THE RELATIONSHIP BETWEEN SOCIALITY AND ALCOHOL CONSUMPTION IN THE PRAIRIE VOLE RODENT MODEL

By

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LIST OF ABBREVIATIONS

AH – anterior hypothalamus

Amyg – amygdala

ANOVA – analysis of variance

AUD – alcohol use disorder

AUDIT – alcohol use disorders identification test

AVP – arginine vasopressin

BEC – blood ethanol concentration

BNST – bed nucleus of the stria terminalis

CeA – central nucleus of amygdala

CNO – clozapine N-oxide

CO_2 – carbon dioxide

CORT – corticosterone

CP – caudate putamen

CRF – corticotropin releasing factor

D1 – dopamine receptor D1

D2 – dopamine receptor D2

DAPI – 4’,6-diamidino-2-phenylindole

DLPAG – dorsal lateral periaqueductal gray

DMPAG – dorsal medial periaqueductal gray

EtOH – ethanol/alcohol

EW ; EWcp – centrally projection Edinger-Westphal nucleus

FDA – food and drug administration

GHS-R1a – growth hormone secretagogue 1a receptor

HICs – handling-induced convulsions

HIPP – hippocampus

ICV – intracerebroventricular

IHC – immunohistochemistry

IL – infralimbic cortex

IR – immunoreactivity

IP ; i.p. – intraperitoneal

LPAG – lateral periaqueductal gray

LS – lateral septum

mRNA – messenger RNA

NAcc – nucleus accumbens

OPRM1 – mu-opioid receptor

PAG – periaqueductal gray

PBS – phosphate buffered saline

PFA – paraformaldehyde

PFC – prefrontal cortex

PP – partner preference

PPT – partner preference test

PVN – paraventricular nucleus of hypothalamus

RAGE – receptor for advanced glycation end-products

RFID – radio frequency identification tags

RI – resident-intruder

SEM – standard error of mean

SON – supraoptic nucleus of hypothalamus

SUD – substance use disorder

V1aR – vasopressin 1a receptor

vBNST – ventral bed nucleus of the stria terminalis

VP – ventral pallidum

VTA – ventral tegmental area

WD - withdrawal

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ABSTRACT

Alcohol use disorder (AUD) affects millions of people each year and is heavily influenced by social networks. On the other hand, social networks are dramatically influenced by alcohol consumption. Understanding the interaction between sociality and alcohol consumption is important to prevent and treat AUD.

Rodent models have been useful to study the interaction between sociality and alcohol consumption. Traditionally the laboratory mouse and rat have been used as the main animal models of study. However, these traditional animal models are difficult to use to model human social relationships because they do not form strong social bonds with conspecifics. Therefore, our laboratory and others have used the prairie vole (*Microtus ochrogaster*) to explore the interactions between sociality and alcohol use because prairie voles have high predictive validity for human social behaviors and will consume high levels of alcohol voluntarily.

The aim of this dissertation is to explore the relationship between alcohol use and sociality and further develop the prairie vole rodent model to study the efficacy of oxytocin as a pharmacotherapy for AUD.

In Chapters 1 and 2 of this dissertation I explore the effects of discordant drinking on established pair bonds in male and female prairie voles. I show that discordant alcohol drinking between opposite-sex partners leads to decreased preference for a partner in male prairie voles, but not in female prairie voles using the partner preference test (PPT). This decrease in partner preference in male prairie voles is accompanied by an increase in FosB immunoreactivity (-IR) in the periaqueductal grey (PAG), traditionally a region known to play a role in pain modulation. I also show that oxytocin-ir is decreased in the paraventricular nucleus of the hypothalamus (PVN) in alcohol consuming male and female prairie voles, regardless of their partner's drinking status. This decrease in oxytocin-ir due to alcohol consumption led me to explore oxytocin as a

potential pharmacotherapy for alcohol use. Therefore, in Chapter 3 I use radiofrequency tracking technology to determine the effects of oxytocin on alcohol consumption in socially housed male and female prairie voles. Voluntary alcohol consumption in these animals results in high daily alcohol intakes, blood ethanol concentrations that are considered intoxicating, and central changes in FosB IR in the centrally-projecting Edinger Westphal (EWcp) nucleus, indicative of changes in neural activity. Prairie voles that receive oxytocin decrease their alcohol consumption compared to control treated prairie voles, regardless of whether their cagemates receive a similar treatment or not.

Together, these studies further our understanding that sociality and alcohol use influence each other heavily and identify potential brain regions that might play a role in the interaction between sociality and alcohol use. In addition, our data shows that the effectiveness of pharmacotherapies can be tested in mixed treated socially-housed animals in a similar manner to clinical studies in humans. Finally, this dissertation shows that the factors, sociality and sex of the animal, must be taken into account when investigating preclinical animal models if AUD.

GENERAL INTRODUCTION

Alcohol Use and Social Relationships in Humans

According to the most recent National Survey on Drug Use and Health, approximately 14.1 million persons aged 18 and older have an alcohol use disorder (AUD) in the United States (SAMHSA, 2017). This includes approximately 9.0 million men and 5.1 million women. AUD is the third leading preventable cause of death in the United States, behind tobacco use and poor diet/physical inactivity (Mokdad et al., 2004). AUD is associated with many mental/psychological disabilities, motor vehicle crashes, fetal alcohol syndrome, financial burdens, and negative social relationships (Hasin et al., 2007; Lemoine et al., 2003; Mokdad et al., 2004; Rehm et al., 2009).

Social networks tend to influence alcohol drinking across the life span. In adolescents, social networks promote to the initiation, escalation, and de-escalation of alcohol drinking (Musher-Eizenman et al., 2003; Prinstein et al., 2001). Alcohol consumption in adults is also highly influenced by social networks (Andrews et al., 2002; Delucchi et al., 2008). Alcohol use also leads to increases in social bonding, the desire to socialize, and increases verbal behaviors in social drinkers compared to nonalcoholic-beverage drinkers (Sayette et al., 2012). In some epidemiological studies, alcohol consumption is associated with increases in intimate partner violence (Leonard and Quigley, 1999; Leonard and Roberts, 1998; Murphy et al., 2001), higher rates of separation (Caces et al., 1999; Collins et al., 2007) and marital dissatisfaction (Halford and Osgarby, 1993; Leonard and Rothbard, 1999; Levinger, 1966). However, when diving further into the literature, researchers began to notice that it’s not just heavy alcohol use that causes these increases in separation, marital dissatisfaction, and intimate partner violence, but that a discrepancy in drinking patterns between partners is more specifically associated with these increases (Homish and Leonard, 2007; Homish et al., 2009; Kelley et al., 2015; Leonard et al., 2014; Mudar et al., 2001; Ostermann et

al., 2005; Torvik et al., 2013; Leadley et al., 2000; Quigley and Leonard, 2000). All together this shows that AUD can be detrimental in many social relationships and that the need for effective treatments is warranted.

Pharmacotherapies for Alcohol Use Disorder in Humans

Evidence-based use of pharmacotherapies did not come into play until 1948. Two scientists discovered that if disulfiram was taken prior to consuming alcohol then it would cause unpleasant or "hangover-like symptoms (i.e. headache, nausea, sweating, vomiting, etc.) (Hald and Jacobsen, 1948). These unpleasant symptoms are caused because disulfiram inhibits the body from converting acetaldehyde to acetic acid (a normal mechanism that occurs after alcohol drinking) and leads to a surge of acetaldehyde in the body. Hald and Jacobsen suggested that disulfiram could be used to sensitize individuals to alcohol and be used as a potential treatment to battle AUD. Following this study, disulfiram was approved by the U.S. Food and Drug Administration (FDA) in the 1950s to treat AUD. Disulfiram is still used today to prevent relapse in abstinent alcoholics.

Even though disulfiram has been used for over 60 years to treat AUD, there have not been many well-controlled studies showing that it is effective. Skinner et al. (2014) ran a meta-analysis exploring the efficacy of disulfiram in the treatment of alcohol dependence. Overall they found that disulfiram was more effective in supporting abstinence compared to control treated individuals. However, when they compared studies where subjects were blind to the treatment vs. subjects who were in an open-label study, they found that open-label trials showed a significant effect over controls, while blind design studies showed no efficacy of disulfiram compared to controls. Therefore, the efficacy of disulfiram in the treatment of alcohol dependence may only occur when individuals knew they were taking the drug.

In 1992, two randomly controlled trials explored the effects of naltrexone, a nonselective opioid receptor antagonist, on the treatment of alcohol dependence. The first study, Volpicelli et al. (1992), found that oral naltrexone decreased craving, mean drinking days, and relapse rates over a 12-week period in alcohol-dependent male U.S. veterans. The second study, O'Malley et al. (1992), replicated the results in the Volpicelli et al. (1992) in individuals who were receiving care on a weekly basis through an outpatient facility. These two clinical studies led to the FDA approving naltrexone as a treatment for AUD in 1994.

Naltrexone exhibits a modest effect in reducing the risk of heavy drinking, but it appears to be less effective in promoting complete abstinence (Jonas et al., 2014; Rosner et al., 2008). However, the effectiveness of naltrexone seems to depend on if an individual is carrying the Asp40 allele of the mu-opioid receptor (*OPRM1*) gene. Oslin et al. (2003) found that if individuals are carrying the Asp40 allele, then naltrexone has a greater effectiveness in reducing drinking when compared to individuals not carrying the Asp40 allele, showing that oral naltrexone might not be effective in treating alcoholism in all patients. More recently, a double-blind, placebo-controlled randomized trial compared the effects of oral naltrexone in a German population (PREDICT study) to a U.S. population (COMBINE study). They found that naltrexone was effective in decreasing the number of heavy drinking days in the COMBINE study, but was not effective in the PREDICT study (Anton et al., 2006; Mann et al., 2013). This further shows that the results of naltrexone’s effectiveness are varied substantially and that results are perhaps driven by the underlying genetics of the populations under study.

To overcome compliance issues of oral naltrexone, the use of sustained-release, intramuscular naltrexone has been approved for treating AUD. A randomly controlled trial with 600 alcohol-dependent individuals, explored the effects of 380 mg of naltrexone, 190 mg of naltrexone, and placebo (Garbutt et al., 2005). They found that at

the 380 mg dose, there was a significant decrease in the number of heavy drinking days compared to placebo. When exploring the 190 mg dose, they saw no significant difference in heavy drinking days compared to placebo. Sustained-release naltrexone may be an effective treatment, but it is known that naltrexone at 300mg causes hepatotoxicity (Pfohl et al., 1986); therefore, more research needs to explore the effects of sustained-release naltrexone on liver damage.

In 2004, the FDA approved acamprosate for the treatment of AUD. AUD patients usually have an imbalance between the inhibitory neurotransmitter GABA and excitatory neurotransmitter glutamate. Acamprosate is thought to act as a NMDA receptor antagonist and positive allosteric modulator of $GABA_A$ receptors. Compared to naltrexone, acamprosate is considered to be better at the maintenance of alcohol abstinence and is most efficient when an alcoholic individual is abstinent prior to treatment onset (Maisel et al., 2013). The approval of acamprosate for the treatment of AUD was driven by three randomized, double-blind European studies. These three European studies found that patients taking acamprosate compared to placebo had longer durations of abstinence and improved rates of complete abstinence (Paille et al., 1995; Pelc et al., 1997; Sass et al., 1996). However, two recent U.S. studies and one German study found that acamprosate was no better at decreasing alcohol abstinence compared to a placebo control group (Mason et al., 2006; Anton et al., 2006; Mann et al., 2013). These differences in efficacy could potentially be due to the varying subject characteristics and the severity of AUD in the populations.

Disulfiram, naltrexone, and acamprosate are currently the only three FDA approved pharmacotherapies for AUD in the United States. All three have shown substantial varying results for treating AUD. The variability in efficacy has led to a continued search for other potential treatments for AUD. One in particular is oxytocin. Oxytocin is a nonapeptide that has been implicated to play an important role in lactation,

parturition, and a wide variety of social behaviors (Lee et al., 2009). In addition, oxytocin is involved in pain and stress reduction, emotional regulation, and the modulation of processes associated with drug use (Leong et al., 2018; Tops et al., 2014). Specifically, oxytocin has shown some promising results in treating AUD in preclinical and clinical studies (Hansson et al., 2018; King et al., 2017; Macfadyen et al., 2016; Mcgregor and Bowen, 2012; Mitchell et al., 2016; Pedersen et al., 2013; Pirnia and Pirnia 2018; Stevenson et al., 2017a). For example, intranasal oxytocin has been effective in reducing alcohol withdrawal symptoms and craving in humans (Mitchell et al., 2016; Pedersen et al., 2013). Similarly, oxytocin has been shown to decrease alcohol consumption and cue-reactivity in rodents (Hansson et al., 2018; Leong et al., 2018). The use of oxytocin as a treatment for AUD will be further discussed in Chapter 3.

Prairie Voles as a Model for Sociality in Humans

Typically, mice and rats are used as models of human sociality in the laboratory. These animal models have been successful in leading the way to understanding the underlying biological mechanisms of human social behaviors, including mating, maternal care, and aggression (Burns-Cusato et al., 2004; Blaustein, 2008; Leckman and Herman, 2002; Ferrari et al., 2005). However, these traditional laboratory rodents do not form long-term social attachments between adults, something that is highly prevalent in humans, leading to the understudy of such behavior. One laboratory model that has emerged to be suitable to study human social behavior and attachment, is the prairie vole (*Microtus ochrogaster*). Prairie voles belong to a group of 3-5% of mammals who display social monogamy, something that traditional rat and mouse animal models do not exhibit (Kleiman, 1977). Prairie voles form long-term attachments called pair bonds. The earliest studies on this subject found that in the wild, prairie voles were repeatedly captured together in male and female pairs during the breeding and nonbreeding

season, indicating long-term male-female associations (Getz et al., 1981). Additionally, male prairie voles has displayed aggression to unfamiliar males and females in the vicinity of their nest and has displayed biparental behaviors toward their offspring (Thomas and Birney, 1979)

Mechanisms of pair bonds in prairie voles has been extensively researched in the laboratory. The formation of a pair bond occurs after a male and female prairie vole are cohabitated (Insel et al., 1995). The strength of a pair bond can be assessed using the partner preference test (PPT). The PPT involves a three-chambered test apparatus that consists of a chamber with a tethered partner animal, another with a tethered unfamiliar animal, and a central chamber (nonsocial) that the subject animal passes through to get to the other two chambers. The standard test is 3 hours and the amount of time the subject animal spends huddling in side-by-side, motionless contact with each stimulus animal is measured. Along with huddling time, aggression towards each animal, amount of time spent in each chamber, and the frequency of chamber entries is measured. When a subject animal spends significantly more time in side-by-side contact with a partner than a stranger, it is called a partner preference (Carter and Getz, 1993; Getz et al., 1981; Williams et al., 1992). Usually 24 hours of cohabitation will reliably induce partner preference formation in male and female prairie voles. Similarly, partner preference has been shown to occur in same-sex conspecific strangers (Devries et al., 1997).

Another behavior that develops after a pair bond is established is selective aggression. Prairie voles are highly affiliative toward strangers before pair bonds are formed. However, after a pair bond is formed prairie voles display aggression toward stranger animals but not their partner, this being termed as “selective aggression”. Selective aggression in prairie voles is analyzed using the resident intruder test (Resendez and Aragona, 2012; Resendez et al., 2012; Winslow et al., 1993; Wang et

al., 1997). During this test stranger animals are placed in the home cage of the subject animal for 5-10 minutes. The frequency of aggressive interactions (lunges, bites, chases, offensive rears) and the duration of affiliative behaviors (olfactory investigation, anogential sniffs, side-by-side contact) towards the stranger animal is analyzed. Selective aggression can be seen in male and females, but usually males will display a higher number of aggressive interactions toward a conspecific stranger compared to females (Williams Jr, 1992; Resendez et al., 2012).

Not only have the behavioral aspects of pair bonds been extensively studied, but several neuropeptides and specific receptors have been identified for playing roles in pair bond formation and maintenance. Specifically, oxytocin, vasopressin, corticotropin releasing factor (CRF), dopamine receptors, and opioid receptors have been identified to play a role in pair bonds. Early pharmacological studies found that intracerebroventricular (ICV) injections of oxytocin have led to partner preference formation in female prairie voles and that an oxytocin receptor antagonist blocked this effect (Williams et al., 1994). Additionally, oxytocin receptors in the nucleus accumbens are necessary for pair bond formation in female (Liu and Wang, 2003) and male prairie voles (Johnson et al., 2016). Furthermore, oxytocin receptor polymorphisms are predictive of individual variations in pair bond formation in prairie voles and social attachment in humans (King et al., 2016; Walum et al., 2012).

Vasopressin 1a receptor (V1aR) and the CRF receptors have been shown to play roles in pair bond formation as well. ICV injections of vasopressin has led to the facilitation of partner preference and V1aR antagonists has led to the inhibition of partner preference in male and female prairie voles (Winslow et al., 1993). Additionally, activation of V1aR in the lateral septum and ventral pallidum has led to the facilitation of partner preference (Lim and Young, 2004; Pitkow et al., 2001; Liu et al., 2001). Lastly,

activation of CRF receptors in the nucleus accumbens was necessary for pair bond formation in male prairie voles (Devries et al., 2002; Lim et al., 2007).

The dopamine and opioid systems are implicated to play a role in the formation and the maintenance of pair bonds. When female prairie voles are injected peripherally with a D2 receptor antagonist they display an inhibition of partner preference compared to D1 receptor antagonist and saline treated prairie voles (Wang et al., 1999). More specifically, when a D2 receptor antagonist is administered directly into the nucleus accumbens, partner preference is blocked; meanwhile, when a D2 receptor agonist is microinjected into the NAcc after 6 hours of cohabitation, female prairie voles display an increased partner preference compared to saline treated animals (Gingrich et al., 2000). Additionally, when a mu-opioid selective receptor antagonist is administered in the dorsal striatum, a reduction in partner preference occurs (Burkett et al., 2011). Interestingly, the D2-like receptors and the mu-opioid receptors seem to not play a role in the maintenance of a pair bond. Using the resident-intruder paradigm it has been shown that pair bond maintenance is mediated by the D1-like dopamine and kappa-opioid receptors in the nucleus accumbens shell (Aragona et al., 2006; Resendez et al., 2012). When a D1 or a kappa-opioid receptor antagonist is administered directly in the nucleus accumbens shell, male prairie voles display a decrease in aggressive behaviors toward a resident intruder. Taken all together, the neurotransmitters and receptors that play a role in pair bonds in prairie voles are homologous to the neurotransmitters and receptors that play a role in human social affiliations (Walum et al., 2012; Walum et al., 2008), thus making prairie voles a great partial model to advance the field of social neuroscience.

Alcohol Drinking in Prairie Voles

As stated above, it is known that there is a strong relationship between alcohol consumption and social relationships in humans. However, a majority of the interactions between sociality and alcohol was mainly studied in the traditional rodent models (i.e. mice and rats). Therefore, the prairie vole model recently emerged as a way to model social alcohol drinking in the laboratory. Our laboratory discovered that prairie voles voluntarily consume high amounts of alcohol similar to C57BL/6J mice, a mouse strain known to consume high amounts of alcohol (Anacker et al., 2011a). Due to the large genetic diversity of outbred prairie voles, there is a high variability in alcohol intake between individuals (ranging from ~5 g/kg/day to over 30 g/kg/day), something that is not as prevalent in the traditional inbred rodent models, but prevalent in humans. Not only do prairie voles voluntarily consume high amounts of alcohol, but these amounts have led to signs of hyperalgesia after an acute withdrawal period (i.e. 24-hours) (Appendix 1; Walcott et al., 2018). This sign of hyperalgesia in prairie voles is similarly seen in humans who are alcohol dependent (Egli et al., 2012). When alcohol dependent humans experience an acute withdrawal period, they display signs of increased pain sensitivity compared to non-dependentfd humans (Jochum et al., 2010). Therefore, prairie voles are a good animal model to study the underlying mechanism of some aspects of physical dependence in human alcoholics.

In humans, alcohol consumption is heavily influenced by social factors. Higher levels of alcohol use in family members is associated with increased alcohol use in adolescents (Dawson, 2000; Needle et al., 1986). One social group that heavily influences alcohol use in young adults is peers. Peer pressure, peer alcohol norms, and socializing with alcohol-using peers is associated with increases in alcohol use in young adults (Patrick et al., 2013; Studer et al., 2014; Varvil-Weld et al., 2014).

It is difficult to model social influences on alcohol use in animal models because the traditional laboratory rodent models show no effects on alcohol consumption due to

the drinking status of a conspecific (reviewed in Anacker and Ryabinin (2010). In contrast, prairie voles consume higher levels of alcohol when housed in same-sex pairs compared to prairie voles housed in isolation (Anacker et al., 2011a). This increase in alcohol drinking in socially housed prairie voles is similar to the social facilitation of drinking occasionally seen in humans (De Castro, 1990). Interestingly, alcohol consumption levels in prairie voles are socially influenced by a stranger drinking peer (Anacker et al., 2011b; Anacker and Ryabinin, 2013). Prairie voles who are high drinkers decrease their alcohol intake and preference when paired with low drinkers. In a few cases, low drinkers will increase their alcohol intake when paired with a higher drinker (Anacker et al., 2011b). However, in opposite-sex partners the social influence of alcohol consumption levels has not been shown until this current dissertation (Hostetler et al., 2012). These studies show that different social environments impact alcohol intake in prairie voles, much like humans.

As stated previously, alcohol can affect social behavior environments in humans (Leonard and Rothbard, 1999; Sayette et al., 2012; Mcleod, 1993). Our laboratory has not only shown that social environments affect alcohol drinking, but we have recently discovered that alcohol can affect a prairie vole's social behavior. Anacker et al. (2014a) explored the effects of alcohol drinking on pair bond formation. Opposite-sex prairie voles cohabitated for 24 hours, while simultaneously having access to 10% alcohol and water. Following this 24-hour period, pair bond strength was tested using the partner preference test. They found that male prairie voles showed no preference for their partner over a stranger when alcohol was made available compared to water drinking males. However, when female prairie voles had access to alcohol, they showed an increase in partner preference compared to females who had access to only water. In sum, prairie voles have proven to be a better animal model than the traditional laboratory rodent models to explore the interactions between sociality and alcohol.

Testing Alcohol Use Disorder Treatments in Prairie Voles

Prairie voles voluntarily consume high amounts of alcohol at varying levels between individuals, and alcohol levels are influenced by social environments and vice versa, similar to alcohol drinking in humans. As a result, prairie voles are emerging as a rodent model to study the effects of pharmacotherapies for AUD that might be affected by social influences. For example, the administration of 8 mg/kg of naltrexone significantly decreases alcohol preference in a limited 2-hr access procedure in semi-socially housed prairie voles (Anacker and Ryabinin, 2010). Naltrexone's effect on alcohol consumption in prairie voles shows that approved FDA pharmacotherapies for AUD can be tested in these highly social animals.

Recently two pharmacotherapies that have not been approved yet for AUD have been tested in prairie voles. In two studies, an antagonist of the growth hormone secretagogue 1 receptor (GHS-R1a) decreased alcohol consumption in an alcohol concentration-dependent manner in semi-socially housed prairie voles (Stevenson et al., 2015; Stevenson et al., 2016). GHS-R1a is a receptor for the orexigenic hormone ghrelin. In other rodent models, an injection of ghrelin increases alcohol consumption and GHS-R1a antagonism decreases alcohol consumption in socially isolated animals (Jerlhag et al., 2009; Gomez et al., 2015; Gomez and Ryabinin, 2014).

Oxytocin is a neuropepetide that is known to be involved in reproductive, maternal, and other social behaviors in humans (Feldman, 2012). Prairie voles have been predictive in identifying the role oxytocin plays in human social bonds (Insel and Hulihan, 1995; Lee et al., 2009; King et al., 2016; Walum et al., 2012). For example, variations in the human oxytocin receptor gene are associated with variations in partner bonding; meanwhile, variations in the prairie vole oxytocin receptor gene is associated with variations in partner preference (King et al., 2016; Walum et al., 2012). Oxytocin is

affected by voluntary alcohol consumption in prairie voles and humans. After alcohol consumption, the number of oxytocin cells in the paraventricular hypothalamus is decreased in semi-socially housed prairie voles (Chapters 1 & 2, (Stevenson et al., 2017b; Walcott and Ryabinin, 2017). Similarly, a rise of oxytocin in serum caused by breast stimulation in humans is inhibited by alcohol consumption (Coiro et al., 1992). On the other hand, oxytocin has been tested as a pharmacotherapy for alcohol consumption in prairie voles. In semi-socially housed prairie voles, oxytocin decreases alcohol consumption and preference in an intermittent alcohol access procedure 1-hr and 24-hrs after treatment. However, in a continuous alcohol access procedure, oxytocin decreases alcohol drinking only 1-hr after treatment in prairie voles (Stevenson et al., 2017a). Meanwhile in a study in humans, intranasal oxytocin treatment has been shown to decrease alcohol consumption (Pirnia and Pirnia 2018). As stated above, prairie voles have a high predictive validity for the mechanism underlying oxytocin's role in human social bonds. So it is likely that prairie voles have a high predictive validity for the effects of oxytocin on alcohol drinking in humans. Therefore, I further explore oxytocin effects on alcohol consumption in prairie voles in Chapter 3 of this dissertation.

Dissertation Goals

The first goal of this dissertation was to investigate the effects alcohol consumption had on social relationships. Anacker et al. (2014a) demonstrated that prairie voles are a good model for studying alcohol's effects on pair bond formation. However, this study was modeling when a person has their first drink during their first encounter with an opposite-sex conspecific. This is not typical of alcohol drinking in humans. Therefore, I wanted to model a more typical experience that occurs in humans. Specifically, I wanted to model the effects discordant drinking had on the maintenance of pair bonds in prairie voles and determine the potential underlying biologically

mechanisms. In Chapter 1, I investigated the effects of discordant alcohol drinking on established pair bonds in male prairie voles, measured by partner preference and selective aggression. In Chapter 2, I proceeded to explore the effects of discordant alcohol drinking on established pair bonds in female prairie voles, measured by partner preference.

I saw that alcohol drinking resembled the human epidemiological data in males in the first goal, so I took the advantage of this model to investigate the effects of a relevant treatment for AUD. Therefore, the second goal of this dissertation was to explore how a non-approved pharmacotherapy would affect alcohol consumption and preference in socially-housed male and female prairie voles. It is known that oxytocin plays a role in alcohol drinking in semi-socially housed prairie voles, therefore in Chapter 3 I explored the effects of oxytocin in fully socially-housed prairie voles. I did this by using a new caging system, HM-2, that uses radiofrequency identification technology to track alcohol consumption on an individual animal level in full social housing. My overall hypothesis for this dissertation is that discordant alcohol use between partners disrupts pair bonds and that peripheral oxytocin treatment will decrease alcohol consumption in social settings. Together these goals provided insight into how alcohol and sociality are intertwined and that prairie voles should further be used to study the efficacy of pharmacotherapies on AUD.

CHAPTER 1: Alcohol's Effects on Pair Bond Maintenance in Male Prairie Voles

(This chapter has been reformatted and minimally edited for inclusion in this dissertation from: Walcott, A. T., & Ryabinin, A. E. (2017). Alcohol's effects on pair-bond maintenance in male prairie voles. Frontiers in psychiatry, 8, 226.)

Introduction

Alcohol is often used as a “social lubricant” to enhance social bonds. On the other hand, alcohol abuse can have detrimental effects on certain social bonds. Among such affected social bonds are long-term relationships between spouses. In a survey exploring the demographic distribution of drinking patterns, 73% of married men and 63% of married women stated that they drink alcohol (Hilton, 1988). Heavy alcohol use during a marriage has been associated with decreased marital satisfaction and increased rate of divorce (Caces et al., 1999; Collins et al., 2007; Leonard and Rothbard, 1999; Leonard and Senchak, 1993; Mcleod, 1993). In fact, alcohol and drug use is the third most commonly reported reason – behind infidelity and incompatibility – for divorce in the United States (Amato and Previti, 2003). However, it has been shown that marital dissatisfaction (Homish and Leonard, 2007; Homish et al., 2009; Mudar et al., 2001) and divorce rates (Leonard et al., 2014; Ostermann et al., 2005; Torvik et al., 2013) tend to increase when there is a discrepancy in husband and wife drinking patterns, but not when the spouses drink in concordance. Given the high prevalence of alcohol consumption in long-term relationships, it is important to understand whether biological mechanisms contribute to the effect of discrepancies in alcohol intake on separation rates.

Using laboratory rodent models can help to elucidate the biological mechanisms regulating long-term relationships. One valuable rodent model of mammalian social monogamy is the prairie vole (*Microtus ochrogaster*). Like humans, prairie voles form socially monogamous bonds between same-sex mates (Devries et al., 1997) and opposite-sex partners (Carter and Getz, 1993), and display biparental care for offspring (Solomon, 1993; Carter et al., 1995). Social attachments, or pair bonds, in prairie voles are mediated by several neurotransmitter and receptor systems that are homologous to

those regulating human social affiliations (Aragona et al., 2006; Insel and Hulihan, 1995; Lee et al., 2009; Pitkow et al., 2001; Walum et al., 2012; Walum et al., 2008; Wang et al., 1999). These similarities between prairie voles and humans make prairie voles a good translational animal model to study social pair bonds in the laboratory.

Researchers began to investigate the effects of drugs of abuse on pair bonding in prairie voles (Hostetler et al., 2016; Liu et al., 2010; Shapiro et al., 1989; Young et al., 2011). Importantly, not only will prairie voles form social bonds, but also they voluntarily self-administer high doses of alcohol without training on a sucrose-fading procedure (Anacker et al., 2011a). Unlike most other rodent models, prairie voles can consume higher levels of alcohol when housed in same-sex pairs compared to prairie voles housed in isolation (Anacker et al., 2011a). This increase in social drinking in prairie voles is similar to the social facilitation of drinking seen in humans (De Castro, 1990). Interestingly, the social facilitation of drinking was previously seen only in same-sex prairie vole pairs, but not between opposite-sex partners (Anacker et al., 2011b; Hostetler et al., 2012). Thus, it is clear that different social environments influence the self-administration of alcohol in prairie voles, much like in humans.

Previously, there has not been an adequate amount of research on the effects of alcohol on opposite-sex pair bonds in rodent models. To our knowledge, only one study has investigated these effects. Anacker et al. (2014a) explored the effects of alcohol on the formation of pair bonds in male and female prairie voles. Briefly, male and female prairie voles were paired for 24 hours while simultaneously receiving access to 10% alcohol and water or only water. To determine their pair bond strength, animals were then tested in the partner preference test (PPT). Males exposed to alcohol showed no preference for their partner when compared to the control group. In contrast, females showed facilitation in the preference for their partner compared to the control group. The opposite effects of alcohol consumption on the formation of partner preference (PP) in

males versus females were accompanied by sex-specific effects of alcohol on neural activity in several brain regions. These findings demonstrated that alcohol's effects on social pair bonds could have biological underpinnings. However, alcohol's effects on pair bond formation do not fully model the disruption that the discrepancy in alcohol intake has on long-term relationships.

In the present study we used male prairie voles to investigate the effects of discrepancies in alcohol intake on established pair bonds. We hypothesized that when there was a discrepancy in alcohol access between partners, the prairie voles would show a decrease in PP compared to voles that had a partner who was given access to alcohol. Our results demonstrate that discordant, but not concordant, alcohol drinking leads to a decrease in PP in male prairie voles. Follow up experiments testing effects of alcohol on immunoreactivity of oxytocin, arginine vasopressin (AVP), and FosB suggest that the effect of discrepant drinking may involve activation of the periaqueductal gray (PAG). To our knowledge, this is the first demonstration of alcohol's effects on pair bond maintenance and the first investigation of neurocircuits that might mediate this effect.

Materials and Methods

Animals

Adult male and female prairie voles (n=150; 76-126 d old) from our breeding colony at the VA Portland Health Care System (VAPORHCS) Veterinary Medical Unit were used in these experiments. All animals were weaned at 21 days and housed in same-sex sibling groups (2-4 animals per cage) in cages (27x27x13cm) under a 14:10 light/dark cycle (6am lights-on), until the start of experiments. All subjects had access to cotton nestlets and ad libitum access to water and a diet of mixed rabbit chow (LabDiet Hi-Fiber

Rabbit; PMI Nutrition International, Richmond, IN), corn (Nutrena Cleaned Grains; Cargill, Inc., Minneapolis, MN), and oats (Grainland Select Grains; Grainland Cooperative, Eureka, IL) throughout the entire experiment. All experiments were conducted in accordance with the Institutional Animal Care and Use Committees at the VAPORHCS and Oregon Health & Science University (OHSU), Portland, Oregon, USA.

Housing Conditions

At the start of experiments, male subjects were placed in a standard plastic housing cage with a female partner for one week to establish a pair bond. The following week, all subjects and opposite-sex partners were placed in a mesh-divided social housing cage (27x27x13 cm). These social housing cages have been described previously (Anacker et al., 2011b; Hostetler and Ryabinin, 2014). Briefly, they contain a mesh divider in the middle of the cage to separate each animal in the pair. These cages prevent animals from mating, but allow olfactory and visual social contact with partners to still occur. These cages also allow the monitoring of individual fluid consumption. It has previously been described that these mesh-divided social housing cages do not affect established pair bonds (Curtis, 2010).

Two-bottle choice test

During the period when animals were housed in mesh-divided cages, all animals were given continuous access to two 25mL glass cylinders fitted with a metal sipper tube and rubber stopper. Three experimental conditions were used in these set of experiments: 1) both male and female partners were given access to one bottle of water and a second bottle containing 10% ethanol (Both EtOH); 2) the male was given access to one bottle of water and a second bottle of 10% ethanol, while the female partner was given access to two bottles of water (Male only EtOH); 3) both male and female partners were given

access to two bottles of water (Control). Bottles were monitored every 24 h for 7 days, and then bottles were refilled and their position was switched to prevent side bias.

Average daily alcohol consumption for each prairie vole was calculated by dividing the grams of alcohol by the kilogram of body weight. Alcohol consumption and preference for alcohol were both analyzed by repeated-measures ANOVA for the effects of days, treatment, and their interaction after testing for normality using the Shapiro-Wilk test (all data sets for alcohol consumption and preference passed the normality test; $p > 0.05$). Significance for all experiments was set at $p<0.05$.

Partner Preference Test

PPT was used as a standard way to test pair bonding in prairie voles (Ahern et al., 2009; Williams et al., 1992). Immediately following the two-bottle choice paradigm (described above), the effect of discordant and concordant alcohol consumption on pair-bond maintenance in male subjects (total n=23) was assessed using a 3-h PPT (test started between 11am-1pm). The PPT was performed in a three-chambered apparatus with the partner stimulus (n=23) tethered in one chamber, the female stranger (n=23) tethered in the opposite chamber, and the subject animal placed in a center, non-social chamber and allowed to move freely throughout the three chambers. The female stranger animals were housed in mesh-divided cages with siblings and were exposed to the same experimental treatment as the female partners. PPT was videotaped and was viewed later for behavioral analysis.

The main outcome of PPT is the duration of time the male subject spends huddling with either the partner or stranger animal; this is a measure of social preference. An experimenter who was blinded to group assignment and trained in detecting huddling behavior used VLC Media Player (Boston, MA, USA) to view the

recorded videos. Behavior Tracker 1.0 software was used to measure the amount of time each animal spent huddling with the partner or stranger at a 5x playback speed. Male huddling time with female partners was analyzed using the Brown-Forsythe test to determine normality. Partner huddling was normally distributed ($F_{2,15} = 0.532$, $p = 0.598$), thus the PPT data met the assumptions required to use parametric test for analysis. PPT data were analyzed by two-way ANOVA with stimulus animal (i.e., partner or stranger) and treatment (i.e., alcohol or water access) as between-subjects factors and followed by a Fisher's LSD post hoc test.

Resident Intruder Test

Another way to measure pair-bond maintenance is through the resident-intruder (RI) test. Previously it has been described that attack frequency toward a same-sex stranger during the RI test can be used to measure the strength of a pair-bond (Aragona et al., 2006; Resendez et al., 2012; Resendez et al., 2016). Therefore, a different set of male animals (total n=27) from the ones described above was used for the RI test. These animals were exposed to the two-bottle choice paradigm and the mesh-divided housing as above, but instead of the PPT; they were put through the RI test. The ten-minute RI test took place in the mesh divided cages (on the subject's side) immediately following the voluntary alcohol intake procedure. The female partner (n=27) was removed from her side and placed in a separate holding cage during the test. The male strangers (n=27) were housed in mesh-divided cages with siblings and were exposed to the same experimental treatment as the male subjects. The RI test was videotaped and was viewed later for behavioral analysis.

The main outcome of the RI test is the frequency of aggressive interactions (lunges, bites, chases, offensive rears) toward the stranger male. An observer blind to experimental conditions used VLC Media Player (Boston, MA, USA) to view the

recorded videos. JWatcher behavioral observation software (V 1.0, Macquarie University and UCLA) was used to measure the frequency of aggressive interactions at a 1x playback speed. To determine if we could use a parametric test to analyze the RI data, we used the Brown-Forsythe test to analyze normality. The Brown-Forsythe test revealed that the RI data was normally disturbed ($F_{2,15} = 2.647$, $p = 0.104$), thus a parametric test was used. RI data were analyzed by one-way ANOVA to determine the effects concordant and discordant alcohol drinking had on aggressive frequency.

Embryo Analysis

After the PPT and RI tests, female partners were euthanized. Embryos were then removed and weighed. The average weights of all apparent embryos in an animal were used for analysis. Embryo weights were analyzed because the stage of pregnancy is positively correlated with measurements of pair bond maintenance of pair bonds (Curtis, 2010). Specifically, male prairie voles that have a female partner that had been pregnant for 10 days or more spend significantly more time huddling with their partner over a stranger, compared to males who have a female partner who had been pregnant for less than 10 days. Average embryo weights that correspond to >0.3 g are considered to be optimal impregnation (greater or equal to 10 days pregnant at the time of testing), while weights <0.3 g are considered suboptimal impregnation (less than 10 days pregnant at the time of testing) (Curtis, 2010; Resendez et al., 2012). Five female partners in the PPT experiment and nine female partners in the RI experiment had suboptimal pregnancies. As a result, in the final analysis, there were 6 animals per group in the PPT experiment and 5-7 animals per group in the RI analysis. Only data from male subjects that had a female partner, who reached optimal pregnancy, were used in statistical analysis within this current study.

Immunohistochemistry

To determine the potential molecular mechanisms involved in effects of discordant and concordant alcohol consumption on established pair bonds, subjects (n= 5-7 per group) from the RI experiment were euthanized by CO2 immediately after the completion of the RI test. Brains were then extracted, fixed in 2% paraformaldehyde/PBS for 24 h, and cryoprotected using 20% and then 30% sucrose/PBS. Brain tissue was sliced at 40-µm coronal sections and stored in 0.1% sodium azide until immunohistochemistry (IHC) assay. Sections containing 18 brain regions were selected for analysis. Regions of interest were determined by using the Paxinos and Franklin (2004) mouse brain atlas. The following primary antibodies were used: anti-oxytocin (1:20,000, Peninsula Laboratories), anti-arginine vasopressin (1:50,000, Peninsula Laboratories), and anti-FosB (1:27,000, Abcam). An anti-rabbit secondary antibody made in goat (Vector Laboratory, Inc) was used and then signal was amplified using a Vectastain ABC kit (Vector Laboratory, Inc). Tissue was then stained using a metal enhanced diaminobenzidine substrate kit (Thermo Fisher Scientific) and visualized using a Leica DM4000 bright-field microscope. All cells that were stained above background were counted using automatic cell counting techniques by ImageJ. An experimenter blinded to the condition of the subjects analyzed the data by one-way ANOVA. Significant effects were followed up by a Fisher's LSD post hoc test.

Results

Effects of concordant and discordant drinking on maintenance of partner preference

To compare effects of concordant and discordant drinking on pair bond maintenance, we examined three groups of adult male prairie voles. Control males were cohabitated with females for two weeks. During the second week, a mesh divider was

introduced between the male and the female allowing the experimenter to monitor fluid consumption of each member of the pair. Males of the Both EtOH group were housed similarly, but during the second week both the male and the female were introduced to a choice between two fluids: water and 10% ethanol. Since both males and females in these pairs were exposed to alcohol, they were considered to experience concordant drinking. Males of the Male only EtOH group were also cohabitated for two weeks, but only male animals had access to a choice between water and 10% ethanol during the second week. Therefore, these males experienced discordant drinking.

When both male and female partners were given access to alcohol, males consumed on average 10.8 ± 0.3 (mean ± SEM) grams of alcohol per kilogram of body weight (g/kg) and females consumed 9.9 ± 0.6 g/kg per day over a seven-day drinking period. Meanwhile, when only the male was given access to alcohol, males consumed on average 6.4 ± 0.5 g/kg of 10% ethanol over the same seven-day period. Depending on day and animal, alcohol consumption ranged from 0.0-30.8 g/kg and 0.9-11.9 g/kg in the Both EtOH and Male only EtOH groups, respectively. Analysis of the alcohol consumption revealed that males in the Both EtOH group significantly increased the amount of 10% ethanol consumed compared to males in the Male only EtOH group ($F_{1,70} = 11.820$, $p = 0.001$; Fig. 1A). There was no significant difference between the amount of 10% ethanol consumed each day ($F_{6,70} = 0.135$, $p = 0.991$) and no significant interaction between day and treatment group ($F_{6,70} = 0.199$, $p = 0.976$). Alcohol preference was not significantly different between the males in the Both EtOH (range: 0.0-98.8%) group and the males in the Male only EtOH (range: 4.9-85.2%) group ($F_{1,70} = 3.127$, $p = 0.081$; Fig. 1B). There was no significant difference in alcohol preference between each day ($F_{6,70} = 0.921$, $p = 0.485$) and no significant interaction between day and treatment group ($F_{6,70} = 0.451$, $p = 0.842$).

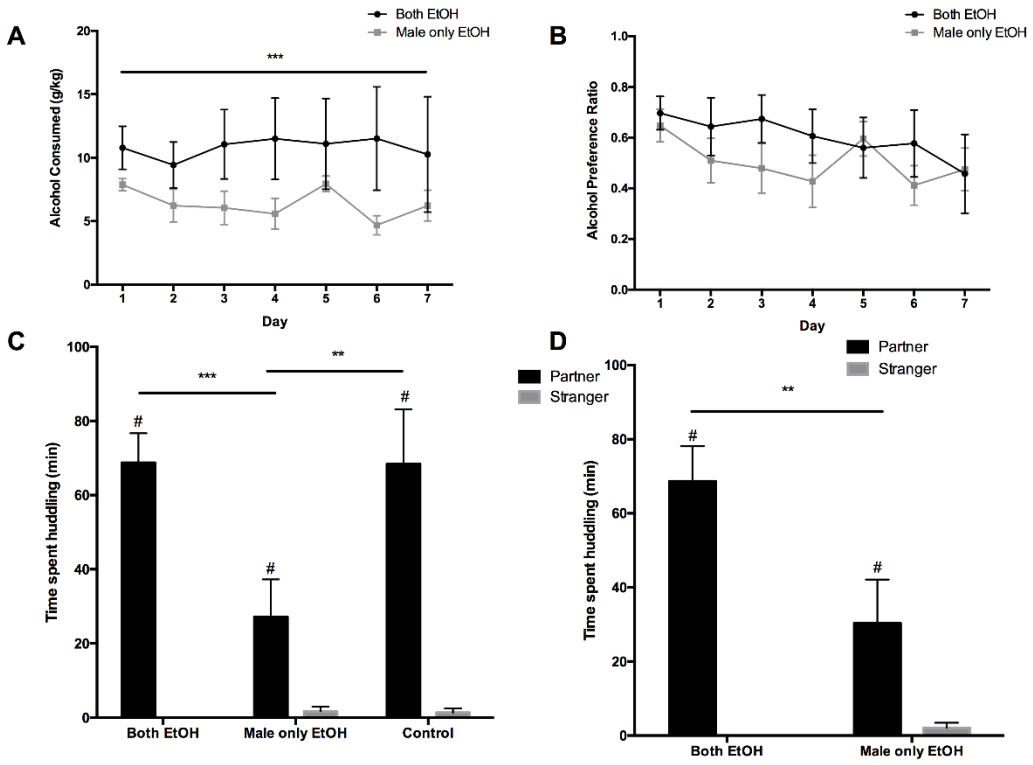


Figure 1. The effects of discordant and concordant alcohol consumption on the PPT in male voles. (A) Males that had a female partner that was exposed to alcohol showed a significant increase in alcohol consumption during the two-choice test over the Male only EtOH group, but showed no difference in (B) alcohol preference. (C) Males showed a PP under all three experimental conditions, but PP was significantly decreased when only the male had access to EtOH compared to when both animals were exposed to EtOH or only water. (D) When the EtOH exposed groups were matched (n = 4 per group) for alcohol consumption and preference, there was still a significant decrease in the amount of time the males in the Male only EtOH group spent huddling with their partners, compared to the males in the Both EtOH group. **p*<0.05; ***p*<0.01; ****p*<0.001; #significant effect of animal (*p*≤0.05). Error bars indicate mean ± SEM (n = 6 per group).

In addition to the analysis of alcohol intake and preference, water intake was analyzed. There was no significant difference in water intake between males in the Both EtOH group and males in the Male only EtOH group ($F_{1,70} = 0.016$, $p = 0.900$). There was no significant difference between the amount of water consumed each day ($F_{6,70} = 1.165$, $p = 0.335$) and no interaction between day and treatment group ($F_{6,70} = 0.378$, $p = 0.891$).

Next, we tested the effects of discordant and concordant drinking on PP in male prairie voles. During the PPT, there was a significant effect of stimulus animal (partner vs. stranger) on huddling time ($F_{1,30} = 67.70$, $p < 0.0001$), a significant effect of treatment (Both EtOH vs. Male only EtOH vs Control) on huddling time ($F_{2,30} = 4.236$, $p = 0.002$), and a significant interaction between treatment and stimulus animal ($F_{2,30} = 4.701$, $p = 0.017$; Fig. 1C). Post hoc analysis revealed that males in all three groups spent significantly more time huddling with their partner compared to the stranger animal ($p < 0.05$). The most important finding was that males in the Both EtOH ($p = 0.0009$) and Control ($p = 0.001$) groups spent significantly more time huddling with their partners compared to the amount of time the males spent huddling with their partner in the Male only EtOH group.

To determine if the difference in alcohol consumption between the Both EtOH and Male only EtOH groups contributed to the difference in PP between these groups, we matched groups for alcohol consumption by eliminating data from four animals (Both EtOH alcohol consumption = 7.7 ± 0.6 g/kg; Male only EtOH alcohol consumption = 6.8 ± 0.4 g/kg). This manipulation eliminated the significant difference in alcohol intake between the Both EtOH and Male only EtOH groups ($p = 0.237$). Reanalysis of the PPT in animals with matched intakes confirmed the significant effect of stimulus animal on huddling ($F_{1,14} = 37.610$, $p < 0.0001$), a significant effect of treatment on huddling time ($F_{1,14} = 5.237$, $p = 0.038$), and a significant interaction between stimulus animal and

treatment ($F_{1,14} = 6.514$, $p = 0.023$; Fig. 1D). Post hoc analysis revealed that both groups had a significant PP ($p < 0.05$) and again showed a significant increase in the amount of the time males in the Both EtOH group spent huddling with their partner compared to the males in the Male only EtOH group. This finding confirmed that the difference in the amount of time the males spent huddling with their partners was not attributed to the difference in alcohol consumption in the Both EtOH and Male only EtOH groups.

Effects of concordant and discordant drinking on selective aggression

When sexually naïve prairie voles are introduced to a novel conspecific they tend to show affiliative behaviors (Insel et al., 1995). These affiliative behaviors start to become directed specifically toward their partner once they formed a pair bond (Aragona et al., 2006; Gobrogge et al., 2007; Resendez and Aragona, 2012). In addition to more affiliative behaviors toward their partner, pair-bonded voles also display more aggressive behaviors toward unfamiliar same-sex stimulus animals. Therefore, we explored if males in the Male only EtOH group would show a change in the amount of aggressive behaviors toward an unfamiliar same-sex stimulus animal during the RI test. A separate group of animals were cohoused for a week and then introduced to the mesh divider cages with each animal receiving the two-bottle choice paradigm, as described above.

When both partners were given access to 10% ethanol, males consumed on average 7.2 ± 0.5 g/kg and females consumed 8.6 ± 1.0 g/kg during the seven-day drinking period. When only the male was given access to 10% ethanol, males consumed on average 7.0 ± 1.0 g/kg. Analysis of alcohol consumption revealed that there was no statistical difference in the amount of alcohol consumed by the males in the Both EtOH and the Male only EtOH groups ($F_{1,70} = 0.012$, $p = 0.915$; Fig. 2A). There was no significant difference between the amount of 10% ethanol consumed each day ($F_{6,70} =$

1.264, p = 0.285) and no significant interaction between day and treatment group (F6,70 = 0.674, p = 0.671). Males in the Both EtOH group showed a 64 ± 4.4% preference for alcohol, while the males in the Male only EtOH group showed a 44 ± 4.7% preference for alcohol; thus, leading to the occurrence of a significant difference between the two groups (F1,70 = 11.10, p = 0.001; Fig 2B). There was no significant difference in alcohol preference between each day (F6,70 =1.204, p = 0.315) and no significant interaction between day and treatment group (F6,70 = 0.895, p = 0.504).

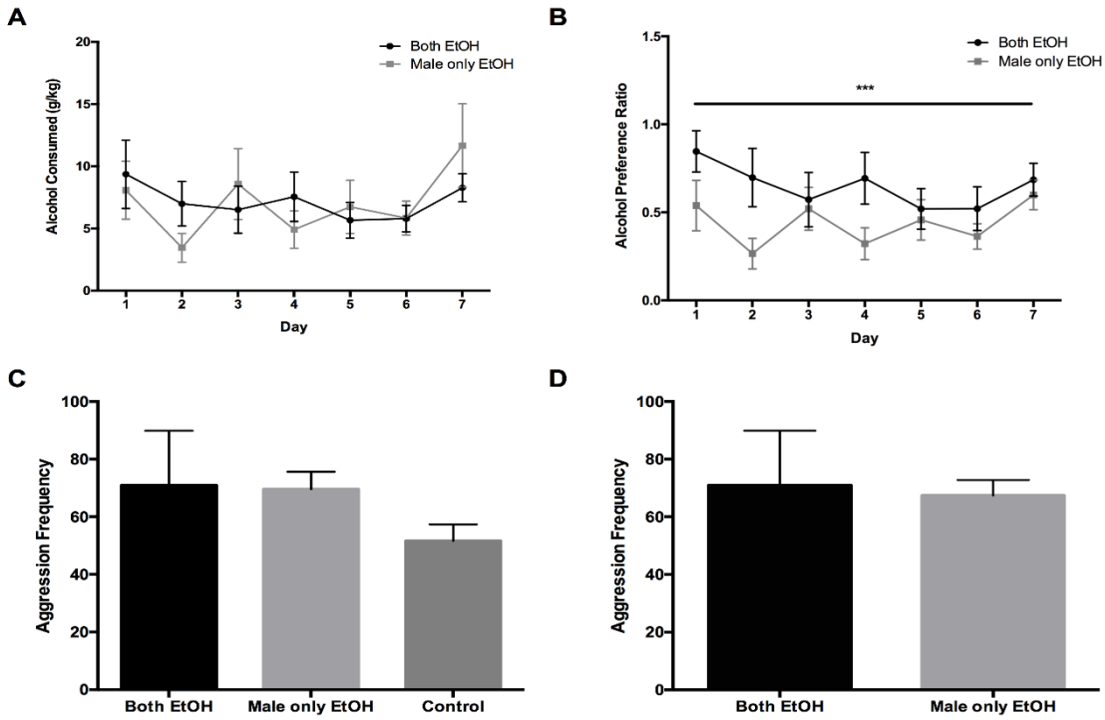


Figure 2. **The effects of discordant and concordant alcohol consumption on aggression frequency during the RI test in male prairie voles**. (A) Alcohol consumption in males did not differ between treatment groups. (B) There was a significant difference between the alcohol preference ratio between the Both EtOH and Male only EtOH groups. (C) Aggression frequency towards a stranger male during the RI test did not differ between male subjects in the three treatment groups. (D) When the alcohol consumption and alcohol preference ratio between the two alcohol consuming groups were matched, there was still no difference in aggression frequency between the two groups. *** $p<0.001$. Error bars indicate mean ± SEM (n = 5-7 per group).

To complement the alcohol intake and preference data, we analyzed the amount of water intake between groups. There was a significant difference between the amount of water consumed between the males in the Both EtOH group and males in the Male only EtOH group ($F_{1,70} = 17.050$, $p < 0.0001$). There was no significant difference in water intake between days ($F_{6,70} = 1.519$, $p = 0.185$) and no significant interaction between day and treatment group ($F_{6,70} = 0.935$, $p = 0.475$).

To determine if discordant alcohol consumption between partners contributes to a change in aggressive behavior, we ran the RI test after seven days of the two-bottle choice paradigm. We found no significant difference in the number of aggressive behaviors towards the unfamiliar same-sex intruder between the three treatment groups ($F_{2,15} = 1.066$, $p = 0.369$; Fig. 2C). To determine if the difference in alcohol preference ratio contributed to the non-significant effect of aggressive behaviors, we matched groups for alcohol consumption and preference and reanalyzed the RI test for the Both EtOH (alcohol consumption = 7.2 ± 0.5 g/kg; alcohol preference = 64 ± 4.4%) and Male only EtOH (alcohol consumption = 8.8 ± 1.3 g/kg; alcohol preference = 53 ± 6.3%) groups. Similarly to the previous results, we found no difference in the number of aggressive behaviors toward the resident intruder ($t_9 = 0.183$, $p = 0.859$; Fig 2D).

Immunohistochemical analysis of potential substrates of alcohol's effects on pair bond maintenance

Oxytocin and AVP play important roles in pair bonding, and oxytocin levels in the neurons of the paraventricular nucleus of hypothalamus (PVN) have been shown to decrease following long-term alcohol consumption (Stevenson et al., 2017b). Therefore, we tested whether the effects of alcohol on PP in the experiment above could be due to changes in the levels of these peptides. Immediately following the RI test, animals were euthanized and brains were cryopreserved for immunohistochemistry. We found a

significant effect of treatment for oxytocin-ir in the PVN ($F_{2,17} = 3.753$, $p = 0.045$; Fig 3A). Post hoc analysis revealed that the males that were given access to alcohol had a significant decrease of the amount of oxytocin-ir cells within the PVN ($p < 0.05$). Photomicrographs of oxytocin-ir in the PVN are shown for all three groups in Figs 3C-E. In contrast, we found no significant difference between the number of AVP-ir cells within the PVN between the three groups ($F_{2,17} = 1.576$, $p = 0.236$; Fig 3B).

Although we identified an effect of alcohol consumption on oxytocin levels, these levels were not different between the Both EtOH and Male only EtOH groups. Therefore, an effect of alcohol on PVN oxytocin levels could not completely explain the difference in PP between these groups, suggesting that other systems are involved in the effects of discordant drinking on pair bond maintenance.

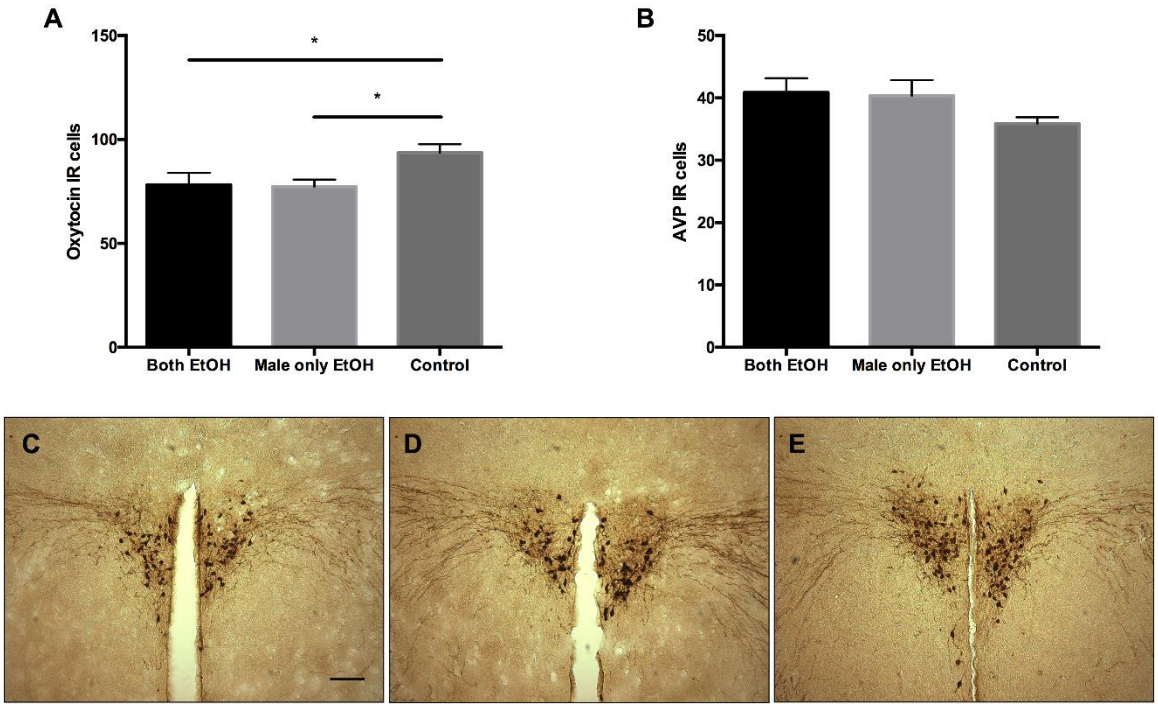


Figure 3. Immunoreactivity for oxytocin and AVP in the PVN. (A) Number of oxytocin-immunoreactive cells within the paraventricular nucleus of the hypothalamus (PVN) is significantly higher in the Control group compared to the Both EtOH and Male only EtOH groups. (B) Number of AVP-immunoreactive cells within the PVN does not differ between the three treatment groups. Representative photomicrographs of oxytocin-immunoreactivity in the PVN in the (C) Both EtOH (scale bar, 0.2um), (D) Male only EtOH, and (E) Control groups. *p<0.05. Error bars indicate mean ± SEM (n = 5-7 per group).

To begin identifying other neural substrates potentially involved effects of discordant drinking on PP, we examined FosB-ir across 18 different brain regions in the slices collected in the experiment above (Table 1). Five of the 18 brain regions showed significant differences between groups (Table 1). The number of positive FosB-ir cells within the PAG was significantly different between the three treatment groups. Specifically, males in the Male only EtOH group had an increase in FosB-ir cells in the entire PAG compared to the males in the Both EtOH and Control groups (Fig. 4,5A). In addition to the effects in the PAG, there were significant between group differences in the nucleus accumbens core (NAcc Core) ($F_{2,17} = 5.227$, $p = 0.017$; Fig. 6A), infralimbic cortex (IL) ($F_{2,17} = 3.808$, $p = 0.043$; Fig. 6B), ventral bed nucleus of the stria terminalis (vBNST) ($F_{2,17} = 3.607$, $p = 0.05$; Fig. 6C), and centrally projecting Edinger-Westphal nucleus (EW) ($F_{2,17} = 6.931$, $p = 0.006$; Fig. 6D). In all four of these regions, post hoc analysis revealed that FosB-ir in the males in the Both EtOH and Male only EtOH groups were not significantly different from each other. However, FosB-ir in Both EtOH and/or Male only EtOH groups was significantly different from males in the Control group. Thus, of all the brain regions examined, only PAG showed patterns of FosB expression different between males exhibiting discordant versus concordant drinking. To investigate if the difference was caused by a global increase in FosB-ir cells in the entire PAG or its particular subregion, we subdivided the PAG into three regions: dorsal medial (DMPAG), dorsal lateral (DLPAG), and lateral (LPAG) (Fig. 4). There were no between group differences in the number of FosB-ir cells in the DMPAG ($F_{2,17} = 0.297$, $p = 0.297$) and DLPAG ($F_{2,17} = 0.441$, $p = 0.65$). However, there was a between group difference in the LPAG ($F_{2,17} = 5.311$, $p = 0.016$; Fig 5B-D). Post hoc analysis revealed that males in the Male only EtOH group had a significant increase in the number of FosB-ir cells in the LPAG compared to the males in the Both EtOH ($p < 0.05$) and Control ($p < 0.01$) groups.

Brain Region	Both EtOH	Male only EtOH	Control	*p*-value
Anterior Cingulate (CG1)	781.3±36.98	701.4±75.5	775.9±36.82	0.2993
Anterior Cingulate (CG2)	911.8±95.26	919.0±103.2	888.7±38.65	0.9676
Agranular Insula (AI)	250.9±30.42	237.9±22.25	254.5±16.65	0.8813
Granular Insula (GI)	378.2±50.85	362.1±35.65	353.7±16.87	0.9038
Infralimbic Cortex	354.9±50.97	418.8±36.44	260.2±23.99	**0.0430**
Retrosplenial Cortex	1395±154.9	1333±79.22	1202±45.40	0.4699
Dorsal Lateral Striatum	953.9±92.69	868.6±149.3	1007±79.68	0.7004
Dorsal Medial Striatum	1383±89.47	1380±104.3	1287±95.07	0.7411
Nucleus Accumbens Core	1385±119.0	1510±83.98	988.6±145.6	**0.0170**
Nucleus Accumbens Shell	945.6±86.02	1021±91.77	821.1±129.0	0.4048
Lateral Septum	384.7±34.97	372.2±32.58	328.6±26.74	0.4666
Dorsal Bed Nucleus of the Stria Terminalis	197.8±33.05	165.1±25.80	112.5±12.63	0.1064
Ventral Bed Nucleus of the Stria Terminalis	204.6±29.55	187.6±19.89	119.1±16.14	**0.0495**
Paraventricular of the Hypothalamus	27.31±5.340	35.94±5.007	47.94±16.74	0.2085
Hippocampus (CA1-3)	198.5±49.06	308.1±53.00	322.9±59.48	0.2484
Dentate Gyrus	592.6±120.3	747.8±124.0	478.5±107.1	0.3065
Periaqueductal Gray (total)	220.3±24.78	290.8±30.10	199.4±5.631	**0.0373**
Periaqueductal Gray (Dorsal Medial)	31.55±3.375	45.12±6.755	38.25±7.754	0.2970
Periaqueductal Gray (Dorsal Lateral)	40.90±6.555	47.38±6.155	39.83±5.581	0.6504
Periaqueductal Gray (Lateral)	149.2±18.13	199.7±19.69	121.3±10.29	**0.0162**
Edinger Westphal Nucleus	11.38±2.051	10.29±1.550	3.139±1.039	**0.0063**

Table 1. The mean ± SEM for positive FosB cells for each experimental group per brain region examined. The *p* value from the ANOVA for each brain region is listed in column 5. Regions with significant *p* values ($\alpha \leq 0.05$) are in bold text.

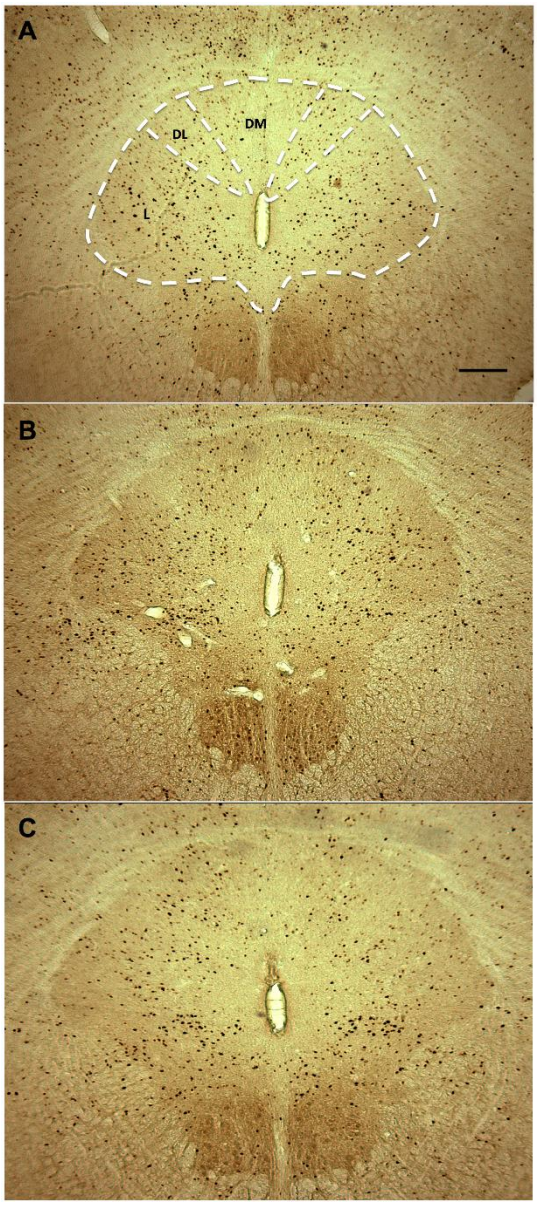


Figure 4. Representative photomicrographs for FosB immunoreactivity in the sub regions of the periaqueductal gray (PAG) in (A) Both EtOH, (B) Male only EtOH, and (C) Control. DM, dorsal medial PAG; DL, dorsal lateral PAG; L, lateral PAG.

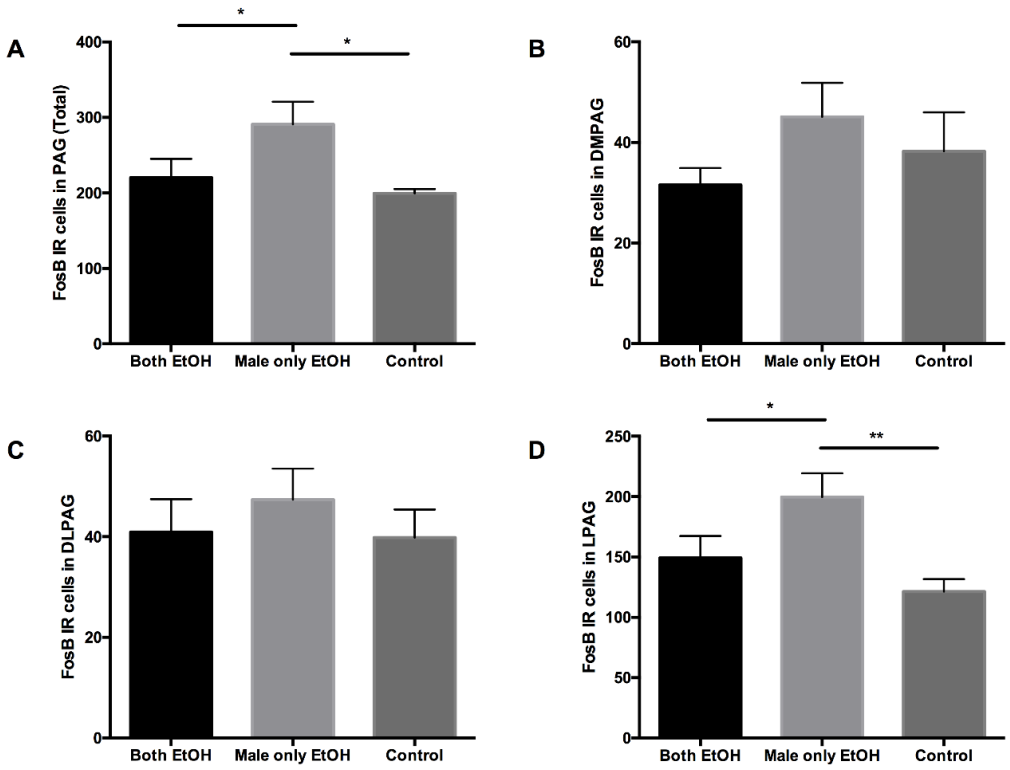


Figure 5. Immunoreactivity for FosB in the sub regions of the periaqueductal gray. (A) The number of immuoreactivity FosB cells within the PAG was significantly increased in the Male only EtOH group compared to the Both EtOH and Control groups. There was no difference in the number of FosB cells in the PAG when both partners were exposed to EtOH compared to when both partners were exposed to only water. The PAG was divided into three sub regions: (B) dorsal medial, (C) dorsal lateral, and (D) lateral. The three different 2-bottle choice conditions had no significant effect on the number of FosB cells within the dorsal medial and dorsal lateral regions of the PAG. The number of FosB cells within the lateral region of the PAG significantly differed between the three treatment groups (D). The Male only EtOH group showed an increase in the number of FosB cells within the lateral PAG when compared to the Both EtOH and Control groups, thus leading to an increase in the total number of FosB cells within the PAG. *$p < 0.05$; **$p < 0.01$. Error bars indicate mean ± SEM (n = 5-7 per group).

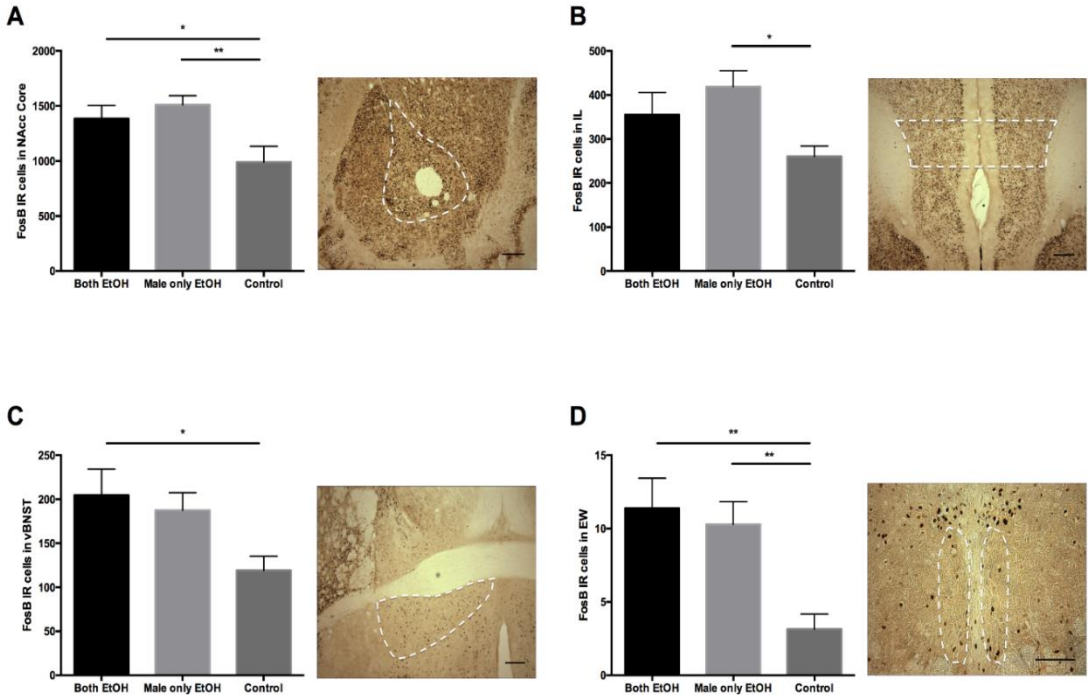


Figure 6. FosB immunoreactivity enhanced in regions within the Both EtOH and Male only EtOH groups. The number of FosB positive cells in four additional brain regions showed a significant difference between the three groups. (A) Number of FosB positive cells within the nucleus accumbens core (NAcc Core) was significantly increased within the Both EtOH and Male only EtOH groups compared to the Control group. (B) Number of FosB positive cells in the infralimbic cortex (IL) was significantly increased in the Male only EtOH group compared to the Control group. (C) Subjects in the Both EtOH group had an increase in the number of FosB positive cells in the ventral bed nucleus of the stria terminalis (vBNST) compared to the Control group. (D) The number of FosB positive cells in the centrally projecting Edinger-Westphal nucleus (EW) was significantly increased in the Both EtOH and Male only EtOH groups compared to the Control group. *$p < 0.05$; **$p < 0.01$. Error bars indicated mean ± SEM (n = 5-7 per group).

Discussion

The present study investigated how discordant and concordant alcohol drinking influences established pair bonds in male prairie voles. We found that male prairie voles had a decreased PP if the drinking was discordant, but not when it was concordant. Specifically, PP was decreased when the males were drinking alcohol, while their female partner was drinking only water. In contrast, when both male and female partners were drinking alcohol, male prairie voles showed no reduction in PP compared to when both partners were exposed to only water. Interestingly, when males were tested for selective aggression we saw no group differences between the amount of aggressive behaviors displayed toward an unfamiliar same-sex prairie vole in the RI test. Previous studies have shown that drugs of abuse administered during the formation of a pair bond can affect PP (Anacker et al., 2014a; Hostetler et al., 2016; Liu et al., 2010; Young et al., 2014). To our knowledge, this is the first study to demonstrate an effect of a drug of abuse on PP when given after a pair bond has been formed.

We chose to investigate the effects of alcohol on maintenance of PP using the voluntary 2-bottle choice drinking procedure because voluntary and involuntary modes of drug administration in rodent models engage different neurocircuits (Chen et al., 2008; Hemby et al., 1997; Mccutcheon et al., 2011; Ryabinin, 2000). This procedure allowed us also to investigate whether female partners would influence alcohol consumption in the males. We found that female drinking status had an inconsistent tendency to influence alcohol self-administration in male prairie voles. Thus, in the first experiment the amount of alcohol consumed was significantly higher in the Both EtOH versus the Male only EtOH group. While this difference was not significant in the second experiment, males in the Both EtOH group showed a significantly higher preference for alcohol than males in the Male only EtOH group. Previous research has shown that same-sex prairie voles will socially facilitate the amount of alcohol each partner

consumes (Anacker et al., 2011b), but opposite sex partners do not significantly influence drinking behaviors (Hostetler et al., 2012). The latter study had methodological differences in relation to the current study, including that males were gonadectomized and partners were exposed to increasing alcohol concentration (3-10%) over a twelve-day period. It is possible that these two methodological differences were the reason why we saw that females can influence males' self-administration in the current study, but not in previous studies. It is also possible that significant effects of alcohol intake would be reached if more animals were used in this study. Importantly for the main result of the current investigation, when the Both EtOH and Male only EtOH groups were matched for alcohol intake and preference, only the Male only EtOH group showed decreased PP. This finding indicated that discordant drinking, but not concordant drinking inhibits maintenance of the pair bond in prairie voles.

Remarkably, the inhibitory effects of discordant drinking on pair bond maintenance observed here parallel epidemiological data on the association between alcohol consumption and marital dissolution in humans. Thus, couples with high alcohol drinking in both spouses are often found to be as stable as abstinent couples and significantly more stable than couples in which only one spouse drinks (Marshal, 2003; Ostermann et al., 2005; Torvik et al., 2013). This observation appears to be very consistent when it is based on the number of separations, and less so when it is based on subjective measures of marital satisfaction (Mcleod, 1993; Haber and Jacob, 1997; Graham and Braun, 1999; Kelly et al., 2002). Interestingly, the effect of such discordant drug taking on marital stability is relatively specific for alcohol, as it is not observed in relations to smoking and marijuana (Leonard et al., 2014).

Importantly, a recent study by Leonard et al. (2014) suggests that effects of discordant drinking on divorce rates could be stronger in heavy drinking wives than in heavy drinking husbands. Specifically, the effect of discordant drinking in husbands was

statistically significant when data were unadjusted for sociodemographic, antisocial personality and depression. When these three factors were adjusted for, heavy drinkers-husband heavy couples displayed a trend for the increase in divorce rates compared to non-using couples. In contrast, the discrepant heavy drinkers-wife heavy group in their study showed a significant increase in divorce rates compared to non-using couples when adjusted or unadjusted for the same factors. Our study for the first time analyzed effects of discordant drinking on pair bond maintenance in voles and initially focused on males. Our future experiments will address how a discrepancy in alcohol consumption affects pair bond maintenance in female prairie voles. In addition, it will be important to investigate whether a different duration of alcohol access or withdrawal (versus intoxication) could modulate the effects of alcohol on pair bond maintenance. Nevertheless, the current findings of disruptive effects of discordant alcohol drinking during one week on pair bond maintenance in male voles, provide evidence that such effects have biological underpinnings. Therefore, prairie voles can be used to investigate neural substrates of the effects of alcohol use and abuse on social monogamous behaviors. Such investigations were initiated in the current study.

Our analysis on PVN, showed that alcohol drinking in prairie voles leads to a decrease in the number of oxytocin-immunoreactive cells within the PVN. This reduction in oxytocin-immunoreactive cells occurred in both alcohol-consuming groups, regardless of female drinking status. This alcohol-mediated decrease in oxytocin levels is in agreement with two previous studies. Silva et al. (2002) found that rats that received an alcohol solution as their only liquid source for 6 or 10 months showed a decrease in the amount of oxytocin-immunoreactive and AVP-expressing cells in the PVN which was attributable to cell death. Interestingly, the surviving cells showed hypertrophy, such that oxytocin mRNA and AVP mRNA levels per cell compensated for the cell loss. A more recent study performed by Stevenson et al. (2017b) in prairie voles showed that seven

weeks of voluntary alcohol consumption of 15% ethanol in a 2-bottle choice procedure resulted in a decrease in the number of oxytocin cells in the PVN of male animals. As in our experiments, there was no significant reduction in the number of PVN AVP neurons. These findings suggest that while a prolonged exposure to alcohol can affect the AVP system, the PVN oxytocin neurons are sensitive to relatively short exposures. In the current study, the decrease in the number of oxytocin neurons was observed after an even shorter (one week) exposure to alcohol than in the Stevenson study. While Stevenson et al. (2017b) and our study did not specifically address whether the reduction in oxytocin-positive neurons is attributable to cell death, the rapid effect observed in our study suggests an effect on oxytocin expression, rather than loss of specific neurons.

Our observation that only one week of voluntary alcohol consumption was required for the significant reduction in oxytocin neurons indicates high sensitivity of this system to alcohol. On the other hand, the fact that both concordant and discordant drinking affected the PVN oxytocin neurons suggests that effects of alcohol on this system alone can't explain the selective effect of discordant drinking on maintenance of PP. Therefore, additional mechanisms involved in this selective effect need to be explored.

We began searching for such involved additional systems by testing levels of FosB immunoreactivity in 18 brain region that could be potentially involved in regulation of social attachment or effects of alcohol. FosB is an immediate early gene. Expression of immediate early genes Fos, FosB, JunB can be used to map acute changes in neural activity (Graybiel et al., 1990; Hope et al., 1992; Young et al., 1991). However, repeated exposure to the same stimulus can attenuate the immediate early gene response in neurons (Melia et al., 1994; Nestler et al., 2001). This decreased sensitivity to repeated treatment makes mapping changes in neural activity following one week of continuous

exposure to alcohol difficult. In contrast to other immediate early genes, FosB also encodes a short deltaFosB protein, which gradually accumulates with repeated treatments (Hope et al., 1994; Kelz and Nestler, 2000; Moratalla et al., 1996; Nye et al., 1995). The anti-FosB antibody used in the current experiments recognizes both the full-length FosB protein and deltaFosB. Therefore, our FosB immunohistochemistry was capable of detecting effects of both acute and prolonged effects of alcohol.

In the present study we detected 5 brain regions in which the number of FosB-immunoreactive cells were regulated by alcohol. We observed that alcohol, independent of the drinking status of the female partner, increased FosB in NAcc Core, IL, vBNST, and EW. NAcc and EW have been previously repeatedly found to respond with immediate early gene induction to either involuntary or voluntary alcohol exposure (Bachtell et al., 1999; Bachtell et al., 2002a; Bachtell et al., 2002b; Ozburn et al., 2012; Ryabinin and Wang, 1998b; Ryabinin et al., 2001). IL and BNST have been found to respond with induction of the immediate early gene c-fos to involuntary alcohol exposure (Bachtell and Ryabinin, 2001; Ryabinin et al., 1997). Importantly in relation to behavior results, we also found that males, who are in the discordant drinking group, have an increase in the amount of FosB-immunoreactive cells in the PAG compared to the males in the concordant and control group. Specifically, the LPAG was driving the increase in FosB-immunoreactivity for the entire PAG region. This result suggests that LPAG could be involved in mediating selective effects of discordant drinking on maintenance of PP.

While previous research has suggested that PAG is involved in social behaviors, most studies focused on its activity in response to a social stress (Depaulis et al., 1992; Vivian and Miczek, 1999). Related to affiliative behaviors, PAG activation was been shown to be associated with exposure to maternal emotional responses in humans (Bartels and Zeki, 2004). In agreement with this idea, Miranda-Paiva et al. (2003) showed that injections of the opioid antagonist naloxone into the rostral lateral PAG

reversed inhibitory effects of morphine on maternal behaviors in rats. While the neurocircuitry of pair bond formation has been elucidated, only a few studies to date have examined the potential contribution of specific brain regions in maintenance of pair bonds. Bales et al. (2007) and Maninger et al. (2017) have mapped changes in glucose metabolism following pair-bonding in monogamous titi monkeys. They found significant increases in glucose metabolism is several brain regions, but not in the PAG. Resendez et al. (2016) have shown that manipulations of the opioid system in the nucleus accumbens regulated maintenance of pair bonds in prairie voles. The potential causal contribution of PAG to maintenance or formation of pair bonds has not been tested. Such studies will need to be performed in the future.

Taken together, we have identified that discordant, but not concordant, voluntary alcohol consumption inhibits maintenance of pair bonds in male prairie voles, as evidenced by decreased PP. This effect is reminiscent of effects of heavy discordant drinking on marital dissolution in humans. We have identified potential neural substrates involved in these effects. Future studies should use comprehensive pharmacological and molecular approaches testing whether these inhibitory effects of discordant drinking can be reversed or prevented.

CHAPTER 2: Effects of Alcohol Consumption on Pair Bond Maintenance and Potential Neural Substrates in Female Prairie Voles

(A modified version of this chapter has been accepted for publication in Alcohol and Alcoholism)

Introduction

Fictional literature and art, as well as human epidemiological research, abound with examples of interactions between excessive alcohol consumption and intimate partner relationships. The epidemiological research confirms a significant association between alcohol abuse and disruptions in these relationships. Alcohol and drug abuse has been shown to be the third most cited reason why couples get divorced in the United States (Amato and Previti, 2003). Heavy alcohol use specifically has been known to lead to higher rates of separation, relationship dissatisfaction, and intimate partner violence (Leonard and Senchak, 1993; Mcleod, 1993; Caces et al., 1999; Leonard and Quigley, 1999; Leonard and Rothbard, 1999; Collins et al., 2007).

The majority of studies on this subject have explored alcohol consumption in one partner without taking into account the amount of alcohol the other partner consumes. A very limited number of studies have explored the potential difference in effects of concordant and discordant heavy alcohol consumption on intimate relationships. Thus, couples in which one spouse drinks heavily, but the other one does not, are found to be unstable. On the other hand, couples with high alcohol drinking in both spouses are often as stable as abstinent couples and significantly more stable than couples in which only one spouse drinks (Marshal, 2003; Ostermann et al., 2005; Torvik et al., 2013; Leonard et al., 2014). Other studies have shown higher rates of separation and higher rates of marital dissatisfaction in heterosexual (Mudar et al., 2001; Homish and Leonard, 2007; Homish et al., 2009) and homosexual couples (Kelley et al., 2015) when there is a discrepancy in alcohol consumption between partners. Several factors could potentially contribute to these statistics, including neurobiological effects of alcohol, human-specific socioeconomic factors, and psychological factors. Several studies indicate that while socioeconomic and psychological factors (i.e. depression and antisocial personality disorder) can impact marital stability, they do not modulate alcohol's effects on this

measure (Kenkel et al., 1994; Leonard et al., 2014). In addition, this idea contradicts greater stability of couples with two heavy drinking spouses versus couples with only one heavy drinker. Therefore, biological factors contributing to alcohol's effects on stability of intimate partner relationships need to be examined.

It is difficult to establish causal relations between factors using only epidemiological studies. Human imaging studies have not yet been performed to evaluate effects of discordant drinking on neural activity. In contrast, animal models are invaluable in understanding neurobiological mechanisms underlying the causal effects of alcohol on physiology and behavior. Unfortunately, modeling human social relationships is difficult in traditional laboratory animals because mice and rats are not socially monogamous. Recently much progress in understanding the neurobiology of social relationships has been made by studying prairie voles (*Microtus ochrogaster*), a socially monogamous species. Prairie voles form life-long pair bonds with opposite-sex (Carter and Getz, 1993) and same-sex (Devries et al., 1997) partners. In the laboratory, pair bonding can be investigated using the partner preference test (PPT). Studies testing partner preference in laboratory-housed prairie voles have identified several mechanisms regulating such bonds, including the causal contribution of central oxytocin and vasopressin systems (Insel and Hulihan, 1995; Pitkow et al., 2001). Subsequent studies confirmed the importance of these systems for human relationships thereby indicating the translational value of studies in prairie voles (Ebstein et al., 2009; Heinrichs et al., 2009; Meyer-Lindenberg et al., 2011).

Based on this translational perspective, previous studies have explored the effects of drugs of abuse on social relationships in prairie voles. In addition to being a translational model for life-long social attachments, prairie voles express preference for alcohol-containing solutions and can voluntarily consume high levels of alcohol without sucrose-fading procedures (Anacker et al., 2011a). Social factors have been shown to

influence alcohol consumption in prairie voles, allowing one to model social facilitation and inhibition of drinking, as well as effects of social hierarchies on drinking, and to subsequently study neural substrates of these effects (Anacker et al., 2011b; Hostetler et al., 2012; Anacker et al., 2014b; Hostetler and Ryabinin, 2014).

More recent studies in prairie voles transitioned to investigating the effects of alcohol consumption on social attachments. Anacker et al. (2014a) explored how alcohol consumption affects the formation of pair bonds in male and female partners. Opposite-sex prairie voles received access to alcohol and water (or only water) during a 24-hour cohabitation period. Pair bond formation was accessed using the PPT. Males exposed to alcohol displayed no preference for their partner over stranger females compared to control males who displayed a partner preference. Interestingly, females exposed to alcohol displayed a facilitation in partner preference compared to the female control group. Thus, alcohol consumption has sex-dependent effects on pair bond formation in prairie voles.

While Anacker et al. (2014a) confirmed the existence of biological mechanisms of alcohol's effects on the formation of social bonds, they did not address effects of alcohol on maintenance of such bonds. Importantly, although less investigated, maintenance of social attachments in prairie voles includes additional mechanisms (i.e. the D1-like dopamine and kappa-opioid receptors) besides those involved in the formation of social bonds (Aragona et al., 2006; Resendez and Aragona, 2012). Therefore, a second study explored the effects of alcohol on established pair bonds in male prairie voles. Briefly, in Chapter 1 we introduced male and female prairie voles into a standard housing cage for one week (Walcott and Ryabinin, 2017). Following the one week of cohabitation, pairs were placed into semi-social housing cages with a mesh divider down the center of the cage. Within these mesh-divided cages, animals were introduced to 10% EtOH and water or continued to drink water for one week. In these

experiments, when only the male, but not the female, consumed alcohol, male prairie voles showed a decrease in partner preference. On the other hand, when both the male and the female concordantly consumed alcohol, male prairie voles showed intact partner preference. This finding indicated that the association between discordant drinking in males observed in human epidemiological studies could be due to the existence of biological effects of alcohol on mechanisms regulating pair bond maintenance. Subsequent experiments identified that alcohol decreased oxytocin and increased FosB immunoreactivity in several brain regions irrespective of the partner's drinking. However, there was an increase in FosB immunoreactivity in the periaqueductal gray only after discordant drinking in male prairie voles, suggesting involvement of this brain region in the effects of discordant alcohol drinking on pair bond maintenance.

In chapter 1 we explored the effects of discordant alcohol consumption between partners on established pair bonds in male prairie voles. However, in humans the highest rate of separation is observed when a female partner is the heavy drinker and the male partner is an abstainer (Ostermann et al., 2005; Leonard et al., 2014). On the other hand, the occurrence of such couples is less frequent than those where the male is the heavy drinker and the female is an abstainer. To clarify the effects of alcohol on pair bond maintenance, it would be valuable to model heavy drinking in females and abstinence in males using animal models. Therefore, in the current study, we explored the effects of discordant alcohol consumption between partners on established pair bonds in female prairie voles. Following the behavioral analysis, we also analyzed levels of oxytocin, vasopressin, and the neuronal activity marker FosB in our experimental subjects to assess the neurobiological effects of alcohol.

Materials and Methods

Animals

Female adult prairie voles 66-109 days old at the beginning of the experiment were used from our breeding colony at the VA Portland Health Care System (VAPORHCS) Veterinary Medical Unit. The animals were weaned at 21 days of age and housed with same-sex siblings in cages (27x27x13 cm) on a 14:10 light/dark cycle with lights on at 6 a.m. All animals had access to cotton nestlets and *ad libitum* access to water and food. Prairie vole diet consist of a mixture of rabbit chow (LabDiet Hi-Fiber Rabbit; PMI Nutrition International, Richmond, IN), corn (Nutrena Cleaned Grains; Cargill, Inc., Minneapolis, MN), and oats (Grainland Select Grains; Grainland Cooperative, Eureka, IL) throughout the entire experiment. Prairie voles are inducible ovulators and do not cycle prior to a prolonged exposure to males. Therefore, virgin females were housed in a room separate from the rest of the colony. All experiments were performed under the approval of the Institutional Animal Care and Use Committees at VAPORHCS and Oregon Health & Science University (OHSU), Portland, Oregon, USA.

Housing Conditions

Female subjects were housed with male partners in a standard housing cage for one week to establish a pair bond. Immediately following this week, female subjects were placed in a mesh-divided cage (27x27x13 cm) for one week with their opposite-sex partners, with each animal on each side of the divider. These cages have been described previously (Chapter 1; Walcott and Ryabinin, 2017). These cages prevent animals from mating, but allow the transfer of visual, olfactory, and tactile cues between animals and allow the experimenter to measure the individual amount of fluid consumed by each animal. Previous studies have shown that these cages do not disrupt established pair bonds (Curtis, 2010).

Two-Bottle Choice

Following the one week in the standard housing cages, pairs experienced a two-bottle choice paradigm in the mesh-divided cages. All animals received continuous access to two 25mL glass tubes fitted with rubber stoppers with metal sippers attached. Fluid consumption was measured every 24 hours for 7 days and the location of the bottles was switched each day to prevent side bias. There were three drinking conditions in this study; 1) both female and male partners received access to one bottle of 10% EtOH and one bottle of H2O (Both EtOH, *n* = 7); 2) female subject received access to one bottle of 10% and one bottle of H2O, while the male partner received access to two bottles of H2O (Female EtOH only, *n* = 6); 3) Both female subject and male partner received access to two bottles of H2O (Control, *n* = 7). Stranger males, who were later used in the PPT, received the same treatment as their male partner counterpart in a separate room.

Partner Preference Test

The partner preference test (PPT) is the standard way to measure pair bonds in voles. PPT has been described previously (Williams et al., 1992; Ahern et al., 2009). Briefly, it occurs in a three-chambered apparatus with the partner animal tethered to one chamber, while a stranger animal is tethered to the other. The subject animal is placed in the center (neutral) chamber and is allowed to move freely through the three chambers. The 3-hour PPT occurred immediately following the two-bottle choice paradigm and was videotaped for later behavioral analysis. An experimenter who was blind to the group assignments and trained in detecting huddling behavior analyzed recorded videos. VLC Media Player (Boston, MA, USA) was used to view the recorded videos. Behavioral analysis software, JWatcher V1.0 (http://www.jwatcher.ucla.edu/), was used to measure the amount of time each animal spent huddling with the partner or stranger stimulus at a 5x playback speed.

Embryo Analysis

Embryo weights were analyzed after PPT. Average embryo weights are known to be positively correlated with measurements of pair bond maintenance (Curtis, 2010; Resendez et al., 2012). Average embryo weights over 0.3g were considered an optimal impregnation (greater or equal to 10 days pregnant at the time of testing), while average embryo weights less than 0.3g were considered a suboptimal impregnation (less than 10 days pregnant at the time of testing). Only data from optimally impregnated female subjects were used in this current study.

Immunohistochemistry

To determine the potential biological mechanisms underlying the interactions between alcohol drinking and social behaviors, brains from female subjects were examined. Immediately after PPT, female subjects were euthanized by CO_2 inhalation. Brains were extracted, fixed in 2% paraformaldehyde/PBS for 24 hours, and cryoprotected in 20% and then 30% sucrose/PBS for 24 hours in each solution. Brains were sliced into 40µm coronal sections. The immunohistochemistry (IHC) protocol was based on previous publications (Ryabinin and Wang, 1998a; Anacker et al., 2014a). The following antibody concentrations were used: anti-oxytocin (1:20,000 for brightfield IHC and 1:1000 for double fluorescence, Peninsular Laboratories); anti-arginine vasopressin (1:50,000, Peninsular Laboratories); anti-FosB (1:27,000, Abcam), and anti-cleaved caspase 3 (1:1000, Cell Signaling). All primary antibodies were polyclonal and made in rabbit, except the oxytocin used in double fluorescence which was made in guinea pig. 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei (1:30,000, Invitrogen) in the double fluorescence test. For brightfield analysis, the tissue was visualized using a diaminobenzidine substrate kit (Thermo Fisher Scientific). For immunofluorescence, the

tissue was visualized with Alexa fluor 488 donkey anti-guinea pig IgG (1:400, Jackson Immuno. Research), or Alexa fluor 555 donkey anti-rabbit IgG (1:400, Invitrogen). Images were obtained on a Leica DM4000 microscope. For quantitative analyses, ImageJ was used to automatically count cells stained above background.

Statistical Analysis

Alcohol consumption was calculated by dividing the grams of alcohol consumed by the weight of each vole in kilograms (g/kg). Additionally, alcohol preference for each vole was calculated based on volume of fluid consumed. Alcohol consumption and preference were both analyzed by repeated-measures ANOVA design using group (Both EtOH, Female only EtOH, and Control) as the between-subject factor and the drinking day as the repeated measure. Significant outcomes were followed by a Fisher's LSD *post hoc* test.

The main measure of PPT is the amount of time the subject animal spends huddling with their partner or stranger stimulus animal. Female partner huddling was analyzed using a one-way ANOVA with treatment (Both EtOH, Female only EtOH, and Control) as the between-subjects factor. We used the Brown-Forsythe test to determine normality of female huddling time with male partners. Partner huddling was normally distributed ($F_{2,17} = 0.76$, $p = 0.48$). We did not use stranger huddling in the analysis because female subjects did not spend anytime huddling with the stranger.

Activity levels, embryo weights, and immunohistochemistry data were analyzed by one-way ANOVA, with treatment as the between-subjects factor. Significant effects were followed up by a Fisher's LSD *post hoc* test.

Results

Female alcohol consumption and preference are affected by the drinking status of male partners

To determine if a discrepancy in alcohol drinking between partners can lead to a decrease in partner preference in female prairie voles, we exposed prairie voles to three treatment conditions: 1) female and male partners both received 10% EtOH and water during the two-bottle choice paradigm (*Both EtOH*); 2) females received 10% EtOH and water, while male partners received two bottles of water during the two-bottle choice paradigm (*Female only EtOH*); 3) female and male partners both received two bottles of water during the two-bottle choice paradigm (*Control*).

Females in the *Both EtOH* group displayed a mean intake of 8.7 ± 0.8 (mean ± SEM) grams of alcohol per kilogram of body weight (g/kg) and their male partners consumed 7.2 ± 0.4 g/kg over the seven-day two-bottle choice paradigm. Meanwhile, females in the *Female only EtOH* group displayed a mean intake of 5.7 ± 0.5 g/kg over the seven-day two-bottle choice paradigm. A repeated-measures ANOVA analyzing alcohol consumption over the seven-day period revealed no significant main effect of time [$F_{6,66} = 0.87$, $p = 0.52$] or treatment [$F_{1,11} = 2.68$, $p = 0.13$]. However, there was a significant interaction between time and treatment [$F_{6,66} = 2.21$, $p = 0.05$]. *Post hoc* analysis revealed that alcohol consumption was significantly different between groups on Days 6 and 7 (Fig. 1A). To confirm that drinking was concordant in the *Both EtOH* group, we also analyzed alcohol consumption between the female and male partners in the *Both EtOH* group. Analysis revealed no significant main effect of time [$F_{6,72} = 1.19$, $p = 0.32$], sex [$F_{1,12} = 0.46$, $p = 0.51$] or interaction [$F_{6,72} = 1.41$, $p = 0.22$; data not shown] confirming that concordant drinking was indeed achieved in the *Both EtOH* group.

Females in the *Both EtOH* group had a mean alcohol preference of 46.2 ± 2.2%, while females in the *Female only EtOH* group had a mean preference of 36.1 ± 4.7% over the seven-day drinking period. A repeated-measures ANOVA revealed a significant

main effect of time [$F_{6,66} = 2.35$, $p = 0.04$] and no effect of treatment [$F_{1,11} = 0.92$, $p = 0.36$]. There was a trend for a significant interaction between time and treatment [$F_{6,66} = 1.89$, $p = 0.09$]. *Post hoc* analysis revealed a trend for significant differences ($p < 0.10$) between treatment groups on Days 6 and 7 for alcohol preference (Fig. 1B) confirming that an opposite-sex partner's drinking status can influence alcohol consumption and preference in female prairie voles.

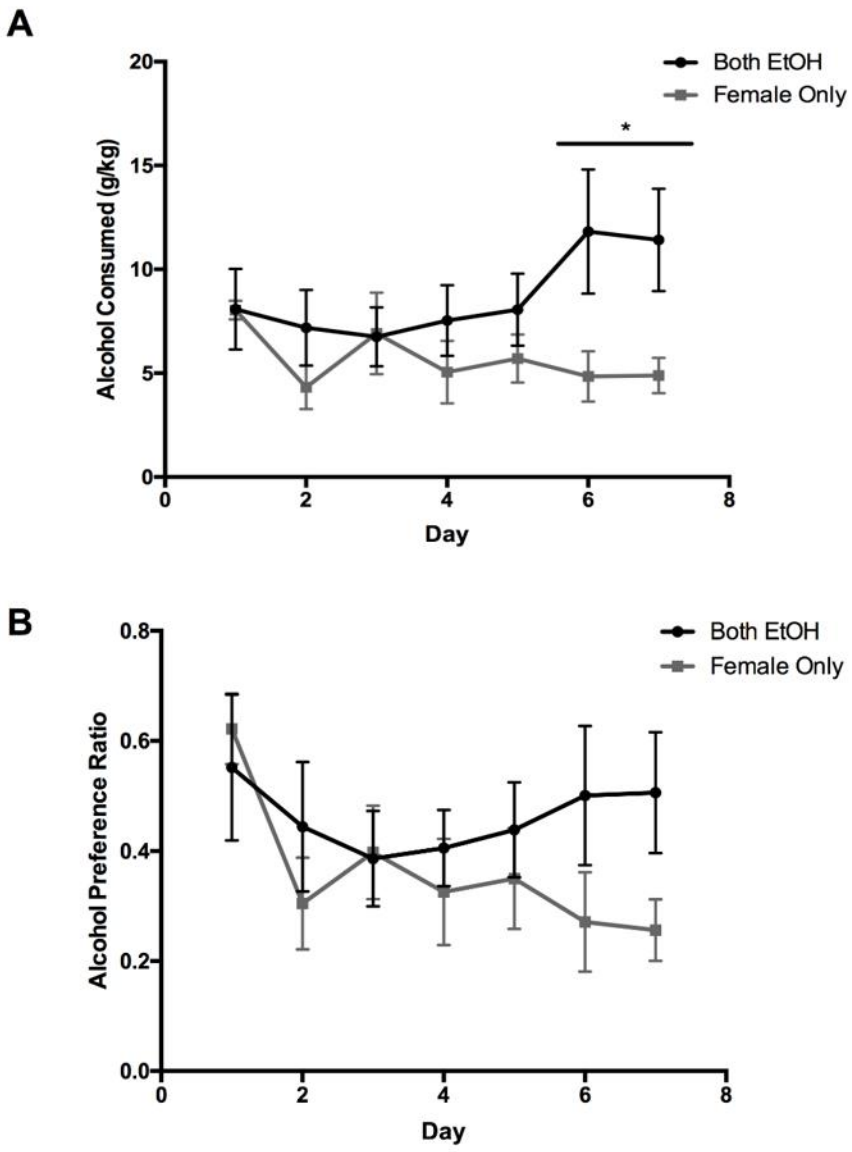


Figure 1. Alcohol consumption and preference across the one week 10% ethanol drinking period. (A) Females in the Both EtOH group had higher alcohol consumption during the last two days of the alcohol access period compared to females in the Female Only group. (B) Alcohol preference for the females in the Both EtOH group approached a significant difference on the last two days of alcohol access compared to the females in the Female Only group. * $p<0.05$ vs Female only EtOH. Error bars indicate mean ± SEM (n = 6-7 per group).

Alcohol drinking does not alter female pair bond maintenance

PPT was used to determine pair bond strength following alcohol consumption. During the test females did not huddle with stranger animals in any treatment group. Therefore, we analyzed the mean amount of time of partner huddling in each treatment group. One-way ANOVA revealed that there was no difference in the time spent huddling with partners between treatment groups [$F_{2,17} = 1.14$, $p = 0.34$; Fig. 2A]. Alcohol consumption can affect locomotor activity (Smoothy and Berry, 1985) and embryo weights (Ghimire et al., 2008) in rodent models; therefore these factors might influence the expression of partner preference. Analyses of these measures in the present study did not identify any significant effects. Specifically, there were no significant differences between treatment groups for locomotor activity [$F_{2,17} = 0.56$, $p = 0.58$; Fig. 2B] during PPT or the average embryo weights [$F_{2,17} = 1.32$, $p = 0.29$; Fig. 2C] post the two-week cohabitation period. These findings demonstrate that drinking alcohol or the drinking status of a male partner does not impact partner preference in female prairie voles.

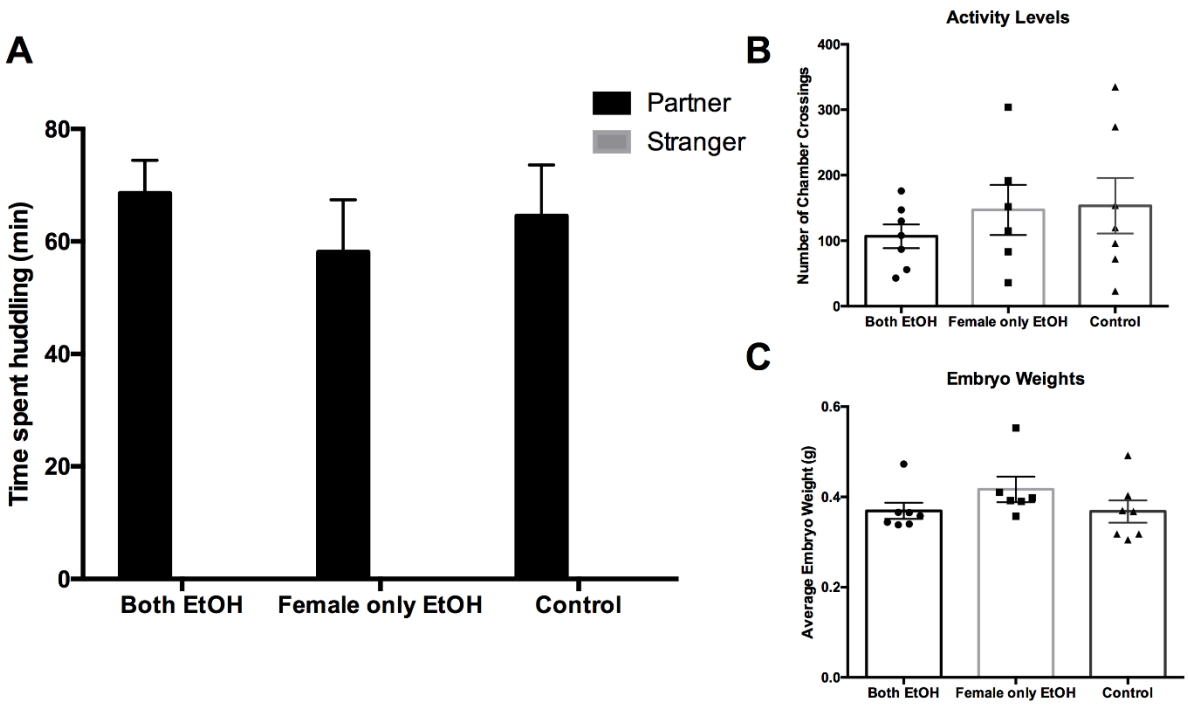


Figure 2. The effects of concordant and discordant drinking on PPT, activity levels, and embryo weights. (A) Females in all three conditions displayed a partner preference during the PPT. However, there was no significant difference in the amount of time female prairie voles spent huddling with their male partner between the three conditions. (B) There was no difference in the number of cage crossings and (C) embryo weights between the three conditions. Error bars indicate mean ± SEM. Symbols in B and C show values in individual animals (n = 6-7 per group).

Effects of alcohol consumption on the brain during pair bond maintenance in female prairie voles

It has been shown that alcohol consumption decreases the number of oxytocin-immunoreactive (-ir) cells within the paraventricular nucleus of the hypothalamus (PVN) in male prairie voles (Chapter 1; Stevenson et al., 2017; (Walcott and Ryabinin, 2017). Therefore, we tested whether female prairie voles would show a decrease in oxytocin- and vasopressin-ir cells in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus after one week of alcohol consumption. We found a significant effect of treatment on the number of oxytocin-ir cells within the PVN [$F_{2,16}$ = 4.44, p = 0.03; Fig. 3A]. *Posthoc* analysis revealed that the females in both the *Both EtOH* and *Female only EtOH* groups had a significantly lower number of oxytocin-ir cells compared to the *Control* group. To determine if this decrease could be caused by apoptosis, we ran IHC for cleaved caspase 3. We found no colocalization of cleaved caspase 3 with oxytocin-ir cells (Fig. 4). We saw no difference in the number of oxytocin-ir cells in the SON between treatment groups [$F_{2,16}$ = 0.17, p = 0.84; Fig. 3B]. We saw a trend for a significant difference in the number of vasopressin-ir cells between treatment groups in the PVN [$F_{2,16}$ = 2.80, *p = 0.09*; Fig. 3C] and no difference between groups in the SON [$F_{2,16}$ = 1.55, p = 0.24; Fig. 3D]. Therefore, alcohol had similar effects on oxytocin-ir cells in the PVN of female prairie voles compared to previously observed effects in males. These effects were unlikely to be due to apoptosis through the cleaved caspase 3 pathway.

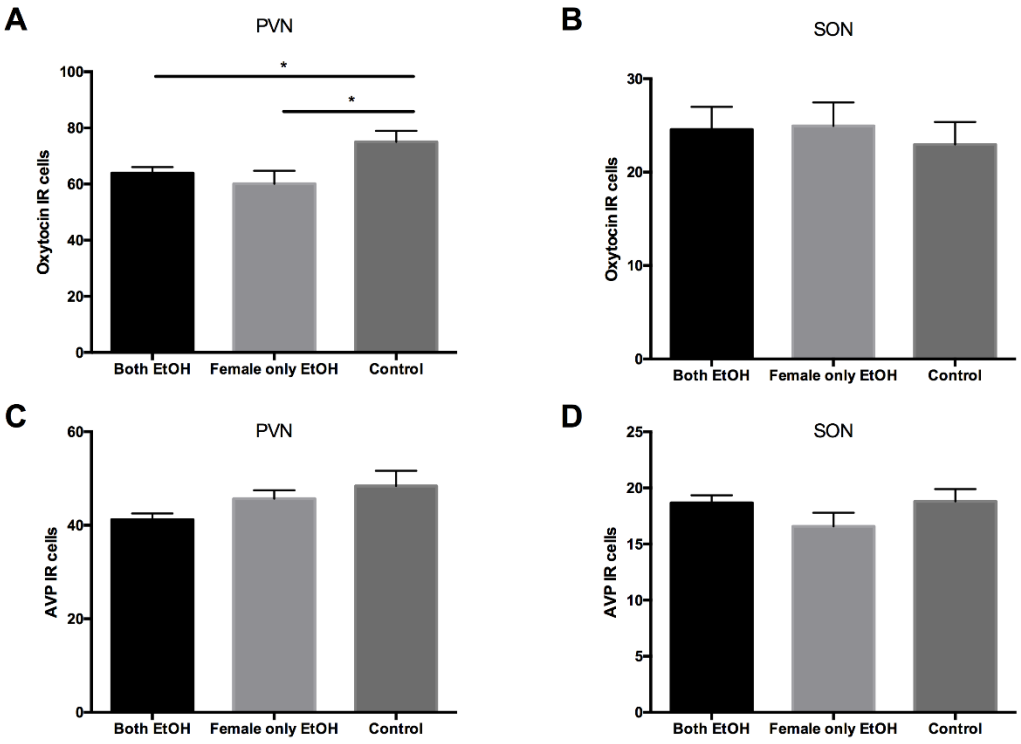


Figure 3. Immunoreactivity for oxytocin and arginine vasopressin in the PVN and SON. (A) The number of oxytocin-immunoreactive (IR) cells in the paraventricular nucleus of the hypothalamus (PVN) was lower in female prairie voles who consumed alcohol compared to females who consumed only water. (B) The number of oxytocin-IR cells in the supraoptic nucleus (SON) did not differ between groups. (C) The number of arginine vasopressin (AVP) –IR cells in the PVN and (D) SON did not differ between the three groups. *$p < 0.05$. Error bars indicate mean ± SEM (n = 6-7 per group).

Since discordant alcohol consumption did not inhibit pair bond maintenance in female prairie voles, we needed to know if alcohol exhibited any effects on neuronal activity across the brain. For this purpose, we analyzed FosB-ir, a marker of both acute and long-term changes in neural activity in the brains of female animals from these experiments (Table 1). Across 18 brain regions, the only region with a significant difference between treatment groups was the centrally projecting Edinger-Westphal nucleus (EWcp) [$F_{2,17} = 5.39$, $p = 0.01$; Fig 4A]. *Posthoc* analysis revealed an increase in FosB-ir within the EWcp in females consuming alcohol compared to females consuming only water [Fig.5A-D]. Combined with the data above, this finding indicates that alcohol consumption in female prairie voles affects neural activity independently from their male counterpart's drinking status.

Table 1. FosB immunoreactivity in analyzed brain regions.

Brain Region	Both EtOH	Female only EtOH	Control	p-value
Anterior Cingulate (CG1)	1014±63.7	903.8±112.9	864.8±61.7	0.5869
Anterior Cingulate (CG2)	1098±89.8	982.3±163.9	1071±99.3	0.7783
Agranular Insula (AI)	549.8±39.8	481±30.3	523.6±39.2	0.4924
Granular Insula (GI)	788.6±40.7	608.3±70.1	681.1±59.7	0.1131
Infralimbic Cortex	622.1±44.9	546.2±96.0	547.1±42.9	0.5705
Retrosplenial Cortex	525.9±89.5	513.5±99.2	486.1±69.9	0.9401
Dorsal Lateral Striatum	732.9±68.8	569.1±99.8	763.5±155.4	0.4759
Dorsal Medial Striatum	1672±102.8	1642±193.2	1658±172.7	0.9912
Nucleus Accumbens Core	1925±179.8	1931±180.9	1918±118.2	0.9983
Nucleus Accumbens Shell	1816±114.0	1619±238.4	1714±110.6	0.6822
Lateral Septum	583.6±76.7	624.8±55.4	574.8±47.2	0.8391
Ventral Bed Nucleus of the Stria Terminalis	224.9±18.8	186.4±24.2	193.0±19.7	0.3918
Dorsal Bed Nucleus of the Stria Terminalis	167.8±24.1	173.8±40.5	167.1±16.6	0.9828
Paraventricular of the Hypothalamus	33.4±5.9	36.9±5.6	38.1±3.8	0.8090
Hippocampus (CA1-3)	403.4±106.3	290.2±29.5	376.5±40.0	0.4108
Dentate Gyrus	280.4±52.5	373.8±22.6	356.7±33.8	0.2242
Periaqueductal Gray	263.0±27.5	250.7±37.0	280.2±35.6	0.8263
Edinger-Westphal Nucleus	14.9±2.5	12.3±1.9	6.4±1.0	**0.0169**

Numbers represent mean ± standard error of the mean.

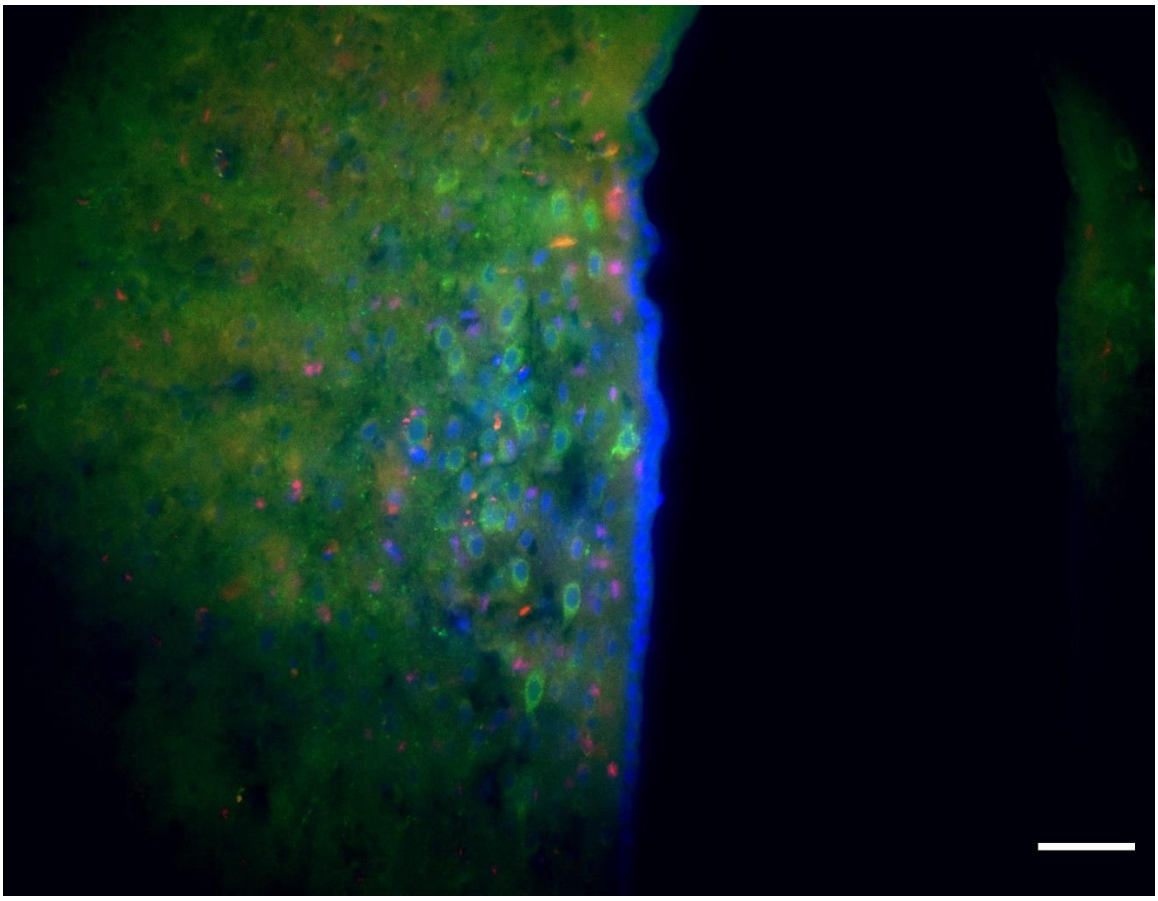


Figure 4. Representative photomicrograph for oxytocin and cleaved caspase 3 in the PVN. There was no colocalization of oxytocin labeled cells (green) and cleaved caspase 3 labeled cells (red). Nuclei were labeled with DAPI (blue). Scale bar = 0.02mm

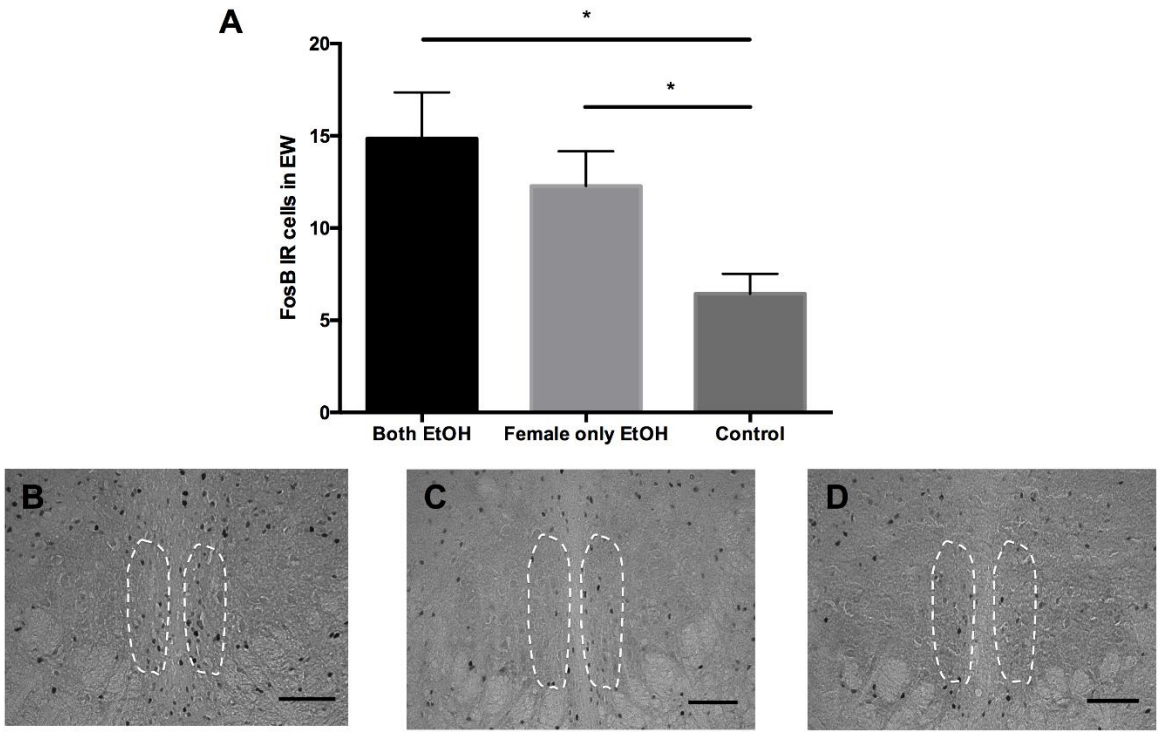


Figure 5. FosB immunoreactivity in the EWcp. (A) The number of FosB-IR cells in the centrally projecting Edinger-Westphal (EWcp) nucleus was significantly higher in female prairie voles in the Both EtOH and Female only EtOH groups compared to the water drinking Control group. Representative photomicrographs of FosB immunoreactivity in the EWcp in the (B) Both EtOH, (C) Female only EtOH, and (D) Control groups. *p < 0.05. Error bars indicate mean ± SEM. Scale bars = 0.1mm (n = 6-7 per group).

Discussion

The present study investigated the effects of discordant and concordant alcohol consumption between partners on established pair bonds in female prairie voles. Consistent with what is expected in a socially monogamous species, and similar to male prairie voles (Chapter 1; (Walcott and Ryabinin, 2017), alcohol- and water-consuming females displayed a preference for their partner over the stranger male. However, in contrast to the previous study in male prairie voles, females showed no inhibition in partner preference after they consumed alcohol, regardless whether there was a discrepancy in alcohol consumption or not. Our finding is in agreement with the idea that maintenance of a pair bond is an evolutionary adaptive behavioral strategy that is not easily disrupted by the presence of alternative rewards. The lack of alcohol's effects in the present study is unlikely due to an insufficient dose of consumed alcohol since withdrawal from alcohol consumption in a similar two-bottle choice procedure results in signs of hyperalgesia, which has been shown to be a sign of dependence in humans (Egli et al., 2012; Appendix 1; Walcott et al., 2018). Therefore, the consumed doses of alcohol are high enough to produce at least some form of alcohol dependence. From an evolutionary perspective, it also appears that the maintenance of pair bonds in females should be more difficult to disrupt by an alternative reward than in males. However, the lack of effect of alcohol on maintenance of pair bonding does not concur with epidemiological observations that discordant heavy drinking in women is associated with disruption of human intimate relationships (Ostermann et al., 2005; Leonard et al., 2014).

The most likely reason for the apparent discrepancy of our finding from the existing epidemiological studies is in the nature of the PPT. In the PPT, the animal is given a choice to select the vicinity of its partner or a stranger. It is the tested female who is initiating the preference behavior in the current study. In contrast, the

epidemiological studies don't assess who among the partners was the initiator of the separation. In other words, the likelihood of a couple's separation also depended on the behavior of the abstinent partner. In contrast, the PPT is mostly targeted towards identifying the motivation of the tested individual. The lack of effect of alcohol on partner preference in the current study indicates that alcohol did not disrupt the motivation of the drinking females to spend their time in the vicinity with the partner.

While our current investigation focused on pair bond maintenance, a previous study from our laboratory found that alcohol inhibits the formation of pair bonds in male prairie voles and facilitates the formation of pair bonds in female prairie voles (Anacker et al., 2014a). Interestingly, an explanation has been put forth that alcohol's disruptive effects in married couples could be mediated by drinking that occurs when the spouses are not together (Roberts et al., 1998; Homish and Leonard, 2005). Paradoxically, in this situation, not only the tendency of alcohol to disrupt formation and maintenance of bonds in males, but also the tendency of alcohol to facilitate pair bond formation in females would promote the likelihood of an established couple's separation. Unfortunately, this situation is even more difficult to model in animals. In the absence of epidemiological studies assessing the initiator of separation in humans, our findings add to the limited body of literature that shows that some drugs of abuse display sexual dimorphic effects in these socially monogamous species (Liu et al., 2010; Anacker et al., 2014a; Young et al., 2014).

In addition to the effects of alcohol on PPT, in the current study we assessed if the drinking status of the male partner would influence the amount of alcohol consumed by the female subject. We observed that when female and male partners were both consuming alcohol, females increased alcohol consumption compared to females whose male partner had no access to alcohol. According to previous studies, same-sex prairie vole siblings can socially facilitate the amount of alcohol consumed by each partner

(Anacker et al., 2011b). Meanwhile, there are mixed results on the ability of opposite-sex partners to socially facilitate the amount of alcohol consumed by each partner in prairie voles. Specifically, in Chapter 1 and Walcott and Ryabinin (2017) we showed that in some cases male prairie voles increased alcohol consumption and preference when an alcohol consuming female partner was present. In contrast, Hostetler et al. (2012), showed that male-female pairs do not socially facilitate the amount of alcohol consumed by each partner. Compared to the current experiments, the latter study had methodological differences that could play a role in the discrepancy in results. In particular, Hostetler et al. (2012) used gonadectomized prairie voles and shorter cohabitation prior to alcohol exposure, and it is not clear whether pair bonding occurred and was maintained during the mesh housing. In contrast, in the current study, animals were able to form and maintain pair bonds, which was confirmed by embryo weight. Together these studies suggest when prairie voles are able to form pair bonds with their partners, social facilitation of alcohol consumption does occur. Importantly for the main aim of the study, it is unlikely that the lower alcohol consumption in the females exposed to discrepant drinking compared to the *Both EtOH* group contributed to the lack of alcohol effects on pair bond maintenance as alcohol consumption in the *Female only EtOH* group is identical to that in the analogous *Male only EtOH* group in our previous study in which effects of alcohol on pair bond maintenance has been observed (Chapter 1; Walcott and Ryabinin (2017).

The present study showed that one week of alcohol consumption can result in a decreased number of oxytocin-IR cells in the PVN. Similar to the effects of alcohol on male prairie voles observed in Chapter 1, this decrease in oxytocin-IR was independent of the drinking status of the female's partner. This decrease in oxytocin-IR is also in agreement with previous studies in rats and in prairie voles that used longer exposures to alcohol (Silva et al., 2002; Stevenson et al., 2017b). Our experiments expand these

previous findings by showing that the effect occurs after a relatively short period (1 week) of voluntary alcohol self-administration. The mechanisms underlying alcohol's effect on oxytocin neurons are currently unknown. Here we tested whether the decrease in oxytocin-IR could be due to apoptosis. One of the common early mechanisms of apoptosis involves cleaving the caspase 3 protein (Porter et al., 1999). Examination of selected brain slices containing PVN did not show any colocalization between cleaved caspase 3-ir and oxytocin-ir. This finding provides evidence against alcohol-induced apoptosis in oxytocin cells of PVN. It appears more likely that the decreased expression of oxytocin contributes to this effect of alcohol. Alternatively, the decreased oxytocin-ir could be due to another, more unusual, form of cell death or due to apoptosis occurring at earlier stages of alcohol consumption. Although a decreased number of oxytocin-containing cells in the PVN seemed not to contribute to any effects on pair bond maintenance, these cells regulate a number of social behaviors (Ross et al., 2009; Smith and Wang, 2014). Therefore, the mechanisms underlying the consistent effects of alcohol on these cells are worthy of further investigations.

Since no significant effects of alcohol on pair bond maintenance were observed in our study, it was important to confirm that alcohol consumption resulted in effects on neural activity. Similarly to the Chapter 1, we did this using an antibody that detects the products of immediate early gene FosB. While acute neural activation leads to temporary expression of the full-length FosB protein, repeated neural activation results in accumulation of the delta-FosB protein (Kelz and Nestler, 2000; Nestler et al., 2001). Therefore, positive FosB-ir serves as a marker for both acute and prolonged effects of treatments on neural activity. Out of the 18 examined brain regions in our study, the only region that showed a difference between groups was the EWcp. Animals that were consuming alcohol, independently of the drinking status of their partner, showed an increase in FosB-IR within the EWcp. Immediate early gene expression in the EWcp is

known to be strongly affected by voluntary and involuntary modes of alcohol exposure (Ryabinin and Wang, 1998a; Bachtell et al., 1999; Ozburn et al., 2012). Interestingly, the studies in Chapter 1 showed that alcohol consumption in male prairie voles affected FosB-ir not only in the EWcp, but also the nucleus accumbens, infralimbic cortex, and bed nucleus of the stria terminalis. One limitation to the current study was that females used in the PPT were also used to explore potential brain regions affected by alcohol exposure. Meanwhile, in the previous male prairie vole study a separate group of males was used to explore the brain regions. Since the antibody used in both studies detects both full-length FosB and delta-FosB, the PPT in the current study could have modified the FosB-ir levels. Importantly, even though there were no significant differences in FosB-ir in other brain regions, the finding that FosB-IR was increased in the EWcp following alcohol consumption indicates that alcohol consumption resulted in physiological changes in the central nervous system.

Overall, we showed that discordant and concordant voluntary alcohol consumption does not affect established pair bonds in female prairie voles measured by the PPT. This effect does not mirror the effects of discordant heavy alcohol consumption on marital separation in humans. We showed that independently of a partner drinking status, alcohol affects neuronal substrates in female prairie voles, specifically in the the PVN and EWcp. Future studies should further explore the relationship between these neuronal substrates and alcohol consumption. In addition, further research should be done to allow closer modeling of alcohol's effects on marital dissolution in humans caused by heavy discordant alcohol consumption by female partners that would allow to determine the underlying biological mechanisms for this phenomenon.

CHAPTER 3: Assessing Effects of Oxytocin on Alcohol Consumption in Socially-Housed Prairie Voles using Radio Frequency Tracking.

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

Introduction

According to the National Survey on Drug Use and Health, 19.7 million people aged 12 and older in the United States have a substance use disorder (SUD) (SAMHSA, 2017) . Of those 19.7 million people, about 14.5 million have an alcohol use disorder (AUD) and 5.2 million have an illicit drug use disorder (i.e. misuse of prescription psychotherapeutics or the misuse of marijuana, cocaine, heroin, hallucinogens, inhalants, or methamphetamine). In 2017, about 109,000 people died from substance use related causes (Centers for Disease Control and Prevention; SAMHSA, 2017) and about 1.5% of people received treatment for a SUD (either pharmacotherapy or psychosocial treatment (SAMHSA, 2017) . Currently only five pharmacotherapies are approved for the treatment of SUD in the United States. For AUD: acamprosate, disulfiram, and naltrexone (oral and extended-release injectable) are approved. For illicit drug use (mainly opioid addiction): buprenorphine, methadone, and naltrexone are approved. These pharmacotherapies have been effective in decreasing alcohol and illicit drug use in rodent animal models of SUD (Volpicelli et al., 1992; Spanagel et al., 1996; He et al., 1997; Macfadyen et al., 2016; Carroll and Lac, 1992; Griffiths et al., 1975). However, in humans these pharmacotherapies have not been successful in treating SUD across all patients and in many cases worked only modestly better than those of placebo (Fuller et al., 1986; Oslin et al., 2003; Mann et al., 2013; Haney and Spealman, 2008). Therefore, development of novel medications to treat SUD in necessary.

It has been suggested that one of the difficulties in development of novel effective therapies for SUD is due to complex interactions with social environment (Ahmed et al., 2018; Heilig et al., 2016; Inagaki, 2018). Thus, a potential medication might be effective in hospital settings, but can fail as the treated individual faces social influences outside of the clinics. Predicting whether a potential medication will be effective in social settings is hampered by the fact that most preclinical studies on

substance abuse are performed in isolate-housed animals. Recognizing this caveat, researchers have developed several approaches to take into account social influences on drug taking. Most common of these approaches are: 1) housing animals socially and calculating the total intake for an entire cage (Anacker et al., 2014a; Deatherage, 1972; Ehlers et al., 2007; Juárez and Vázquez-Cortés, 2003); 2) housing an animal socially, but testing drug intake in a probe trial in isolated condition (Rockman et al., 1989; Rockman et al., 1988); 3) housing animals in semi-social conditions by separating them within a cage by a mesh divider (Hostetler et al., 2012; Tomie et al., 2007; Smith, 2012); 4) housing animals socially, calculating total cage intake and dividing it by the time each individual animal spends near a drinking spout through video tracking (Logue et al., 2014; Varlinskaya et al., 2015). These methods are a step forward in assessing social aspects of substance abuse, but either do not provide high resolution needed to measure individual substance consumption in social settings or introduce additional factors capable of affecting drug intake (for example, stress of mesh-separation). For additional discussion of these caveats see (Ryabinin and Walcott, 2018).

Advances in technology have allowed the use of radio frequency tracking in behavioral neuroscience, especially in ethological settings (Schneider et al., 2012; Kays et al., 2011; Bonter and Bridge, 2011). Relatively recent studies also started to use this technology in studies on substance abuse, mostly focusing on alcohol. Radio frequency tracking allowed the investigation of the effects of social hierarchies on alcohol drinking in rats (Pohorecky, 2006; Pohorecky, 2008; Pohorecky, 2010). However, these initial studies were hampered from imprecise measures of individual alcohol drinking and overall low drinking of alcohol. A study combining radio frequency tracking and lickometer procedures using the automated Intellicage® did not measure alcohol intake but demonstrated very low preference for alcohol solution in C57BL/6 mice, which made it incompatible with future studies on medications to decrease alcohol intake (Holgate et

al., 2017). The only study to date that used radio frequency to test effects of a potential medication adapted HM-2, a novel system developed to measure fluid or food intake in socially housed animals. The system consists of a large enclosure with protruding channels allowing access to a fluid or food source which is connected to a precision balance. Radio frequency detection of individual animals in the channels allows to track exact amounts of food or fluid consumed at any particular time of testing. Thomsen et al. (2017) used this system to demonstrate the effects of glucagon-like peptide 1 receptor agonist on alcohol consumption in socially-housed male C57BL/6 mice. While this development presented an important advancement to alcohol research, the study assigned the same condition to all animals in each individual cage (all animals in a cage were either treated or untreated). Therefore, the question whether a potential medication could be effective in the presence of peers not receiving the medication remained unanswered.

The goal of the present study, therefore, was to test whether one of the new promising medications to treat alcohol use disorder would be effective in socially housed animals unrestrained in their ability to interact with other peers. Specifically, we adapted a recently developed HM-2 system to explore effects of oxytocin on alcohol consumption. Oxytocin is a nine amino acid peptide implicated to play a role in lactation, parturition, social behaviors, as well as the modulation of processes associated with drug use (Lee et al., 2009; Leong et al., 2018; Lee et al., 2016). There has been a substantial amount of research in rodents and a growing interest of research in humans on the effects of oxytocin on substance abuse. Preclinical studies have shown that oxytocin treatment peripherally can reduce voluntary alcohol consumption and preference in isolated or semi-socially housed rodents (King et al., 2017; Mcgregor and Bowen, 2012; Stevenson et al., 2017a). Meanwhile, the limited number of clinical studies have shown that intranasal oxytocin decreases alcohol craving, cue reactivity,

withdrawal symptoms, and consumption (Hansson et al., 2018; Mitchell et al., 2016; Pedersen et al., 2013; Pirnia and Pirnia 2018).

It is known that oxytocin plays an important role in regulating social behaviors and that alcohol use is heavily influenced by social environments in humans. However, a majority of studies exploring oxytocin's effects on alcohol consumption in animal models of AUD have been completed in rodents who strongly differ in their social behaviors from humans. Therefore, we decided to explore the effect of oxytocin on alcohol consumption in a socially monogamous rodent model, the prairie vole (*Microtus ochrogaster*). Prairie voles display many similarities in social behaviors with humans, including the ability to form long-term emotionally-based attachments, known as pair-bonds, between adult individuals (Devries et al., 1997; Carter and Getz, 1993). Moreover, mechanisms first identified as regulating pair bonding in this species have been subsequently found to be involved in social behaviors in humans, demonstrating translational validity of the prairie vole model of social attachment (Insel and Hulihan, 1995; Pitkow et al., 2001; Wang et al., 1999; Walum et al., 2012). Oxytocin has been shown to inhibit alcohol consumption in prairie voles housed in semi-social mesh-divided cages. Here we used radio frequency detection to investigate effects of oxytocin on alcohol consumption in socially housed prairie voles in two different experiments. In the first experiment (Across experiment), all animals in each cage were treated with saline or oxytocin. In the second experiment (Mix experiment), half of the animals in each cage were treated with oxytocin and the other half with saline to determine if the effectiveness of oxytocin was altered when animals receiving treatment were housed with animals not receiving oxytocin. Following the behavioral analysis, we assessed whether alcohol consumption or oxytocin treatment in this new system produced central effects.

Materials and Methods

Animals

Experimentally naïve adult female and male prairie voles (n = 91) ranging between 77 – 141 days old at the start of the experiment were used from our breeding colony at the VA Portland Health Care System (VAPORHCS) Veterinary Medical Unit. Voles were weaned at 21 days and housed in same-sex groups in standard housing cages (27x27x13 cm) under a 14:10-h light/dark cycle (lights on at 6am) until the start of the experiment. Before experiments occurred, females were housed in a separate room to prevent induction of ovulation because prairie voles are induced ovulators. All subjects had access to cotton nestlets and *ab libitum* access to water and a diet of mixed rabbit chow (LabDiet Hi-Fiber Rabbit; PMI Nutrition International, Richmond, IN), corn (Nutrena Cleaned Grains; Cargill, Inc., Minneapolis, MN) and oats (Grainland Select Grains; Grainland Cooperative, Eureka, IL) throughout the duration of the experiment. Experiments were approved by the Institutional Animal Care and Use Committee at the VAPORHCS and performed in accordance to the NIH Guidelines for the Care and Use of Laboratory Animals.

Apparatus

To monitor individual fluid consumption in socially housed animals we used a fully automated rodent drinking system (HM-2; MBrose, Faaborg, Denmark). The HM-2 recorded fluid consumption from two bottles (water and 5% EtOH) at the level of individual prairie voles, based on the use of radio frequency identification tags (RFID). Each bottle is at the end of a 11 cm channel, that was customized to allow only one prairie vole to drink from the bottles at a time. In each channel photocells are present at the beginning of the channel and near the bottle spout, to detect the presence of an animal. When the presence of an animal was detected, an RFID reader at the spout of the bottle determined which animal was drinking and the raw drinking data was sent to a

computer. Fluid consumption was based on the weight displacement when an animal was present at the spout. A drip tray was suspended under each fluid bottle to catch any spillage, which is automatically subtracted from the displacement drinking bout. Along with spillage, evaporation was not a factor because fluid displacement was only measured when an animal was present in the channel and consuming fluid from the spout. These channels were attached to a Techniplast 1500U eurostandard type IV S (48x37.5x21 cm) rodent housing cage. Each cage was custom modified to include a set of stairs leading to each channel, the same bedding as used in homecages, and a 10” Habitrail OVO tube (Habitrail, Hagen Inc.) to build a nest in (Fig. 1).

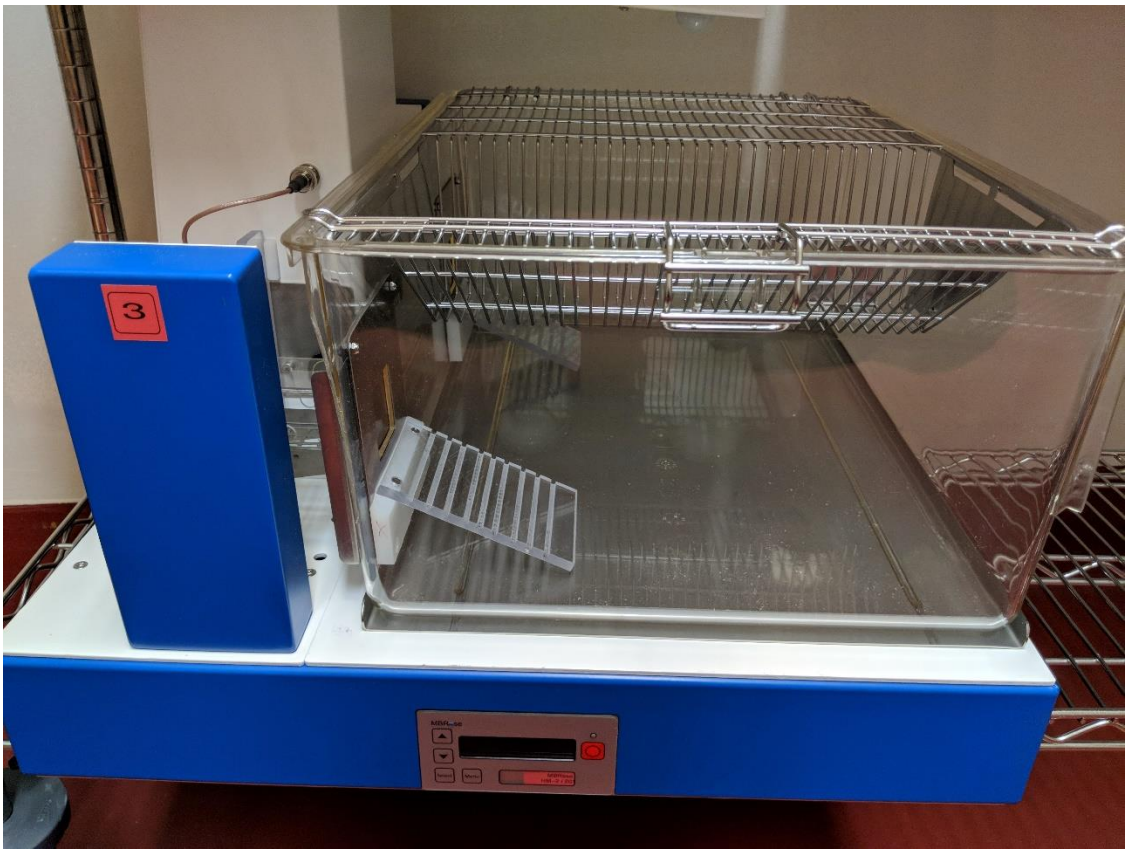


Figure 1: Photograph of the HM-2 system cage.

Training and Experimental Timeline

Animals were implanted with RFID chips (UNO MICRO ID/8, ISO Transponder 2.12 x 8 mm) under isoflurane anesthesia and then placed back into their homecage to recover for 2-3 days. After animals recovered from RFID chip implantation, 3-5 same sex prairie voles were put into each HM-2 cage for 2 days to habituate. Most animals assigned to a cage were sibling offspring from the same breeding pair. Some cages contained offspring from two breeding pairs. Follow-up comparison between these pairings of animals did not detect any difference in drinking behaviors. During the habituation period, access to the channels was closed to allow animals to establish nests in the cages and not the channels. Five 25mL sipper tubes were placed in the cage top to allow the animals access to water. All animals built nests in the cages during this two-day period. After nest building, sipper tubes were removed and animals were given access to the two channels with a bottle of water at the end of the channel for 5 days. The following 12 days, all cages received access to one bottle of 5% EtOH and one bottle of water. Bottles stayed on the same side throughout the experiment, but were counterbalanced between cages. During this 12-day period, habituation injections of saline occurred on days 5, 6, and 7 between 11am-12pm. Following habituation injections, animals received either one injection of saline or oxytocin on days 8, 9, 10, 11, and 12. In the first experiment (Across experiment; n= 10-15 per treatment/sex, 3-4 animals per cage), all the animals in a cage received the same treatment (saline or oxytocin). In the following experiment (Mix experiment; n= 9-10 per treatment/sex, 4-5 animals per cage), half of the animals in a cage received saline, while the other half received the oxytocin treatment. The assignment to the two treatment groups was based on drinking on the last baseline day (day 4) trying to match the drinking measures as much as possible between the treatment groups. In the Across experiment, the matching was done according to average drinking per cage, in the Mix experiment, the matching

was done based on individual drinking. The HM-2 system allows to analyze the fluid consumption at any chosen time point. Therefore, drinking was analyzed at 1, 3, 6, 12 and 24 hours post-injection time. The 1-hour time point showed variability independent of treatment from day to day. This was most likely due to the fact that prairie voles evenly spread their drinking behaviors throughout 24 hours without strong peaks at any particular time point (Anacker et al., 2011a; Anacker and Ryabinin, 2013). Therefore, behavioral analysis of treatment's effects was performed at 3, 6, 12 and 24 hours. All animals were euthanized 2 hours after the last treatment injection on day 12 and brains and blood were collected for later processing.

Drugs

Oxytocin acetate salt (3.0 mg/kg, i.p., Bachem, Torrence, CA, USA) was dissolved in 0.9% saline. This dose was chosen as the medium dose between 3 doses producing effects in previous studies on alcohol drinking in prairie voles(Anacker et al., 2011a; Hostetler et al., 2012). 95% ethyl alcohol was diluted in water to make 5% EtOH.

Immunohistochemistry

To determine if peripheral oxytocin treatment affected central oxytocin and whether alcohol consumption affected the central nervous system, animals were euthanized by CO_2 inhalation 2 hours after the last treatment injection. Brains were extracted and fixed in 2% PFA in PBS overnight. Brains were then transferred to 20% sucrose in PBS for 24 hours prior to being stored in 30% sucrose in PBS until immunohistochemical (IHC) staining.

Brain tissue was sliced at 40-um coronal floating sections and preserved in 0.1% sodium azide in PBS until IHC staining. The following primary antibodies were used: anti-oxytocin (1:20,000, Peninsula Laboratories) and anti-FosB (1:27,000, Abcam). Both

primary antibodies were polyclonal and made in rabbit. An anti-rabbit secondary antibody (Vector Laboratory, Inc.) made in goat was applied, and antibody signal was amplified with a Vectastain ABC kit (Vector Laboratory, Inc.). Tissue was stained using a metal enhanced diaminobenzidine substrate kit (Thermo Fisher Scientific) and visualized using a Leica DM4000 bright-field microscope. All cells that were stained above background were automatically analyzed by ImageJ.

Since the behavioral experiments included a large number of animals, we focused IHC analysis on subsets of samples from animals optimally chosen for each analysis. Specifically, for the oxytocin immunohistochemistry experiment, animals that received oxytocin treatment with the highest average alcohol consumption over the treatment period were used (n = 8 per treatment). There was no difference in alcohol consumption between the males and females chosen, so analysis was collapsed across sex. For the FosB immunohistochemistry experiment, only animals that received saline injections during the treatment period were used. The highest and lowest alcohol consuming prairie voles who received saline treatment (n = 8 per group) were used and there was no significant difference in alcohol consumption between the sexes, so analysis was collapsed across sex.

Blood Ethanol Concentration (BEC) Determination

Trunk blood samples (n = 6-8 per group) were spun down in a centrifuge and plasma supernatant was removed and stored at -20°C until processing occurred. BEC was determined using an Analox Analyzer (Analox Instruments, Luneburg, MO, USA) and is reported in milligrams per deciliter (mg/dL).

Statistical Analysis

Alcohol intake for each prairie vole was calculated by dividing the grams of alcohol consumed by the kilogram of body weight. Alcohol preference for each prairie vole was calculated by dividing the amount of alcohol consumed by the amount of total fluid consumed. Analysis of variance (ANOVA) was used for all data. For alcohol consumption and preference in each experiment (Across or Mix), a repeated-measures ANOVA design used sex (Female vs. Male) and treatment (Saline vs. Oxytocin) as the between-subject factors and the day (days 1 – 12) as the repeated measure. If Mauchly's test of sphericity was significant, a Greenhouse-Geisser test for repeated measures was used. Significant outcomes were followed by a Fisher's LSD *post hoc* test.

An unpaired Student's t-test was used to calculate the difference between oxytocin-ir within the two treatment groups. A linear regression analysis was used to determine the relationship between FosB-ir and alcohol consumption 1 hour and 24 hours after treatment. A linear regression analysis was also used to determine the relationship between FosB-ir and BEC levels within each prairie vole. All the statistical analyses were performed using SPSS software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp) and all figures were made in GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

Results

Oxytocin's effects on alcohol intake when all animals in a cage are receiving the same treatment.

To determine if multiple oxytocin treatments would affect alcohol consumption/preference, we peripherally treated socially-housed male and female prairie voles with oxytocin or saline. In the first experiment (Across experiment), all

animals in a cage received the same treatment (oxytocin or saline) during days 8 – 12. A repeated measures ANOVA revealed a main effect of sex ($F_{1,48}$ = 8.73, *P* = 0.005), showing that female prairie voles in the first 3-hrs of each day consumed higher amounts of alcohol over the entire 12-day period compared to male prairie voles. Additionally, there was a significant day by treatment interaction ($F_{6.5,311.7}$ = 2.57, *P* = 0.016). Post hoc analysis confirmed a significant decrease in alcohol consumption on the first day of oxytocin treatment in female, but not male voles (Fig. 2A & 2C). Analysis of the 6- and 12-hour time points revealed that females consumed more alcohol during the first 6-hours and 12-hours compared to males (Table 1 and Figs. 3 and 4). However, there was no significant difference in the amount of alcohol consumed at the 24-hour time point (Fig. 5). Additionally, there was no significant day × treatment interaction for the 6-, 12-, and 24-hour time points in both sexes.

At the 3-hr time point we found no main effect of sex ($F_{1,48}$ = 2.43, *P* = 0.13) and treatment ($F_{1,48}$ = 1.20, *P* = .28) on alcohol preference, but there was a significant interaction of sex by treatment ($F_{1,48}$ = 6.19, *P* = 0.02). Additionally, we found a 2-way interaction of day by treatment ($F_{6.0,289.5}$ = 2.20, *P* = 0.04). Post hoc analysis revealed that there were significant differences between treatment groups (in male and females) during the baseline period (Fig. 2B & 2D). Therefore, effects of oxytocin treatment on preference were difficult to interpret. Similarly, we saw group differences between treatment groups at the 6- and 12-hour time points regardless of the treatment (Table 2 and Figs. 3 & 4), again making comparisons during the treatment period not reliable. Such observed differences in preference preceding treatment are most likely due to the difficulty of experimental matching of this measure between cages. Such matching is easier to achieve when taking into account individual drinking of animals, as was done in the next experiment.

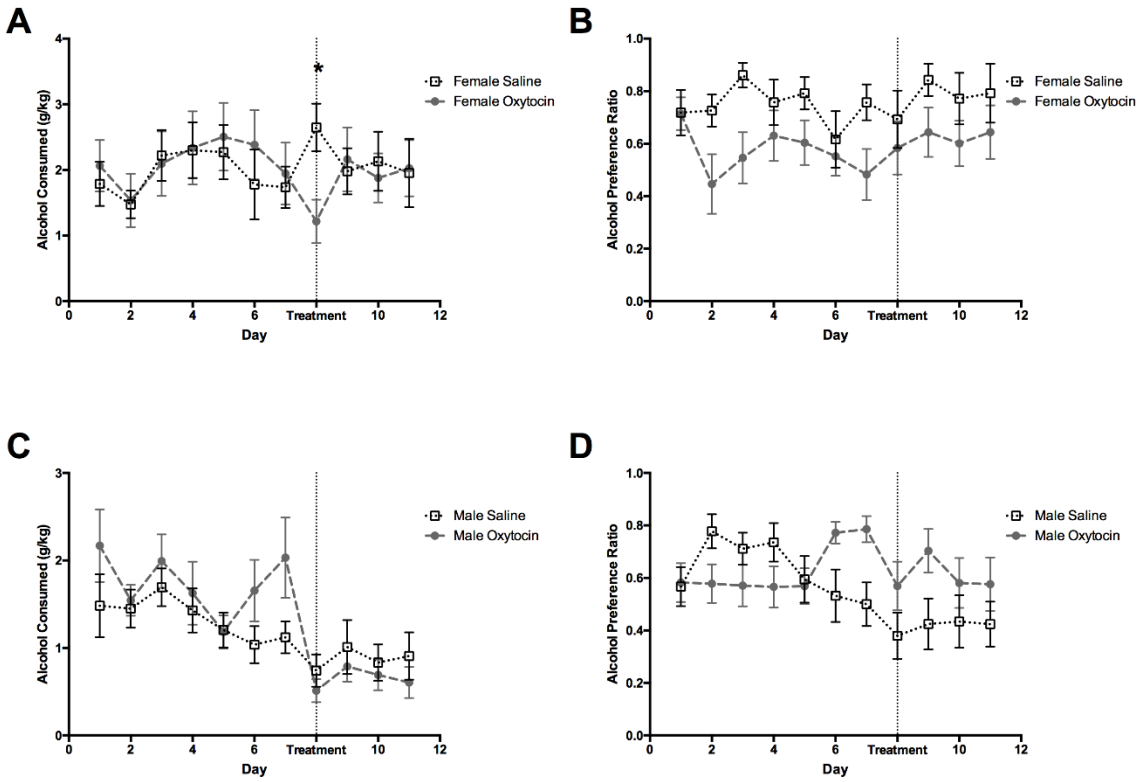


Figure 2: Alcohol consumption and preference in the first three hours of alcohol access following oxytocin treatment when all animals in a cage receive the same treatment. (A) Oxytocin reduces alcohol consumption in female prairie voles for the first day of treatment relative to control treated females (; meanwhile, (C) there was no difference in alcohol consumption between saline and oxytocin treated males. There was a difference between treatment groups for alcohol preference during the baseline drinking in (B) female and (D) male prairie voles. Therefore, alcohol preference during the treatment period could not be reliably inferred. *p<0.05. Error bars indicate mean ± SEM. (n= 10-15 per treatment/sex, 3-4 animals per cage).

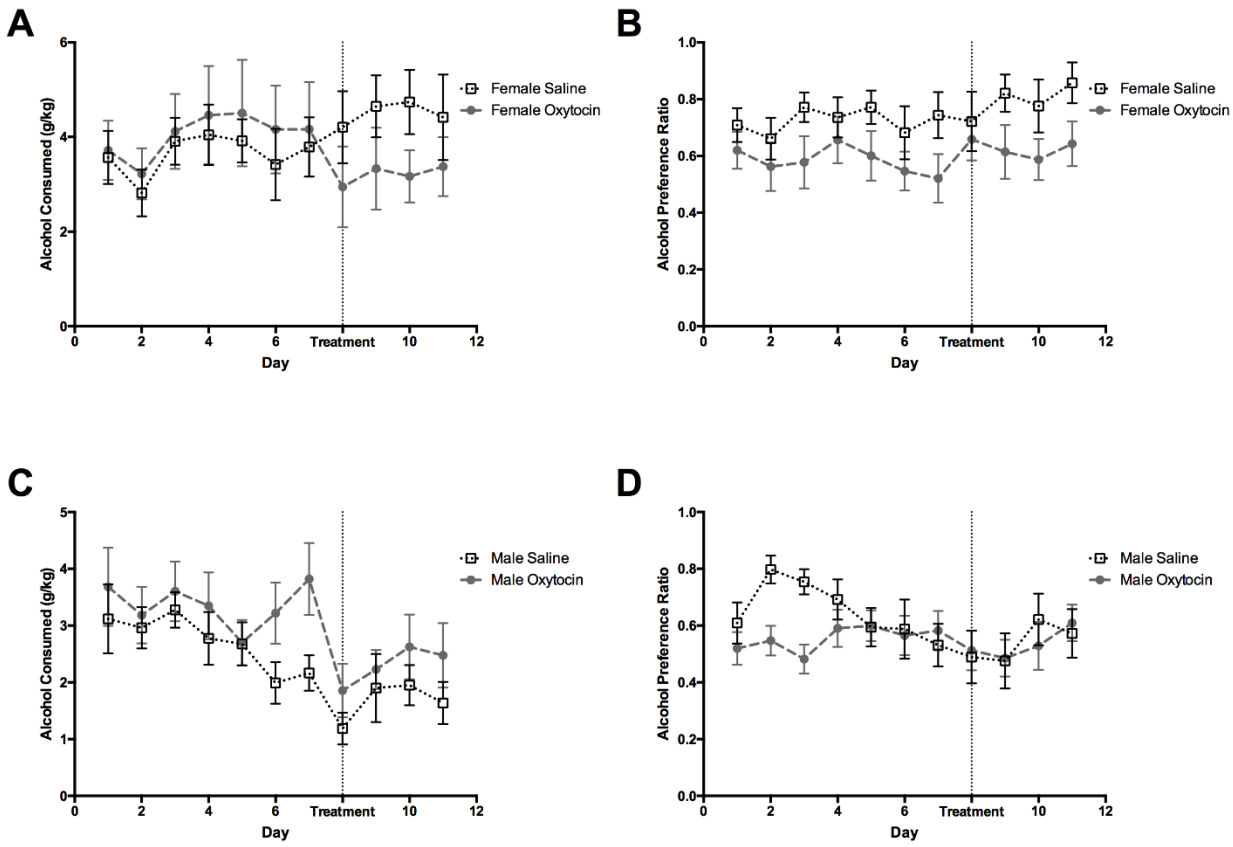


Figure 3: Alcohol consumption and preference after 6 hours of alcohol access following oxytocin treatment when all animals in a cage receive the same treatment. Alcohol consumption in both (**A**) females and (**C**) males did not differ when animals received oxytocin or saline during the treatment days. (**B**) There was a significant difference in alcohol preference between females treated with saline or oxytocin prior to receiving treatment; therefore, any differences during the treatment period are not reliable. (**D**) There was no significant difference between saline treated males and oxytocin treatment males. Error bars indicate mean ± SEM for the number of voles in Fig.2.

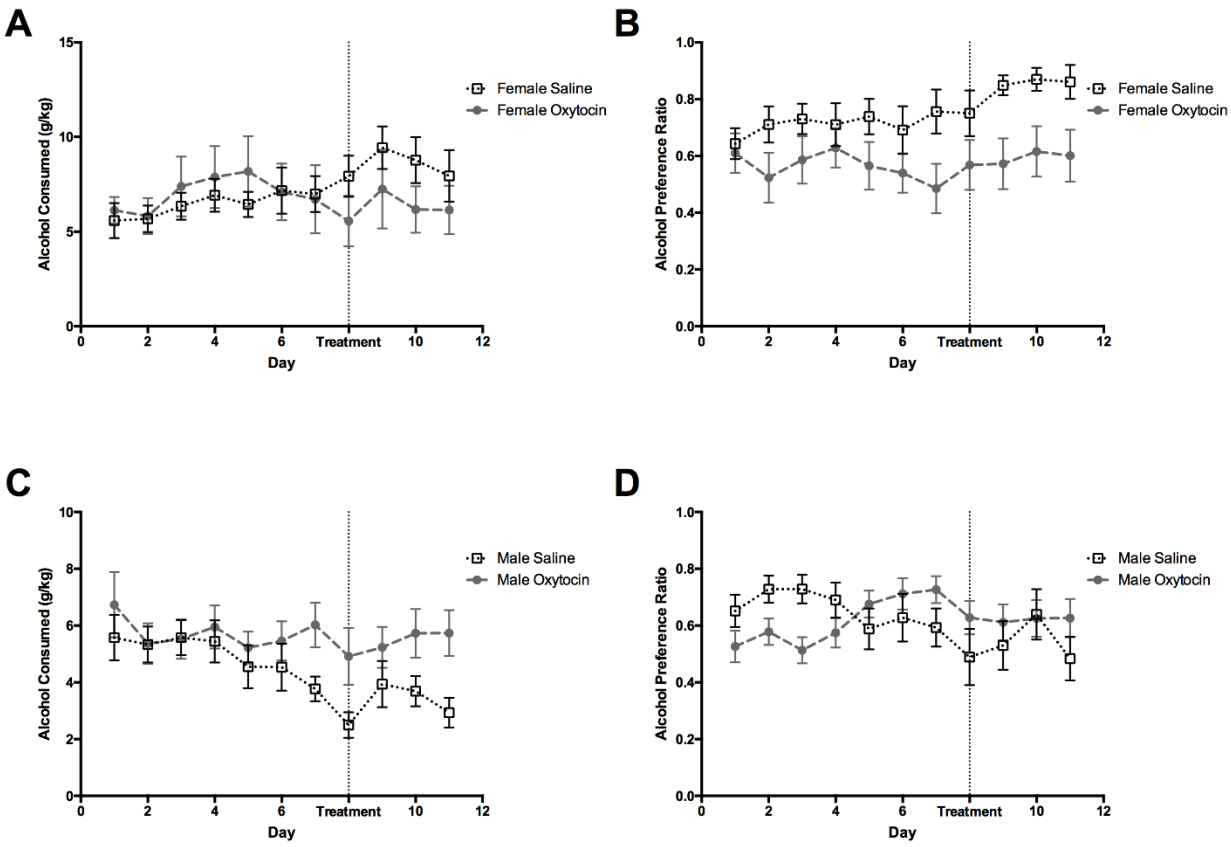


Figure 4: Alcohol consumption and preference after 12 hours of alcohol access following oxytocin treatment when all animals in a cage receive the same treatment. Alcohol consumption in both (**A**) females and (**C**) males did not differ when animals received oxytocin or saline during the treatment days. (**B**) Across all days oxytocin treated females had a lower alcohol preference compared to saline treated females. (**D**) There was no significant difference between saline and oxytocin treated males. Error bars indicate mean ± SEM for the number of voles in Fig.2.

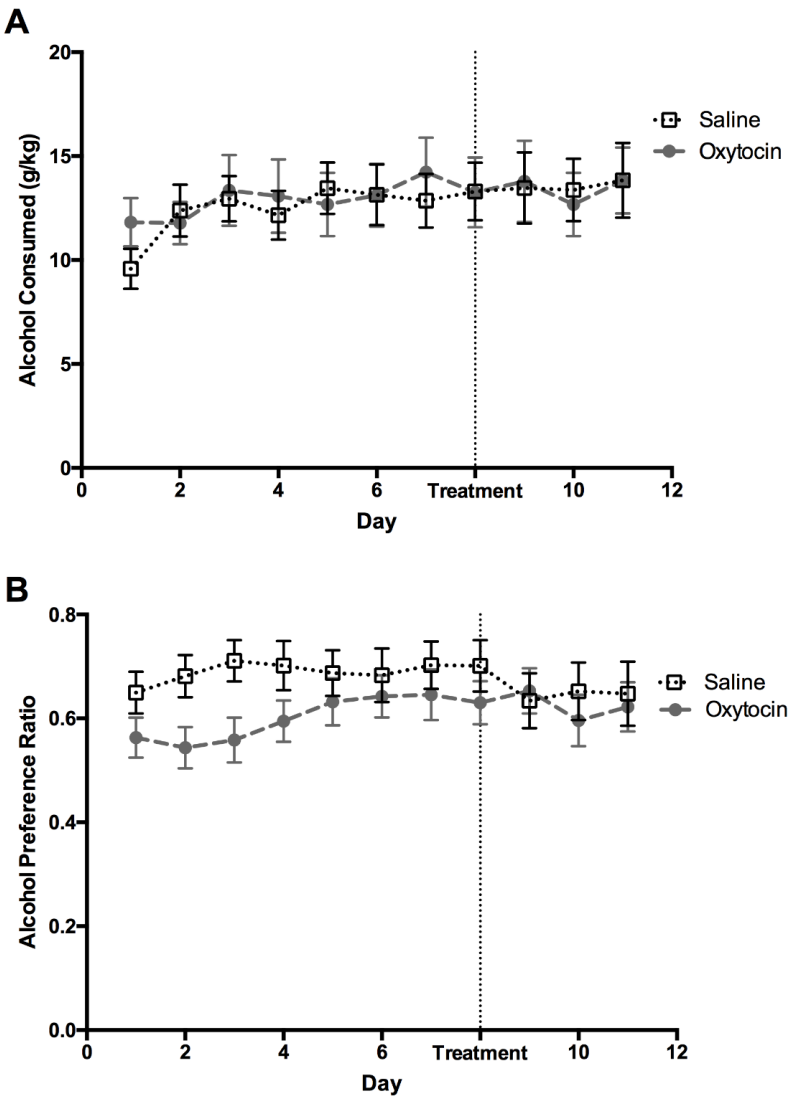


Figure 5: Alcohol consumption and preference each 24-hour period when all animals received the same treatment within a cage. There was no difference between females and males, therefore sex was collapsed across each day. Oxytocin treatment did not affect alcohol (**A**) consumption or (**B**) preference over the 24-hour period when all animals received the same treatment in a cage. Error bars indicate mean ± SEM. (n = 24-28/group).

Table 1: Statistics for Alcohol Consumption at All-Time Points for Both Experiments.

Time Point	Factor	F-Value	*p*-Value
3hr Across	Day	3.714	0.001
	Day * Sex	3.709	0.001
	Day * Treatment	2.567	0.016
	Day * Sex * Treatment	0.834	0.552
	Sex	8.733	0.005
	Treatment	0.018	0.893
	Sex * Treatment	0.333	0.567
6hr Across	Day	3.047	0.007
	Day * Sex	3.024	0.008
	Day * Treatment	1.739	0.115
	Day * Sex * Treatment	1.475	0.189
	Sex	5.333	0.025
	Treatment	0.074	0.786
	Sex * Treatment	1.156	0.288
12hr Across	Day	1.755	0.114
	Day * Sex	4.352	< 0.001
	Day * Treatment	0.76	0.617
	Day * Sex * Treatment	3.994	0.001
	Sex	5.561	0.022
	Treatment	0.23	0.634
	Sex * Treatment	1.096	0.3
24hr Across	Day	5.937	< 0.001
	Day * Sex	3.257	< 0.001
	Day * Treatment	0.647	0.622
	Day * Sex * Treatment	1.169	0.326
	Sex	1.539	0.221
	Treatment	2.55	0.117
	Sex * Treatment	0.825	0.368
3hr Mix	Day	3.004	0.011
	Day * Sex	1.771	0.116
	Day * Treatment	2.755	0.003
	Day * Sex * Treatment	0.646	0.674
	Sex	0.549	0.464
	Treatment	1.823	0.186
	Sex * Treatment	0.016	0.899
6hr Mix	Day	2.656	0.019
	Day * Sex	3.051	0.008
	Day * Treatment	2.646	0.004
	Day * Sex * Treatment	0.739	0.61
	Sex	1.632	0.21
	Treatment	0.751	0.392
	Sex * Treatment	0.727	0.4
12hr Mix	Day	1.879	0.096
	Day * Sex	3.947	< 0.001
	Day * Treatment	1.552	0.172
	Day * Sex * Treatment	0.725	0.701
	Sex	0.019	0.892

	Treatment	1.008	0.322
	Sex * Treatment	0.037	0.849
24hr Mix	Day	1.14	0.341
	Day * Sex	1.347	0.252
	Day * Treatment	1.061	0.392
	Day * Sex * Treatment	0.469	0.777
	Sex	0.045	0.834
	Treatment	0.626	0.434
	Sex * Treatment	0.956	0.335

The time points for alcohol consumption across the 11 days is listed in column 1. The between- and within-subject factors is listed in column 2. The F-value from the repeated ANOVA for alcohol consumption is listed in column 3 for the factors of day, sex, treatment, and the interactions between these factors. The p-value from the repeated ANOVA for alcohol consumption is listed in column 4 for the factors of day, sex, treatment, and the interactions between these factors.

Table 2: Statistics for Alcohol Preference at All-Time Points for Both Experiments.

Time Point	Factor	F-Value	*p*-Value
3hr Across	Day	1.029	0.407
	Day * Sex	2.019	0.063
	Day * Treatment	2.198	0.043
	Day * Sex * Treatment	1.753	0.108
	Sex	2.428	0.126
	Treatment	1.198	0.279
	Sex * Treatment	6.186	0.016
6hr Across	Day	1.027	0.419
	Day * Sex	1.451	0.199
	Day * Treatment	0.709	0.635
	Day * Sex * Treatment	1.594	0.153
	Sex	3.76	0.058
	Treatment	5.108	0.028
	Sex * Treatment	0.874	0.354
12hr Across	Day	0.672	0.582
	Day * Sex	1.661	0.174
	Day * Treatment	0.944	0.427
	Day * Sex * Treatment	4.01	0.007
	Sex	1.418	0.24
	Treatment	3.165	0.082
	Sex * Treatment	3.473	0.069
24hr Across	Day	1.503	0.197
	Day * Sex	2.186	0.065
	Day * Treatment	1.81	0.121
	Day * Sex * Treatment	4.074	0.002
	Sex	0.149	0.701
	Treatment	1.501	0.226
	Sex * Treatment	0.453	0.504
3hr Mix	Day	1.201	0.306
	Day * Sex	2.091	0.053
	Day * Treatment	0.442	0.856
	Day * Sex * Treatment	1.022	0.413
	Sex	0.063	0.804
	Treatment	2.417	0.129
	Sex * Treatment	0.922	0.343
6hr Mix	Day	1.824	0.094
	Day * Sex	2.982	0.008
	Day * Treatment	0.556	0.769
	Day * Sex * Treatment	1.124	0.349
	Sex	0.1	0.754
	Treatment	1.843	0.183
	Sex * Treatment	0.176	0.677
12hr Mix	Day	1.374	0.228
	Day * Sex	2.269	0.039
	Day * Treatment	0.556	0.762
	Day * Sex * Treatment	0.538	0.776
	Sex	0.005	0.945

	Treatment	2.91	0.097
	Sex * Treatment	0.363	0.551
24hr Mix	Day	1.473	0.202
	Day * Sex	3.78	0.003
	Day * Treatment	0.418	0.832
	Day * Sex * Treatment	0.705	0.618
	Sex	0.004	0.953
	Treatment	3.525	0.069
	Sex * Treatment	0.503	0.438

The time points for alcohol preference across the 11 days is listed in column 1. The between- and within-subject factors is listed in column 2. The F-value from the repeated ANOVA for alcohol preference is listed in column 3 for the factors of day, sex, treatment, and the interactions between these factors. The p-value from the repeated ANOVA for alcohol preference is listed in column 4 for the factors of day, sex, treatment, and the interactions between these factors.

Oxytocin's effects on alcohol intake when half of the animals in the same cage receive oxytocin treatment.

Treatment for alcohol use is usually taken in settings outside of the clinic and in social settings (i.e. around others who are not prescribed the same medication). This (Mix) experiment modelled this condition. In a separate group of animals, half of the voles in each cage received the oxytocin treatment, while the other half received the saline treatment during days 8 – 12. At the 3-hour time point, there were no main effects of sex ($F_{1,35}$ = 0.59, P = 0.46), treatment ($F_{1,35}$ = 1.8, P = 0.19), and no sex × treatment interaction ($F_{1,35}$ = 0.02, P = 0.90) for alcohol consumption. However, there was a significant 2-way interaction of day by treatment ($F_{5.3,185.8}$ = 2.76, P = 0.02). Post hoc analysis revealed that oxytocin reduced alcohol consumption for the first three days of treatment compared to the saline-treated animals (Fig. 6A). Similarly, at the 6-hour time point, there was no main effect of sex ($F_{1,35}$ = 1.63, P = 0.21), treatment ($F_{1,35}$ = .75, P = .39), and no sex by treatment interaction ($F_{1,35}$ = 0.73, P = 0.40), but there was a significant 2-way interaction of day by treatment ($F_{5.6,196.1}$ = 5.6, P = 0.02). Post hoc tests confirmed that oxytocin decreased alcohol consumption during the first day of treatment (Fig. 7A). Oxytocin treatment did not significantly decrease alcohol consumption at the 12- and 24-hour time points (P > 0.05, Table 1 and Figs. 8A & 9A).

There were no significant effects of sex or treatment or any interactions between these factors on alcohol preference at any of the analyzed time points (P > 0.05). This lack of effect reflected that there were no visible differences in alcohol preference at either baseline or during treatment (Fig 6B/7B/8B/9B, and Table 2), revealing that oxytocin treatment decreased both alcohol and water consumption and the effect was not specific to alcohol.

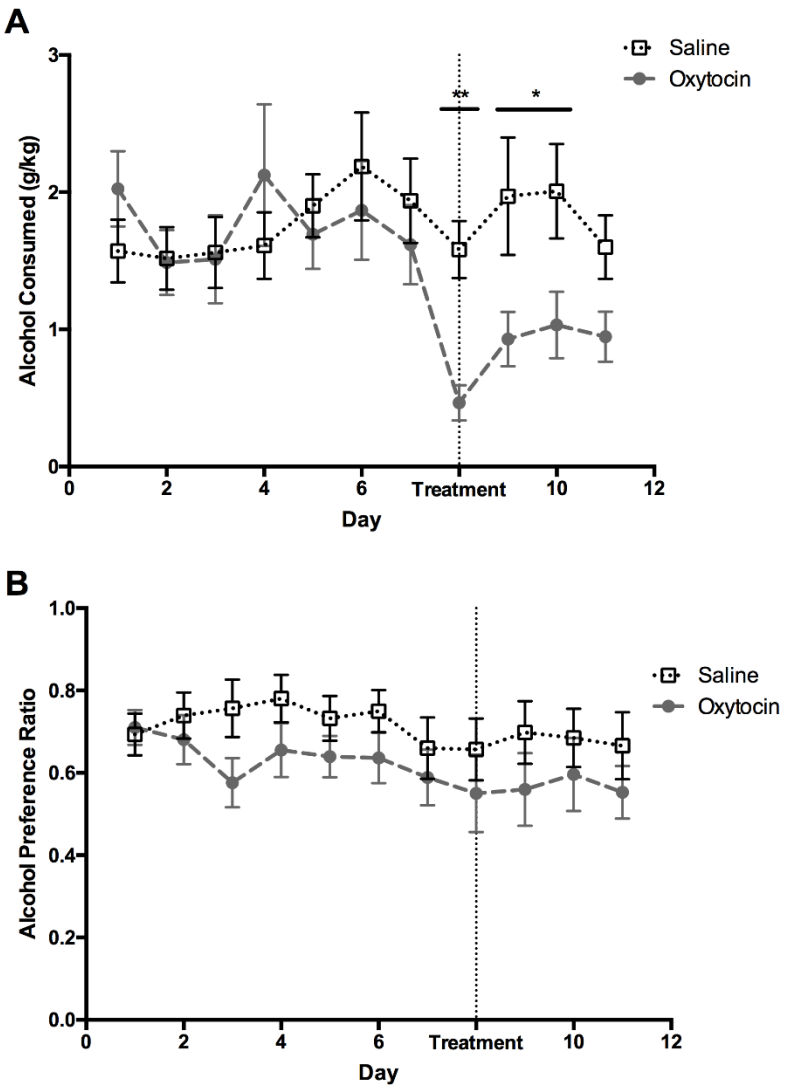


Figure 6: Alcohol consumption and preference in the first three hours of alcohol access following oxytocin treatment when animals in the same cage receive different treatments. Sex was collapsed across treatment for both (A) alcohol consumption and (B) preference. (A) Oxytocin decreased alcohol consumption during the first three hours of alcohol access only for the first three days of treatment compared to saline treated animals. However, (B) oxytocin did not affect alcohol preference. *p<0.05, **p<0.01. Error bars indicate mean ± SEM. (n = 19-20/group, 4-5 animals per cage).

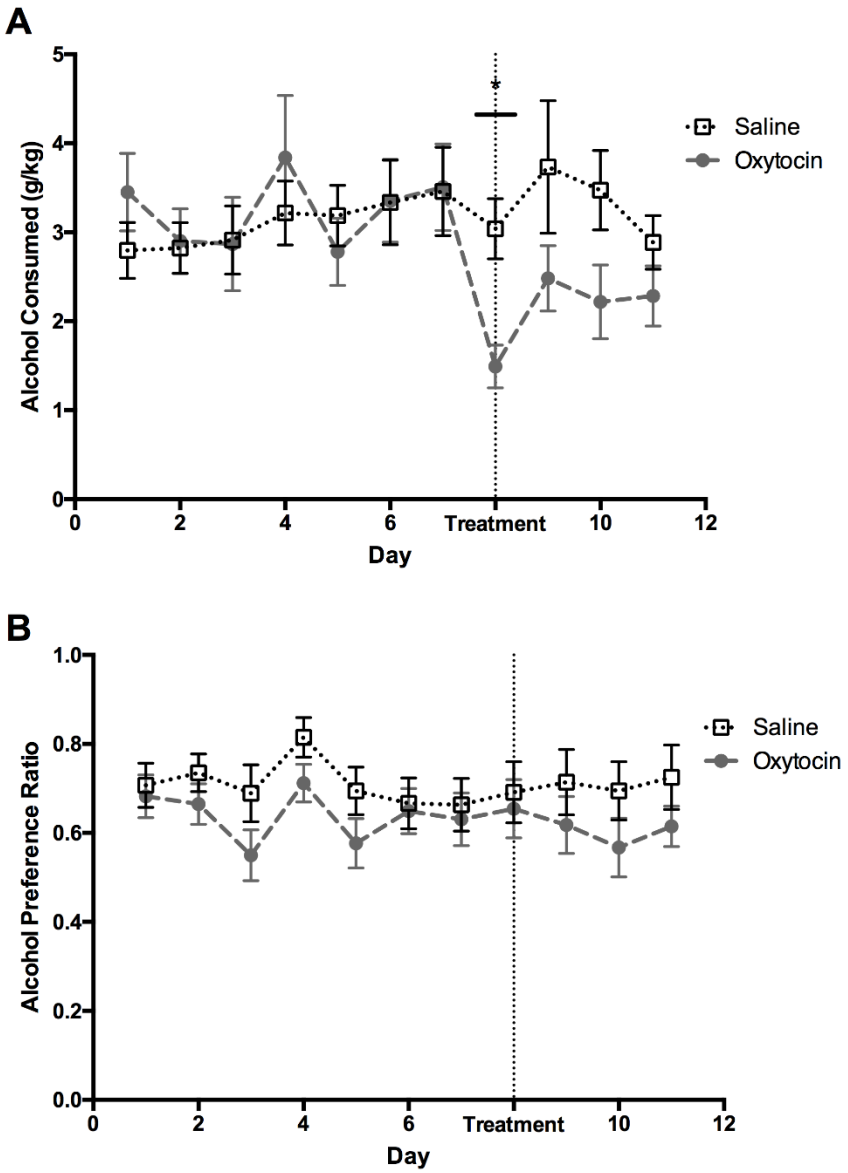


Figure 7: Alcohol consumption and preference in the first six hours of alcohol access following oxytocin treatment when animals in the same cage receive different treatments. (A) Oxytocin decreased alcohol consumption during the first six hours of alcohol access only for the first day of treatment compared to saline treated animals. (B) Alcohol preference did not differ between treatment groups during any day of alcohol access. *$p<0.05$. Error bars indicate mean ± SEM for the number of voles in Fig. 6.

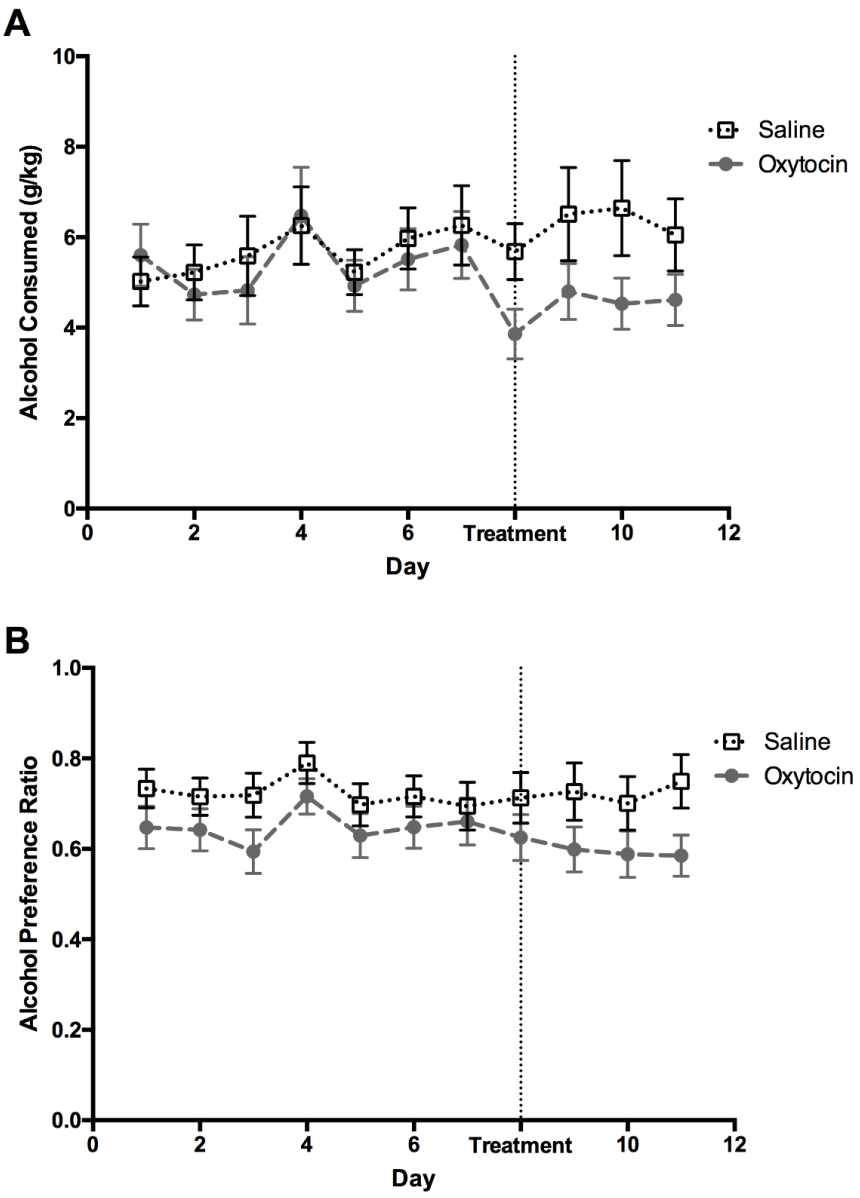


Figure 8: Alcohol consumption and preference over the 12-hour period when treatment was mixed in each cage. There was no overall difference between females and males, therefore sex was collapsed across each day. Oxytocin treatment did not affect alcohol (**A**) consumption or (**B**) preference over the 12-hour period when animals received different treatments within a cage. Error bars indicate mean ± SEM for the number of voles in Fig. 6.

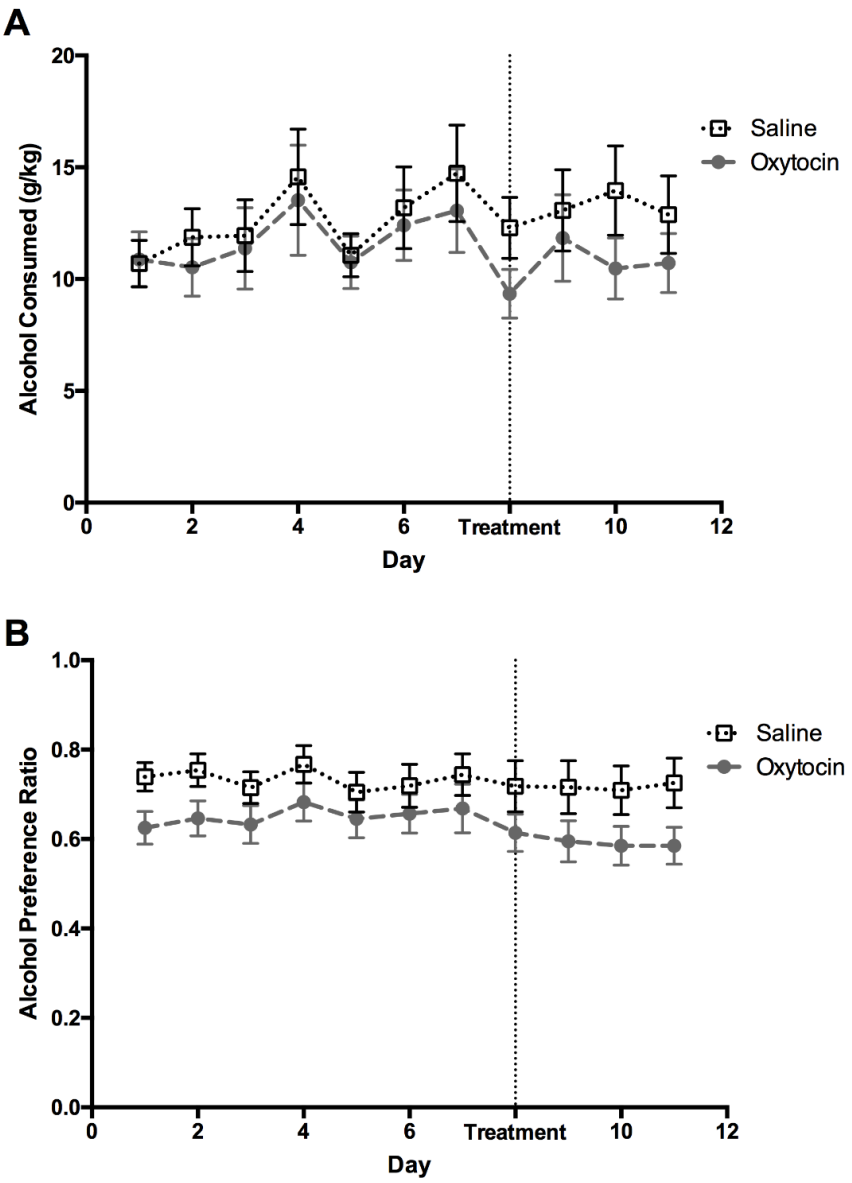


Figure 9: Alcohol consumption and preference over the 24-hour period when treatment was mixed in each cage. There was no difference between females and males, therefore sex was collapsed across each day. Oxytocin treatment did not affect alcohol (**A**) consumption or (**B**) preference over the 24-hour period when animals received different treatments within the same cage. Error bars indicate mean ± SEM for the number of voles in Fig. 6.

Oxytocin and alcohol's effects on neural substrates.

There is a controversy whether peripherally-administered oxytocin crosses the blood brain barrier (Ermisch et al., 1985; Leng and Ludwig, 2016). However, previous research showed that peripherally administered oxytocin can increase activation in oxytocin-synthesizing neurons in the PVN and SON (Carson et al., 2010). We explored if peripheral oxytocin treatment would affect oxytocin neurons in the brain in a subset of high drinking animals. High drinking animals were used because previously it has been shown that voluntary alcohol consumption will decrease the number of oxytocin-ir cells in the PVN, and the treatment groups had to match their intake to make this comparison meaningful (Silva et al, 2002; Stevenson et al., 2017b; Chapter 1; Walcott and Ryabinin, 2017). The average alcohol consumption for the high drinking saline animals was 20.8 ± 1.1 g/kg and for the high drinking oxytocin animals was 22.2 ± 2.2 g/kg. Consumption were collapsed across sex because we saw no difference in alcohol consumption between males and females. We found that peripheral oxytocin treatment had no effect on the number of oxytocin-ir cells in the PVN (t_{14} = 0.19, P = 0.85) and the SON (t_{14} = 1.41, P = 0.18), an observation arguing that peripheral oxytocin treatment did not affect oxytocin expression in the PVN or SON (Figs. 10 & 11).

Alcohol metabolism in prairie voles is significantly faster than in humans (Anacker et al., 2011a). To confirm that consumed alcohol affects the activity of the central nervous system in our animals, we analyzed levels of FosB-ir in the lowest and highest alcohol consuming animals that received only saline. Specifically, we analyzed FosB-ir within the NAcc, CeA, and EWcp because previous research has shown that immediate early gene expression in these brain regions can be affected by voluntary alcohol consumption (Ozburn et al., 2012; Sharko et al., 2013; Chapter 1). Alcohol consumption and BECs did not significantly correlate with FosB-ir in the NAcc core or shell and CeA (results not shown). However, FosB-ir in the EWcp (Fig. 12) positively

correlated with alcohol consumption at the 1 hour ($r = 0.5$, $F_{1,14} = 4.7$, $P = 0.05$; Fig. 13A) and 24 hour time points ($r = 0.56$, $F_{1,14} = 6.4$, $P = 0.02$; Fig. 13B). Similarly, FosB-ir in the EWcp positively correlated with BECs *(*$r = 0.64$, $F_{1,12} = 8.1$, $P = 0.01$; Fig. 13C). These results add to the growing literature showing that alcohol consumption is associated with neuronal activation in the EWcp.

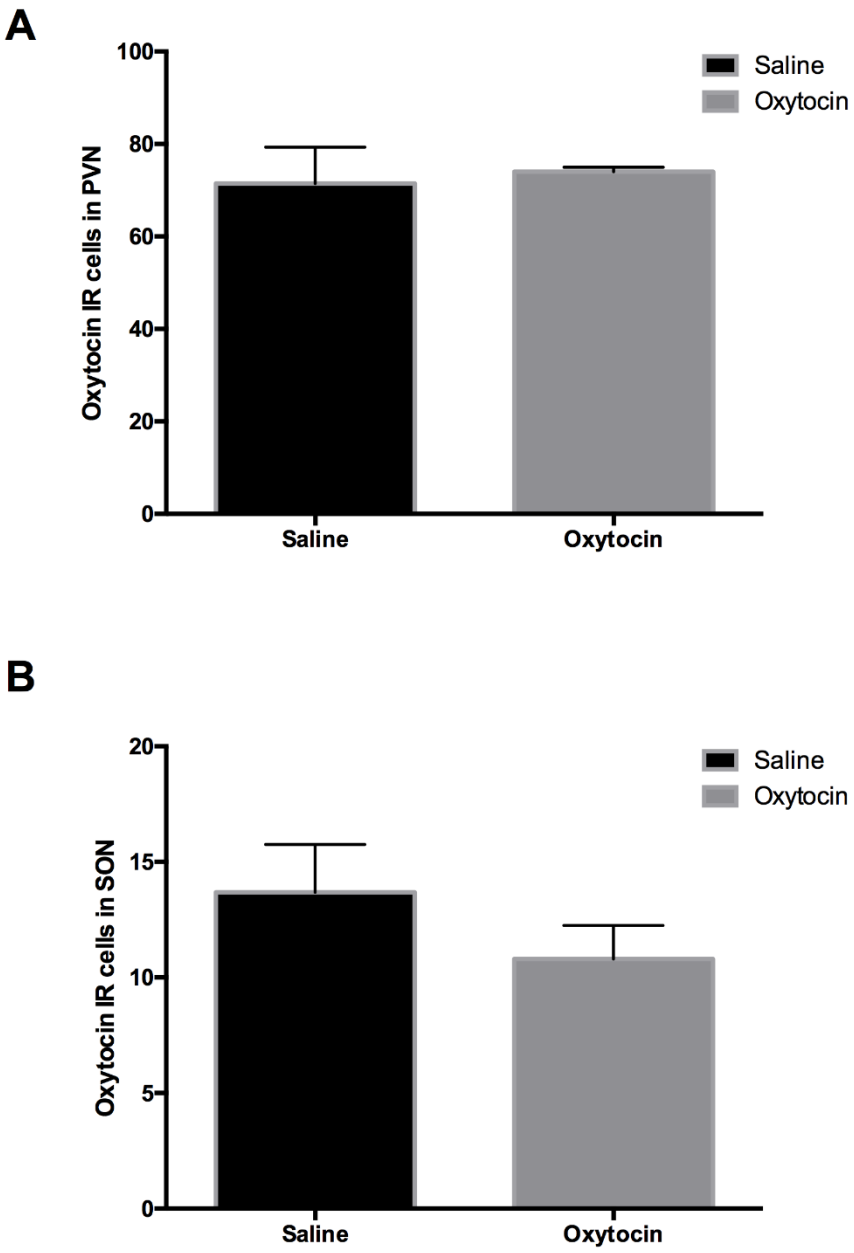


Figure 10: Immunoreactivity for oxytocin in the PVN and SON. There was no difference between sexes; therefore, data was collapsed across sex. There was no significant difference between the number of oxytocin-ir cells in the (**A**) PVN or (**B**) SON between the saline and oxytocin treated high drinking animals (High drinking saline animals drank 20.8 ± 1.1 g/kg and the high drinking oxytocin animal drank 22.2 ± 2.2 g/kg. Error bars indicate mean ± SEM. (n = 8/treatment).

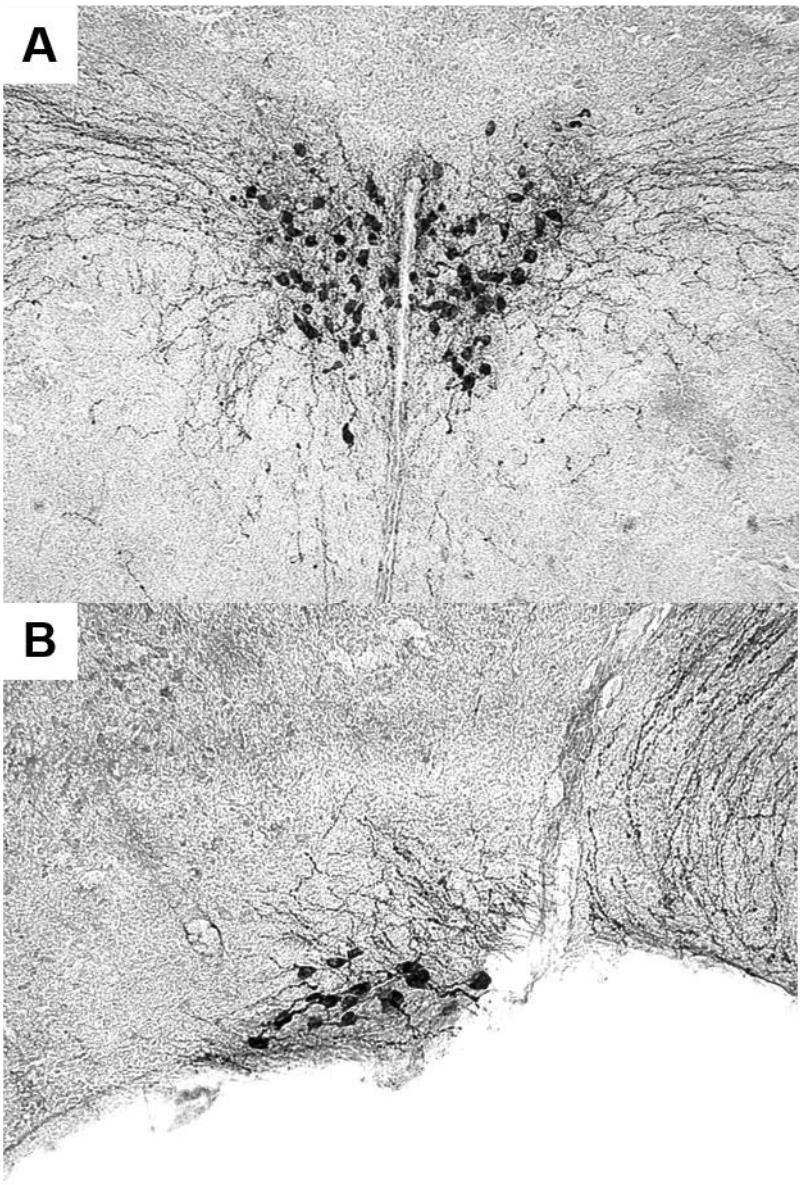


Figure 11: Representative photomicrographs. Oxytocin immunoreactivity in the (A) paraventricular nucleus and (B) supraoptic nucleus of the hypothalamus**.**

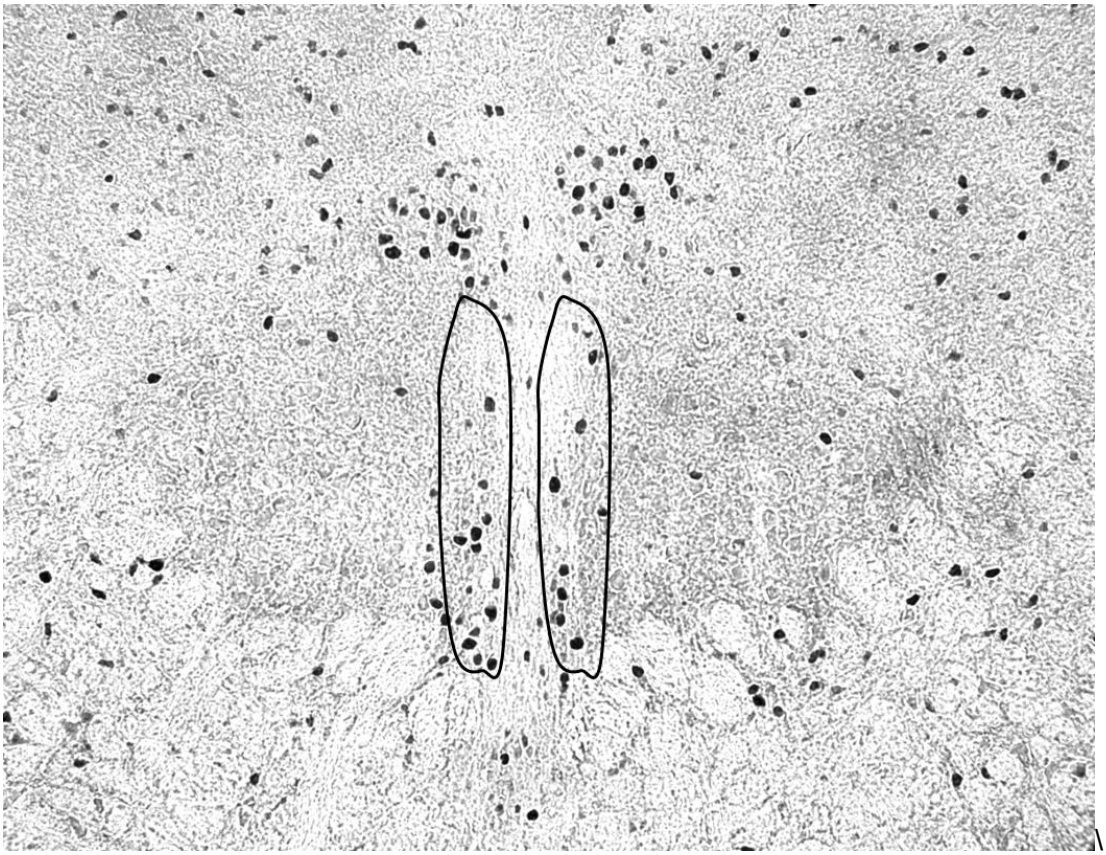


Figure 12: Representative photomicrograph. FosB immunoreactivity in the centrally-projecting Edinger Westphal nucleus.

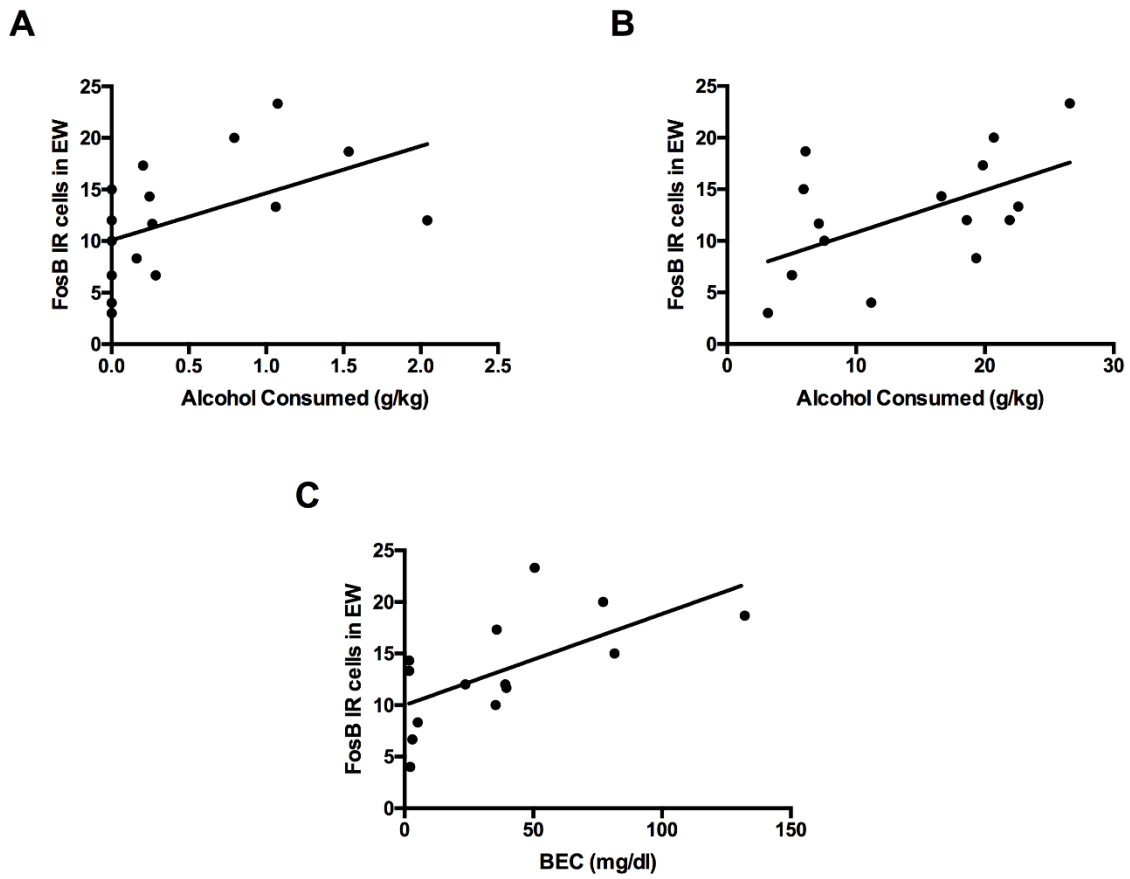


Figure 13: Alcohol consumption is associated with neural activation in the EWcp. FosB activation in the EWcp was significantly positively correlated with average alcohol intake during the (**A**) 1-hour and (**B**) 24-hours of alcohol consumption. (n= 8/sex). (**C**) Similarly, blood ethanol concentration was positively correlated with FosB activation in the EWcp (n= 8 females, n= 6 males).

Discussion

In the described experiments oxytocin treatment decreased alcohol consumption under social settings in male and female prairie voles. Moreover, alcohol intake was decreased in oxytocin-treated animals despite their interactions with untreated cagemates. These results not only show effectiveness of a potential therapy for substance abuse disorder despite counteractions from peers, but also demonstrates that other potential medications can be tested for effectiveness in similar circumstances.

We have administered oxytocin via an intraperitoneal injection. Earlier reports have suggested that oxytocin is too large of a molecule to cross the blood-brain barrier (Ermisch et al., 1985). However, it has been shown that peripheral oxytocin treatment rapidly increases oxytocin levels in brain dialysates and plasma during the first 30 minutes after treatment, which then subsequently return to baseline (Neumann et al., 2013). One potential alternative explanation for the increased central levels of oxytocin following a peripheral administration, is that peripheral oxytocin could be stimulating central production of this peptide. While this possibility can't be completely ruled out, our observation of lack of changes in oxytocin-ir in the PVN and SON suggests that this mechanism does not play a major role in central effects of oxytocin. Importantly, a recent study discovered that oxytocin is actively transported through the blood-brain barrier by a Receptor for Advanced Glycation End-products (RAGE) (Yamamoto et al., 2019). Specifically, mice lacking RAGE don't show increases in oxytocin in the brain following peripheral administration of this peptide and mimic behavioral profiles of mice lacking oxytocin. Meanwhile, animals that have RAGE show an increase in oxytocin in several brain regions for 60-90 minutes following its peripheral administration. Therefore, it seems most likely that peripheral treatment with oxytocin in the current study decreased alcohol consumption through direct central effects.

Importantly, our study assessed effects of oxytocin on consumption of physiologically relevant amounts of alcohol with a few animals reaching BECs of over 75 mg/dL, which are considered intoxicating (Niaaa 2018). These levels are reached despite rapid ethanol elimination rates in this species (Anacker et al., 2011a). Alcohol consumption in our study was also associated with changes in central nervous activity as reflected in a significant correlation between BECs and FosB-ir in the centrally-projecting EWcp. The FosB antibody used here measures levels of both the full length FosB protein and a truncated FosB protein, deltaFosB. Full length FosB is usually rapidly expressed after an acute stimulation, while deltaFosB gradually accumulates after repeated stimulations (Nestler et al., 2001). Therefore, our study suggests that both forms of FosB protein are present in the EWcp with the increased deltaFosB correlated with prolonged alcohol drinking (24hr) while the full length FosB correlated with acute (1hr) alcohol drinking is related to full length FosB activation. The EWcp has been shown not only to be sensitive to alcohol, but also to be involved in regulation of alcohol consumption (Giardino et al., 2017; Bachtell et al., 2003; Bachtell et al., 2004). However, the design of our study does not allow to distinguish whether activation of EWcp was regulating alcohol consumption or responding to consumed alcohol. Further studies on this subject are needed.

Our study expands the growing literature showing that oxytocin can be a potential treatment for alcohol use disorder. Specifically, Mcgregor and Bowen (2012) found that a single administration of oxytocin (1mg/kg, i.p.) led to a long-lasting decrease in preference for a sweetened alcohol solution over the sweetener in male and female rats. Peters et al. (2013) observed a decrease in alcohol consumption and preference at 24 hours, but not 48 hours after a single icv injection of oxytocin in rats. Additionally, King et al. (2017) found that peripheral oxytocin administration reduced alcohol consumption in male C57BL/6J mice in a no-choice binge-like alcohol self-administration

and operant self-administration procedures for alcohol with higher doses also being capable of decreasing sucrose preference. Similarly, Stevenson et al. (2017a) reported that peripheral oxytocin administration decreased alcohol consumption and preference over a 24-hour period in an intermittent two-bottle choice paradigm in semi-socially housed male and female prairie voles. In contrast, under a continuous access two-bottle choice paradigm, oxytocin decreased alcohol consumption in only the first hour after treatment but had no effect on consumption over a 24-hour period. In post mortem brains of human AUD patients, it has been shown that hypothalamic oxytocin immunoreactivity is altered (Sivukhina et al., 2006) and oxytocin receptors are upregulated (Hansson et al., 2018), meaning that the oxytocin system is affected by AUD. Additionally, intranasal oxytocin treatment in AUD patients decreased withdrawal symptoms and alcohol cue-reactivity (Pedersen et al., 2013; Hansson et al., 2018). A more recent case report found that intranasal oxytocin decreased alcohol consumption and depression symptoms in a cancer patient (Pirnia and Pirnia 2018).

In the majority of preclinical studies, peripheral oxytocin treatment decreased both alcohol consumption and preference across a variety of voluntary alcohol drinking models. Interestingly, in the current study oxytocin did not alter alcohol preference. In the Across experiment, it was difficult to interpret the results because during the baseline period preference was significantly different between treatment groups. However, in the Mix experiment, alcohol preference between the treatment groups did not differ during the baseline and treatment periods, thus oxytocin reduced water consumption as well. This decrease in total fluid consumption is consistent with oxytocin's reported effects on food consumption. In rats, oxytocin, administered IP or intracerebroventricularly, dose-dependently reduces food consumption and time spent eating (Arletti et al., 1989). Similarly, intranasal oxytocin treatment reduced reward-driven food intake and caloric intake in humans (Lawson et al., 2015; Ott et al., 2013). Nevertheless, the described

above previous alcohol studies indicated that oxytocin had preferential effects on consumption of alcohol versus sweetener-based solutions.

One clear procedural difference in the current studies from the previous ones is that here we for the first time allowed animals to socially interact. The consequences of this advance in experimental design have to take into account the prosocial effects of oxytocin. Indeed, oxytocin treatment in mammals has been shown to increase their prosocial behaviors (Heinrichs et al., 2009; Insel, 2010; Striepens et al., 2011). Specifically, repeated peripheral oxytocin treatments increased partner preference formation in female prairie voles compared to females that received a single injection of oxytocin (Cushing and Carter, 2000). In naked mole rats, an eusocial rodent, a 1mg/kg or 10mg/kg IP injection of oxytocin increased huddling behavior when animals were placed back in their home cage with familiar conspecifics (Mooney et al., 2014). It seems likely that alcohol preference was decreased in previous studies because oxytocin treatment was given to animals that were either consuming alcohol in isolation or in semi-social housing environments (i.e. two animals in a cage divided by a mesh divider) (King et al., 2017; Mcgregor and Bowen, 2012; Stevenson et al., 2017a). In this current study animals were drinking alcohol and receiving oxytocin treatments in cages where they had full social contact with their cagemates, similarly to clinical treatment conditions in humans. Therefore, increased prosocial behavior (i.e., huddling) could have competed with consumption of both alcohol and water in our experiment. Increased prosocial behavior is not likely to be of negative consequence as a side effect for the potential use of a AUD medication. Further work investigating oxytocin's ability to decrease fluid consumption moderated by an increase in prosocial behavior is warranted.

Importantly, this study to our knowledge is the first to explore the effects of a potential alcohol treatment in a mixed treatment group setting. We showed that the current procedure is sensitive enough for effects to be discovered. In the future, these

mixed treatment group setting procedures should be used when studying other treatments of AUD and other drugs of abuse. The combination of mixed treatment group settings and radio frequency identification tracking will ultimately improve the translational value of animal studies on treatment for addiction in humans.

GENERAL DISCUSSION

Social relationships affect alcohol use disorder (AUD). However, at the same time AUD affects social relationships. The majority of preclinical alcohol research focuses more on the individual, instead of taking into account the social aspects of alcohol use. The issue with this is that alcohol use and social relationships rely on overlapping mechanisms. This dissertation shows that sociality should be taken into account when investigating preclinical alcohol animal models. In addition, this dissertation characterizes an animal model that can be used to explore the biological factors that underlie the intertwining of AUD and social relationships from the early alcohol use stage to the early treatment phase.

Alcohol's Effects on Pair Bond Maintenance

In Chapters 1 and 2, I explored the effects discordant drinking had on pair bond maintenance in male and female prairie voles. I found that discordant drinking has a sex-dependent effect on partner preference. In males, discordant alcohol drinking led to a decrease in partner preference, while females showed no effect of discordant alcohol drinking on partner preference in comparison to water controls and concordant drinking partners.

In the human literature discordant alcohol drinking between couples is associated with an increase in separation and divorce. However, when the wife is the heavy alcohol drinker it is more robustly related to separation and divorce compared to when the husband is the heavy alcohol drinker (Leonard et al., 2014; Torvik et al., 2013; Ostermann et al., 2005). One major issue with the human literature is that the number of couples where the wife is the heavy drinker and the husband is the abstainer is extremely small compared to when the husband is the heavy drinker and the wife is the abstainer. Specifically, in Ostermann et al. (2005), there were 9 times more couples in

the group where only the husband is the heavy drinker compared to when only the wife is the heavy drinker. These epidemiological studies of discordant drinking involve many more couples in which the husband, and not the wife, is the heavy drinker, such that a separate analysis on heavy drinking in females could be underpowered. Therefore, couples involving the wife as the heavy drinker might not lead to increases in separation. If this is true, then it could be possible that we did not see a change in PP in the discordant female drinking study because couples with heavy drinking females does not affect marital status.

Another possible reason why I saw no difference in partner preference between the three treatment groups in females in Chapter 2 is that pregnancy could have been masking the effects of discordant alcohol drinking. It is well known that pregnancy in rodents causes increases in ovarian steroid hormones (Virgo and Bellward, 1974). Ovarian hormones have been shown to alter several behavioral functions including aggression, maternal behavior, depression, and memory (Fraile et al., 1987; Bloch et al., 2000; Spiteri and Agmo, 2009; Sandstrom and Williams, 2001; Numan et al., 1999). Specifically, Spiteri and Agmo (2009) showed that estradiol and progesterone increase social recognition in ovariectomized female rats compared to rats treated with only oil. It could be possible that discordant alcohol drinking female prairie voles in Chapter 2 showed no decrease in partner preference because they had an enhanced social recognition for their partner, thus masking the effects of discordant drinking between partners.

In Chapter 1, I saw that partner preference in male prairie voles was affected by the drinking status of their female partner. Discordant alcohol consuming males was accompanied by an increase in FosB immunoreactivity in the periaqueductal gray (PAG), a region that is mainly known for its role in the modulation of stress and pain. However, a few studies have found that the PAG might potentially play a role in maternal

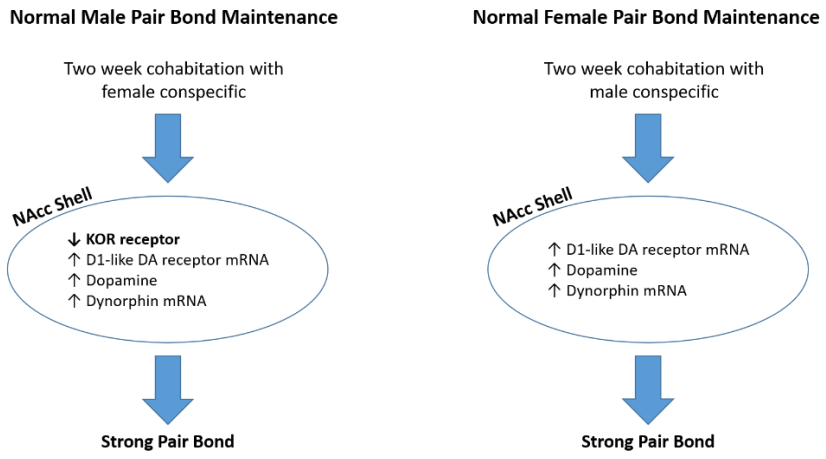
and romantic love (Acevedo et al., 2012; Bartels and Zeki, 2004). The PAG has a high density of vasopressin fibers (De Vries and Al-Shamma, 1990). More recently, it has been discovered that there is high vasopressin 1a receptor (V1aR) density in the PAG (reviewed in Smith et al., 2019). As stated throughout this dissertation, it is well known that the V1aR plays a role in pair bond formation in male prairie voles (Lim and Young, 2004; Pitkow et al., 2001; Cho et al., 1999). To my knowledge, V1aR in the PAG has not been studied in the context of pair bonds. In mice, the Alston's brown mice exhibits a complex vocal system and these mice have a high density of V1aR binding in the PAG (Campbell et al., 2009). Additionally, a microinjection of vasopressin in the PAG increases flank marking, a form of social communication, in Syrian hamsters (Hennessey et al., 1992). Finally, in a socially monogamous primate, the titi monkey, the activation of the PAG is altered when a male monkey is separated from his female pair mate for a 48-hour period (Hinde et al., 2016). These studies show that the PAG plays a role in social behavior, but the specific role of the PAG in the context of pair bonds is unknown.

The PAG receives many inputs from several different brain regions. One specific region that projects to all regions of the PAG is the anterior hypothalamus (AH) (Semenenko and Lumb, 1992; Vianna and Brandao, 2003). In prairie voles, selective aggression leads to an increase in neuronal activation in the AH, specifically in the vasopressin expressing neurons, and an increase in vasopressin release (Gobrogge et al., 2007; Gobrogge et al., 2009). Activation of the V1aR in the AH in sexually-naïve prairie voles leads to an increase in selective aggression; meanwhile, when V1aRs are pharmacologically blocked in the AH it leads to a decrease in pair bonds in male prairie voles (Gobrogge et al., 2009). Additionally, when comparing V1aR binding density in the AH, pair bonded males exhibit an increase compared to sexually naïve males (Gobrogge et al., 2009). Because the AH and PAG are highly connected and the AH plays a role in

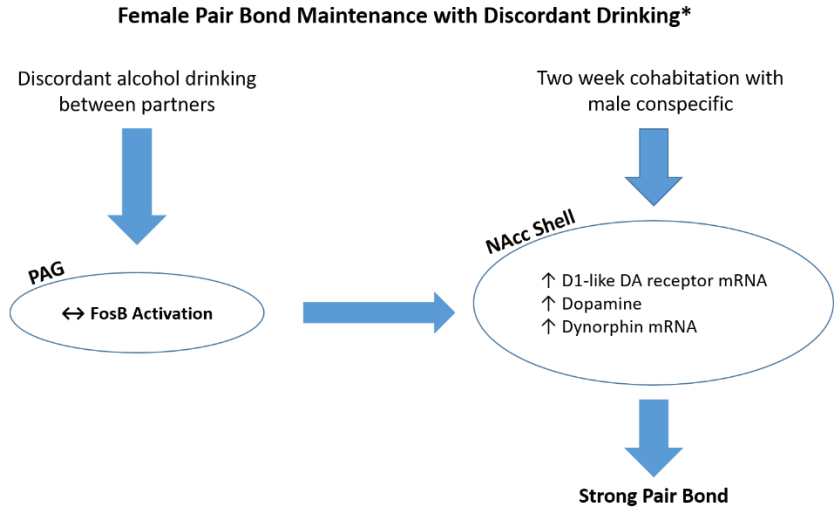
selective aggression and affiliative behavior, it seems possible that the two regions together play a role in pair bond maintenance and the effects of discordant drinking on this behavior.

Another region the PAG projects to is the nucleus accumbens (NAcc), specifically the NAcc shell (Hasue and Shammah-Lagnado, 2002). The NAcc shell has been shown to play a role in the maintenance of pair bonds in prairie voles (Resendez et al., 2016; Resendez et al., 2012). Specifically, the interaction between the dopamine and the kappa opioid receptor/dynorphin systems within the NAcc shell mediate the maintenance of pair bonds. When a female prairie vole is pair bonded there is an increase in the D1-like dopamine receptor, increase in dopamine release, and an increase in dynorphin mRNA. When a male prairie vole is pair bonded there is an increase in the D1-like dopamine receptor, increase in dopamine release, increase in dynorphin mRNA, and a **decrease** in kappa opioid receptor binding in the NAcc shell (Resendez et al., 2016). This decrease in kappa opioid receptor binding is necessary for pair bond maintenance in male prairie voles, but is not necessary in female prairie voles. Therefore, it is possible that when discordant alcohol drinking occurs in male prairie voles, that the PAG becomes hyperactive, and then causes a disruption in this kappa opioid receptor binding, which then leads to disruptions in the dopamine system in the shell. Not much is known about the role the PAG plays in social behaviors or pair bond maintenance in general; therefore, it is impossible to provide a thorough biological mechanism of how the PAG mediates the interaction between discordant drinking and pair bond maintenance. However, I provide here a schematic representation of a possible mechanism for this in Figure 1. I did not explicitly test any causal mechanisms involved in the effects of discordant drinking on pair bond maintenance, but I can speculate about a possible mechanism.

A


Normal Male Pair Bond Maintenance
Two week cohabitation with female conspecific
NAcc Shell
↓ KOR receptor
↑ D1-like DA receptor mRNA
↑ Dopamine
↑ Dynorphin mRNA
Strong Pair Bond
Normal Female Pair Bond Maintenance
Two week cohabitation with male conspecific
NAcc Shell
↑ D1-like DA receptor mRNA
↑ Dopamine
↑ Dynorphin mRNA
Strong Pair Bond

B


Female Pair Bond Maintenance with Discordant Drinking*
Discordant alcohol drinking between partners
PAG
↔ FosB Activation
Two week cohabitation with male conspecific
NAcc Shell
↑ D1-like DA receptor mRNA
↑ Dopamine
↑ Dynorphin mRNA
Strong Pair Bond

C

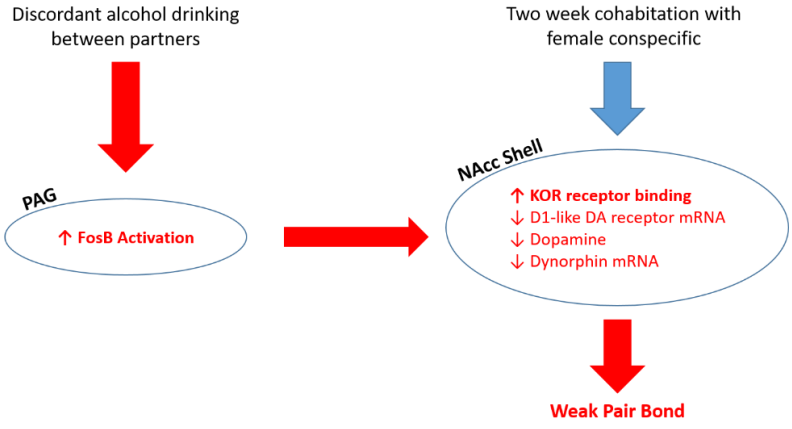

Male Pair Bond Maintenance with Discordant Drinking*
Discordant alcohol drinking between partners
PAG
↑ FosB Activation
Two week cohabitation with female conspecific
NAcc Shell
↑ KOR receptor binding
↓ D1-like DA receptor mRNA
↓ Dopamine
↓ Dynorphin mRNA
Weak Pair Bond

Figure 1. The mechanism of pair bond maintenance with and without discordant drinking between partners. (A) The maintenance of a pair bond in male prairie voles requires a decrease in kappa opioid receptor (KOR) binding, an increase in D1-like dopamine (DA) mRNA expression, an increase in dopamine, and an increase in dynorphin mRNA expression in the nucleus accumbens (NAcc) shell. In female prairie voles an increase in D1-like DA receptor mRNA, an increase in dopamine, and an increase in dynorphin mRNA is required for pair bond maintenance NAcc shell. (B) When a discrepancy in alcohol drinking occurs, female prairie voles show no change in FosB activation in the periaqueductal gray (PAG), therefore there is no change in the mechanism occurring in the NAcc shell. *Untested hypothesis. (C) When a discrepancy in alcohol drinking occurs, male prairie show an increase in FosB activation in the PAG. This activation potentially causes an increase in KOR receptor binding, a decrease of D1-like DA receptor mRNA, a decrease in dopamine, and a decrease in dynorphin mRNA in the NAcc shell. *Untested hypothesis.

Alcohol and the Oxytocin System

In Chapters 1 and 2, in addition to behavioral outcomes, I examined the potential effect of alcohol on the oxytocin system in both pair bonded male and female prairie voles. Specifically, I tested oxytocin-ir in the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) of the hypothalamus. Oxytocin quantification in these two brain regions showed that alcohol drinking, independent of their partner's drinking status, led to a decrease in oxytocin-ir in the PVN compared to water only drinking prairie voles. On the other hand, alcohol drinking in females did not significantly alter oxytocin-ir in the SON.

My results added to the limited field of research exploring the effects of voluntary alcohol consumption on oxytocin-ir levels in the PVN. To my knowledge, only one other study has shown that completely voluntary alcohol consumption affects the oxytocin cells in the PVN of voles (Stevenson et al., 2017b). The major difference between the Stevenson et al. (2017b) study and the current ones is the amount of time animals were given access to alcohol. The current studies showed that the decrease in the number of oxytocin-ir cells in the PVN can occur within 7 days compared to the Stevenson et al. (2017b) study, where they showed oxytocin-ir cells in the PVN decreases after 7 weeks of alcohol drinking. Animals in both studies drank comparable amounts of alcohol. None of these studies determined the underlying mechanism, however, in Chapter 2 I determined that the decrease in oxytocin-ir cells was not due to cell death through the caspase 3 pathway. It appears possible that alcohol consumption leads to a decrease in oxytocin mRNA or protein expression in the PVN. The latter seems more plausible because it has been shown that after 6 or 10 weeks of forced alcohol consumption, rats do not show a decrease in oxytocin mRNA, but still show a decrease in the number of oxytocin-ir cells in the PVN (Silva et al., 2002). Therefore, I find it likely that alcohol consumption decreases the expression of oxytocin peptide in the PVN in prairie voles.

Due to the effects alcohol had on oxytocin-ir in Chapters 1 and 2, I explored the effects of oxytocin on alcohol consumption in social housing cages in Chapter 3. I discovered that peripheral oxytocin treatment decreases alcohol consumption 3- and 6-hours post treatment injection in a “mixed” treatment setting. However, alcohol preference was not decreased after oxytocin treatment because both alcohol and water consumption decreased, thus showing that oxytocin does not have alcohol specific effects in social housing cages where prairie voles have physical access to familiar conspecifics. This decrease in alcohol consumption is in line with previous studies exploring peripheral oxytocin’s effects on alcohol consumption in rodents (King et al., 2017; Peters et al., 2013; Macfadyen et al., 2016; Mcgregor and Bowen, 2012; Stevenson et al., 2017a). However, all of these studies show that oxytocin also decreases alcohol preference. In these prior studies, animals are housed in isolation or semi-socially. It is possible that the reason why I saw no decrease in alcohol preference in Chapter 3 was because oxytocin treatment led to an increase in prosocial behavior (i.e. huddling) and created a “competition” between huddling and consuming fluid. This “competition” is very possible because peripheral oxytocin treatment increases prosocial behaviors in other studies (Cushing and Carter, 2000; Mooney et al., 2014).

The mechanism of how oxytocin decreased fluid consumption in our study is unknown. Previous studies have shown that oxytocin decreases food and fluid consumption (Arletti et al., 1990; Arletti et al., 1989), however the studies described above focusing on oxytocin’s effects on alcohol consumption vs. water or sweetener-based solution have shown that oxytocin has preferential effects on alcohol consumption. It has been shown that when mice receive an IP injection of oxytocin, they show a rapid increase in microdialysates from both the hippocampus and amygdala and an increase in oxytocin plasma concentration with a peak occurring around the first 30 minutes after treatment (Neumann et al., 2013). However, it is debated that oxytocin is

too large of a molecule to cross the blood brain barrier. In Chapter 3 I provided the explanation that oxytocin could have passed through the blood brain barrier by a Receptor for Advanced Glycation End products (RAGE) (Yamamoto et al., 2019). Another possibility is that alcohol could have caused the blood brain barrier to dysfunction. When alcohol metabolizes in brain endothelial cells, this results in the production of acetaldehyde and reactive oxygen species. These two chemicals can cause significant damage to the blood brain barrier, thus leading to a decrease in blood brain barrier integrity in brain endothelial cells (Haorah et al., 2005). Potentially alcohol consumption in my study decreased the blood brain barrier's integrity, allowing oxytocin to pass the barrier and acting on the central system directly.

Oxytocin has the potential to be an approved pharmacotherapy for AUD. As stated in Chapter 3, oxytocin can decrease alcohol consumption in mixed treatment social settings. However, a majority of clinical studies explore how oxytocin affects alcohol craving, alcohol-cue reactivity, and alcohol withdrawal (Hansson et al., 2018; Mitchell et al., 2016; Pedersen et al., 2013). To my knowledge, only one study has explored the effect of oxytocin on an indirect measure of alcohol consumption in a clinical setting. Pirnia and Pirnia (2018) found when a patient was using an intranasal oxytocin spray treatment, the patient showed a decrease in his Alcohol Use Disorders Identification Test (AUDIT) score compared to the baseline periods when the oxytocin treatment was not used. This shows that oxytocin has the potential to be used in clinics to decrease alcohol drinking; however, the effects of oxytocin on alcohol consumption needs to be repeated in a larger population to determine the efficacy in clinics.

Future Directions

Future studies exploring the interactions between sociality and alcohol use may help understand how social relationships affect current pharmacological treatments for

all drugs of abuse and lead to the development of more effective pharmacological treatments for AUD.

Further investigation of heavy alcohol use on divorce rates needs to occur. The issue with the current epidemiological studies in humans is that couples that consist of wives who drink heavy and husbands who abstain from drinking are not highly prevalent. In one study, the number of discordant-husband heavy drinking couples is twice as many as the discordant-wife heavy drinking couples (Leonard et al., 2014). It could be possible that in epidemiological studies a separate analysis of discordant-wife heavy drinking couples is underpowered, thus potentially leading to discordant-husband heavy drinking couples being the only true group that leads to increased rates of marital separation. If this is the cause, then it could be possible that we are modeling this in prairie voles (i.e. having only discordant-male drinking prairie voles leading to weaker partner preference and discordant-female drinking prairie voles leading to no difference). Therefore, future epidemiological studies need to increase the representation of couples that consist of the wife as the heavy drinker and the husband as the abstainer or low drinker in their analysis.

It is necessary to understand the biological mechanisms that are controlling alcohol's effects on partner preference during the maintenance of a pair bond. In Chapter 1, I discovered that the lateral PAG is activated in male prairie voles when there is a discrepancy in alcohol intake between partners. The next logical step is to test whether the PAG is causally involved in the effects of discordant drinking on pair bonding. This could be done by inactivation of the lateral PAG using designer receptors exclusively activated by designer drugs (DREADDs). Males would be injected with DREADDs prior to being paired with their partner in the PAG. I have shown that DREADDs can be activated in the prairie vole brain and that prairie voles will consume water with clozapine N-oxide (CNO) voluntarily (data not shown). Therefore, CNO could

be administered in two ways. First, CNO can be diluted in the alcohol drinking fluid. When male prairie voles are consuming alcohol, they will receive CNO at the same time and it would potentially lead to alcohol not affecting partner preference. Second, CNO can be injected I.P. prior to the partner preference test. This way would enhance the expression of partner preference in discordant drinking males that show a decrease in partner preference. These studies would show that the PAG plays a key role in the effects alcohol has on pair bond maintenance and future studies could target the PAG as a region to study for pair bond maintenance.

Further investigation needs to be done to understand why discordant alcohol consumption in males (not tested in females in this dissertation) did not affect aggression frequency using the resident intruder (RI) test. One possibility is that because there was a mesh divider between the male and female partners for one week (to determine individual drinking levels) that it was not a true resident intruder test to measure pair bond maintenance. The experiment took place only on the male's side of the cage. Future studies, using the HM-2 cages, should be done with both male and female partners living in the same cage with full social capability during the experiment and then introducing the intruder to the male partner during the RI test.

The sex-dependent effects of discordant alcohol drinking on pair bond maintenance is important to consider. In humans, wives who are the heavy drinker in a couple is a better predictor of marital separation and divorce compared to when the husband is the heavy drinker. But, in humans it is not known who is the initiator of the divorce. I may not have seen an effect in our female study of pair bond maintenance because the females were the subject animals choosing with whom they wanted to huddle. A future study should explore what would happen if the female prairie vole is the alcohol consumer and the male prairie vole is the abstainer, but the males are the

subject animals. This could help us gain a better understanding of how discordant drinking leads to higher rates of separation in humans.

Based on Chapter 3, the new HM-2 cages can be used to explore the efficacy of other pharmacotherapy treatments for all drugs of abuse. However, first it is necessary to explore the behavioral mechanism that is leading to a decrease in total fluid intake after oxytocin treatment. Based on existing literature I hypothesize that total fluid intake is decreased because oxytocin administation leads to increases in prosocial behaviors (i.e. huddling) (Mooney et al., 2014). One simple way to determine if an increase in huddling is leading to decreases in total fluid intake is by videotaping animals in the HM-2 cages for the first three hours post oxytocin treatment. Videos can be behaviorally analyzed to determine changes in huddling behavior in a cage, due to oxytocin treatment. If animals who receive oxytocin treatment have decreased fluid intake and an increase in huddling behavior, then I can say that a potential “competition” between prosocial behaviors and fluid consumption is occurring and that prosocial behaviors could be more rewarding in the prairie vole model.

Overall this dissertation has explored the interactions between sociality and alcohol use in the prairie vole animal model. It has laid the groundwork for future studies to explore biological mechanisms underlying pair bond maintenance and help develop future approved pharmacotherapies for AUD in humans.

Appendix: Social Transfer of Alcohol Withdrawal-Induced Hyperalgesia in Female Prairie Voles

(This appendix has been reformatted and minimally edited for inclusion in this dissertation from: Walcott, A. T., Smith, M. L., Loftis, J. M., & Ryabinin, A. E. (2018). Social transfer of alcohol withdrawal-induced hyperalgesia in female prairie voles. Social neuroscience, 13(6), 710-717.)

Introduction

Pain is considered to be a “biopsychosocial” phenomenon (Gatchel et al., 2007; Lumley et al., 2011), because the experience of pain is dramatically influenced by social and environmental factors. Moreover, the relationship between pain and the social environment is bidirectional, such that the experience of persistent pain negatively affects not only the patient, but also individuals that are in close contact. For example, it is well documented that spouses of chronic pain patients can report increased pain (Block et al., 1980; Mohamed et al., 1978; Saarijärvi et al., 1990; Shanfield et al., 1979). However, we do not currently have a neurobiological explanation for this type of phenomena.

Pain is influenced by a variety of social and sensory cues, and it is communicated to nearby conspecifics via several sensory modalities. For example, rodents demonstrate heightened responses to painful stimuli (hyperalgesia) following visual observation of a cage mate experiencing pain (Langford et al., 2006; Li et al., 2014). Recent studies also demonstrate that mice experiencing pain induce an indistinguishable state of hypersensitivity in mice housed and tested within the same room, and that this “social transfer” of pain is likely communicated via olfactory cues (Laviola et al., 2017; Smith et al., 2016).

As a social cue, pain is beneficial in providing a warning through multiple sensory modalities to conspecific animals about potential injury. For example, mice that witnessed other mice being attacked by biting flies, displayed self-burying behaviors when exposed to flies that were unable to bite (Kavaliers et al., 2001). In another experiment, non-stressed rats could discriminate between odors produced by stressed and non-stressed rats and avoid the odor produced by the stressed rats (Mackay-Sim and Laing, 1981).

Recently, neuroanatomical experiments demonstrated activation of the anterior cingulate and anterior insula during the social transfer of hyperalgesia (Smith et al., 2017), regions which have been implicated in the experience of pain and empathy in humans (Bernhardt and Singer, 2012; Gu et al., 2012; Singer et al., 2004). The involvement of this neurocircuitry suggests that the social transfer of hyperalgesia may be a phenomenon related to empathy. Furthermore, female mice displayed a stronger effect in the social transfer of pain compared to male mice (Smith et al., 2016), which matches stronger empathy responses observed in females versus males in humans (Christov-Moore et al., 2014; O'brien et al., 2013).

Currently, the social transfer of hyperalgesia has only been demonstrated using inbred mouse strains. It is unknown whether social transfer can be observed in other rodent species. Of particular interest would be the examination of this phenomenon in species with unique social phenotypes like the prairie vole (*Microtus ochrogaster*). Similar to humans, and in contrast to mice, prairie voles develop long-term attachments between adult individuals, i.e., pair bonds (Carter and Getz, 1993). Moreover, neurochemical mechanisms mediating the development of pair bonds in prairie voles also have been shown to play a role in social attachments in humans, demonstrating homologies in mechanisms regulating social behaviors between humans and prairie voles (Aragona et al., 2006; Insel and Hulihan, 1995; Lee et al., 2009; Pitkow et al., 2001; Walum et al., 2008; Wang et al., 1999). Therefore, to test whether social transfer of hyperalgesia can be observed beyond inbred mouse strains, the current studies explored this phenomenon in female prairie voles.

Materials and Methods

Subjects

Adult female prairie voles (n=60) ranging from 74-121 days at the start of the experiment were used from our breeding colony at the VA Portland Health Care System (VAPORHCS) Veterinary Medical Unit. Animals were weaned at 21 days and housed in same-sex sibling groups in cages (27x27x13 cm), with females and males housed in different rooms. All subjects had access to cotton nestlets and *ad libitum* access to water and a diet of mixed rabbit chow (LabDiet Hi-Fiber Rabbit; PMI Nutrition International, Richmond, IN), corn (Nutrena Cleaned Grains; Cargill, Inc., Minneapolis, MN), and oats (Grainland Select Grains; Grainland Cooperative, Eureka, IL) throughout the duration of the experiment. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the VAPORHCS.

Housing Conditions

Voles were housed in a social housing cage (27x27x13 cm) with a wire mesh divider down the center of the cage, which kept each of the paired voles in one half of the cage (Fig. 1). These cages allow the monitoring of individual fluid consumption, while simultaneously allowing for olfactory, visual, and auditory contact between animals. Previous studies indicated that these cages do not strongly affect social or drinking behaviors (Anacker et al., 2011b, Curtis, 2010; Chapters 1 and 2).

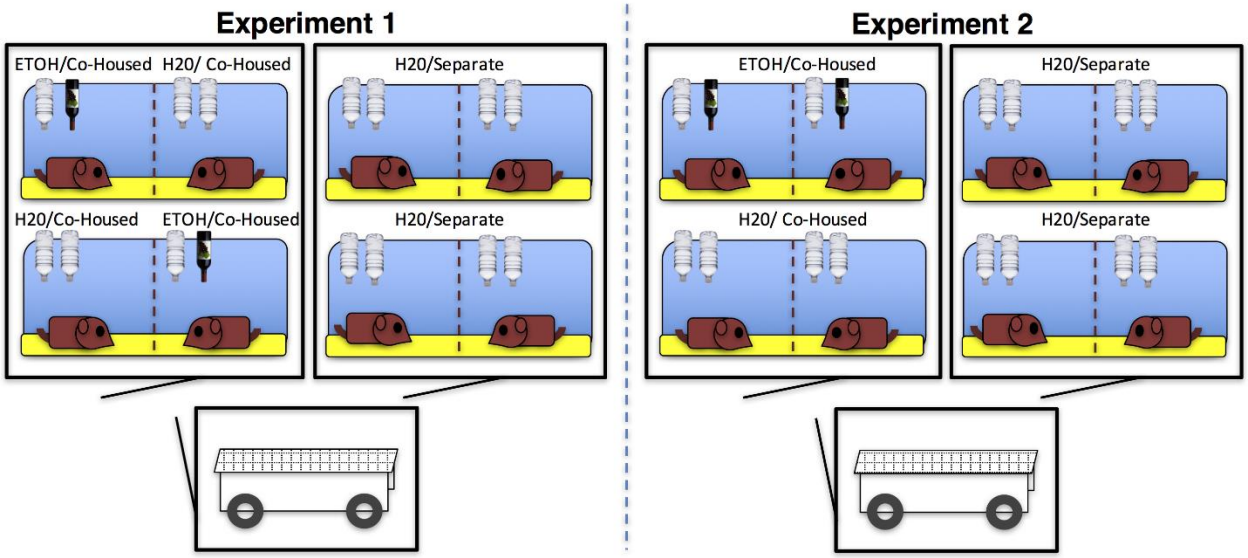


Figure 1: Schematic representation of the two experiments. In both experiments animals were socially-housed, separated by a mesh divider. In each experiment, one room contained both Co-Housed groups of voles and another room contained the H20/Separate group. Mechanical sensitivity using von Frey fibers was performed in a different room (illustrated by a room with a rack below). In Experiment 1 (left), EtOH/Co-Housed and H2O/Co-Housed animals were housed in the same cage. In Experiment 2 (right), EtOH/Co-Housed and H2O/Co-Housed animals were housed in separate cages.

Two-Bottle Alcohol Drinking and Withdrawal

The protocol for alcohol drinking and withdrawal was modeled after previous studies in mice (Smith et al., 2016; Smith et al., 2017), with the exception that all voles were socially housed as described above, rather than isolated (as in the mouse studies). Voles on each side of the mesh were given continuous access to two 25 mL glass tubes with metal sippers attached to rubber stoppers. Animals that received access to ethanol had one bottle containing tap water and one bottle containing increasing concentrations of unsweetened ethanol (3-10% v/v) dissolved in tap water. Fluid levels were measured every 24 hours and the locations of the bottles were switched every day to prevent side bias. Voles were given continuous access to two bottles for two weeks. Once a week, the ethanol bottles were removed and replaced with bottles containing water. During the first week (Monday – Friday) of drinking all voles received 3% ethanol for 2 days, 6% ethanol for 2 days, and 10% ethanol for 1 day followed by a 24-hour withdrawal period (WD1). In the second week (Monday – Saturday), all voles received 10% ethanol for 6 days followed by a second 24-hour withdrawal period (WD2). Average alcohol preference over water for each vole was calculated by dividing the total volume of alcohol consumed by the total volume of fluid consumed. Additionally, the average daily alcohol consumption was calculated 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 each vole in kilograms (g/kg).

Mechanical Sensitivity

Mechanical sensitivity was measured using the von Frey up-down technique 24 hours after alcohol access was removed from cages (Chaplan et al., 1994). We focused on mechanical sensitivity as a test of nociception because our previous study in mice demonstrated similar social transfer when we measured either mechanical sensitivity,

thermal sensitivity, or nocifensive behaviors to a chemical irritant (Smith et al., 2016). Responses were elicited by mechanical stimulation by von Frey hairs (0.04 to 6.0g of plastic fibers) to the plantar surface of left hindpaw. Hindpaw withdrawal, shaking, or licking from the fiber stimulation was considered a response. This method uses stimulus oscillation around the response threshold to determine the median 50% threshold of the response. As previously described in mice (Smith et al., 2016), voles were allowed to acclimate to a Plexiglas box located on top of a wire mesh testing rack for 2 days for 40 minutes prior to the start of the experiment. Prior to experimental treatment, basal mechanical thresholds were measured (baseline) and animals were assigned to treatment groups in a counterbalanced manner. Testing occurred 24-hours after the start of each withdrawal session. Before the start of each mechanical test session, voles were put into the Plexiglas box for 10-20 minutes to acclimate. All testing sessions occurred during the light cycle, but testing occurred in a room only lit by a dim red lamp. Testing occurred in a separate room from the housing room. In our previous experiments in mice, when the animals were tested in the same room in which they were housed, the experimenter performing the von Frey test was blind to the conditions of the group (Smith et al., 2016). Due to our decision to perform the test in a separate room from housing rooms in order to avoid potential visual mimicry, the current study was run in a semi-blinded fashion. During both experiments, the investigator was aware whether animals came from the Co-Housed or Separate room. The investigator was aware whether the animals belonged to the EtOH or H20 Co-Housed group in Experiment 2, but not in Experiment 1.

Statistical Analysis

The dependence variable is mechanical sensitivity. When the mechanical threshold decreases, then the mechanical sensitivity is increased and vice versa.

Mechanical sensitivity was analyzed by repeated-measures ANOVA design using group (EtOH/Co-housed, H_2O/Co-housed, and H_2O/Separate) as the between-subjects factor and mechanical threshold test (week/WD session) as the repeated measure. Significant outcomes were followed by a Tukey's *post hoc* test. Significance threshold was set at $p < 0.05$ for all analyses. All statistical analyses were performed using GraphPad Software Prism 6. The number of animals in each group was determined by previous mouse studies (Smith et al., 2016; Smith et al., 2017). One animal was removed from all analyses due to incomplete mechanical threshold measurements during the baseline test.

Results

The social transfer of alcohol-withdrawal induced hypersensitivity within the same cage

To determine whether alcohol-withdrawal would affect sibling prairie voles within the same cage, we examined three different groups of female prairie voles. As described above, females in the *EtOH/Co-housed* group (n=10) were exposed to increasing concentrations of ethanol (3-10%) for two weeks, followed by a 24-hour withdrawal period. Females in the *H_2O/Co-housed* group (n=9) were housed in the same cage as the females in the EtOH/Co-housed group, but were separated by a mesh divider. H_2O/Co-housed females were given access to only water for two weeks and mechanical threshold was measured weekly. Lastly, *H_2O/Separate* females (n=10) were socially housed in a separate room in mesh divided cages with another H_2O/Separate female and received access to only water. All groups were tested weekly for mechanical sensitivity on the same day, in a separate room from their housing cages 24 hours after the start of the withdrawal period (i.e. removal of alcohol access from cage).

EtOH/Co-housed females voluntarily consumed 5.4 ± 0.5 g/kg (Fig. 2A) and had a 48 ± 4.1% preference (Fig. 2B) for alcohol over the two-week period. A repeated-measures ANOVA comparing mechanical sensitivity over time revealed a significant main effect of week ($F_{2,52} = 28.01$, $p < 0.0001$) and treatment ($F_{2,26} = 5.60$, $p = 0.01$). There was no significant interaction between week and treatment ($F_{4,52} = 1.36$, $p = 0.26$; Fig. 2C). To test our *a priori* hypothesis if there were treatment differences over time, we included the *post-hoc* results showing that females in the EtOH/Co-housed and H_2O/Co-housed group showed a significant increase in mechanical sensitivity from baseline after WD1 and WD2. Meanwhile females in the H_2O/Separate group showed no significant difference in mechanical sensitivity after WD1 and WD2 relative to baseline. These findings confirm that 24-hour withdrawal from alcohol leads to mechanical hypersensitivity in female prairie voles that is socially transferred to female siblings housed in the same cage.

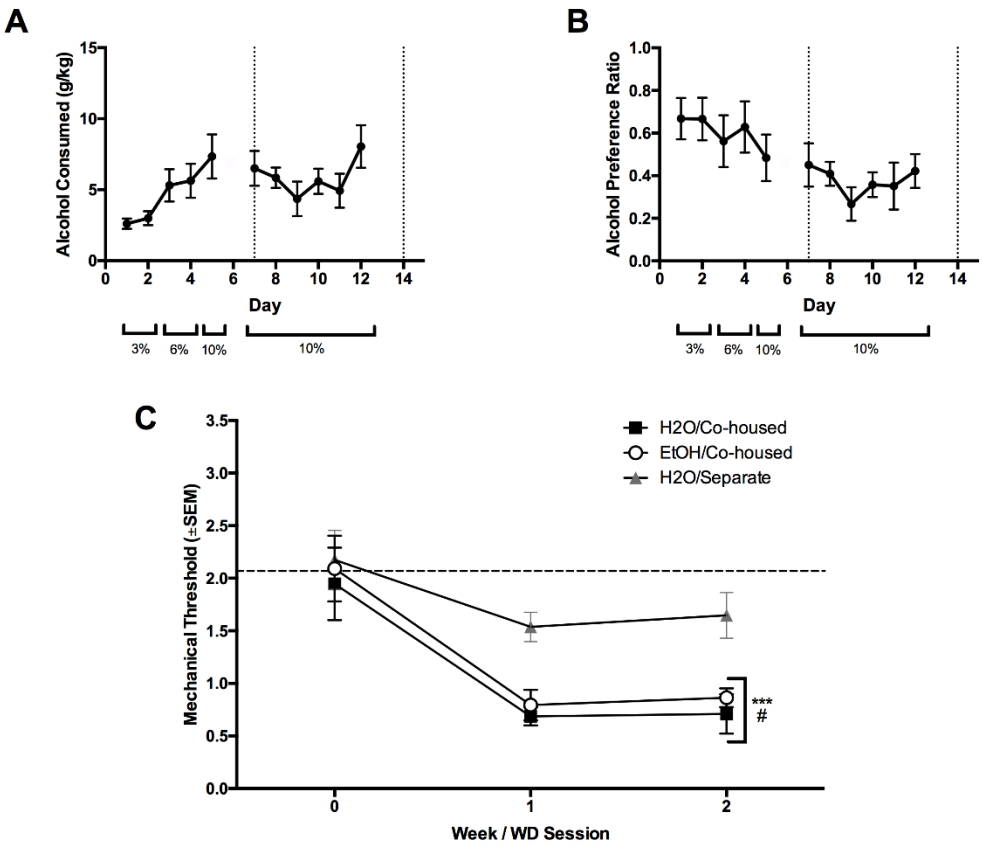


Figure 2: Alcohol withdrawal's effect on mechanical threshold within same cages. Females in the EtOH/Co-housed group were exposed to increasing alcohol concentrations (3-10%) for two weeks, but showed no difference in (A) alcohol consumption or (B) alcohol preference between the day before and after alcohol withdrawal. (C) EtOH/Co-housed (n=10) and H_2O/Co-housed (n=9) females showed a significant decrease in mechanical threshold by the second withdrawal session when compared to females in the H_2O/Separate (n=10) group and baseline. Significant differences compared to the H_2O/Separate group ($p < 0.001$) are represented by ***. Significant changes from baseline ($p < 0.05$) are represented by #. Error bars indicated mean ± SEM. Mechanical threshold testing is represented by a dotted line (A, B). Mean basal responses of all groups are represented by a dashed line (C).

The social transfer of alcohol-withdrawal induced hypersensitivity between different cages

We next examined whether social transfer occurs between non-sibling animals housed in separate cages within the same room. Therefore, with a different set of female voles we conducted the same social transfer paradigm as above, with the exceptions that: (1) animals in this experiment received the same treatment as their cage mate, and (2) animals in the three treatment groups were tested separately, to eliminate visual mimicry between voles in different treatment groups.

Females in the EtOH/Co-housed group self-administered on average 7.3 ± 0.7 g/kg of alcohol (Fig. 3A) and showed an average 60 ± 3.0% preference (Fig. 3B) for alcohol over the two-week period. Analysis of the mechanical threshold after each withdrawal revealed a mean effect of week ($F_{2,54}$ = 12.74, p < 0.0001), treatment ($F_{2,27}$ = 19.44, p < 0.0001), and a significant interaction between treatment and week ($F_{4,54}$ = 4.58, p = 0.003; Fig. 3C). A Tukey's *post hoc* analysis revealed that females in both EtOH/Co-housed (n=10) and H_2O/Co-housed (n=10) groups showed a significant decrease in mechanical threshold after WD1 and WD2. Meanwhile, females in the H_2O/Separate (n=10) group showed no hypersensitivity at any point in time. These data further indicate that alcohol-withdrawal induced hypersensitivity is socially transferred not only within the same cage, but also between female prairie voles house in different cages within the same room.

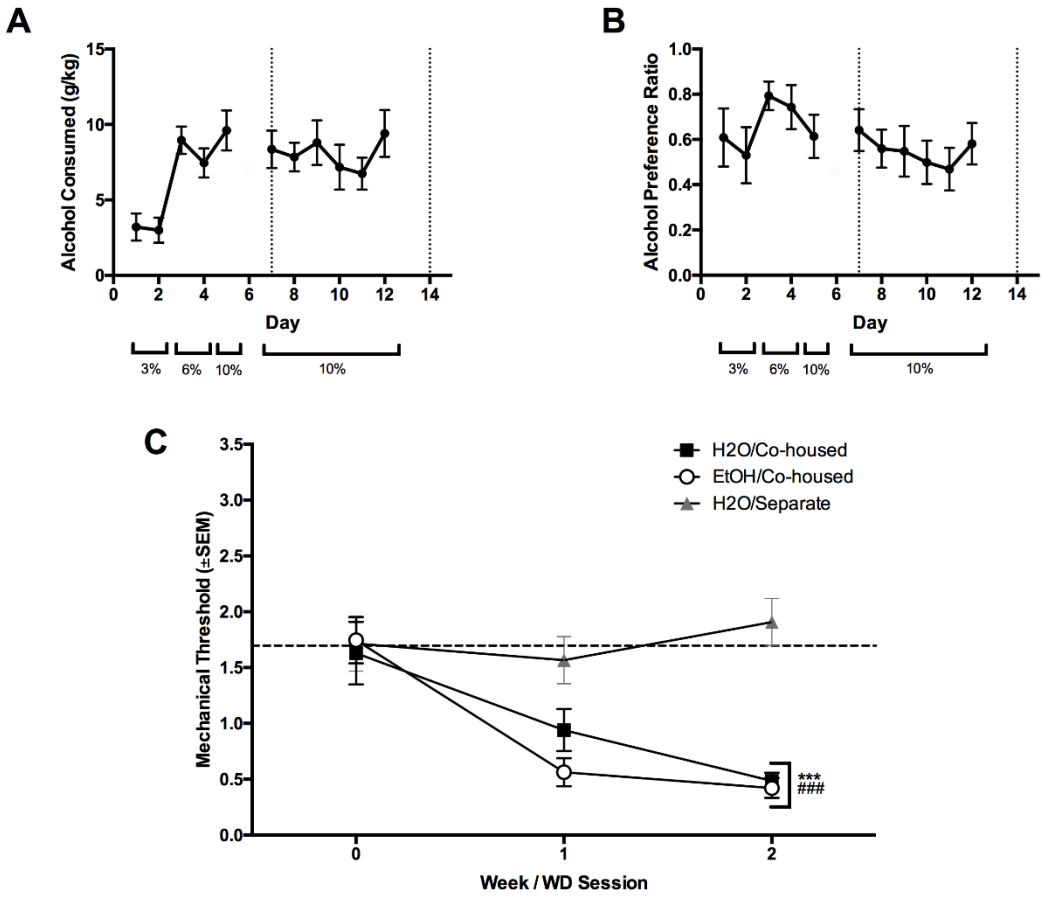


Figure 3: Alcohol withdrawal's effect on mechanical threshold across cages.

(A) Alcohol consumption and (B) alcohol preference ratio did not significantly differ between the day prior and the day after ethanol withdrawal. (C) Female prairie voles in the EtOH/Co-housed (n=10) and H_2O/Co-housed (n=10) groups showed a decrease in mechanical threshold from the H_2O/Separate (n=10) group and baseline after two withdrawal sessions. Significant differences compared to the H_2O/Separate group ($p < 0.001$) are represented by ***. Significant changes from baseline ($p < 0.001$) are represented by ###. Error bars indicated mean ± SEM. Mechanical threshold testing is represented by a dotted line (A, B). Mean basal responses of all groups are represented by a dashed line (C).

Discussion

The present study demonstrated female prairie voles show mechanical hypersensitivity after an acute 24-hour withdrawal from several days of voluntary alcohol consumption. This withdrawal induced mechanical hypersensitivity is socially transferred to conspecifics. Females that were cohoused in the same cage displayed a level of hyperalgesia that roughly matched females that were experiencing the acute withdrawal. This display of hypersensitivity in females that were exposed to just water not only occurred when their cage mate was experiencing withdrawal, but it also occurred when other animals in different cages - within the same room - experienced alcohol withdrawal. Previous studies have shown that naïve mice acquire socially transferred hyperalgesia when housed in separate cages within the same room as conspecifics experiencing direct hyperalgesia (Smith et al., 2016; Smith et al., 2017). The present study is the first to show that socially transferred and alcohol-withdrawal induced hyperalgesia occurs in another species besides the traditional laboratory mouse models.

Until the current study, alcohol withdrawal has not been behaviorally demonstrated in prairie voles. The traditional way to measure alcohol withdrawal in mouse models is by measuring handling-induced convulsions or HICs (Crabbe et al., 1980; Crabbe et al., 1991). HIC severity is normally rated on an ordinal scale. For example, 0 to 7, where 7 represents a convulsion occurring spontaneously or elicited by a mild environmental stimulus and 0 represents no convulsion. HIC onset normally require mice to be initially suspended in the air by their tails and sometimes requite an additional rotation before a spontaneous convulsion occurs. Running this behavioral test in prairie voles is not feasible because the tail of a prairie vole is relatively short and extremely fragile. The von Frey mechanical threshold test provides an alternative and sensitive method to evaluate withdrawal- and socially-transferred hyperalgesia. The

decreased mechanical threshold in prairie voles undergoing withdrawal provides additional evidence for the relationship between pain and alcohol withdrawal.

Importantly, our findings indicate that the social transfer of hyperalgesia initially observed in inbred mice, is also not specific to just this rodent species (Smith et al., 2016; Smith et al., 2017). In evolutionary terms, voles belong not only to a different genus, but a different family of rodents from mice and rats. Moreover, since prairie voles are genetically heterogeneous, this is the first demonstration that social transfer of hypersensitivity occurs between individuals of different genotypes. The present study also differs from the previous studies in that here the animals were housed in social conditions. Previous studies on social transfer of withdrawal-induced hypersensitivity used socially isolated mice housed in the same room. There is increased evidence that rodents can display empathy-like consolation behavior (Burkett et al., 2016; Rice and Gainer, 1962). Therefore, it was possible social housing would prevent or mask social transfer of hyperalgesia. Contrary to this possibility, we observed social transfer of hypersensitivity indicating consistency of this phenomenon across housing conditions. This finding is in agreement with the demonstration that olfactory cues are sufficient for transfer of hyperalgesia in mice (Smith et al., 2016).

The present study also differed from experiments in mice in the context in which the testing occurred. Thus, in the previous study, testing occurred in the same room in which the mice were housed, allowing for the possibility that the mice influenced each other's behavior during testing (for example, through visual mimicry (Langford et al., 2006). In contrast, in the current experiments, testing occurred in a room that was separate from the housing room, removing the voles from any cues that were specific to the housing room. While previous studies in mice included experiments that suggested that visual mimicry does not play a role in social transfer of hyperalgesia (Laviola et al., 2017; Smith et al., 2016), the current experiments definitively eliminate this possibility.

Since prairie voles were not able to observe each other during testing, hyperalgesia in the co-housed animals can only be explained by transfer of a hyperalgesic state, and not by visual mimicry.

Alcohol withdrawal can be accompanied by increased anxiety and disruption of sleep cycles in rodents and humans (Brager et al., 2010; Landolt and Gillin, 2001; Rassnick et al., 1993). It could be theorized that the increase in mechanical sensitivity in the H_2O/Co-housed group was in part due to changes in anxiety or sleep cycles in these animals. Disruption of sleep increases corticosterone (CORT) levels and affects anxiety measures in rodents (Legates et al., 2012; Silva et al., 2004). Our previous studies in mice have not found elevated plasma CORT levels, and no changes behavior in elevated plus maze and acoustic startle tests following identical procedures (Smith et al., 2016). Therefore, we reason that the social transfer observed between rodents is more specific to nociception, rather than explained by general anxiety, arousal or sleep disruption. Interestingly, Smith et al. (2016) have also shown that nociception can be socially transferred after inflammatory type of pain induced by complete Freund's adjuvant, indicating that the transfer in not limited to chronic withdrawal-induced hyperalgesia. It seems likely that similar mechanisms of social transfer are engaged in voles and in mice, but the exact nature of the transfer of hyperalgesia need to be addressed in future studies.

Even though multiple treatments have been developed and used in preclinical models of pain, many treatment options have not been effective in clinical research. This translational problem could be due to the lack of the "social" aspect in the models of "biopsychosocial" phenomenon of pain. The prairie vole is one animal model that can provide an advantage over traditional laboratory rodents. For example, prairie voles show consolation behavior towards a conspecific that experienced a stressful situation. Interestingly, when meadow voles (a non-social species of voles) were tested they

showed no consolation behavior and social buffering towards a stressed conspecific (Burkett et al., 2016; Smith and Wang, 2014). Prairie vole and human social relationships show similarity and are controlled by homologous biologically mechanisms. Therefore, our future studies will investigate the biological mechanisms that play a role in the social transfer of hyperalgesia in prairie voles. Nevertheless, the current findings support that the use of prairie voles in pain research will help close the current translational gap.

As a cautionary note, our studies used a semi-blinded procedure because it was impossible to conceal the identity of housing arrangements of the Co-housed groups versus the Separate group. Indeed, early studies have noted that different raters using manual von Frey testing can deviate in their assessment of hyperalgesia (Chaplan et al., 1994). That said, three experimenters working independently in this laboratory have repeatedly observed the social transfer of hyperalgesia in mice or prairie voles. As an alternative interpretation, two potential scenarios can be theorized: an experimenter would not notice a deviation from threshold baseline due to repeated testing in the H_20/Separate group or would notice a non-existent decrease in threshold in the H_20/Co-Housed group. Our analysis of the limited hyperalgesia literature that mentions the terms “baseline” and “von Frey” in the publication abstracts identifies no deviation from baseline due to repeated testing across multiple mouse strains and in rats (Banik et al., 2006; Macolino et al., 2014; Young et al., 2016). Interestingly, studies in mice indicating the use of non-bias procedures show a decrease from baseline in control groups equivalent to our H_20/Co-Housed group in mice (Macolino et al., 2014; Marquez De Prado et al., 2009). It is possible, however, that such deviation would be more difficult to detect in larger animals, such as rats (Nirogi et al., 2012). Therefore, we believe that our results were not influenced by our semi-blinded testing procedure. Nevertheless, future

studies comparing Co-Housed and Separated animals will need to be specifically designed to avoid any potential testing bias.

Taken together, our studies expand existing examples of empathy-like behaviors in rodents. They indicate that such behaviors can be relatively resistant to influence by social context. In addition, they further suggest that laboratory rodents housed in separate cages within the same room can influence each other's physiological states, and thereby prevent detecting differences between experimental groups and controls. This possibility needs to be taken into account when designing future animal experiments.

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