apparatus was modified from the apparatus designed by Ford et al. and a schematic diagram is pictured in Fig. 1A-B. The plastic cage bottom

that surrounded the wire rack was 16.8 x 27.6cm and 5.4cm high (Flair Plastic Products, Inc., Portland, USA) and had bedding, food, and a nestlet available, so the subjects were not required to be on the wire rack when they were not drinking. The plastic cage top was 17cm high and in addition to the holes for the drinking spouts, there were holes in the lid and openings along the bottom for air circulation (Flair Plastic Products, Inc.). The cages used for pair housing were identical except that they were twice as wide, with separate lids for each half, and a wire mesh down the center that divided the cage into two equal compartments but allowed the subjects visual, olfactory, vocal, and some tactile contact, similar to what has been described by us previously (Chapter 1, Anacker, et al., 2011; Chapter 2, Anacker, Loftis, & Ryabinin, 2011; Hostetler, Anacker, et al., 2012). Wire dividers were isolated from the wire racks and drinking spouts and did not interfere with lickometer data collection.

Procedure

At the beginning of the experiment, voles were placed in individual lickometer cages and given access to water in the drinking tubes for five days, to habituate to the apparatus. After habituation, subjects were presented with alcohol in one drinking tube (10% ethanol by volume in tap water) and water in the other, and they had continuous access to these solutions throughout the rest of the experiment. Fluid volumes were recorded every 24 hours, and the position of the bottles relative to one another was counterbalanced across pairs and switched every two days. Fluids were replaced every two days. After changing and recording fluids each day, the lickometer recording began and continued for 22 hours.

After four days of access to alcohol in isolation, subjects were categorized as high, medium, or low drinkers, dependent on the dose of alcohol they consumed (g/kg/day) and the preference ratio for alcohol over water, as in a previous study (Chapter 2, Anacker, Loftis, & Ryabinin, 2011). Slightly more than 1/3 of the subjects were classified as low drinkers, with equal numbers of high drinkers, and so a smaller proportion of subjects were classified as medium drinkers than in that previous experiment. Also as in other studies (Chapter 2, Anacker, Loftis, & Ryabinin, 2011; Hostetler, Anacker, et al., 2012), high drinkers were paired with low drinkers and moved into the double cages with mesh dividers, where continuous access to alcohol and water continued for four days. After pairing, subjects were again moved into isolation and had access to alcohol and water for a final four days. The total number of animals used in all three phases was 64 (28 female and 36 male). Each phase of the experiment is noted in Figure 1.

Following the final isolation period, voles were euthanized by CO_2 inhalation, and tail tissue samples were taken for genetic analysis.

Drinking analyses

Alcohol dose and preference were calculated for each day based on fluid volumes consumed. Average measures for each housing period were compared by repeated measures ANOVA with high and low drinkers as a between-subjects variable. Further analyses were done by splitting high drinkers into a group of animals that decreased their drinking level category during the four days of pair housing with a low drinker (n=14) and a group of animals that did not (n=18), and comparing alcohol intake on the first and

last days of isolation and the last day of pair housing. Bonferroni post-hoc tests were used to determine specific group differences.

To validate the lickometer, water and alcohol volume consumed were each compared with the number of licks registered for each subject, and analyzed using a Pearson's correlation.

The lickometer data were analyzed as described previously (Ford, et al., 2005) by custom software for bout frequency, bout size, interbout interval, bout length, lick rate, and latency to first bout. In addition to comparing these features of alcohol drinking between high and low drinkers, we also wished to assess the individual variability in propensity to change intake levels, and so the high drinkers were split into two groups: those that remained high drinkers and those that decreased their drinking level when paired with a low drinker. There were approximately equal numbers of each type of high drinkers, similar to the variation that we have reported previously (Chapter 2, Anacker, Loftis, & Ryabinin, 2011). Averages were compared by repeated measures ANOVA with three groups, (high drinkers that remained high, high drinkers that decreased drinking level, and low drinkers) as a between-subjects variable and three days (isolation day 1, isolation day 4, and pair day 4) as the repeated measure. Fisher's protected least significant difference test was used for post-hoc analysis.

The lickometer data were then processed using custom-designed software (u2615, Portland, USA) which first rescaled the data from 10 ms to 1 second resolution. Cumulative lick plots for each pair on the last day of isolation and pairing were examined and the number of bouts occurring in temporal proximity was determined using a standardized visual assessment. Close bouts were later determined to be less than or equal

to 15 minutes apart. The number of close bouts, and the number of close bouts normalized to the lowest number of bouts exhibited by one member of the pair, were compared using repeated measures ANOVA with change in drinking as a between subjects factor and housing as the repeated measure.

The data processed through u2615 were then analyzed for each pair by a cross-correlation analysis (R for Mac OS). The correlations were compared between isolation day 4 and pair day 4. The presence or absence of a significant correlation for each day was noted, as well as the lag time and degree of correlation (autocorrelation, ACF) for each significant correlation. The lag time range was limited to ±10 minutes, in order to analyze only correlations that occurred close together in time. This metric indicated which subject followed the other in drinking, and was examined in conjunction with individual pair data on which subject changed intake.

Microsatellite length analysis

DNA was extracted from each subject's tail tissue sample using a DNeasy Blood and Tissue Extraction Kit (Qiagen, Valencia, USA), and stored at -20°C until use.

The V1aR microsatellite sequence was amplified using a variation of a previously published PCR technique (Hammock, et al., 2005). We used the same sequences of primers but the forward primer was labeled with a 5-FAM fluorophore (Eurofins MWG Operon, Huntsville, USA). We also used a touchdown PCR protocol to increase the specificity of the reaction, with a HotStarTaq DNA polymerase (Qiagen). The reactions were heated to 94°C for 15 minutes to activate the polymerase, and then had 28 cycles of 30 seconds denaturing (94°C), 45 seconds annealing, and one minute for elongation (72°C). The annealing temperature started at 71°C on the first cycle and decreased by

1°C in each of the following 12 cycles. The last 25 cycles all had an annealing temperature of 58°C. The reaction was ended by a 5 minute elongation at 72°C and cooling to 4°C.

The samples were each read by a 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, USA), by the Oregon Clinical and Translational Research Institute Core Laboratory at OHSU. The microsatellite length was determined for each allele for each subject with approximately 3 base pair resolution.

Microsatellite allele lengths were not normally distributed due to a highly leptokurtotic sample, which could not be normalized by any transformation. Thus, correlations could not be conducted using the collected data. Instead, a median split was applied to the data and t-tests were performed to compare between animals that had short or long average microsatellite length. A number of dependent variables were tested (baseline preference and dose, change in preference and dose for high or low drinkers between isolation 1 and pairing, or pairing and isolation 2, or overall from isolation 1 to isolation 2) and a Bonferroni correction for multiple comparisons was applied.

Results

High drinkers were paired with low drinkers, leading to a total of 32 pairs that completed the experiment (14 female pairs and 18 male pairs, combined for analysis since there were no significant effects of sex or interactions with other variables), while medium drinkers did not continue past the initial isolation phase. As was seen in past experiments (Chapter 2, Anacker, Loftis, & Ryabinin, 2011), high drinkers had a significantly higher preference for alcohol than low drinkers ($F_{(1,62)}$=45.71; p<0.0001) and consumed higher doses ($F_{(1,62)}$=40.85; p<0.0001), due to their categorization. There was a significant effect of housing conditions on preference ($F_{(2,124)}$=4.91; p=0.009) but

not dose ($F_{(2,124)}$=0.82; p=0.441). Importantly, there was a significant interaction between drinking category and housing condition on preference ($F_{(2,124)}$=6.94; p=0.0014) and dose ($F_{(2,124)}$=4.48; p=0.013). Planned Bonferroni post-hoc tests revealed that high drinkers decreased their alcohol preference from baseline (isolation 1) to paired housing and isolation 2 (t=3.93 and 3.26 respectively; df=15; p<0.001), as well as the dose consumed (t=2.76 and 2.44 respectively; df=15; p<0.05), while low drinkers did not significantly change (Fig. 2).

Analysis of drinking on individual days showed that all high drinkers had greater alcohol preference and intake than low drinkers on the first and last day of the first isolation period, while only those high drinkers that altered their drinking under social conditions decreased their preference and intake to the level of the low drinkers by the last day of pair housing. There was a main effect of drinking category on alcohol preference ($F_{(2,122)}$=36.38, p<0.0001) and dose ($F_{(2,122)}$=28.03, p<0.0001), a main effect of housing on alcohol preference ($F_{(2,122)}$=4.70, p<0.0001) and dose ($F_{(2,122)}$=5.04, p<0.0001), and an interaction between the group and housing on preference ($F_{(4,122)}$=7.69, p<0.0001) and dose ($F_{(4,122)}$=10.35, p<0.0001). Post-hoc tests revealed that the low drinkers had significantly lower alcohol preference and intake than the high drinkers on both days of isolation (p<0.001 for all comparisons), while both the high drinkers that changed and the low drinkers had lower alcohol intakes than high drinkers that did not change on the last day of pair housing (p<0.001 for both comparisons; Fig. 3). Furthermore, the high drinkers that changed exhibited an intermediate preference for alcohol on the final day of isolation housing, which was significantly lower than the high group that didn't change (p<0.05). However, the alcohol preference and intake were not

significantly different between high drinkers that did or did not change when analyzed over the entire four-day isolation ($p>0.05$).

Volumes of alcohol and water consumed each day correlated very well with the number of licks recorded for each subject. One cohort (the first 10 animals tested) is shown here (Fig. 4), and is representative of the entire experiment (alcohol: $r=0.694$, $n=40$, water: $r=0.815$, $n=40$; $p<0.0001$ for each).

Analysis of the features of bouts of alcohol consumption revealed several differences between high drinkers that did not change alcohol intake when paired with low drinkers, high drinkers that did change, and low drinkers. While all the high drinkers had similar features on the first day of alcohol drinking, by day four of isolation the high drinkers who later decreased drinking exhibited bout features similar to low drinkers (Fig. 5). Since the software could not analyze data from subjects with one or fewer drinking bouts, ten low drinkers and one high drinker were not included in the analysis. There was a main effect of group on bout frequency ($F_{(2, 100)}=3.35$; $p=0.043$), interbout interval ($F_{(2, 100)}=3.54$; $p=0.036$), lick rate ($F_{(2, 100)}=4.26$; $p=0.02$), and time to first lick ($F_{(2, 100)}=5.62$; $p=0.0063$). There was a main effect of housing on bout frequency ($F_{(2, 100)}=18.38$; $p<0.0001$), bout size ($F_{(2, 100)}=12.06$; $p<0.0001$), interbout interval ($F_{(2, 100)}=8.04$; $p=0.0006$), and lick rate ($F_{(2, 100)}=14.16$; $p<0.0001$).

Post-hoc tests revealed specific group differences and differences between days. Bout frequency was significantly higher in the high-no change group than the low group ($p=0.015$), and on isolation day 1 than other days ($p<0.0001$). Bout size was significantly lower on isolation day 1 than other days ($p\leq0.001$). Interbout interval was shorter in the high-no change group than the low group ($p=0.012$) and on isolation day 1 than the other

days ($p \leq 0.002$). Lick rate was significantly lower in the high-no change group than the low group ($p=0.0054$) and on isolation day 1 than the other days ($p \leq 0.0007$). The latency to the first lick was lower in the high-change group than the low group ($p=0.0016$). Furthermore, inspection of these bout features across each of the four days of initial isolation suggested that the high drinkers that later changed drinking level exhibited characteristics of drinking that were more similar to high drinkers on some days and more similar to low drinkers on others. The occurrence of these differences and similarities were mostly unpredictable, rather than consistently shifting toward the low drinkers' levels over time (data not shown).

Visual analysis of the cumulative lick graphs (Fig. 6) revealed that while there were occurrences of alcohol drinking bouts close together in time for pairs of animals, the frequency of close bouts was not significantly different between isolation and pair housing ($F_{(1,30)}=1.80$; $p=0.190$) (Figs. 6, 7), or between pairs that didn't change drinking levels compared to those who did ($F_{(1,30)}=1.71$; $p=0.202$), and there was no interaction between the two factors when either the number ($F_{(1,30)}=1.12$; $p=0.298$) (Fig. 7A) or proportion ($F_{(1,25)}=0.340$; $p=0.565$) (Fig. 7B) of close bouts was assessed.

Cross-correlation analyses revealed that over two-thirds of the pairs exhibited a significant correlation between alcohol drinking patterns regardless of whether they were physically isolated (Fig. 8A) or housed together (Fig. 8B). Additionally, there was no consistent difference in the presence or absence of correlations between pairs that exhibited changes in drinking behavior and those that didn't (Table 1). Contrary to our hypothesis, there was no consistent directionality of the lag time of cross-correlations in pairs that changed their drinking level: in pairs where high drinkers changed to low

drinkers, there was not a greater presence of a negative lag time that would indicate the low drinker 'leading' the high drinker (Fig. 8B panels 2 and 3).

There was no significant correlation between the number of close bouts by visual assessment and the strength of cross-correlations (ACF value) ($r=0.083$; $n=19$; $p=0.734$). However, there was a statistical trend for a positive correlation between the *proportion* of close bouts by visual assessment and the strength of cross-correlations ($r=0.444$; $n=18$; $p=0.065$).

The region containing the V1aR microsatellite was successfully amplified and lengths were determined for 59 subjects. The rate of homozygosity was 47%. The length of the amplified region ranged from 669 to 736 base pairs. The mean, median and mode for all alleles were 703, 699, and 698bp, respectively. The allele lengths were not normally distributed (Fig. 9).

There was no significant difference in drinking behavior between subjects with short or long average microsatellite lengths on any measure of behavior (Table 2): initial alcohol preference, initial alcohol dose consumed, change in alcohol preference or dose from isolation to pair housing, pair housing to subsequent isolation, or overall change from the beginning to the end of the experiment. There was a difference within high drinkers, where subjects with long alleles had a greater decrease in alcohol preference from the beginning to the end of the experiment than those with short alleles ($t=2.27$; $df=26$; $p=0.031$), but this difference did not remain significant when adjusted for multiple comparisons.

Discussion

Prairie voles drinking high levels of alcohol paired with low drinkers in the lickometer apparatus exhibit a decrease in drinking similar to what we have previously

demonstrated in home cage drinking (Chapter 2, Anacker, Loftis, & Ryabinin, 2011). These changes in alcohol drinking that replicate previous work are not dependent on peers drinking together at the same time, or following specific patterns of consumption. This finding is in agreement with our previous results which showed that no changes in saccharin drinking occurred when high drinkers were paired with low drinkers (Chapter 2, Anacker, Loftis, & Ryabinin, 2011), which indicates that the act of consumption is not sufficient to influence one another in voles. We report evidence that variability in specific drinking behaviors, such as bout frequency, among a subset of high drinkers were predictors of a predisposition to decrease alcohol intake under the social influence of a low drinker.

These findings indicate that there may be predictors of whether an individual is likely to be responsive to social influence or pressure to decrease alcohol drinking. It is widely known that different types of therapies work for only subsets of people with alcohol use disorders (Anton, et al., 2006). Some people may be responsive to social support groups, others to drug therapies, and others to cognitive or behavioral therapy, while still others benefit from a combination. Unfortunately, there is currently no way to identify what would be the best form of treatment for a given individual, and regrettably the only way to learn what doesn't work is by trial and error, with failure resulting in relapse. Thus, if an assessment based on past drinking patterns could predict whether someone might be particularly responsive to social support to decrease or stop drinking, it would save time, effort, money, and considerable distress.

We found that there were distinctions between high drinkers that did or did not alter their alcohol intake when paired with a low drinker. Interestingly, these distinctions,

such as differences in the number of alcohol drinking bouts, were evident during drinking in isolation (Figure 5A,C), while the actual change in alcohol intake did not occur until after the high drinkers were paired with low drinkers (Figure 3B). This indicates that specific signs such as changes in bout frequency may predict whether an animal is susceptible to peer influence. While there was no significant interaction between effects of group and housing on bout parameters that would allow us to examine specific differences, it appears that the high drinkers that change drinking behaved more similarly to other high drinkers during the first day of drinking, but more similarly to low drinkers by the end of isolation, giving them an overall intermediate level that was not distinct from either group by statistical analysis. This indicates a degree of change or flexibility in features of drinking behaviors, such as bout frequency. This change doesn't appear to directly affect drinking levels, since on the same day (isolation day 4) that bout parameters change, the dose of alcohol consumed does not change (although there is a significant decrease in preference compared to stable high drinkers). Thus, flexibility in drinking behaviors is linked to propensity to be influenced by a peer, while not affecting drinking level directly.

However, this peer influence itself was not detected by any comparisons of drinking patterns undertaken here. The visual assessment of the cumulative lick records and the cross-correlation analyses both indicated that subjects often have drinking bouts that are close together in time. We initially hypothesized that these coincident drinking bouts would occur more often when pairs were housed together than when they were in isolation, since they may synchronize their ultradian rhythms to be awake and feeding

and drinking at the same time. However, this was not the case; nearly equal numbers of pairs had significant correlations in isolation and in paired housing.

While we found that neither cumulative lick record nor cross-correlation analyses revealed evidence of consistent patterns of linked alcohol intake in pairs, we also found that these different analyses did not exhibit strong correlations with one another. In particular, we would have expected a large number of close drinking bouts in a visual assessment of drinking patterns to be associated with a stronger ACF value in the cross-correlation, but this positive correlation did not reach statistical significance. There are many possible reasons for this. One explanation is that the lag time between bouts would have to be nearly identical within a pair in order to produce a strong ACF by cross-correlation. If the time between paired subjects' drinking bouts varied even by 30 seconds for each bout, it is possible that a significant ACF value would never be produced by cross-correlation: each lag time would be cataloged, but would have such a low frequency of occurrence that none would be considered significant. In this case, with animal behavior having the potential to be extremely variable even within a framework of a consistent pattern, cross-correlational analyses may not be optimal for detecting such patterns.

Given the evidence from the various types of pattern analyses performed in this study, it appears that prairie voles do not alter their alcohol drinking behavior by following a peer's drinking patterns. Therefore, another mechanism must be at work to explain the peer-dependent change in drinking levels observed in the present study and previous work, where most often the high drinker decreases its intake when paired with a low drinker. Thus, it is an open question whether the low drinker is typically the

dominant vole within the pair and, if so, how it may dictate alcohol intake or changes in alcohol intake. Another possible explanation is that the voles try to match one another's intoxication levels, perhaps through visual observation or vocal interactions. This explanation addresses the specificity of behavioral changes for alcohol but not saccharin, another rewarding substance, but one which does not lead to intoxication.

In addition to the behavior, we also analyzed a genetic polymorphism. The length of the vasopressin receptor 1a (V1aR) microsatellite fragment was different than what has previously been reported by others. Hammock and Young (2005) and Solomon et al. (2009) reported a range of allele lengths from 723-760 and 703-798 base pairs, respectively, which are considerably longer and show very little overlap with our sample. Additionally, they observed between 75-100% heterozygosity and a normal distribution while almost half of our sample was homozygous, leading in part to a highly leptokurtic distribution. We used the same primer sequences as both groups and the same genetic analyzer as Solomon et al., so we would not expect any of the sampling differences to be due to procedural differences. Since the subjects in our study and Hammock and Young's study arose from two different colonies of prairie voles, it is possible that our samples represent two different but overlapping subsamples of the wild prairie vole population, and that in our colony we have a larger presence of similarly-sized alleles due to random sampling or accidental inbreeding leading to a higher presence of particular alleles and homozygosity.

In addition to differences in allele length in the samples, the present experiment did not find effects of the microsatellite length on any alcohol-related behaviors, while others have shown effects on social behaviors previously. Since vasopressin is involved

in social behaviors and alcohol drinking, and since others have shown that the length of the V1aR microsatellite in the regulatory region of the gene is correlated with expression levels and certain social behaviors in the lab or in the field, we hypothesized that the length would also be correlated with socially-influenced alcohol drinking behaviors. However, we found no significant effect of a short average microsatellite compared to longer microsatellite lengths on any measure of prairie voles' alcohol drinking behavior, or on the propensity to change alcohol intake when paired with a peer. There was an indication of an effect of the longer microsatellite length corresponding to a greater change in alcohol preference following the effect of a peer influence, but this effect did not remain significant following adjustment for multiple comparisons. Thus, this should only be considered suggestive of the potential for the V1aR polymorphism to modulate the social influence on alcohol drinking.

The effects of the V1aR microsatellite length reported by others appear to be very specific to particular tests and environments. For example, microsatellite length was correlated with the receptor expression level in various brain regions, and several of these regions were then correlated with measures of partner preference in the laboratory test (Hammock, et al., 2005), but not when laboratory-bred voles were tested for social monogamy in semi-natural enclosures (Ophir, Campbell, et al., 2008; Solomon, et al., 2009), or in wild populations of prairie voles (Mabry, et al., 2011). In contrast, the length was correlated with genetic monogamy in the wild, but not in semi-natural enclosures.

One possible reason for effects of the microsatellite on behavior that may be difficult to detect has previously been proposed by others (Ophir, Campbell, et al., 2008): While there are several ways in which microsatellite length may influence expression

levels (Hammock & Young, 2005), it is likely that the length is actually an imperfect marker of different alleles possessing sequence differences that more directly affect gene expression and, ultimately, behavior. If particular single nucleotide polymorphisms (SNPs) are demonstrated to directly affect social behaviors, although no published work has established this to date, it would be possible to sequence the microsatellite and reanalyze the data from the present study to show whether SNPs that influence one form of social behavior also influence social alcohol drinking.

In summary, the present study shows that while high drinkers decrease their alcohol intake when paired with low drinkers, it is not due to matching patterns of drinking, and the behavioral changes cannot be predicted by the length of the microsatellite polymorphism in the vasopressin receptor 1a. Other behaviors and specific genetic polymorphisms that may affect peer-influenced alcohol drinking may be studied in the future. This study demonstrates new methods for examining data from fluid consumption studies where social influences can be assessed using visual and cross-correlational analyses. Most importantly, these findings demonstrate that certain features of alcohol drinking behavior and a flexibility to change in isolation may predict ability to be influenced by a peer. In this case we demonstrate that high drinkers with a tendency to change bout frequencies on their own are particularly susceptible to a positive influence of the low drinkers. It is unclear from the present studies whether this flexibility in behavior is specific to alcohol or would extend to more consummatory or other behaviors. It is possible to test in humans whether flexibility in drinking patterns before attempts to quit may predict susceptibility to peer influence, or whether inflexibility is linked with successful pharmacotherapy, perhaps even with existing data sets. Thus we

conclude by suggesting that future studies in humans may examine drinking patterns and behaviors as potential indicators of responsiveness to different treatment types, particularly social support.

Table 1. Number of pairs exhibiting significant correlations in drinking patterns

The total number of pairs (32) is divided into groups where at least one subject changed the drinking level and those that did not (change, no change). For each group, the number of pairs exhibiting a significant correlation or not, when analyzed by the cross-correlation function within a lag time of 10 minutes is shown for each alcohol and water drinking patterns, in isolation and pair housing. There are clearly no substantial differences between groups that did or did not change, between isolation and pairing, or between alcohol and water, although a statistical test could not be applied due to inadequate numbers (<5) of subjects in some cells.

		Alcohol Isolation1	Alcohol Pair	Water Isolation1	Water Pair
Change	Correlation	11	12	14	14
	No Correlation	6	5	3	3
No Change	Correlation	12	12	12	15
	No Correlation	3	3	3	0

Table 2. Effect of V1aR microsatellite length on alcohol drinking behaviors

Subjects possessing a short or long average microsatellite length were compared for each of the behaviors listed in the column on the left. The results of the t tests are listed in the middle column, with the unadjusted p value in the right. Significant ($p<0.05$) values prior to correction are shown in bold. Baseline preference and dose were based on the average alcohol (and water) intakes of the first four days of access, during isolation. 'High' indicates a comparison between only the high drinkers, and 'Low' indicates only the low drinkers. 'Change 1' indicates the change in drinking from the average during isolation 1 to the average during pair housing. 'Change Overall' indicates the change in drinking from the average during isolation 1 to the average during isolation 2 following pair housing.

Behavior	t, df	unadjusted p value
Baseline Preference	t=0.151 df=57	0.880
Baseline Dose	t=1.37 df=57	0.176
High Preference Change 1	t=1.30 df=26	0.207
High Preference Change Overall	t=2.27 df=26	**0.0314**
Low Preference Change 1	t=0.257 df=29	0.799
Low Preference Change Overall	t=0.638 df=29	0.529
High Dose Change 1	t=0.166 df=26	0.870
High Dose Change Overall	t=0.441 df=26	0.663
Low Dose Change 1	t=0.000571 df=29	1.00
Low Dose Change Overall	t=0.0418 df=29	0.967

Figure 1. Schematic diagram of lickometer cages and procedure

Custom-designed cages were made to house voles individually (A) or in pairs separated by a mesh divider (B). In both cases, plastic cages with air holes in the top surrounded wire metal racks that covered most of the cage floor. Voles had to step on the wire rack to reach the metal sipper tubes to obtain fluid, completing an electrical circuit to register each lick on the drinking tube.

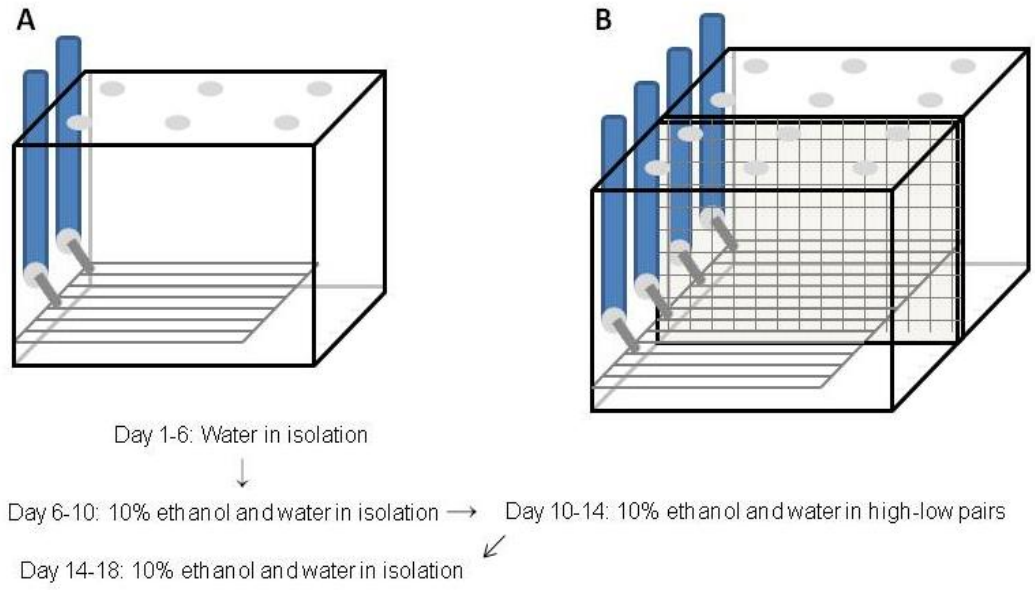


Figure 2. Alcohol preference and dose in different housing conditions

Alcohol preference (A) and dose consumed (B) by high (black) and low (white) drinkers in each housing condition. n=16 per drinking category. *Significant difference between isolation 1 and subsequent housing conditions for high drinkers; $p<0.05$.

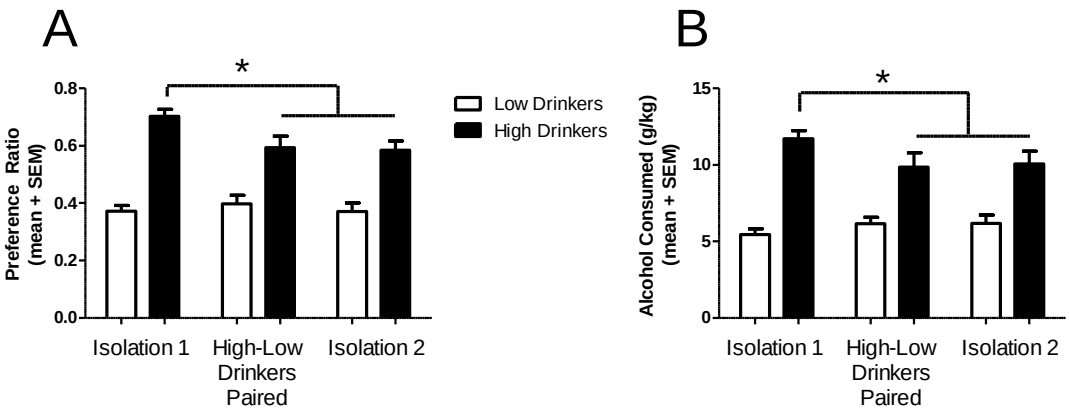


Figure 3. Alcohol preference and dose on different days

Alcohol dose consumed by high drinkers that did (checkered, n=14) or did not (black, n=18) change alcohol intake when paired with low drinkers (white, n=32). Dose on the first and last day of isolation as well as the last day of pair housing are shown.

*Significant difference between group indicated and both others; $p<0.05$.

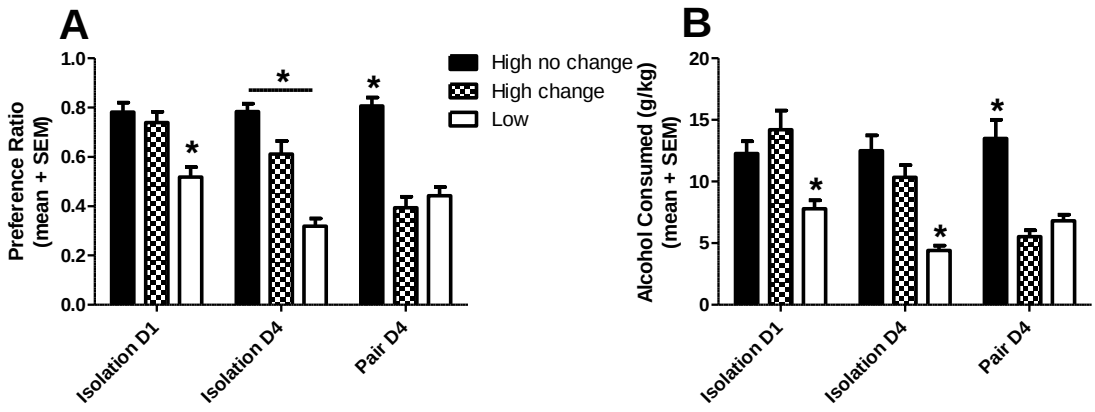


Figure 4. Correlation of recorded licks with fluid volume consumed

One cohort (n=10) is represented in this graph showing the relationship between the number of recorded licks from each drinking tube on the X-axis with the volume of water (O) or alcohol (X) consumed on the Y-axis, for each of four days in isolation. There was a strong positive correlation for both water ($r=0.815$, $n=40$; $p<0.0001$) and alcohol ($r=0.694$, $n=40$; $p<0.0001$).

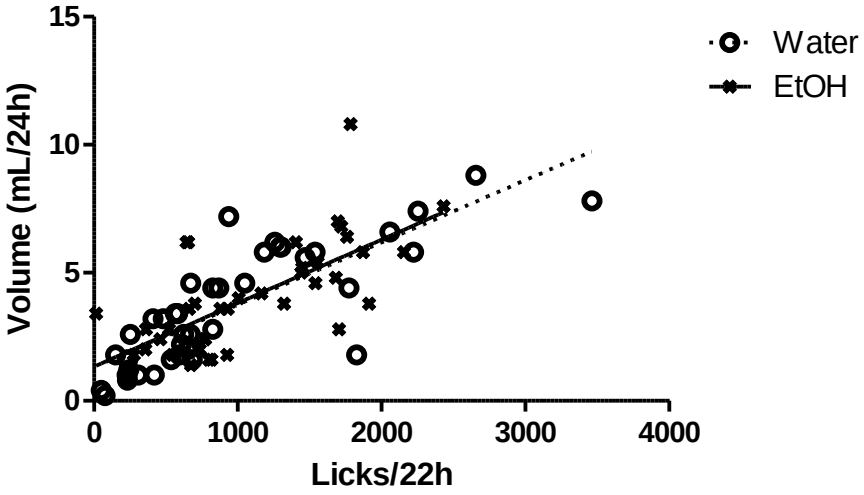


Figure 5. Alcohol drinking bout features

High drinkers that did (checked, n=13) or did not (black, n=18) change alcohol intake when paired with low drinkers (white, n=22) are shown on the first and last day of isolation as well as the last day of pair housing. The number of bouts of alcohol drinking (A), the average length of bouts as measured by number of licks (B) and time (D), the average time between bouts (C), the rate of licks within a bout (E), and the average time to until the first lick on the alcohol drinking spout was recorded (F) is shown for each 22 hour period. *Significant difference between high-no change group and low group; # significant difference between Isolation D1 and all other days.

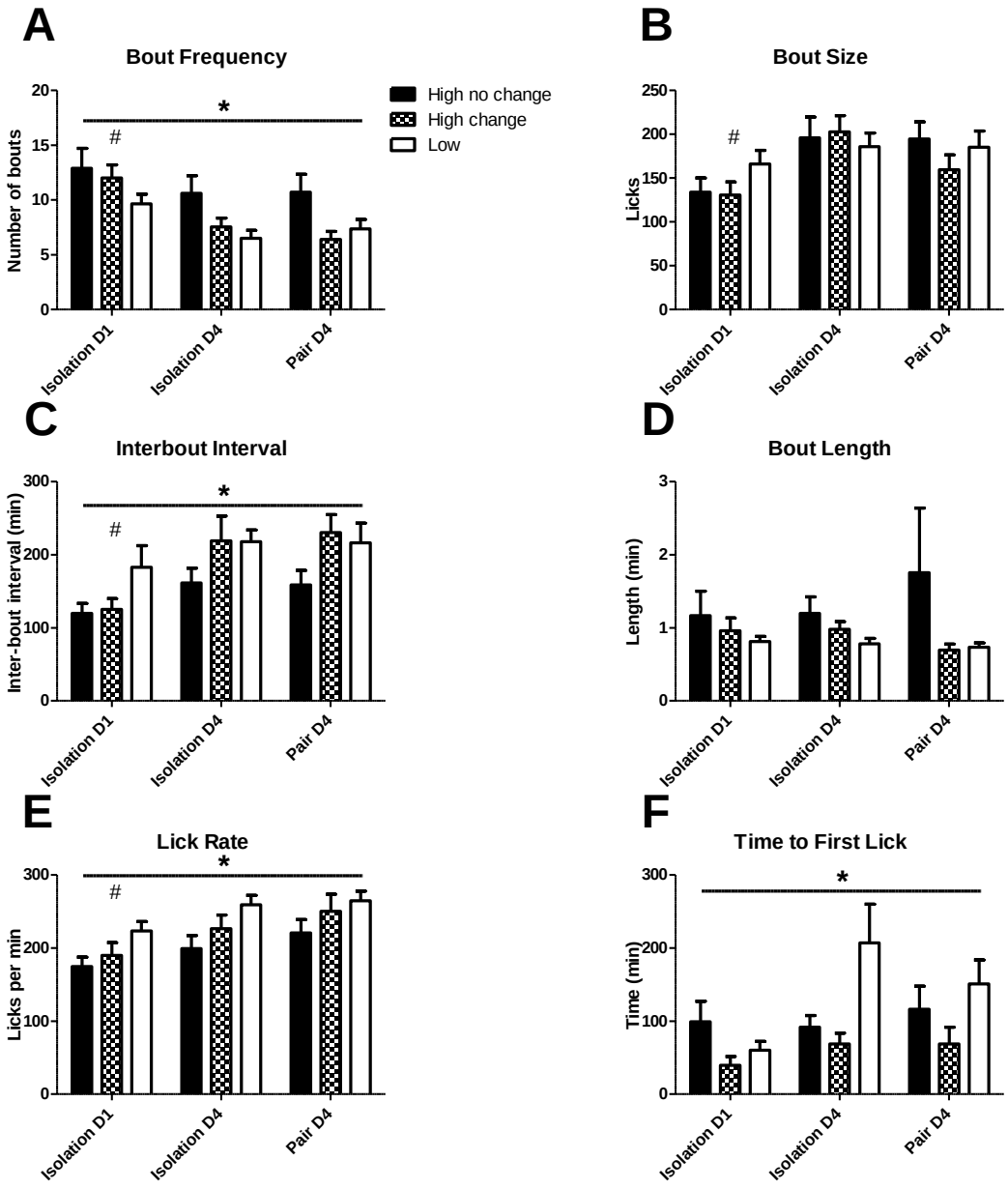

A
Bout Frequency
Number of bouts
High no change
High change
Low
*
#
Isolation D1
Isolation D4
Pair D4
B
Bout Size
Licks
C
Interbout Interval
Inter-bout interval (min)
D
Bout Length
Length (min)
E
Lick Rate
Licks per min
F
Time to First Lick
Time (min)

Figure 6. Cumulative number of licks of alcohol over 22 hours for an example pair A) The drinking patterns for subjects in pair 9 on the last day of isolation. B) The drinking patterns for subjects in pair 9 on the last day of pair housing. The high drinker is shown in blue and the low drinker is shown in black. Each 'step up' in the graph indicates a bout of drinking while each horizontal line indicates a time when no drinking occurred. The red indicates bouts that occurred close together in time, within the applied threshold.

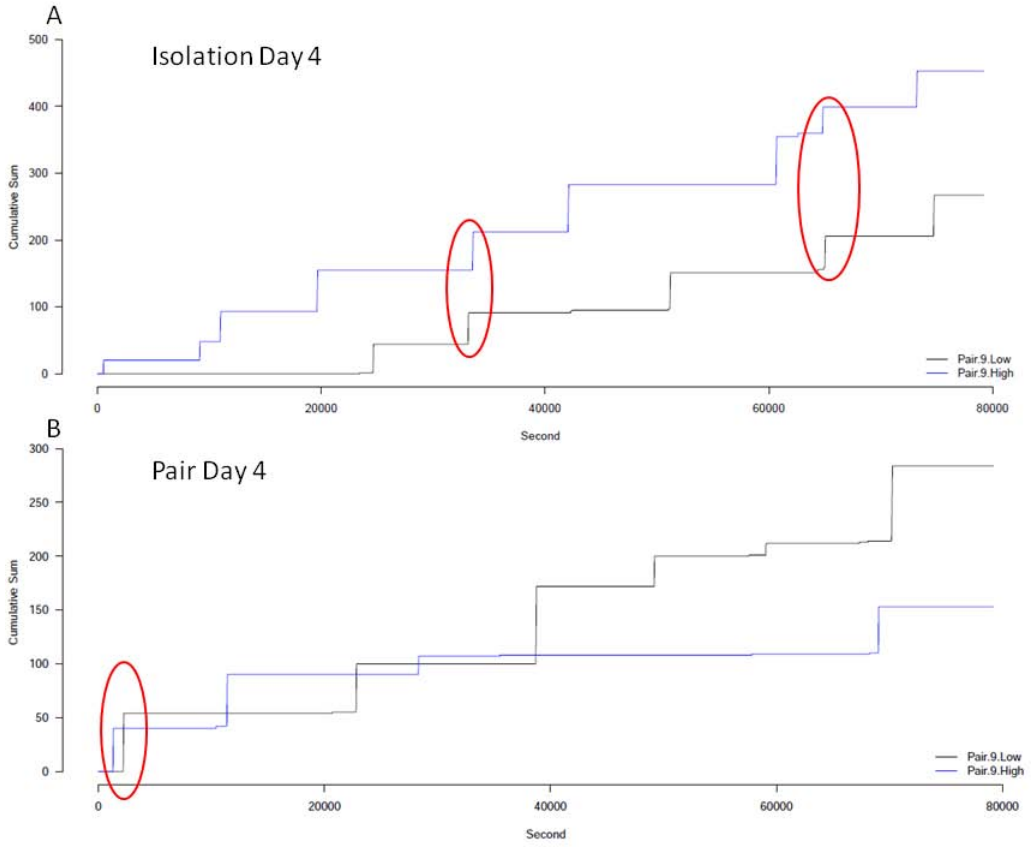


Figure 7. Visual assessment of close alcohol drinking bouts between partners in isolation and pair housing

A) The number of close bouts does not significantly differ between housing conditions or group changes, and there is no significant interaction of effects. B) The proportion of close bouts relative to the lowest number of bouts one subject exhibited does not significantly differ between housing conditions or group changes, and there is no significant interaction of effects.

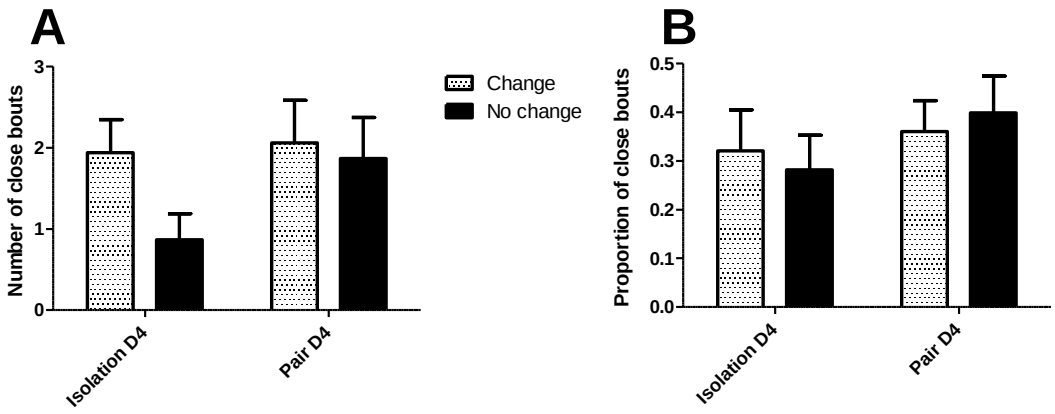


Figure 8. Cross-correlations between alcohol drinking patterns of peers

A) Correlations between high and low drinkers in isolation, before they have been housed together. B) Correlations between high and low drinkers in pairs on the fourth day of pair housing. The Y-axis represents the strength of the correlation (autocorrelation function; ACF), and each graph has a different upper limit shown, so they are not to scale relative to each other. Horizontal dashed lines represent the threshold for significance, which are set at the same level in all graphs. The X-axis represents 'lag' time, in seconds, between drinking events. A significant correlation at a lag time 'h' indicates that h seconds after the high drinker licks, the low drinker is likely to lick; a positive h value indicates that the high drinker leads the low drinker, and a negative value indicates that the low drinker leads the high drinker. Pairs shown are examples of each type of pair observed: those where both subjects changed drinking levels when paired (top panels), those where the high drinker changed to match the low drinker (middle panels show two of many variations of outcomes), and those where neither subject changes (bottom panels).

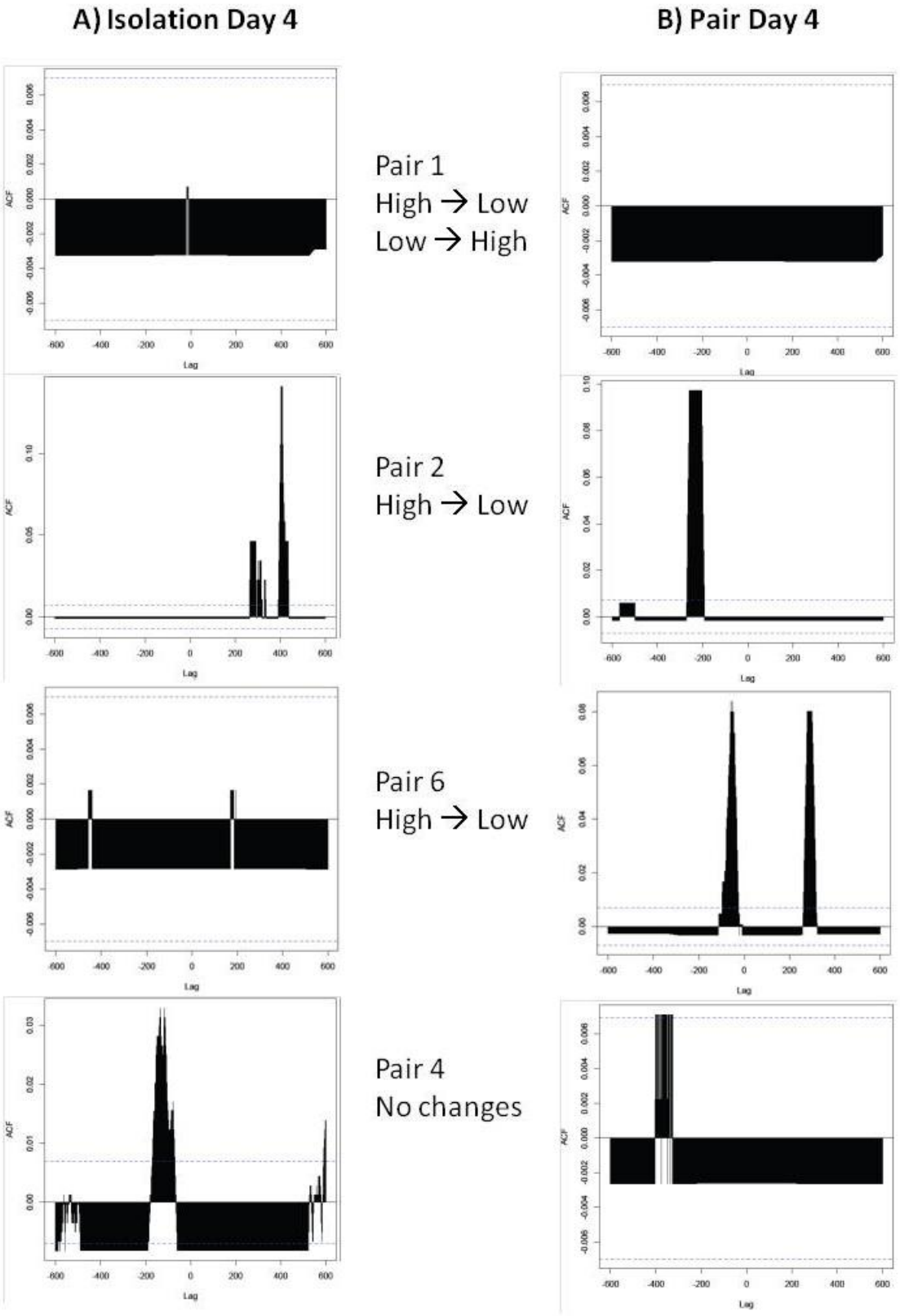
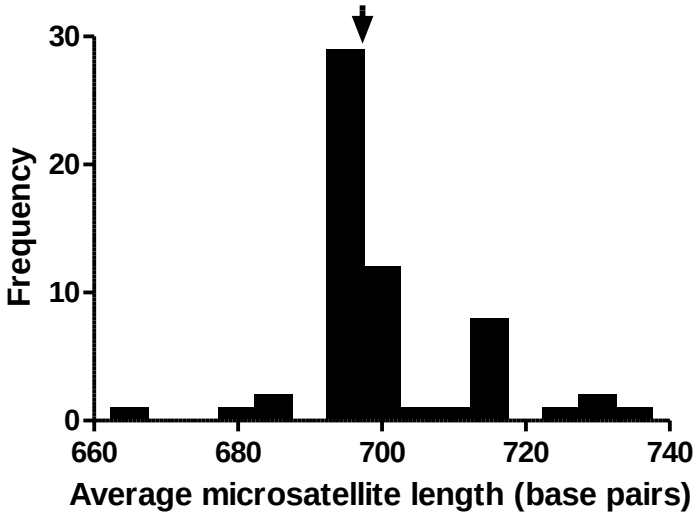

A) Isolation Day 4
B) Pair Day 4
Pair 1
High → Low
Low → High
Pair 2
High → Low
Pair 6
High → Low
Pair 4
No changes
ACF
Lag

Figure 9. Histogram of microsatellite length frequencies

The average microsatellite length of the two alleles of each individual was calculated and subjects were grouped into 5-base pair bins (X-axis). The number of subjects in each bin (frequency) is shown by the height of the black bar. The sample was non-normal (leptikurtic) due to the high number of lengths in the 695-700 base pair range. The arrow indicates where the median split occurred.



CHAPTER 4: Drinking Alcohol Has Opposite Effects on Social Affiliation in Male and Female Prairie Voles

(A modified version of this chapter has been submitted for publication.)

Introduction

Alcohol (ethanol) abuse often leads to devastating effects on many aspects of life, including important social relationships. Greater alcohol use is often linked to greater marital dissatisfaction and divorce (Halford & Osbarby, 1993; Leonard & Rothbard, 1999; Levinger, 1966; McLeod, 1993). However, couples usually report greater levels of dissatisfaction with their marriage when the spouses' alcohol intake levels are discordant (Floyd, et al., 2006; Homish & Leonard, 2007; Homish, et al., 2009; Kearns-Bodkin & Leonard, 2005), and some couples also report positive effects of alcohol on their relationships (Leonard & Eiden, 2007; Marshal, 2003). Alcohol drinking is often found to play a role in intimate partner violence, which can be destructive for important social bonds (Kachadourian, et al., 2011; Leonard, et al., 1985; Testa, et al., 2012). The disruption of these social bonds as a direct or indirect effect of alcohol abuse is difficult for the alcohol dependent individual as well as for the others in his or her life, from friends and co-workers to parents, spouse and children.

Various forms of recovery from alcoholism include a focus on repairing and maintaining healthy relationships, which are important factors in sustaining lower alcohol drinking levels (Bond, Kaskutas, & Weisner, 2003; Gordon & Zrull, 1991; Humphreys, Moos, & Cohen, 1997; Hunter-Reel, Witkiewitz, & Zweben, 2012; Kaskutas, Bond, & Humphreys, 2002; Kelly, et al., 2011; Longabaugh, 2003; Polcin, Prindle, & Bostrom, 2002; Wu & Witkiewitz, 2008). Greater understanding of alcohol's effects that lead to disruption of social bonds would help target therapies to improve this essential aspect of life and recovery. However, it is difficult to determine from studies in humans how much of the effect of alcohol on relationships may be due to innate biological responses and how much could come from other effects such as cultural influences and perceptions or

expectations. Understanding the neurobiological mechanisms underlying formation and destruction of bonds would help elucidate potential pharmacotherapies that could also aid recovery from alcohol dependence and the reestablishment of social bonds. These issues are difficult or impossible to assess in humans, and until now an adequate animal model for examining the effects of alcohol intake on social bonds has not been developed.

Prairie voles are a valuable animal model of mammalian social monogamy as they form strong bonds between males and females as well as parents and offspring (Carter, DeVries, & Getz, 1995; Getz, Carter, & Gavish, 1981; Gruder-Adams & Getz, 1985). A variety of neurochemical substrates such as vasopressin, oxytocin and dopamine have been shown to be necessary for adult bond formation in voles (Wang, et al., 1999; Williams, Carter, & Insel, 1992; Winslow, et al., 1993; recently reviewed in K. A. Young, Liu, & Wang, 2008). Many studies have also demonstrated the importance of these molecules in human social behaviors, and as potential therapies for disorders involving social dysfunction (Dai, et al., 2012; Guastella, et al., 2012; Guastella & MacLeod, 2012; Kosfeld, et al., 2005; L. J. Young & Flanagan-Cato, 2012; Zink & Meyer-Lindenberg, 2012).

Parallels between social reward and drug reward have been noted by others (Burkett & Young, 2012; Dackis & O'Brien, 2001; Esch & Stefano, 2004; Hostetler & Ryabinin, 2012; Liu, et al., 2010; Schultz, 2000; Wise, 1996a, 1996b; L. J. Young & Wang, 2004), and the interactions between social relationships and drug abuse in prairie voles have begun to be explored (Anacker, et al., 2012; Chapter 1, Anacker, et al., 2011; Chapter 2, Anacker, Loftis, & Ryabinin, 2011; Aragona, Detwiler, & Wang, 2007; Curtis & Wang, 2007; Gobrogge, et al., 2009; Liu, et al., 2010; Liu, et al., 2011; K. A. Young,

et al., 2011). We previously demonstrated that prairie voles exhibit social facilitation of excessive alcohol intake and direct peer influence in same-sex pairs (Chapter 1, Anacker, et al., 2011; Chapter 2, Anacker, Loftis, & Ryabinin, 2011). The same effect was not seen in male-female pairs (Hostetler, Anacker, et al., 2012).

Alcohol's mechanisms of action in the brain and on behavior are varied and not completely understood. A number of neuropeptides are affected by alcohol, and these same molecules also affect social behaviors. For example, vasopressin is a neuropeptide necessary for bond formation in male prairie voles. Although acute administration of alcohol is commonly thought to decrease peripheral vasopressin levels in humans and rats (Colbern, et al., 1985; Eisenhofer & Johnson, 1982; Guillaume, Gutkowska, & Gianoulakis, 1994; Helderman, et al., 1978; K. M. Ogilvie, S. Lee, & C. Rivier, 1997), there are many reports of no effect, or the opposite effect, which may be linked to time since administration (Colbern, et al., 1985; R. G. Cooper & Musabayane, 2000; Inder, et al., 1995; Linkola, et al., 1978; Taivainen, et al., 1995; Wigle, et al., 1993). In addition, there are mixed reports on the effects of acute (or chronic) alcohol exposure on vasopressin levels in the brain, and the effects appear to be region-specific (reviewed in Madeira & Paula-Barbosa, 1999; Madeira, et al., 1993; K. M. Ogilvie, S. Lee, & C. Rivier, 1997; Rivier & Lee, 1996). Similarly, alcohol decreases peripheral oxytocin levels (Coiro, et al., 1992; Mennella & Pepino, 2006), but it is unclear what effects it may have on specific brain regions known to be important in pair bonding (Madeira, et al., 1993; Silva, et al., 2002).

In the present experiment, we hypothesized that alcohol would have an effect on pair bond formation that would result in a change in the expression of partner preference,

but we did not have initial hypotheses for the direction of the effect; in humans, there is a common perception that alcohol can act as a 'social lubricant', but it can also lead to negative social effects such as aggression and destruction of bonds, so we thought that either social facilitation or inhibition was a possibility in prairie voles. Typically the effect of a molecule is tested by administration of a substance by the experimenter immediately prior to the cohabitation period where bond formation takes place (DeVries, et al., 1995; Wang, et al., 1999; Williams, Carter, & Insel, 1992; Winslow, et al., 1993). However, to improve the face validity of a model of addiction-related behavior, we chose to use self-administration of alcohol throughout the cohabitation period in these experiments. In addition, we tested levels of neuropeptides that are likely to play a role in mediating alcohol's effects on bond formation. Finally, we tested the hypothesis that a particular microsatellite sequence in the promoter region of the vasopressin 1a receptor (V1aR) gene was related to any of the alcohol drinking or pair bonding behaviors tested here, as the length of this polymorphism has been suggested to relate to a variety of social behaviors, as described in the previous chapter (Hammock, et al., 2005), but this has not been validated by others (Mabry, et al., 2011; Ophir, Campbell, et al., 2008; Solomon, et al., 2009).

Materials and Methods

Animals

Adult male and female prairie voles (67-115 days old) from our breeding colony at the Portland Veterans Affairs Medical Center Veterinary Medical Unit were used in these experiments. Voles were weaned at 21 days of age and housed with same-sex siblings. Male and female weanlings were housed in separate rooms, under a 14:10 hour

light:dark cycle with lights on at 0700 and lights off at 2100. The animals were given *ad libitum* access to food and water throughout the experiments, except where noted.

Surgeries

To control hormonal status and sexual receptivity, all female prairie voles used as stimulus animals to test male partner preference formation were ovariectomized approximately two weeks prior to the start of experimentation. Surgery was conducted under isoflurane anesthesia (3% for induction, 1.5-2% for maintenance), with carprofen administered intraperitoneally (5 mg/kg in saline), both immediately and 24 hours following surgery. Females were monitored daily following the surgery, and in all cases incisions appeared clean, dry, and healed after 10 days. Post-operative housing was in a cage with a wire mesh divider that allowed two females to be housed together with visual, olfactory, auditory, and some tactile contact, while allowing physical isolation so that they could not affect each other's incision sites. After wound clips were removed on the tenth day following surgery, each pair was housed in a typical home cage with full contact until the experiment began. Female stimulus animals for one of the male experiments were given priming injections of estradiol benzoate (EB) to induce sexual receptivity, while another group was not, since mating is not typically necessary for pair bond formation, but can facilitate it (Insel, Preston, & Winslow, 1995; Williams, Carter, & Insel, 1992; Winslow, et al., 1993).

Female test subjects were not surgically altered or primed with EB, in order to test them in as close to a natural state as possible during the process of pair bonding.

Drugs and drinking solutions

Estradiol benzoate (EB; Sigma-Aldrich Co., USA) was first dissolved in a 4mg/mL solution of 100% ethanol and then diluted to 20µg/mL in sesame oil (Acros Organics, Thermo Fisher Scientific, USA). EB in sesame oil vehicle was injected subcutaneously at a dose of 2µg, in 0.1mL. Injections occurred at 20:45 daily for 3 days prior to the start of the experiment, i.e. 60, 36, and 12 hours prior to cohabitation. This dose and similar timing of injections has been shown to reliably induce mating behavior (Insel & Hulihan, 1995; Modi & Young, 2011).

Ethanol drinking solution was made by diluting 95% ethanol (Pharmco-Aaper, USA) in tap water to 10% by volume. This concentration was chosen because most prairie voles will self-administer large enough doses of alcohol at this concentration to likely produce intoxicating effects while also showing a preference for this solution over water (Chapter 1, Anacker, et al., 2011).

Each pair of voles was given access to four 25mL tubes containing drinking solutions. For the alcohol-exposed group, two tubes contained 10% ethanol and two tubes contained tap water. For the water control group, all four tubes contained tap water. The position of the alcohol tubes relative to the water tubes was counter-balanced across pairs. Each stranger stimulus animal was given similar access to either one drinking tube of 10% ethanol and one tube of tap water, or two tubes of tap water, to match the condition of the pair it would be tested with.

Effects of ethanol self-administration on partner preference in females

On day 1, stimulus males were weighed and a small area of fur was shaved off for identification. Males assigned to be partners were shaved on the dorsal, posterior side; males assigned to be strangers were either shaved in a different shape or ‘sham’ shaved,

which consisted of touching the activated trimmer to the body without removing any fur. Males were then habituated to tethering within the partner preference chamber for one hour.

On day 2, female test subjects (n=15 per group) were weighed and placed in clean homecages, followed within minutes by a dorsoposterior shaved 'partner' male. Once paired, both animals were given access to tubes containing ethanol and water or only water. The volume of each tube was recorded 0, 22, and 24 hours from the beginning of cohabitation. The total volume of alcohol or water consumed per cage for each time period was divided by two and assigned to each partner. 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 body weight).

The other shaved and 'sham' shaved males were also placed in clean homecages, but they did not cohabitate with a female. These 'stranger' males received ethanol and water or water only to match the condition of the subject with which they would later be tested. Alcohol preference and dose consumed were also determined for each stranger.

Female prairie voles are induced ovulators and are not usually sexually receptive until after being exposed to a male for 24 hours (Carter, et al., 1989). Thus, mating was not expected during the 24-hour cohabitation period for these pairs. However, to detect any potential differences in mating behavior, the last two hours of cohabitation were digitally video recorded.

Following 24 hours of cohabitation, the partner preference formation was assessed with a 3-hour partner preference test (PPT) on day 3. 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 (Ahern, et al., 2009; Williams, Carter, & Insel, 1992). The PPT lasted three hours, during which time the voles did not have access to food, water, or alcohol. The entire three-hour test was digitally video recorded for later analysis.

All animals were euthanized by carbon dioxide inhalation immediately following the partner preference test. Tail tissue samples were taken, placed immediately on ice, and then stored at -20°C for later genetic analysis. Brains were extracted and fixed for immunohistochemistry.

Effects of ethanol self-administration on partner preference in males

The test for partner preference in males was conducted in two experiments, with or without mating. The tests were conducted the same way 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 as described above so that they were not sexually receptive. For this experiment we used 16 subjects in the alcohol group and 15 in the water control group.

In the second male experiment, female stimulus animals were ovariectomized and given priming doses of EB to induce sexual receptivity, as described above. Since mating was expected under these conditions, the first two hours of cohabitation were recorded, in addition to the last two hours, in order to detect whether mating occurred in all pairs, and to assess whether there were differences in mating behaviors between groups.

After the first experiments in males and females were completed, we observed that the habituation to tethering was not necessary in that the voles apparently became accustomed to the tether and acted normally within five minutes of being placed in the partner preference chamber. Subsequently, and with approval of the PVAMC IACUC, we did not expose the stimulus animals to tethering habituation sessions in the second male experiment. For this experiment we used eight test subjects per group. Of these eight subjects, one subject from the water control group was removed from analysis for fighting with the partner.

Immunohistochemistry

In order to assess potential neuropeptide involvement in the effects of alcohol on partner preference, and to rule out any effects of the PPT itself, a separate group of animals (18 male-female pairs) was given similar access to 10% ethanol and water or water only during a 24-hour cohabitation period. Immediately following the cohabitation, they were euthanized by carbon dioxide inhalation. Brains were extracted, fixed in paraformaldehyde (2% in 10mM phosphate-buffered saline [PBS]) for 24 hours, and cryoprotected by sucrose (20% in PBS with 0.1% sodium azide followed by 30% in PBS with 0.1% sodium azide) for immunohistochemical staining. Thus, the time of analysis of neuropeptide levels was identical to the placement into the PPT in the previous behavioral experiments.

Brain tissue was sliced in 40 μm coronal floating sections and preserved until immunohistochemistry (IHC) in PBS with sodium azide. Prior to starting IHC, slices from relevant brain regions were chosen and placed in PBS. The IHC protocol was based on previous publications (Bachtell, et al., 2003; Cservenka, et al., 2010; Giardino, et al.,

2011; Weitemier, Tsivkovskaia, & Ryabinin, 2005). Floating sections were first placed in 0.3% hydrogen peroxide to quench endogenous peroxidase activity, followed by PBS rinses. They were then placed in blocking solution containing 2% bovine serum albumin, 5mg/mL heparin, and Triton X-100 (3%) in PBS. Following blocking, sections were incubated overnight with the primary antibody diluted as follows in PBS with Triton X-100 and bovine serum albumin. Anti-oxytocin antibodies (Peninsula Laboratories, San Carlos, CA) were diluted 1:20,000; anti-vasopressin antibodies (Peninsula Laboratories) were diluted 1:50,000; anti-corticotropin releasing factor (CRF) antibodies (Peninsula Laboratories) were diluted 1:10,000; anti-neuropeptide Y (NPY) antibodies (Sigma-Aldrich Co., USA) were diluted 1:50,000; anti-urocortin 1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were diluted 1:5,000; anti-c-Fos antibodies (Santa Cruz Biotechnology, Inc.) were diluted 1:2,000. All primary antibodies were polyclonal, made in rabbit against mouse, with the exception of the urocortin antibodies made in goat. Unbound primary antibodies were washed in PBS, and the anti-rabbit secondary antibodies made in goat or anti-goat secondary antibodies made in rabbit (Vector Laboratory Inc., Burlingame CA, USA) were applied in PBS with Triton X-100. Following PBS washes, the antibody signal was amplified with a Vectastain ABC kit (Vector Laboratory, Inc.). After final washes in PBS, the tissue was processed for visualization using metal-enhanced diaminobenzidine (Pierce, Rockford IL, USA), and the reaction was quenched in water. Slices were immediately mounted onto gelatin-coated slides, which were dehydrated and covered with coverslips the next day.

Immunoreactive cells and fibers were visualized using a Leica DM4000 brightfield microscope (Bartels and Stout, Inc., Bellevue WA, USA). All cells stained

above background in each region were manually counted. Fibers in some regions were manually counted, and fiber density was determined in other regions using Image J software (Rasband, 1997).

Microsatellite length analysis

DNA was extracted from each tail tissue sample and the V1aR microsatellite was amplified and analyzed using the same procedures described in Chapter 3.

Analyses and statistics

The main outcome variable of the PPT was time spent huddling with the partner and stranger. Huddling is a species-specific behavior in which the two voles are in motionless physical contact. The videos were scored by an experimenter unaware of group assignment of animals and trained to detect huddling behavior, analyzing one subject at a time, at 3X speed using Quicktime Player Version 7.6.9 (Apple). Stopwatch+ (Atlanta GA, USA) was used to measure the amount of time spent huddling with the partner and stranger stimulus animals, and values were multiplied by three to convert to real time for analysis. These data were compared with a mixed-model ANOVA with treatment (water or ethanol) as a between-subjects factor, and time with each stimulus animal (partner or stranger) as a within-subjects factor. Each experiment was analyzed separately, since they were tested in separate experiments and under slightly different conditions, as described. To test our a priori hypotheses that the time spent with partner versus stranger would be different depending on alcohol exposure, Bonferroni-corrected t-tests were used to compare huddling times for each group.

We were also interested in what other behaviors subjects exhibited in the PPT, particularly to see if changes in other behaviors could explain between-group differences

in huddling time. Thus, we also analyzed these behaviors in the experiments that showed effects of alcohol (tests of females and males with mating). We assessed locomotor activity by the total number of chamber crossings, and also counted the number of mating bouts and aggressive events. These behaviors were analyzed by t-tests comparing the two treatment groups (water or ethanol), with the exception of mating, which was compared by ANOVA, using the stimulus animal (partner or stranger) or cohabitation time as another factor.

IHC results were analyzed by ANOVA with sex and alcohol access as between-subjects factors. Significant interactions were followed up by post-hoc t-tests within each sex.

The short, long, and average allele lengths were each analyzed for Pearson's correlation with alcohol intake and preference, and time spent huddling with the partner and stranger either with all subjects or with non-alcohol exposed subjects only. Since each measure produced similar results, we report only the results from the analyses of average microsatellite lengths.

All data are reported as mean ± standard error of the mean (SEM).

Results

Effects of ethanol self-administration on partner preference

Female prairie vole subjects drank on average 12.48 ± 1.03 g/kg ethanol during the 24 hour cohabitation period, and the preference for ethanol over water was 0.58 ± 0.057. Male test subjects that did not mate drank on average 11.2 ± 0.812 g/kg ethanol during the 24 hour cohabitation period, and the preference for ethanol over water was 0.74 ± 0.028. Male test subjects that mated drank on average 14.3 ± 1.87 g/kg ethanol during the 24 hour cohabitation period, and the preference for ethanol over water was

0.73 ± 0.072. However, these figures are not precise due to the fact that the volume consumed by both the male and female partners was averaged between the two. Nevertheless, these doses and preference scores are consistent with what we have previously observed in voles drinking under circumstances where we could distinguish individual drinking (Chapter 1, Anacker, et al., 2011). Partner and stimulus animals' alcohol volume, dose intake and preference are also shown in Table 1. There were no significant differences between the ethanol intake of subjects, partner, and stranger between any experiments.

Female prairie voles exhibited a significant effect of the stimulus animal on huddling time ($F_{(1,56)}$=26.86; p<0.0001), where they spent more time huddling with the partner than the stranger. There was no main effect of alcohol on total huddling time ($F_{(1,56)}$=0.02; p=0.89), but there was a significant interaction between alcohol and the stimulus animal ($F_{(1,56)}$=4.25; p=0.044). Planned t-tests for each group revealed that there was a non-significant trend toward a partner preference in the water control group (t=1.90, df=28, p=0.068), but there was a strong significant partner preference in the females that had access to alcohol during cohabitation (t=6.34, df=28, p<0.0001; Fig. 1A).

Male prairie voles with sexually non-receptive females exhibited no significant effects of the stimulus animal ($F_{(1,58)}$=2.57; p=0.11) or alcohol ($F_{(1,58)}$=0.37; p=0.54) on total huddling time, and there was no significant interaction between alcohol and the stimulus animal ($F_{(1,58)}$=0.13; p=0.72). Planned t-tests for each group revealed that there was no significant partner preference in the water control group (t=1.36, df=28, p=0.18),

or in the males that had access to alcohol during cohabitation (t=0.90, df=30, p=0.38; Fig. 1B).

Male prairie voles with sexually receptive stimulus females exhibited a significant effect of the stimulus animal on huddling time ($F_{(1,26)}$=11.23; p=0.0025), where they spent more time huddling with the partner than the stranger. There was no main effect of alcohol on total huddling time ($F_{(1,26)}$=1.24; p=0.28), but there was a significant interaction between alcohol and the 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 a significant partner preference in the water control group (t=5.98, df=12, p<0.0001), but there was no significant partner preference in the males that had access to alcohol during cohabitation (t=0.13, df=14, p=0.90; Fig. 1C).

To better understand the effects of alcohol on individual partner preference expression, we analyzed which subjects expressed a partner preference (indicated by more than twice as much time spent with the partner as the stranger (Lim, et al., 2007)), a stranger preference (twice as much time spent with the stranger as the partner), or no preference. We found that while all of the male subjects in the water group expressed a preference for the partner, approximately equal numbers of male subjects in the alcohol group expressed each type of preference (Table 2), thereby yielding the overall group effect of exhibiting no preference.

To examine whether the potential effects of alcohol on mating behavior could explain the differences in partner preference, we assessed these behaviors in the experiments with females, and males with mating. As expected, no mating was observed in either the first or last two hours of cohabitation with unprimed female test subjects.

During the female PPT there were few occurrences of mating, though one female in the water group exhibited a very high number of mating bouts. There was no significant effect of alcohol on mating bouts ($F_{(1,56)}$=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 2A). For males cohabiting with primed females, mating was observed in all subjects. The number of mating bouts in the first and last two hours of the 24-hour cohabitation period was not different between water and alcohol groups ($F_{(1,12)}$=0.15; p=0.71), although the number of mating bouts was significantly greater in the last two hours of cohabitation compared to the first two ($F_{(1,12)}$=33.74; p<0.0001), and there was no significant interaction between time and treatment ($F_{(1,12)}$=0.15; p=0.70; Fig. 2B). The number of mating bouts during the three-hour PPT was not significantly different between treatment groups ($F_{(1,28)}$=0.24; p=0.57). In agreement with reports of the social, but not genetic nature of monogamy in prairie voles (Ophir, Phelps, et al., 2008; Solomon, et al., 2004), there was no main effect of stimulus animal on number of mating bouts ($F_{(1,28)}$=0.035; p=0.85), and 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. 2C).

There was no difference in the number of chamber crossings made between treatment groups ($F_{(3,30)}$=0.54; p=0.66; Fig. 2D). There was no difference in the number of bouts of aggression made between treatment groups by males during the first two or last two hours of cohabitation ($F_{(1,14)}$=0.46; p=0.51; Fig. 2E), or the PPT ($F_{(1,26)}$=2.79; p=0.11; Fig. 2G), although there were a larger number of aggressive bouts exhibited between male test subjects and stranger stimulus females compared to the partners

($F_{(1,26)}$=13.37; p=0.001), and there was no interaction between treatment and stimulus animal ($F_{(1,26)}$=2.49; p=0.13). Female aggression was very low, and there was no difference in the number of bouts made between treatment groups by females during the first two or last two hours of cohabitation ($F_{(1,28)}$=0.13; p=0.72; Fig. 2F). There was a trend for an effect of alcohol increasing aggression in the PPT ($F_{(1,56)}$=3.78; p=0.057; Fig. 2H), and for a larger number of aggressive bouts exhibited between female test subjects and stranger stimulus males compared to the partners ($F_{(1,56)}$=2.92; p=0.093), and there was no interaction between treatment and stimulus animal ($F_{(1,56)}$=0.04; p=0.85).

Effects of ethanol self-administration during cohabitation on neuropeptide and transcription factor levels

The brain regions examined for number of cells, fibers, or density of fibers expressing each neuropeptide or Fos are listed in Table 3, along with the mean ± SEM values for each group, and p-values from the ANOVA for the effects of sex and alcohol treatment, as well as interactions.

There were no significant effects of sex or alcohol on oxytocin-positive cell number. There was a significant effect of alcohol on oxytocin-positive fiber number in the lateral dorsal tegmental area where alcohol-exposed subjects had fewer oxytocin fibers than water-drinking controls.

There was a significant effect of sex on vasopressin-positive cell number in the ventral bed nucleus of the stria terminalis (vBNST), with females having greater numbers than males. There were no significant differences for vasopressin-positive fiber numbers.

There was a significant effect of sex on the number of NPY-positive cells and total density of IR in the ventral pallidum (VP), with females having more than males.

There was a significant interaction between sex and alcohol on NPY-IR fiber density in the medial amygdala (MeA). Post-hoc tests revealed that males with alcohol exposure had higher levels of NPY-IR than males without alcohol, but there were no other group differences (Fig. 3C, 4C). There was an effect of sex on NPY density in the ventral lateral septum (LSV), with males having greater NPY-IR than females.

There was a significant effect of sex on the number of CRF-positive cells in the medial geniculate nucleus and median raphe, with males having more in both regions. There was also a significant effect of sex on the density of CRF-positive fibers in the median raphe, with males having more than females. There was a significant effect of alcohol on CRF-positive fibers in the BNST, specifically driven by the vBNST, where alcohol drinkers exhibited less fiber density (Fig. 3D, 4D).

There was a significant interaction between sex and alcohol for the number of Fos-IR cells in both the arcuate nucleus of the hypothalamus (Arc) and the centrally-projecting Edinger Westphal nucleus (EWcp). In the Arc, there were greater numbers of Fos-IR cells in females who had access to alcohol than those who did not (Fig. 3A, 4A). In the EWcp, there was also a main effect of sex on Fos levels, where males had greater IR cell numbers than females; post-hoc tests showed only a trend for an increase in Fos-positive cells in alcohol-exposed males compared to water controls, and no difference in females dependent on alcohol access (Fig. 3B, 4B). There were no group differences in the number of urocortin-positive cells in this region. Representative photomicrographs of neuropeptides that did not exhibit statistical interactions between sex and alcohol (oxytocin, urocortin, and vasopressin) are shown (Fig. 5).

Microsatellite length and behavior

The range and mean of microsatellite lengths observed were similar to what we have seen previously (see Chapter 3), while the subject groups tested here conformed to a normal distribution.

There were no significant correlations of microsatellite length with any of the behaviors measured ($-0.261<r<0.512$), regardless of alcohol exposure. The correlations of behaviors with the average microsatellite length are shown for female subjects (Fig. 6) and male subjects allowed to mate (Fig. 7). In addition, there was no significant correlation between microsatellite length and behavior when only control subjects (those that did not have access to alcohol) were analyzed.

Discussion

In these experiments we discovered sex-dependent differences in the effects of alcohol on formation of social bonds. We found that alcohol self-administration facilitates the development of social bonds in female prairie voles, while it inhibits the development of these bonds in males. This difference will allow us to learn more about alcohol's effects on social relationships in two different model procedures. There are many examples of alcohol's effects on human relationships that parallel the observations in voles. For example, alcohol is often viewed as a 'social lubricant,' perhaps due to its effects or expectations of decreasing social anxiety and inhibition (See reviews: Book & Randall, 2002; Carrigan & Randall, 2003), and the facilitative effects of alcohol on forming social bonds have been shown to be strengthened when more females are present (Sayette, et al., 2012). On the other hand, alcohol often plays a negative role in marital happiness, particularly when drinking levels of partners do not coincide (Homish & Leonard, 2007; Homish, et al., 2009), and alcohol is often associated with fleeting social relationships such as 'one-night stands' without attachment (Justus, Finn, & Steinmetz,

2000; Testa & Collins, 1997). With this novel model of alcohol's effects on social relationships, we may be able to understand the factors that contribute to abnormal bonding behavior.

The design of these studies was based on published methods from other laboratories showing that 24 hours of cohabitation was sufficient for formation and expression of a partner preference even without mating (Williams, Carter, & Insel, 1992). However, there is great variation in reported cohabitation times needed for establishment of the partner preference (DeVries, et al., 1995; Williams, Carter, & Insel, 1992), and in our studies without mating, partner preference in control animals was not statistically significant, which enabled us to see enhancement of this behavior in animals consuming alcohol. The relatively large sample size used in these control groups (n=15) ensures greater power to detect a partner preference if there was one.

Compared to females, the effect of alcohol on partner preference in males appeared to be in the opposite direction in the first study with ovariectomized, sexually unreceptive stimulus females, but the lack of partner preference in the control group could not allow us to show that alcohol had an inhibitory effect. Thus, we conducted another experiment using EB-primed, sexually receptive females and showed that males formed a strong partner preference, and conclusively showed that alcohol access inhibited this preference. Further, the weak partner preference in the control group of the male study without mating allowed us to compare directly to the female study and show that the effects of alcohol on partner preference are not dependent on the strength of the bond; alcohol did not have the same effect on weak bonds in females and males. Thus, the

difference in the effects of alcohol must be due to sex differences rather than a facilitative effect on weak preferences and an inhibitory effect on strong preferences.

The lack of correlation between the length of the vasopressin receptor microsatellite and various behaviors tested here adds to the growing evidence that there is not a direct functional role of the length of the microsatellite in behavior (Mabry, et al., 2011; Ophir, Wolff, & Phelps, 2008; Solomon, et al., 2009). Instead, some of the previous literature supporting positive correlations between behavior and microsatellite length (Hammock, et al., 2005) might actually be due to using the length as a proximate measure for specific sequences that may influence expression levels and behavior, as has been suggested previously (Ophir, Campbell, et al., 2008).

We hypothesized that alcohol would directly affect the neural mechanisms required for pair bonding, but first wished to eliminate the possibility that alcohol affected other behaviors that independently affected bonding. We found that alcohol did not affect mating levels either in cohabitation or in the partner preference test for either sex (Fig. 2 A-C). Very little mating was observed in the female test, which was expected since female prairie voles reportedly begin to become sexually receptive about 24 hours following presentation of a male. Thus, differences in partner preference between alcohol-exposed voles and water-drinking controls could not be due to differences in mating behavior.

Subjects that self-administered alcohol during cohabitation also showed no differences from controls in activity level or aggression (Fig. 2). Male subjects did show more aggression toward the stranger stimulus animal than the partner, and females showed a trend toward the same effect, in accordance with other studies showing

selective aggression in prairie voles (Aragona, et al., 2006; Gobrogge, et al., 2007; Gobrogge, et al., 2009; Gobrogge & Wang, 2011; Insel, Preston, & Winslow, 1995; Wang, Hulihan, & Insel, 1997; Winslow, et al., 1993; K. A. Young, et al., 2011). Thus, while partner preference is affected by alcohol, selective aggression, another feature of the pair bond, is not affected. This indicates that alcohol acts on a specific and dissociable part of the pair bonding neurocircuitry to interfere with the development of partner preference but not selective aggression. This is also in accordance with studies showing that aggression requires similar but not entirely overlapping neural substrates: vasopressin and the V1aR is required for both, but the receptors are activated in the anterior hypothalamus for aggression and in the ventral pallidum and lateral septum for partner preference (Gobrogge, et al., 2007; Gobrogge, et al., 2009; Lim, Hammock, & Young, 2004; Liu, Curtis, & Wang, 2001). Importantly, the selective aggression following alcohol indicates that the lack of partner preference observed in males is not due to an inability to remember the partner or to distinguish the partner from the stranger.

The lack of effects of alcohol on behaviors other than partner preference that could have potentially interfered with bond formation supports our hypothesis that alcohol acts directly on neurobiological substrates involved in bond formation. To address this hypothesis, we assessed levels of a transcription factor and several neuropeptides that play a role in pair bond formation in prairie voles or other social behaviors, and are affected by alcohol intake. We performed these experiments in a separate group of paired prairie voles that had 24 hours of cohabitation with or without access to alcohol. We expected to find at least one brain region that showed different levels of immunoreactivity in alcohol drinkers compared to water drinkers, which was

dependent on the sex of the subjects (a statistical interaction between sex and alcohol treatment).

We examined levels of oxytocin and vasopressin, the two most commonly studied peptides in prairie vole pair bonding and in social behaviors in general, and found no effects of alcohol on regions known to be important for bonding behavior, and no sex-specific effects of alcohol.

NPY has been implicated in a variety of social behaviors including mating (Clark, Kalra, & Kalra, 1985; Painsipp, Herzog, & Holzer, 2008; Poggioli, et al., 1990; Sajdyk, et al., 2008; Sajdyk, Schober, & Gehlert, 2002; Walker, et al., 2009), as well as in alcohol use (Badia-Elder, et al., 2001; Ciccocioppo, et al., 2009; Kauhanen, et al., 2000; Thiele, et al., 1998), and in interactions between social and sexual behaviors and alcohol intake (Gilpin, et al.; Lindell, et al., 2010; Shohat-Ophir, et al., 2012 (NPF, homolog of NPY in Drosophila)). In addition, we have recently found differences in NPY levels in prairie voles compared to non-monogamous meadow voles that may be related to differences in social behaviors (Hostetler, Hitchcock, et al., 2012). There was an interaction between the effects of sex and alcohol treatment on NPY levels in the medial amygdala (MeA), with males showing greater fiber density with alcohol treatment compared to controls, but the decrease in alcohol-exposed females was not significantly different. These results were in the opposite direction of the partner preference expression, indicating that greater NPY levels in the MeA may be linked with lower partner preference. NPY in the amygdala is thought to be anxiolytic, and produced in response to stress to facilitate stress coping (see reviews: Heilig, et al., 2004; Kask, et al., 2002), which leads to the stress-related hypothesis explored below.

CRF is also important in the stress response through the hypothalamic-pituitary-adrenal (HPA) axis, but also acts in other brain regions. Here we found that alcohol decreased CRF levels in the ventral BNST. This was not sex-dependent, but there are sex differences in CRF receptor levels in the BNST. Lim et al. (2005) reported higher CRF receptor 2 binding in the BNST of male prairie and meadow voles compared to females, although this was in the medial caudal BNST. Thus, there is potential for the same change in CRF levels following alcohol exposure to have differential effects in males and females. The effect of CRF (intracerebroventricular: DeVries, et al., 2002; intra-nucleus accumbens: Lim, et al., 2007) on partner preference in males is a facilitation, opposite the effect of alcohol observed in these studies, in agreement with the lower levels of CRF observed in the BNST.

Other studies demonstrating the effects of stress on partner preference are sex-specific. Basal corticosterone levels are very high in prairie voles (Taymans, et al., 1997), and typically decrease in both sexes when male and female prairie voles meet (DeVries, Taymans, & Carter, 1997). This reduction of the corticosterone level is necessary for partner preference formation in female prairie voles, but a subsequent increase or return to baseline later during cohabitation is necessary for partner preference formation in males. Swim stress immediately prior to cohabitation, or administration of corticosterone prevents this partner preference in females but facilitates it in males (DeVries, et al., 1996), which is the opposite of what we observe in the present studies. Removal of the corticosterone response via adrenalectomy reverses the effect of stress, leading to the same behavioral effects observed in the present studies, where PP formation is facilitated

in female prairie voles but inhibited in males, in which case a stranger preference actually develops (DeVries, et al., 1995; DeVries, et al., 1996).

When we examined levels of Fos protein, an early transcription factor that can indicate cellular activation, we found two regions expressing an interaction between sex and treatment: the arcuate nucleus of the hypothalamus (Arc) and the centrally-projecting Edinger-Westphal nucleus (EWcp). The effect in the Arc indicated greater activity in alcohol-exposed females than controls, while the decrease in alcohol-exposed males was not significantly different than controls. This effect is in the same direction as the partner preference levels from the first set of experiments, indicating that greater Arc activation may be linked with greater partner preference. This finding indicates that a number of peptides highly expressed in the Arc, beyond those studied here, may be of interest in future studies. Importantly, there was no difference between groups in the number of NPY-expressing cells in the Arc, so any differences in activation indicated by Fos are not likely due to differences in cell number.

The effect in the EWcp indicated greater Fos immunoreactivity in alcohol-exposed males than controls, and a decrease in alcohol-exposed females, though these differences were not statistically significant. This effect is in the opposite direction as the partner preference levels, indicating that greater EWcp activation and release of neuropeptides produced in this region, such as urocortin 1, may be linked with lower partner preference. Importantly, there were no differences in the number of urocortin-positive cells in the EWcp, indicating that differences in Fos levels were in fact due to differences in activity rather than cell number. It should be noted that Fos induction following a stimulus is extremely time-sensitive and protein levels peak around two hours

after presentation of the stimulus. In addition, Fos induction is not sensitive only to a particular stimulus. Thus, it is possible that timing of the stimulus presentation (such as alcohol self-administration) was different between groups or sexes, leading to the observed interactions, rather than a real biological reaction, particularly since we did not observe a sex difference in Fos expression following alcohol drinking in a previous study (Chapter 1, Anacker, et al., 2011). For these reasons, we place more weight on the results from experiments with neuropeptides in forming our explanations and hypotheses for future experiments.

Based on our results and previous literature on pair bonding in prairie voles, we can formulate a general hypothesis for the mechanism of effects of alcohol on pair bond formation, with other minor hypotheses for specific molecular mechanisms.

Alcohol acts as an anxiolytic, facilitating partner preference for females and inhibiting it for males. The findings from immunohistochemistry indicate effects that also relate to the stress/anxiety response. We further hypothesize that the increase in NPY in the amygdala of alcohol-exposed males acts as an anxiolytic, thereby inhibiting their ability to form a pair bond; similarly, the increase in Fos in the EWcp of males may indicate greater levels of urocortin production, which is also thought to be a stress-coping mechanism; finally, the decreased levels of CRF within the BNST after alcohol exposure may indicate decreased activation of the stress pathway in response to alcohol, leading to facilitation of partner preference for females and inhibition for males.

The generation of these hypotheses leads to clear new directions for research in the fields of alcohol and social bond formation, and especially for the interaction between the two. In addition to testing these potential neuropeptidergic mechanisms for

influencing behavior, a number of questions can be raised for future behavioral experiments. For example, it is unclear from these studies whether alcohol affects the formation of the pair bond or the expression of the partner preference; it is possible that withdrawal from alcohol at the time of the partner preference testing led to the alteration in expression of partner preference. However, we do not believe that withdrawal plays a role since voles typically consume alcohol in bouts throughout the day and night and were unlikely to experience a large enough binge to result in acute withdrawal or 'hangover' symptoms. Another interesting test would be to examine the effects of alcohol on an established pair bond. Most of what is known about pair bonds relates to the formation of the bond, which was the reason we targeted the formation period in these experiments. However, the effects of alcohol on established social bonds are far more prevalent and devastating among humans than effects on forming relationships, and so it will be important to better understand these interactions and how to facilitate rehabilitation of bonds in the future. It will also be important to test whether the effects on social affiliations are specific to alcohol or would extend to other drugs or rewarding substances.

With these and future studies of the effects of alcohol on pair bonds in prairie voles, we aim to model important aspects of human alcohol abuse that require attention. By understanding the neural and behavioral mechanisms underlying problematic effects of alcohol in this animal model, we will be able to conceive of and test potential drug, behavior, and combination therapies to decrease alcohol abuse and improve social functioning.

Table 1. Alcohol consumption during 24 hour cohabitation

*Subject and partner were housed together so the preference and dose were determined by dividing the total volume consumed in half and assigning it to each animal.

	Females			Males (no mating)			Males (with mating)		
	EtOH Volume (mL)	Preference	Dose (g/kg)	EtOH Volume (mL)	Preference	Dose (g/kg)	EtOH Volume (mL)	Preference	Dose (g/kg)
Subject*	5.1 ± 0.5	0.58 ± 0.057	12.5 ± 1.0	5.8 ± 0.5	0.74 ± 0.028	11.2 ± 0.81	7.3 ± 1.1	0.73 ± 0.072	14.3 ± 1.9
Partner*	5.1 ± 0.5	0.58 ± 0.057	8.93 ± 0.66	5.8 ± 0.5	0.74 ± 0.028	12.6 ± 0.77	7.3 ± 1.1	0.73 ± 0.072	17.4 ± 2.5
Stranger	4.7 ± 0.8	0.55 ± 0.087	10.0 ± 1.4	5.3 ± 0.5	0.67 ± 0.053	6.44 ± 1.1	4.2 ± 0.7	0.48 ± 0.088	10.2 ± 2.0

Table 2. Number of subjects exhibiting a preference (at least twice as much time spent huddling with one stimulus animal as the other).

	Females			Males (no mating)			Males (mating)		
	Partner Pref	No Pref	Stranger Pref	Partner Pref	No Pref	Stranger Pref	Partner Pref	No Pref	Stranger Pref
Water	10	2	3	8	3	4	7	0	0
Ethanol	14	0	1	9	2	5	3	2	3

Table 3. Means and statistical effects of alcohol during cohabitation on neuropeptides and Fos

Regions examined are listed on the left, with corresponding levels of immunoreactivity (mean ± SEM) for each group (M, male; F, female; W, water, E, ethanol). The p-value generated from a two-way ANOVA is listed for the effect of either sex, alcohol, or the interaction between these factors, on the level of immunoreactivity. For cells (left), the number of immunoreactive cells was counted. For fibers (right), the number of fibers was counted except in the case of CRF and NPY, where the density of immunoreactivity was measured using ImageJ. Significant p values ($\alpha=0.05$) are highlighted in yellow, while statistical trends ($0.05<p<0.1$) are highlighted in orange. Black regions indicate that either cells or fibers were either not present or not analyzed for the given region. The direction of the effect determined by post-hoc t-tests is listed in the column on the far right.

Abbreviations of brain regions: AHA, anterior hypothalamic area; Arc, arcuate nucleus of the hypothalamus; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis (d, dorsal; v, ventral); CeA, central nucleus of the amygdala; CTX, cortex (a, anterior; p, posterior); EWcp, centrally-projecting Edinger-Westphal nucleus; LDT, laterodorsal tegmental area; LS, lateral septum (D, dorsal; I, intermediate; V, ventral); MeA, medial amygdala; MGN, medial geniculate nucleus; Nac, nucleus accumbens; OB, olfactory bulb; PVN, paraventricular nucleus of the hypothalamus; MPA, medial preoptic area; MR, median raphe; SON, supraoptic nucleus; VMH, ventral medial hypothalamus; VP, ventral pallidum.

	Mean ± SEM CRF-positive cells				CRF cell effects			Mean ± SEM CRF-positive fiber density				CRF fiber effects			*Direction of significant effect*
Brain Region	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	*F: Female; M: Male;*
OB	58.5 ± 12.2	37.4 ± 4.59	54.4 ± 15.8	45.1 ± 9.47	0.18	0.874	0.596								*E: Ethanol; W: Water*
CeA	4.10 ± 1.13	1.75 ± 0.323	6.30 ± 2.00	5.50 ± 1.61	0.32	0.0727	0.62	151 ± 4.14	145 ± 3.22	148 ± 2.73	145 ± 2.86	0.21	0.651	0.597	
BNST	6.88 ± 1.42	14.6 ± 9.74	10.3 ± 1.44	8.94 ± 2.80	0.554	0.833	0.402	317 ± 7.58	312 ± 3.52	301 ± 5.22	301 ± 5.61	0.662	0.0227	0.72	*W>E*
dBNST								159 ± 6.06	154 ± 3.29	151 ± 3.82	148 ± 2.88	0.381	0.123	0.804	
vBNST								158 ± 3.10	158 ± 1.88	150 ± 2.50	150 ± 4.79	0.947	0.0129	0.905	*W>E*
MGN	10.1 ± 1.35	9.07 ± 1.30	14.5 ± 1.94	8.17 ± 1.12	0.0174	0.245	0.0815								*M>F*
MPA	18.1 ± 3.90	12.8 ± 3.01	15.7 ± 3.16	19.7 ± 6.35	0.883	0.601	0.298	147 ± 2.98	151 ± 2.41	142 ± 3.35	142 ± 3.41	0.569	0.0357	0.57	
PVN	41.7 ± 7.27	36.4 ± 6.20	36.9 ± 7.14	41.8 ± 7.94	0.984	0.963	0.485								
MR	18.2 ± 3.21	12.1 ± 3.16	21.9 ± 3.69	13.3 ± 3.10	0.0338	0.47	0.716	125 ± 5.87	117 ± 2.52	121 ± 2.12	110 ± 4.21	0.0283	0.181	0.713	*M>F*
Cortex	150 ± 11.3	130 ± 8.83	145 ± 15.4	151 ± 19.2	0.616	0.597	0.381								
aCTX	147 ± 12.0	125 ± 9.03	140 ± 14.6	140 ± 19.7	0.436	0.774	0.461								
pCTX	137 ± 10.9	137 ± 10.9	150 ± 18.1	165 ± 21.9	0.967	0.462	0.342								

	Mean ± SEM NPY-positive cells				NPY cell effects			Mean ± SEM NPY-positive fiber density				NPY fiber effects			
Brain Region	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	
Nac Core	55.8 ± 10.3	50.8 ± 5.16	50.1 ± 6.46	42.6 ± 4.83	0.392	0.345	0.863	53.8 ± 8.49	51.0 ± 5.95	41.8 ± 5.91	44.4 ± 6.52	0.991	0.177	0.694	
Nac Shell	52.8 ± 6.79	72.9 ± 10.3	52.4 ± 9.16	56.1 ± 8.22	0.183	0.334	0.357	47.6 ± 7.95	57.8 ± 8.00	50.4 ± 8.63	53.9 ± 6.10	0.393	0.945	0.671	
LS								86.3 ± 9.87	92.1 ± 13.5	93.6 ± 10.9	75.2 ± 9.43	0.582	0.671	0.29	
LSD								11.3 ± 2.47	16.8 ± 3.00	14.8 ± 3.60	10.4 ± 2.34	0.861	0.635	0.111	
LSI								40.0 ± 8.71	44.8 ± 8.74	47.1 ± 6.83	40.6 ± 5.85	0.912	0.849	0.469	
LSV								36.2 ± 5.92	27.1 ± 3.12	31.7 ± 2.78	24.2 ± 2.63	0.036	0.331	0.821	*M>F*
VP	28.8 ± 5.19	39.9 ± 5.13	25.6 ± 5.85	37.6 ± 4.41	0.0348	0.607	0.929	30.9 ± 6.23	41.1 ± 4.01	28.6 ± 3.87	39.1 ± 5.13	0.041	0.66	0.974	*F>M*
CeA								228 ± 14.1	235 ± 10.9	230 ± 7.97	228 ± 14.1	0.809	0.892	0.754	
BLA								238 ± 11.4	237 ± 10.9	241 ± 6.45	238 ± 9.14	0.825	0.814	0.889	
MeA								229 ± 8.47	263 ± 10.1	275 ± 15.8	248 ± 13.5	0.779	0.225	0.0278	*ME>MW*
BNST								573 ± 16.8	548 ± 12.2	555 ± 19.1	555 ± 14.6	0.462	0.739	0.433	
dBNST								283 ± 8.12	270 ± 5.31	273 ± 9.55	270 ± 6.46	0.299	0.464	0.513	
vBNST								290 ± 9.56	278 ± 7.86	282 ± 10.3	285 ± 7.56	0.617	0.967	0.43	
PVN								306 ± 12.0	307 ± 11.0	290 ± 20.8	278 ± 17.2	0.691	0.152	0.678	

	Mean ± SEM oxytocin-positive cells				Oxytocin cell effects			Mean ± SEM oxytocin-positive fibers				Oxytocin fiber effects			
Brain Region	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	
Nac Core								16.6 ± 4.31	13.8 ± 3.47	8.61 ± 1.71	10.2 ± 2.96	0.859	0.0836	0.513	
LS								25.3 ± 4.70	19.7 ± 4.58	17.1 ± 3.84	19.7 ± 3.68	0.72	0.334	0.334	
LSD								1.94 ± 0.586	2.67 ± 0.745	1.89 ± 0.470	1.39 ± 0.470	0.849	0.258	0.299	
LSI								22.3 ± 4.01	16.3 ± 4.01	15.1 ± 3.45	16.9 ± 3.48	0.582	0.392	0.3045	
LSV								1.11 ± 0.371	0.722 ± 0.237	0.889 ± 0.361	1.33 ± 0.577	0.946	0.635	0.312	
VP								13.2 ± 3.99	8.39 ± 1.43	10.3 ± 2.69	10.5 ± 2.48	0.411	0.89	0.378	
BNST	2.67 ± 0.682	2.44 ± 0.643	2.28 ± 0.590	2.78 ± 0.693	0.833	0.966	0.584	113 ± 23.2	88.2 ± 15.4	77.2 ± 14.9	77.6 ± 17.4	0.505	0.207	0.491	
dBNST	1.00 ± 0.236	1.44 ± 0.562	0.833 ± 0.264	1.17 ± 0.408	0.366	0.549	0.85	16.2 ± 3.02	13.3 ± 1.75	11.1 ± 1.90	10.2 ± 1.98	0.408	0.0751	0.664	
vBNST	1.67 ± 0.692	1.00 ± 0.425	1.44 ± 0.460	1.81 ± 0.707	0.798	0.614	0.378	96.8 ± 20.4	74.9 ± 14.7	69.8 ± 12.4	68.8 ± 15.5	0.48	0.31	0.521	
MPA								42.1 ± 7.66	36.9 ± 7.28	36.8 ± 7.37	29.9 ± 6.89	0.418	0.409	0.91	
PVN	58.5 ± 5.14	54.7 ± 5.3	58.8 ± 7.37	53.3 ± 8.09	0.485	0.934	0.9								
SON	15.9 ± 2.44	19.2 ± 3.17	17.3 ± 3.27	15.7 ± 2.88	0.784	0.713	0.41								
AHA	0.778 ± 0.602	0.00 ± 0.00	0.00 ± 0.00	0.167 ± 0.118	0.326	0.326	0.133								
LDT								66.7 ± 9.79	45.9 ± 8.08	37.3 ± 5.16	39.6 ± 9.75	0.281	0.0412	0.179	*W>E*

	Mean ± SEM urocortin-positive cells				Urocortin 1 cell effects		
Brain Region	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction
Ewcp	52.4 ± 5.59	45.9 ± 5.38	51.5 ± 4.55	50.2 ± 4.43	0.443	0.734	0.61

	Mean ± SEM vasopressin-positive cells				Vasopressin cell effects			Mean ± SEM vasopressin-positive fiber number				Vasopressin fiber effects			
Brain Region	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	
LS								15.9 ± 6.42	10.1 ± 3.05	22.8 ± 9.55	11.9 ± 4.26	0.195	0.495	0.686	
LSD								1.94 ± 1.13	1.11 ± 0.570	1.22 ± 0.641	0 ± 0	0.156	0.204	0.785	
LSI								13.9 ± 6.66	9.00 ± 2.77	21.6 ± 9.30	11.9 ± 2.77	0.252	0.403	0.702	
LSV								0.056 ± 0.056	0 ± 0	0 ± 0	0 ± 0	0.325	0.325	0.325	
VP								0.222 ± 0.147	0.278 ± 0.147	0.556 ± 0.227	0.333 ± 0.167	0.637	0.275	0.434	
BNST	1.06 ± 0.460	1.61 ± 0.462	1.00 ± 0.479	2.33 ± 0.777	0.102	0.556	0.493	16.3 ± 5.24	8.22 ± 2.60	16.9 ± 4.07	10.7 ± 1.62	0.0595	0.679	0.792	
dBNST	0.667 ± 0.167	0.722 ± 0.290	0.667 ± 0.400	0.889 ± 0.498	0.703	0.819	0.819	7.22 ± 3.02	3.17 ± 0.682	5.94 ± 1.37	4.39 ± 0.832	0.117	0.987	0.478	
vBNST	0.389 ± 0.389	0.889 ± 0.398	0.333 ± 0.186	1.50 ± 0.582	0.0467	0.495	0.414	9.11 ± 2.50	5.06 ± 2.58	10.9 ± 3.28	6.33 ± 1.46	0.0976	0.544	0.914	*F>M*
MPA								2.00 ± 0.417	2.00 ± 0.559	2.67 ± 0.692	2.00 ± 0.736	0.591	0.591	0.591	
PVN	41.8 ± 8.85	46.1 ± 7.45	29.3 ± 4.98	37.9 ± 5.77	0.359	0.143	0.756								
SON	19.0 ± 3.71	14.3 ± 2.43	11.7 ± 1.68	16.0 ± 2.58	0.951	0.302	0.105								
AHA	6.33 ± 3.38	2.17 ± 0.786	1.33 ± 0.702	2.28 ± 1.04	0.389	0.195	0.176								
LDT								2.33 ± 1.00	2.06 ± 1.05	2.56 ± 0.503	0.889 ± 0.298	0.223	0.55	0.381	

	Mean ± SEM Fos-positive cells				Fos cell effects			
Brain Region	M-W	F-W	M-E	F-E	Sex	Alcohol	Interaction	
Nac Shell	38.3 ± 9.90	23.8 ± 4.80	49.2 ± 15.0	30.8 ± 6.42	0.103	0.37	0.845	
LSV	46.9 ± 18.0	27.2 ± 7.76	25.6 ± 12.0	32.2 ± 14.2	0.634	0.55	0.338	
MeA	15.9 ± 5.20	17.4 ± 3.26	26.2 ± 7.44	14.1 ± 7.40	0.397	0.57	0.28	
vBNST	14.6 ± 5.75	18.3 ± 5.16	17.3 ± 3.88	17.3 ± 5.88	0.724	0.871	0.724	
MPOA	28.5 ± 5.14	30.3 ± 12.5	30.8 ± 5.84	19.1 ± 7.69	0.54	0.581	0.405	
PVN	5.75 ± 2.08	4.88 ± 1.89	7.67 ± 2.38	11.6 ± 5.94	0.683	0.249	0.519	
VMH	22.9 ± 10.6	23.1 ± 10.3	11.9 ± 4.33	8.00 ± 3.58	0.812	0.094	0.785	
Arc	2.00 ± 1.16	1.29 ± 0.969	1.22 ± 0.662	9.50 ± 3.78	0.091	0.096	0.047	*FE>FW*
Ewcp	5.00 ± 2.13	5.33 ± 2.67	13.9 ± 4.39	0.667 ± 0.553	0.0278	0.456	0.021	*M>F*

Figure 1. Partner preference in female and male voles exposed to water or alcohol during cohabitation

A) Females showed a trend for a partner preference under control conditions (n=15), and this effect was enhanced in pairs exposed to alcohol (n=15). B) Males paired with sexually non-receptive females did not show a significant partner preference with (n=16) or without (n=15) alcohol. C) Males that mated with estrogen-primed females during cohabitation (n=7) showed a significant partner preference, while males that mated and had access to alcohol did not (n=8). *p<0.0001

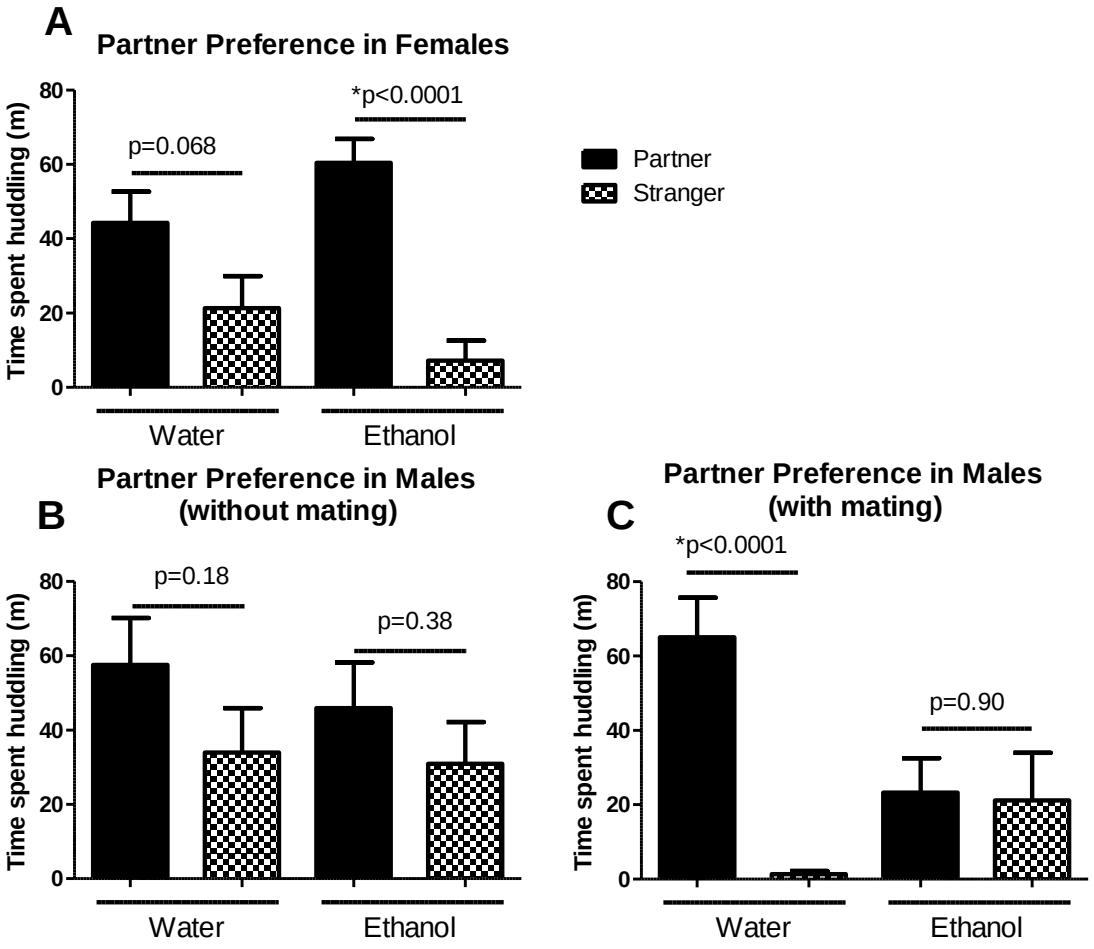


Figure 2. Mating, activity, and aggression during 24 hour cohabitation and PPT did not differ with alcohol access

A) Mating bouts by female subjects during the PPT. B) Mating by male subjects during the first and last two hours of cohabitation, and C) PPT. D) Activity levels in males and females in the PPT. E,F) Bouts of aggression during the first and last two hours of cohabitation and G,H) PPT in males (E,G) and females (F,H).

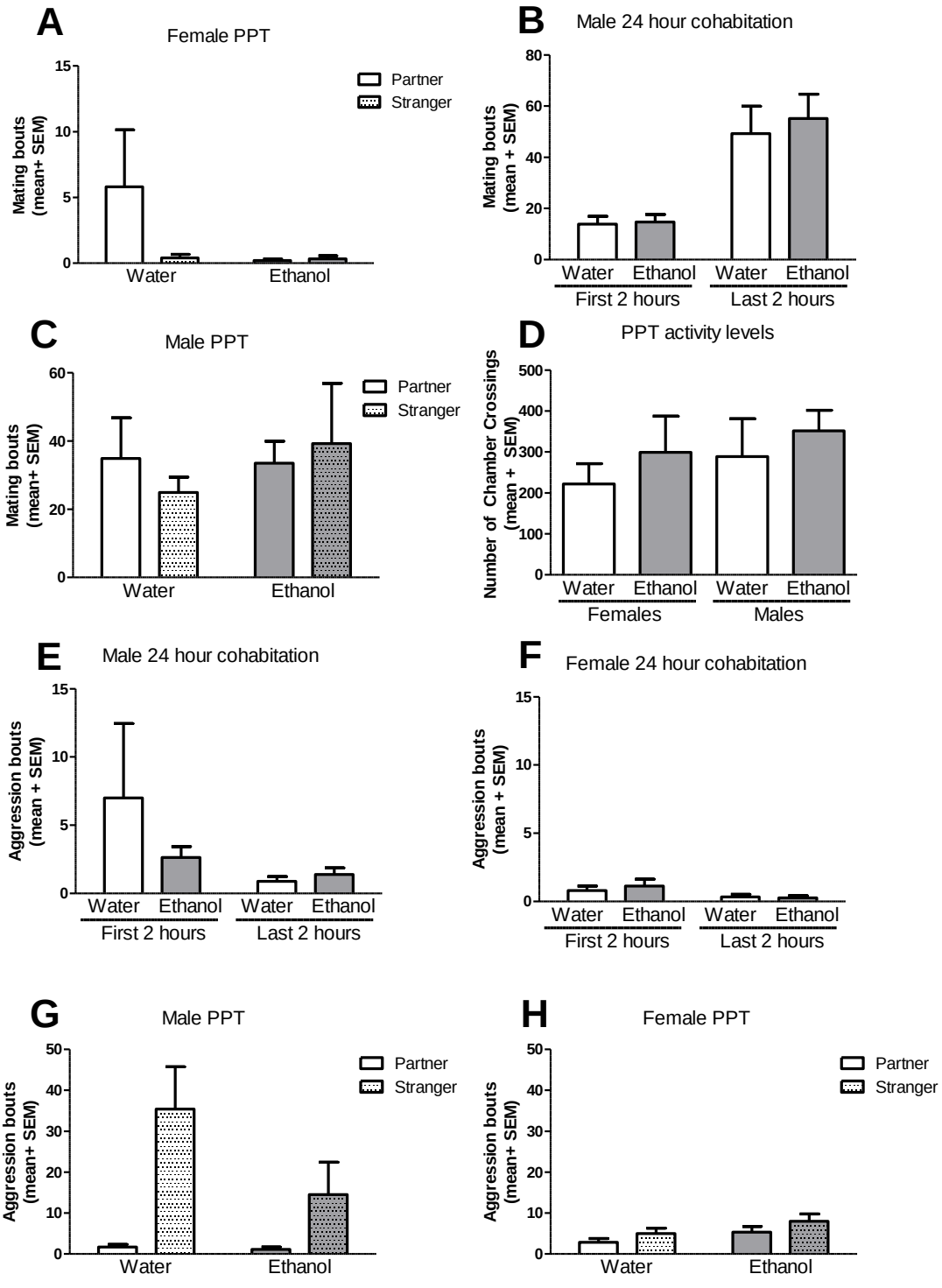

A
Female PPT
Mating bouts (mean+ SEM)
Partner
Stranger
Water
Ethanol
B
Male 24 hour cohabitation
Mating bouts (mean + SEM)
Water
Ethanol
First 2 hours
Last 2 hours
C
Male PPT
Mating bouts (mean+ SEM)
D
PPT activity levels
Number of Chamber Crossings (mean + SEM)
Females
Males
E
Male 24 hour cohabitation
Aggression bouts (mean + SEM)
F
Female 24 hour cohabitation
G
Male PPT
Aggression bouts (mean+ SEM)
H
Female PPT

Figure 3. Results of immunohistochemistry for Fos or neuropeptides

Regions shown exhibit a significant interaction between sex and alcohol, or a significant effect of alcohol in a region with known sex differences in receptor levels. A) The number of Fos cells is significantly higher in the arcuate nucleus of females with access to alcohol. B) The number of Fos cells in the EW is significantly higher in males and there is a statistical trend toward greater cell numbers in the EW of males with access to alcohol than those without. C) The density of NPY fibers in the medial amygdala is higher in males with access to alcohol than males without. D) The density of CRF fibers in the vBNST is significantly lower in voles with access to alcohol. *p<0.05

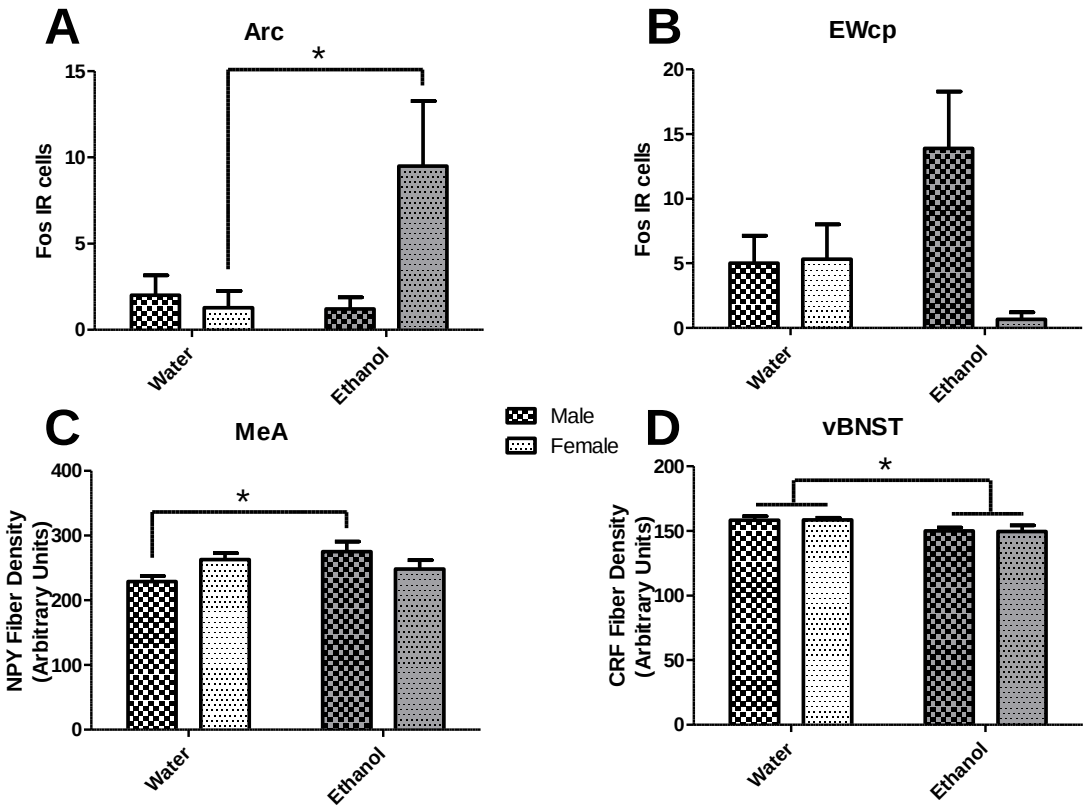


Figure 4. Representative photomicrographs

A) The number of Fos cells is greater in the arcuate nucleus of females with access to alcohol (right) than without (left). B) The number of Fos cells in the EWcp is greater in males with access to alcohol (right; arrows indicate where Fos-positive cells are located in the EW) compared to males without (left), and are significantly higher in males (top) than females (bottom). C) NPY immunoreactivity in the medial amygdala is greater in males with access to alcohol (right) than males without (left). D) The density of CRF fibers in the vBNST is significantly lower in male voles with access to alcohol (right) than without (left). White dashed lines outline the regions quantified for density of immunoreactivity.

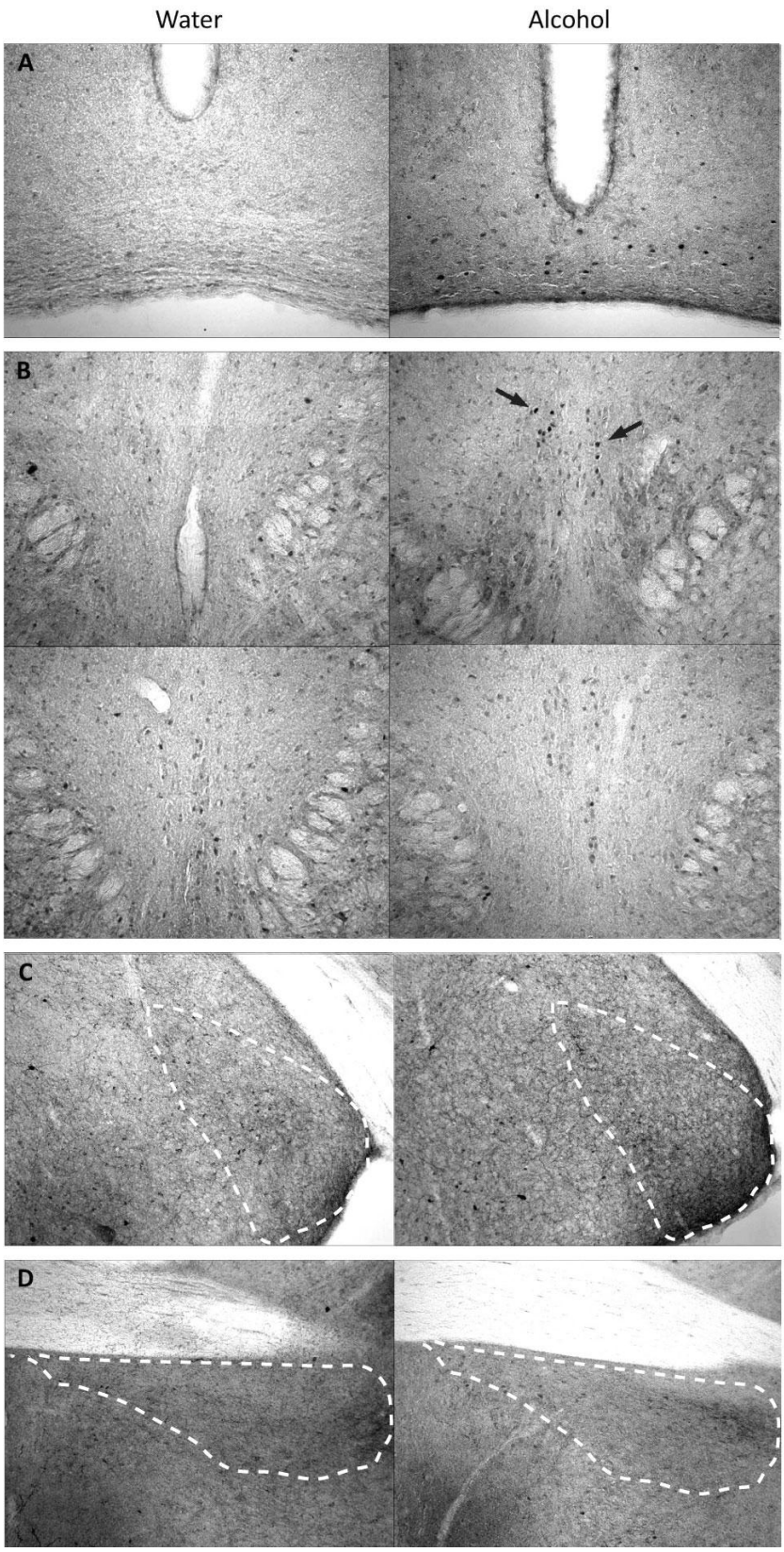

Water
Alcohol
A
B
C
D

Figure 5. Representative photomicrographs of oxytocin, urocortin, and vasopressin

There were no statistical interactions between sex and alcohol for any regions quantified for oxytocin, urocortin, or vasopressin-positive cells or fibers. Examples of high-expression regions are depicted. A) Oxytocin cells and projections from the PVN in female voles with (right) or without (left) access to alcohol. B) Urocortin cells in the EWcp in male voles with (right) or without (left) access to alcohol. C) Vasopressin cells and projections from the PVN in male voles with (right) or without (left) access to alcohol.

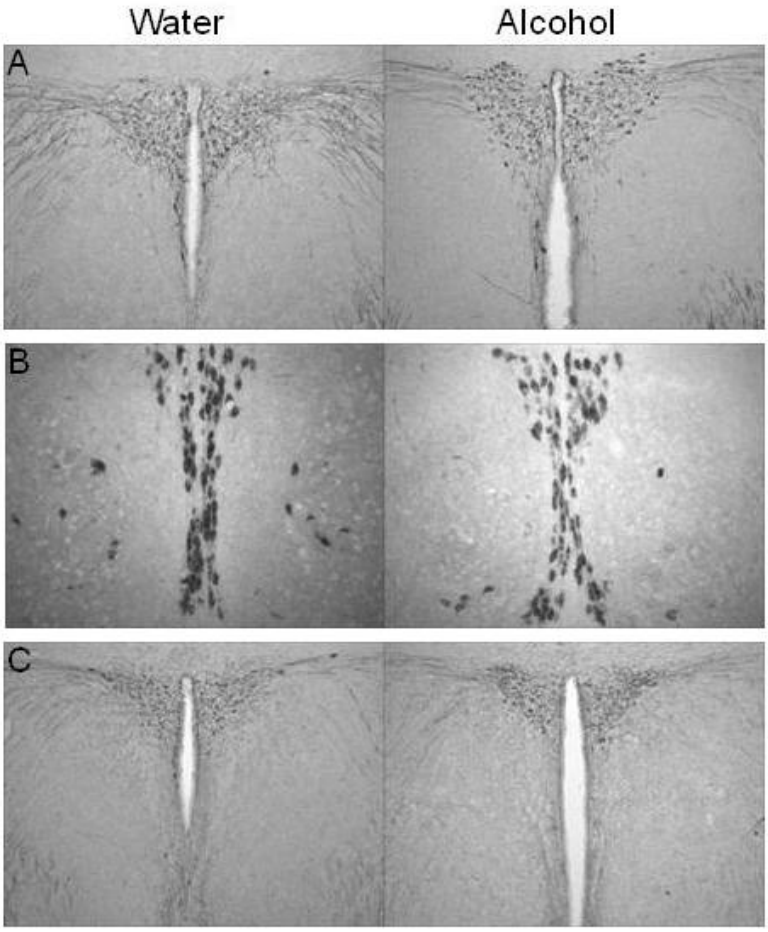


Figure 6. Average microsatellite length and behavior in female experiment

A) No correlation between MS length and dose of alcohol consumed during cohabitation among subjects, partners and strangers in the female test. B) No correlation between MS length and preference for alcohol during cohabitation among subjects, partners and strangers in the female test. C) No correlation between MS length and time female subjects spent huddling with the partner in the female test. D) No correlation between MS length and time female subjects spent huddling with the stranger in the female test.

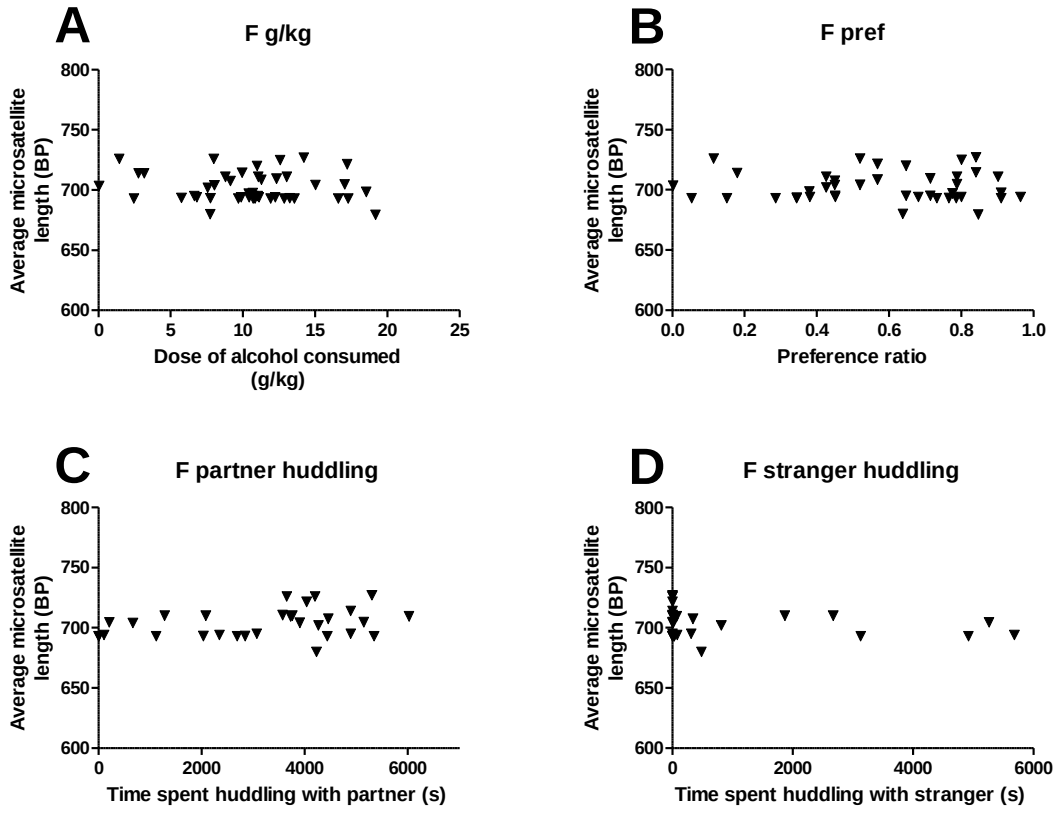
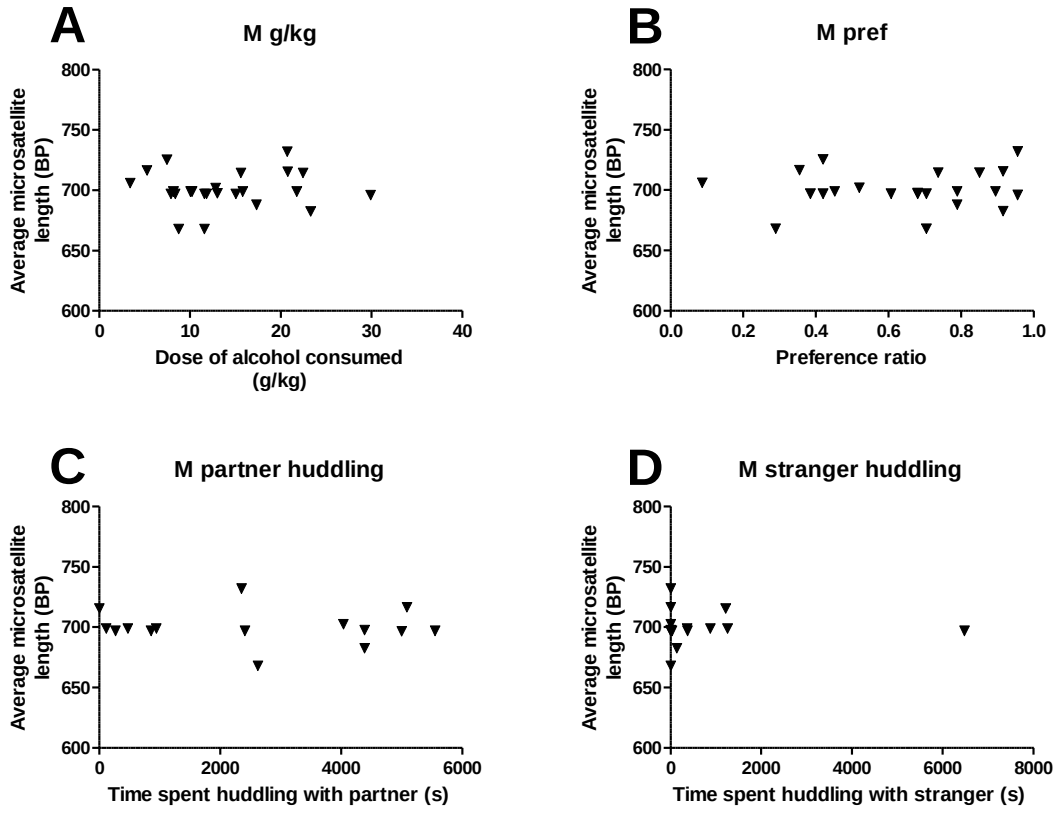


Figure 7. Average microsatellite length and behavior in male experiment with mating A) No correlation between MS length and dose of alcohol consumed during cohabitation among subjects, partners and strangers in the male test. B) No correlation between MS length and preference for alcohol during cohabitation among subjects, partners and strangers in the male test. C) No correlation between MS length and time male subjects spent huddling with the partner in the male test. D) No correlation between MS length and time male subjects spent huddling with the stranger in the male test.



GENERAL DISCUSSION

The main goal of this dissertation was to explore the interactions between social behaviors and alcohol drinking in prairie voles, in order to characterize the types of human behaviors that can be modeled in this species. A further goal was to examine the first of many possible genetic and neurobiological factors that could play a role in these behaviors. The third goal was to determine whether circumstances of the social environment could positively change alcohol drinking in prairie voles. These goals were achieved through a number of experiments, some focusing on the effects of peer social relationships on alcohol intake (Chapters 1-3), and others focusing on the effects of alcohol drinking on the development of male-female pair bonds (Chapter 4).

Effects of social affiliations on alcohol

Multiple lines of evidence indicate that adult same-sex sibling or non-sibling pairs of prairie voles can directly influence one another's alcohol intake (Figure 1). First, we demonstrated that prairie vole siblings prefer alcohol more when they are housed together than when they are separated and housed in isolation. In the same experiment, we observed that the siblings matched their average alcohol intake, but only when housed together so that they could directly influence each other. In further studies we found that high drinkers paired with low-drinking peers would typically decrease their alcohol intake, although in some pairs the low drinker would increase alcohol intake instead.

At first these latter findings may seem contradictory since it would indicate a lower overall level of drinking when voles are paired, contrary to the findings of the first study. However, there is one major procedural difference between these studies that may account for this difference: in the first study, prairie voles were introduced to alcohol

either in isolation or in pair housing, while in the second study they were all introduced to alcohol in isolation and only paired after establishing their own drinking patterns. It is possible that the environment in which a subject is introduced to alcohol can affect the perception of the reward or other stimulus properties of alcohol. Therefore, the effect of a peer may be different at initiation and continued drinking in pairs than as an influence after drinking has been established. In these studies it appears that the presence of a peer during initial exposure to alcohol increases the reward value and/or the propensity to drink, while the presence of a peer after drinking levels have been established modulates the existing level of drinking, perhaps even decreasing the perceived value of alcohol. These hypotheses can be tested by manipulating whether certain aspects of the peer (i.e. whether the peer has access to alcohol or not) change the subject's alcohol drinking, or testing the rewarding properties of alcohol under different social circumstances (i.e. using conditioned place preference for alcohol which has recently been demonstrated in prairie voles using a 2 g/kg dose (Stevenson, et al., 2012)). The effects of these different circumstances will be important to consider for making distinctions between prevention and treatment strategies.

We have determined that the mechanism for changing or matching drinking levels is not through mimicking drinking patterns and must be due to some other form of social communication or observation, or to an environmental cue or stimulus. Further, the likelihood of decreasing alcohol intake when paired with a low drinker appears to be dependent on the flexibility or instability of alcohol drinking behaviors established in isolation. This finding is particularly important to consider for implications for treatment. If flexible drinking patterns are also indicative of the propensity to decrease alcohol

drinking with positive social influence in humans, this could lead to detection and intervention strategies for certain individuals. First, an interview-based experiment could determine whether sub-populations of people with alcohol use disorders exhibit characteristic variation in drinking patterns, such as fewer drinks some days or weeks compared to others, even without a conscious effort to decrease. Then a subsequent experiment could determine whether social influences in support of reduced drinking are more effective at decreasing alcohol intake in these sub-populations. If this was the case, it would be a way for clinicians to assess and prescribe a treatment with the most potential for certain patients.

Interestingly, other studies from our laboratory have demonstrated that the peer influences on alcohol drinking described in this dissertation are not present in male-female prairie vole pairs, indicating that there is a difference in the way that different types of social affiliations can influence alcohol intake (Hostetler, Anacker, et al., 2012). The strong peer influence is similar to behavior observed in humans. Peers and friend networks, particularly in adolescence and young adulthood, can have a direct impact on alcohol drinking levels (Norton, Lindrooth, & Ennett, 1998; Park, Sher, & Krull, 2008). While circumstances such as marriage or divorce can impact alcohol drinking (reviewed by Leonard & Rothbard, 1999), as can the level of a spouse's drinking, the peer networks remain a significant factor for both spouses (Homish & Leonard, 2008; Leonard & Homish, 2008; Leonard & Mudar, 2000).

It is important to note that in all of our studies of the effects of social influences on alcohol drinking, we have not tested whether subjects within a pair exhibit evidence of a bond. In some cases such as Chapter 2-3 where the subjects within a pair were

introduced for the first time within the experiment, they were not expected to have formed a bond immediately. Under these circumstances, the social influences on drinking were evident even without the existence of an established bond. Indeed, in a pilot study for Chapter 2, we found that whether voles were paired with a stranger or with a sibling they exhibited the same social facilitation of alcohol drinking compared to isolated voles, and this was evident from the first day of paired drinking (Anacker and Ryabinin, unpublished data). These data suggest either that social bonds form very quickly, even in same-sex pairs of prairie voles, or that the social effects on alcohol drinking do not require formation of a bond, *per se*. Therefore, in other cases such as Chapter 1 where siblings were housed together since weaning and the study by Hostetler et al. (2012) where the male and female were paired for five days before testing, it is also possible for the subjects to experience social influences without having developed a specific pair bond. Combined, these studies demonstrate that it is not the strength of the bond, but rather the type of social relationship that determines the degree of influence on alcohol drinking. Interestingly, while the male-female pairs studied by Hostetler et al. (2012) did not show the same social facilitation of alcohol drinking as did the sibling pairs studied in Chapter 1, they did exhibit similar decreases in preference for quinine compared to isolated animals. Therefore, we concluded that the intake of some substances may be responsive to the general social environment and not to specific types of influences, especially for an aversive substance that may be advantageous to avoid (Hostetler, Anacker, et al., 2012).

In addition to behavioral interactions, these experiments examined some of the biological aspects of alcohol drinking in these social animals. When given injections of

alcohol, prairie voles reach a similar peak in blood ethanol concentration as do C57BL/6J mice and have comparable rates of ethanol elimination from the blood. In addition, prairie voles given short access to alcohol show increased levels of the transcription factor c-Fos in the centrally-projecting Edinger-Westphal nucleus, a reliable sign of alcohol exposure in other species. In a separate study, we also showed that administration of naltrexone, an opioid receptor antagonist, before access to alcohol, decreased alcohol intake and Fos levels in the EWcp (Anacker & Ryabinin, 2010). Naltrexone decreases alcohol intake in humans (Kiefer, et al., 2003; Volpicelli, et al., 1992), as well as in other laboratory animal species (Kamdar, et al., 2007). These results indicate that alcohol acts through similar mechanisms and pathways in prairie voles and other mammals studied, and that they are responsive to pharmacological treatments to reduce alcohol drinking with comparable efficacy as well. These findings demonstrate a certain degree of construct and predictive validity for the prairie vole model of alcohol drinking.

Taken together, we have demonstrated that prairie voles model specific aspects of human behavior that have not previously been modeled in the laboratory, while also showing similarities in the physiological effects of alcohol when compared to humans or other mammalian species. Specifically, prairie voles can be used to model social facilitation of alcohol drinking and the effect of a direct peer influence, especially to decrease alcohol intake.

Effects of alcohol on social affiliations

In examination of the effects of alcohol on the formation of pair bonds between male and female prairie voles, we demonstrated that alcohol has opposite effects on each sex. Access to alcohol during cohabitation led female prairie voles to form a stronger preference for the partner over a stranger, while leading the males to demonstrate no

preference for the partner, in contrast with their water-consuming controls. These sex-specific effects are similar to the effects of adrenalectomy, which leads to decreased levels of corticosterone and hypothalamic-pituitary-adrenal (HPA) axis activity that facilitate partner preference formation in female prairie voles but prevent it in males (DeVries, et al., 1995; DeVries, et al., 1996). In contrast, these are opposite the effects of stress or corticosterone administration, which prevent partner preference formation in females but facilitate it in males. These combined findings lead in part to the hypothesis that alcohol acts as an anxiolytic to produce the observed behaviors. The anxiolytic effects of alcohol are often cited as a reason people choose to drink (Book & Randall, 2002; Carrigan & Randall, 2003).

It should be noted that many studies have shown that administration of alcohol in fact increases HPA axis activity in rodents and humans (Cicero, 1981), and even to a greater degree in females than males (Ogilvie & Rivier, 1996, 1997), which would contradict our hypothesis that alcohol decreases stress levels. However, in our studies prairie voles were allowed to self-administer alcohol, and there is evidence that this mode of alcohol administration does not elicit the stress response that other modes (intraperitoneal injection or intragastric administration) do (K. Ogilvie, S. Lee, & C. Rivier, 1997).

A schematic representation of the results of these studies with some possible underlying mechanisms, including the stress and anxiety hypothesis put forth, is shown in Figure 2. While we did not explicitly test any causal mechanisms involved in stress, anxiety, or alcohol drinking, we can speculate about the possibilities. First, as suggested in Figure 2, it is possible that alcohol self-administration during cohabitation decreases

activity of the HPA axis (Pohorecky, et al., 1980; Sillaber & Henniger, 2004), which shows high basal activity in prairie voles (Taymans, et al., 1997). This would lead to a decrease in release of CRF and corticosterone, opposing the effect of a typical stressor. In this case, it would be expected that these effects would cause the observed sex differences in the response to alcohol, since a decrease in corticosterone facilitates pair bonding in females and inhibits it in males (DeVries, et al., 1995; DeVries, et al., 1996; DeVries, Taymans, & Carter, 1997).

Another possibility, which is not mutually exclusive with the first, is that alcohol affects other modulators of stress and anxiety in the brain, independently of the HPA axis. A prime candidate for further analysis is NPY, which is known to be affected by alcohol intake and is involved in a variety of social behaviors. Here we saw greater levels of NPY immunoreactivity in the medial amygdala of males drinking alcohol during cohabitation than without. It is possible that this increase in NPY in the amygdala leads to anxiolysis (Heilig, et al., 1993; Kask, et al., 2002), thereby decreasing bonding ability.

The observed behaviors in this study parallel certain behaviors in humans, and thus prairie voles may be used to model these aspects of alcohol's effects. First, we demonstrated that in female prairie voles, alcohol can facilitate social bonds, similar to effects observed in humans (Sayette, et al., 2012). Greater knowledge of this phenomenon can help researchers gain understanding of the environment created within social networks that drink together, and perhaps how those bonds may influence future drinking patterns. Second, we demonstrated that in male prairie voles, alcohol can prevent formation of social bonds. We see similarities between this behavior and fleeting social connections in 'one-night stands' in people (Justus, Finn, & Steinmetz, 2000; Testa

& Collins, 1997). While there is no reported evidence for such sex-dependent effects of alcohol in humans, and individuals of both sexes may exhibit social facilitation or inhibition following alcohol drinking, it is tempting to consider whether these effects are strictly biological and if they would indeed extend to humans. Based on observation and experience in various cultures, many people would not be surprised to learn that alcohol leads to fleeting attachment in males or to persistent affection in females, although there is no scientific research to support this.

In addition, it is possible that the findings observed in male prairie voles may extend to detrimental effects of alcohol on established social bonds which are often observed in people who abuse alcohol, as evidenced by decreased marital satisfaction rating, increased divorce, and incidence of intimate partner violence. Modeling the harmful effects of alcohol on social bonds will be important to help understanding and assisting of people trying to restore their lives during recovery from alcohol abuse.

Future directions

Future studies of the interactions between social relationships and alcohol drinking in prairie voles may further investigate the behavioral and biological mechanisms underlying the observed behaviors, develop new procedures for modeling different aspects of human behavior, or use the models established here to test potential therapeutic strategies.

Further investigation of the behavioral basis of peer influence affecting behavior is certainly warranted, since that is a major factor in initiation of alcohol drinking and establishment of drinking patterns (Norton, Lindrooth, & Ennett, 1998; Park, Sher, & Krull, 2008). Particularly since the age of alcohol initiation is currently the best predictor of future development of alcohol use disorders (Clapper, et al., 1995; Morean, Corbin, &

Fromme, 2012; Prescott & Kendler, 1999), it is important to understand these behaviors in order to know how to target them to prevent problems from starting. While the transition of a low drinker to become a high drinker was relatively rare and not a focus of the studies here, it would be interesting to study this individual variability and perhaps identify specific risk factors for the development of alcohol abuse. Studies of the vocal communications between peers when they are drinking together may be informative, as would analysis of social dominance within pairs. Based on studies of social dominance hierarchies and alcohol drinking in other animals (Blanchard, et al., 1987; Blanchard, Yudko, & Blanchard, 1993; McKenzie-Quirk & Miczek, 2008; Wolffgramm & Heyne, 1991), we would expect the dominant animals to consume lower levels of alcohol than subordinates, and, in a way perhaps unique to prairie voles among animal models, we would expect these dominant low drinkers to influence the high drinkers to decrease their alcohol intake. Experiments to analyze these behaviors could confirm that the influential drinker is actually dominant in other ways, and potentially indicate how they exert their influence.

Additional testing of social influences with an eye to translational studies will be particularly important. Studies in humans have demonstrated that the addition of a non-drinker to an abstinent alcoholic's social network helps maintain reduced drinking or abstinence (Litt, et al., 2007; Litt, et al., 2009); greater understanding of the precise circumstances and mechanisms behind this trend could inform future behavioral therapy. Future studies should elucidate whether development of a bond is necessary to affect peers' drinking, or whether simply the presence of a social partner is sufficient. Increased efficacy of behavior or drug therapy by supplementing with the other is a particularly

important approach. Since the role of social support in drinking management for harm reduction or abstinence is so essential to success, future experiments should address the potential buffering effect of a peer on relapse-like behavior. In fact, current studies in our laboratory use a procedure that induces the "alcohol deprivation effect." Pilot studies have shown that the presence of a peer, regardless of the drinking status of the peer, may decrease the escalation of alcohol intake normally observed after a period of deprivation (Hostetler and Ryabinin, unpublished data).

There is much more to be done toward a complete understanding of the processes by which alcohol can affect social bonds. This is especially significant for human alcoholics who can ruin established bonds in part due to alcohol abuse, but who need the support from interpersonal relationships that is vital to recovery. Thus, advancement of the prairie vole model from both behavioral and neurobiological perspectives is important. The hypothesis that alcohol acts as an anxiolytic, which thereby facilitates development of partner preference in females and inhibits it in males, should be followed up by testing whether administration of the stress hormone corticosterone during cohabitation reverses the effects of alcohol consumption during cohabitation. Further development of the procedures established here should examine the effects of alcohol on established bonds, in addition to the formation of bonds studied here. This can be done by allowing pairs to cohabitate for extended periods of time (i.e. two weeks) before introducing alcohol to one or both members of the pair.

Based on the findings of these studies, there are many neurobiological mechanisms and interactions that could potentially mediate the effects of alcohol on bond formation. The first step toward pinpointing a mechanism should be verification of

changes in regional neuropeptide levels during alcohol access and cohabitation, for example using microdialysis combined with an enzyme-linked immunosorbent assay (ELISA). In the experiments performed for this dissertation, the neuropeptide levels were examined following 24 hours of cohabitation since that time was representative of the state at the beginning of the partner preference test; however, it is possible that changes occur during the early stages of cohabitation that affect the development of partner preference and then return to normal, and thus would not be detected with the methods used here. Microdialysis performed at different points throughout cohabitation and partner preference testing could determine changes that affect the formation or expression of a partner preference with better resolution. After discovering a particular change in neuropeptide levels in the targeted region dependent on alcohol intake interacting with cohabitation, then receptors in that region could be targeted by drug microinjections to confirm whether a particular receptor system is necessary or sufficient for the observed behavioral effects of alcohol.

Another approach to discovery of the mechanisms underlying social and alcohol-related behaviors is to reveal common genetic underpinnings. One advantage to studying prairie voles over inbred mouse or rat lines is that they are genetically diverse, and accordingly exhibit a high degree of variability in behaviors. Examination of gene expression during alcohol drinking, cohabitation, or both together could help determine similarities and differences between the mechanistic effects of these stimuli, and expose effects of their interactions. Further, selective breeding for high and low alcohol drinking and subsequent testing for social affiliation could reveal potential pleiotropic effects of

specific genes. The sequence of the prairie vole genome is imminent, and will facilitate future work in this arena.

The sex differences observed in the effect of alcohol drinking on partner preference are important to consider. While there is no evidence that there is a similar sex difference in these particular behaviors in human males and females, there is evidence that alcohol has different biological and behavioral effects between the sexes, and that different types of therapy have different success rates for each sex. Thus, once specific mechanisms for the sex-dependent effects of alcohol are elucidated, similar mechanisms should also be assessed in humans if possible. This could help us gain a better understanding of the basis of sex differences in humans' response to alcohol, and inform more personalized treatments.

Finally, it will be important to test in these procedures what types of behavioral or drug therapies can facilitate recovery of bonds that have been damaged by alcohol, again with an eye to translational work that could be applied to humans recovering from alcohol use disorders.

SUMMARY AND CONCLUSIONS

The experiments described in this dissertation all confirm the importance of interactions between social relationships and alcohol drinking. Each behavior can affect the other, and both rely on overlapping neural mechanisms. In particular, oxytocin, CRF, and dopamine release in the nucleus accumbens, vasopressin in the ventral pallidum and both oxytocin and vasopressin in the lateral septum are all required for pair bond formation and are all involved in alcohol or other drug reward as well (Fig. 3). These

observations support the theory that drugs of abuse exhibit their rewarding properties through activation of natural reward pathways such as those used for social bond formation, one of the hallmarks of mammalian evolution.

This body of work has revealed some remarkable new findings that allow us to model at least four aspects of human behavior in prairie voles:

1) Social facilitation of excessive alcohol intake
2) Peer influence to decrease drinking
3) Facilitation of social affiliation by alcohol
4) Inhibition of social affiliations by alcohol

Importantly, this work has also demonstrated some biological similarities between prairie voles and humans:

1) Alcohol intake leads to intoxicating blood alcohol levels
2) Alcohol affects the central nervous system and specific neuropeptide systems

Related work has also demonstrated that alcohol drinking in prairie voles is decreased when they are given treatments of naltrexone (Anacker & Ryabinin, 2010), a pharmacotherapy that has been shown to decrease alcohol drinking in humans and other animals.

Combined, these findings are promising for future work to model these various interactions between alcohol drinking and social relationships in animals, and to test the effects of socio-behavioral or drug therapies on alcohol drinking under different conditions.

Figure 1. Effects of social influence on alcohol drinking levels

A) When introduced to alcohol in isolation (left), drinking levels are lower than when introduced in pairs (right), as indicated by the direction of the large arrows. Further, the drinking level of paired animals is typically matched. B) When introduced to alcohol in isolation, prairie voles' drinking levels can be sorted into high or low drinkers after establishment of individual intake, as indicated by the direction of the large arrows. High drinkers may exhibit static drinking (solid gray arrow) or flexible behaviors such as variations in the frequency of drinking bouts (arrow with gradient). The former group is unlikely to be affected by a social influence, while the latter is more likely to be influenced when paired with a low drinker and to decrease their own intake (right).

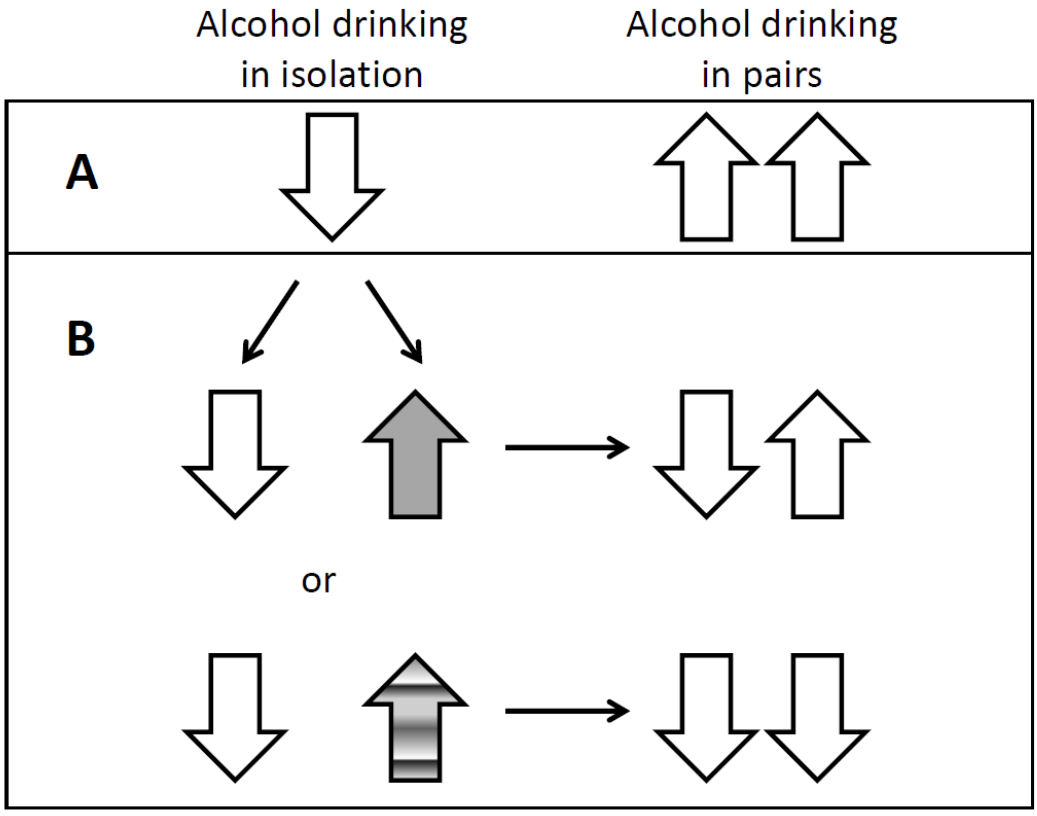


Figure 2. The process of pair bond formation with or without alcohol

A) Female prairie voles require activation of the oxytocin receptor (OTR), dopamine D2 receptor (D2R), and μ opioid receptor (MOR) for pair bond formation. Further, they require a decrease in the level of corticosterone (CORT) and no activation of glucocorticoid or mineralocorticoid receptors (GR/MR). In the present studies, 24 hours of cohabitation with a male was insufficient to produce a strong partner preference through these mechanisms in the brain. B) Male prairie voles require activation of the vasopressin 1a receptor (V1aR), dopamine D2 receptor (D2R), and corticotropin releasing factor receptors type 1 and 2 (CRFR1/2) for pair bond formation, along with an increase in the level of corticosterone (CORT). In the present studies, 24 hours of cohabitation with a sexually receptive female produced a strong partner preference through these mechanisms in the brain. C) When given alcohol, female prairie voles exhibit a strong partner preference, most likely through enhancing one of the mechanisms listed. One possibility is that alcohol acts as an anxiolytic, decreasing CORT and facilitating bond formation. *Untested hypothesis. D) When given alcohol, male prairie voles exhibit no partner preference, most likely through inhibition of one of the mechanisms listed. One possibility is that alcohol acts as an anxiolytic, decreasing CORT and thereby inhibiting bond formation. *Untested hypothesis.

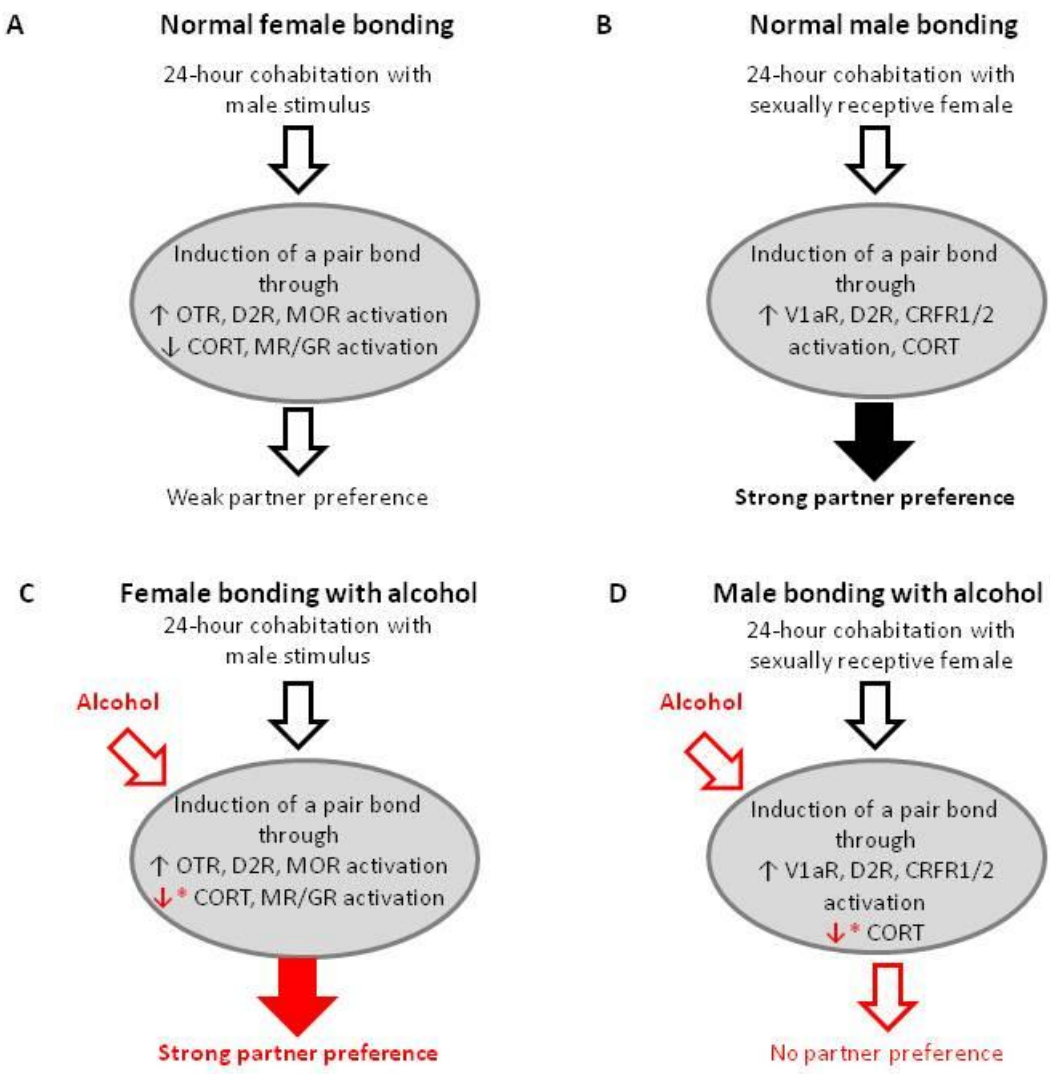
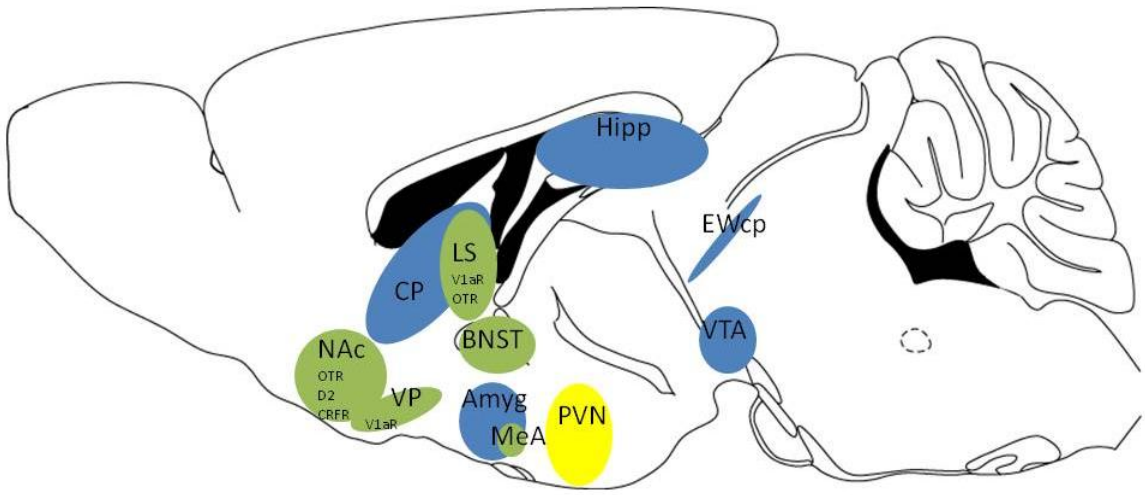

A
Normal female bonding
24-hour cohabitation with male stimulus
Induction of a pair bond through
↑ OTR, D2R, MOR activation
↓ CORT, MR/GR activation
Weak partner preference
B
Normal male bonding
24-hour cohabitation with sexually receptive female
Induction of a pair bond through
↑ V1aR, D2R, CRFR1/2 activation, CORT
Strong partner preference
C
Female bonding with alcohol
24-hour cohabitation with male stimulus
Alcohol
Induction of a pair bond through
↑ OTR, D2R, MOR activation
↓* CORT, MR/GR activation
Strong partner preference
D
Male bonding with alcohol
24-hour cohabitation with sexually receptive female
Alcohol
Induction of a pair bond through
↑ V1aR, D2R, CRFR1/2 activation
↓* CORT
No partner preference

Figure 3. Overlap of alcohol/drug and social reward pathways in the rodent brain Regions involved in alcohol and other drug reward are shown in blue. Caudate-putamen (CP); amygdala (Amyg); hippocampus (Hipp); centrally-projecting Edinger-Westphal nucleus (EWcp); ventral tegmental area (VTA). Regions involved in social behaviors are shown in yellow. Paraventricular nucleus of the hypothalamus (PVN); other hypothalamic regions not shown. Regions involved in both are shown in green. Nucleus accumbens (NAc); ventral pallidum (VP); lateral septum (LS); bed nucleus of the stria terminalis (BNST); medial amygdala (MeA). Receptors at which activation is known to be necessary for pair bond formation are listed in relevant brain regions. Vasopressin 1a receptor (V1aR); oxytocin receptor (OTR); dopamine receptor 2 (D2); corticotropin releasing factor receptors type 1 and 2 (CRFR).



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