The Lmo3 Gene: A Regulator Of Anxiety And Behavioral Responses To Alcohol

BY

ANTONIA M. SAVARESE

B.S., Loyola University Chicago, 2008

THESIS

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Defense Committee:
Amy Lasek, Advisor
Mark Brodie, Chair
Subhash Pandey
Amynah Pradhan
Jamie Roitman, Psychology

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CONTRIBUTION OF AUTHORS

Portions of the published work "Increased behavioral responses to ethanol in *Lmo3* knockout mice" (*Genes, Brain and Behavior*, 2014) have been included in this dissertation. The two experiments/figures that have been included in this dissertation are (1) the beta-galactosidase staining of *Lmo3* heterozygous mice and (2) the drinking-in-the-dark experiment in the $Lmo3^{Z}$ mice and their wild type littermates. I conducted both of these experiments in their entirety, under the guidance of my advisor, Amy Lasek, the senior author of the paper. Dr. Kharazia, a contributing author of the published paper, had previously conducted the beta-galactosidase staining and contributed to the development of that protocol. The other contributing authors of the published paper (Drs. Zou and Maiya) were responsible for conducting additional experiments in the published paper that have not been included in this dissertation work (Figures 2 and 4 of that paper).

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LIST OF ABBREVIATIONS

AD	Anxiety disorder
AUD	Alcohol use disorder
BLA	Basolateral amygdala
BNST	Bed nucleus of the stria terminalis
CeA	Central nucleus of the amygdala
CeL	Lateral portion of the central nucleus of the amygdala
CeM	Medial portion of the central nucleus of the amygdala
Crh	Corticotropin releasing factor (hormone) gene
CRF	Corticotropin releasing factor protein
Crhr1	Corticotropin releasing factor (hormone) receptor 1 gene
GABA	Gamma-aminobutyric acid
Gabra1	$GABA_A$ receptor subunit alpha1 gene
Gabra4	$GABA_A$ receptor subunit alpha4 gene
Gabrd	$GABA_A$ receptor subunit delta gene
LMO1	Lim-domain-only 1 protein
Lmo3	Lim-domain-only 3 gene
LMO3	Lim-domain-only 3 protein
Lmo4	Lim-domain-only 4 gene
LMO4	Lim-domain-only 4 protein
NAc	Nucleus accumbens
PFC	Prefrontal cortex
SSRIs	Serotonin selective reuptake inhibitors

SUMMARY

Anxiety disorders and alcohol use disorders share common neural circuitry and molecular mechanisms that may underlie a shared pathology, including alterations in CRF signaling in the extended amygdala. Because both disorders are highly heritable, finding target genes that may be implicated in alcohol-related and anxiety-like behaviors is critical toward targeting novel and effective treatments. Genes that regulate CRF signaling in the extended amygdala may be especially promising, given the alterations in this system in both anxiety and alcohol use.

LMO proteins are transcriptional regulators that can also function in the cytosol to regulate protein activity levels. Recent work has implicated several LMO proteins in behaviors involved in both alcohol/substance abuse and anxiety. LMO3 is involved in central nervous system development and is highly expressed throughout the brain in adulthood, making it an excellent candidate for regulation of neural signaling and behavior. Indeed, recent work in our lab has supported a role for *Lmo3* in the regulation of several alcohol-related phenotypes, including sensitivity to the sedative effects of alcohol and low-to-moderate alcohol consumption.

In this dissertation, I sought to characterize the role of *Lmo3* in regulation of anxiety-like behavior and high-risk binge-like alcohol consumption. Utilizing the *Lmo3* null ($Lmo3^Z$) mouse and its wild type littermates, *Lmo3* was found to promote anxiety-like behavior and inhibit excessive alcohol consumption. In an effort to target a brain region in which *Lmo3* could be acting to produce these behaviors, viral-mediated RNA interference was utilized to knockdown *Lmo3* expression in brain regions in two areas of the amygdala and in the nucleus accumbens (NAc). Knockdown of *Lmo3* in the basolateral amygdala (BLA) replicated the anxiolytic phenotype observed in $Lmo3^Z$ mice, but *Lmo3* knockdown in the neighboring central nucleus of the amygdala (CeA) did not, suggesting that *Lmo3* regulates anxiety-like behavior via its actions in the BLA. Targeted knockdown of *Lmo3* in the BLA and the NAc did not replicate the binge drinking phenotype observed in $Lmo3^Z$ mice. The mechanism driving elevated binge drinking in these mice is yet unknown. In an effort to identify potential downstream targets of *Lmo3*, brain tissue from $Lmo3^Z$ and wild type mice was collected to measure differences in mRNA and protein expression of relevant genes: *Crh*, *Crhr1*, *Gabra1*, *Gabra4*, and *Gabrd*. $Lmo3^Z$ mice had a reduction of *Crhr1* mRNA and CRF1R protein expression in the BLA. Additionally, $Lmo3^Z$ mice showed a reduction of *Crhr1* mRNA in the CeA, increased *Gabra4* mRNA in the BLA, and a sex-specific increase in *Gabrd* mRNA in the BLA of only females. No corresponding changes in protein expression were observed for these genes. In addition to basal anxiety and binge drinking, $Lmo3^Z$ mice showed sex-specific alterations in ethanol conditioned place preference (CPP, a measure of alcohol reward), cocaine CPP, and ethanol-induced anxiolysis. Female $Lmo3^Z$ mice failed to develop ethanol CPP or ethanol-induced anxiolysis, though they showed an enhanced response to the rewarding effects of cocaine.

Taken together, these results suggest a novel role for *Lmo3* in the regulation of both anxiety and alcohol abuse and suggest that *Lmo3* may be a good candidate gene to further understand the neural mechanisms driving these pathologies.

1. INTRODUCTION

1.1 Comorbidity of Anxiety and Alcohol Use Disorders

The comorbidity of anxiety disorders (AD) with alcohol use disorders (AUD) has been recognized and extensively studied for decades. The Epidemiological Catchment Area survey of 1990 reported a 50% increased risk of an AUD diagnosis in individuals with an AD (Regier, Narrow, & Rae, 1990). Additionally, according to the International Consortium in Psychiatric Epidemiology, nearly 45% of individuals with alcohol dependence in the United States met lifetime criteria for an AD (Merikangas et al., 1998). Although this last value varies with the country of origin (anywhere from 27-40%), the association between AD and AUD is still a significant and robust global phenomenon. Importantly, the rate of comorbidity of AUD and AD exceeds that which would be expected by chance, suggesting that the two disorders have either a shared genetic/neurobiological/environmental vulnerability, or the incidence of one drives the occurrence of the other (Kushner, Sher, & Beitman, 1990). Although the co-occurrence of AUD and AD has long been acknowledged, and the neurobiology underlying this comorbidity is well studied, there still remains very little consensus as to its etiology and, consequently, very few effective treatment options exist (Ipser, Wilson, Akindipe, Sager, & Stein, 2015).

1.1.1 Anxiety disorders

A certain amount of anxiety is normal, and indeed, adaptive. The fifth edition of the Diagnostic Statistical Manual of Mental Disorders (DSM-V) defines anxiety as the anticipation of future threat. Because anticipation of potential threats allows for their avoidance, anxiety is therefore a form of learning that is crucial to the survival of the human species. However, anxiety becomes maladaptive when an individual perceives threats where there are none, or experiences an exaggerated perception of the threat, or alternatively spends a disproportionate amount of time

worrying about future threats. Anxiety disorders, therefore, are characterized by excessive worrying and persistent avoidant behavior that interferes with daily functioning, and is out of proportion to the danger of the perceived threat (Craske et al., 2009; Craske & Stein, 2016).

Unfortunately, the percentage of the population living with an AD is inordinately high in the United States. Nearly a third of individuals in the United States will experience an AD at some point in their lifetime, and this number is even higher in individuals ranging in age from 30-44 years (Kessler et al., 2005). Globally, the prevalence of AD is lower than in the United States, but still significant, with roughly one in nine people in the world meeting diagnostic criteria in a given year (Baxter, Scott, Vos, & Whiteford, 2013).

Given the high rate of occurrence, it is perhaps not surprising that AD imposes a significant burden on society. According to the World Health Organization Global Burden of Disease Study, over 7% of global disability-adjusted life years (DALYs, or years lost to disability) are caused by mental and behavioral disorders, with AD carrying the second heaviest burden in this category behind mood disorders (alone accounting for over 1% of global DALYs) (Murray et al., 2012). The economic burden of AD in the United States is difficult to accurately determine, but conservative estimates project an annual cost of $42-47 billion (DuPont et al., 1996; Greenberg et al., 1999).

Anxiety disorders very rarely exist in isolation – they are highly comorbid with both physical disorders/diseases (Bystritsky, Danial, & Kronemyer, 2014; Fond et al., 2014; Munger Clary, 2014; Panagioti, Scott, Blakemore, & Coventry, 2014) and other psychiatric disorders (Braga, Reynolds, & Siris, 2013; Friborg, Martinussen, Kaiser, Overgard, & Rosenvinge, 2013; Pasche, 2012). Indeed, several studies report comorbidity rates that exceed 90% of cases

(Kaufman & Charney, 2000), and multiple medical comorbidities is, in fact, an important risk factor for generalized anxiety disorder (Moreno-Peral et al., 2014).

If left untreated, anxiety disorders are often chronic (Craske & Stein, 2016). Although there is a range of pharmacological agents that have been shown to be efficacious in the treatment of AD, comorbidities may limit treatment options. While benzodiazepines exhibit excellent anxiolytic properties, their addictive potential (Tan et al., 2010) and lack of antidepressant effects often places them behind SSRIs as primary treatment agents. Additionally, anxiolytic agents are not one-size-fits-all, with treatment options tending to be selective for anxiety disorder type. Panic disorder, for instance, has not been shown to be responsive to either benzodiazepines (N. Watanabe, Churchill, & Furukawa, 2009) or azapirones (Imai et al., 2014) above that of a placebo effect. More importantly, the existence of certain comorbid psychiatric conditions can often exacerbate anxiety symptoms and is a critical consideration when choosing the type and timing of treatment. This is especially true in the case of alcohol use disorder.

1.1.2 Alcohol use disorder

Aside from its high rate of comorbidity with anxiety disorders, alcohol use has a significant impact on society. In the United States alone, excessive alcohol consumption accounts for approximately 88,000 deaths each year, with each death averaging about 28 years of potential life lost (Stahre, Roeber, Kanny, Brewer, & Zhang, 2014). Globally, alcohol consumption was responsible for 3.3 million deaths in 2012, or nearly 6% of deaths that year, according to the World Health Organization's 2014 Global Status Report on Alcohol and Health. In addition to the cost in human lives, the estimated economic cost of excessive alcohol consumption in the U.S. in 2006 was over $200 billion, 42.1% of which was borne by federal, state, and local governments (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011). Long-term

heavy alcohol consumption often leads to the development of an alcohol use disorder (AUD). A diagnosis of AUD requires the presence of at least two of a list of eleven criteria (i.e., "alcohol is often taken in larger amounts or over a longer period of time than was intended", "craving, or a strong desire or urge to use alcohol", etc.). The severity of the AUD is then defined by how many criteria an individual meets (mild: 2-3 symptoms, moderate: 4-5 symptoms, severe: 6 or more symptoms) (American Psychiatric Association. & American Psychiatric Association. DSM-5 Task Force.).

The harmful effects of alcohol are not due purely to the amount of alcohol consumed; rather, the pattern of drinking can serve as an indicator of an individual's risk for developing an AUD. For instance, an individual consuming eight alcoholic beverages in a month can either fall into a low-risk category (if those drinks were consumed with meals twice a week across the month) or high-risk category (if those drinks were consumed in a single setting, an occurrence termed "heavy episodic drinking"). Additionally, binge drinking, defined as a pattern of drinking that brings a person's blood alcohol concentration above 0.08 grams percent, is associated with an increased risk of experiencing alcohol-related problems and of developing an AUD (Jennison, 2004). On its own, binge drinking can be a liability – it accounted for $170 billion, or 76% of the total economic cost of alcohol consumption, in 2006 (Bouchery et al., 2011). And yet, this pattern of drinking is prevalent. In 2011, 18.4% of adults in the United States had engaged in binge drinking, with a significantly higher rate among those aged 18-24 years (30.0%) and 25-34 years (29.7%) (Kanny et al., 2013). This pattern of drinking has short- and long-term health consequences – it has been linked to increased risk of hypertension (Fan, Russell, Stranges, Dorn, & Trevisan, 2008) and type 2 diabetes (Pietraszek, Gregersen, & Hermansen, 2010) – and has

been associated with an increased proclivity to engage in risky behaviors (Naimi et al., 2003). What's more, binge drinking is an integral part of the addiction cycle, and serves as the transition point for impulsive drinking to become compulsive drinking and dependence (Koob & Volkow, 2010).

Unfortunately, while AUDs have been shown to have significant detrimental impacts on society, few viable chronic treatments are available for afflicted individuals (Bouza, Angeles, Munoz, & Amate, 2004). The most common chronic treatment options include counseling, cognitive behavioral intervention, and pharmaceutical intervention. Disulfiram was the first pharmaceutical treatment for alcoholism to be discovered over 75 years ago, but its effectiveness is heavily debated (Ellis & Dronsfield, 2013). Due to its side effects profile (Berlin, 1989) and the potential need for supervised treatment in order for it to be effective, disulfiram use has recently declined while newer treatments like naltrexone and acamprosate, which target the reinforcing effects of alcohol, are more readily prescribed. Yet even these newer treatments have limited efficacy. In a large randomized clinical trial, naltrexone significantly decreased the likelihood of heavy drinking and increased the number of abstinent days, but these effects only lasted the duration of the treatment and failed to induce any long-term benefits. In the same study, cognitive behavioral intervention (CBI) and acamprosate showed no advantage over placebo during or after treatment (Anton et al., 2006).

The inefficacy of current treatments for AUD highlights how little the biology of this complex disorder is understood. The need to better characterize alcohol dependence becomes even more important when considering the prevalence of alcohol use – over half of adults in the

United States identify as regular drinkers, with an additional 14% identifying as infrequent drinkers (National Center for Health Statistics, 2011).

1.1.3 Treatment of comorbid anxiety disorder and alcohol use disorder

The lack of available and effective treatments for AUD is especially problematic within the context of comorbidity with AD, because co-occurrence of these disorders is associated with more severe symptoms and poorer response to treatment. In a prospective study of alcohol outpatient treatment, individuals with comorbid anxiety disorders showed greater functional impairment and consumed more alcohol at baseline than those individuals with only AUD, and this effect persisted even after treatment for AUD (Burns, Teesson, & O'Neill, 2005). Additionally, AUD can worsen the course of anxiety disorders – individuals with alcohol dependence were more likely to continue to meet AD criteria at a 2-year follow-up than those individuals without comorbid alcohol dependence (Boschloo et al., 2012).

Despite the significant public health burden of comorbid AD and AUD, and the severity with which they present together, treatment options are bleak. Clinical trials for AD or AUD treatment often exclude for comorbid psychiatric disorders, and very few clinical trials exist that specifically examine this comorbidity. One small clinical trial found that patients with social anxiety disorder and an AUD that were treated with paroxetine (an SSRI) reported a reduction in anxiety symptoms and a reduction in the desire to self-medicate in social settings with alcohol, but no significant difference was observed in actual drinking behavior (Thomas, Randall, Book, & Randall, 2008). Further, a recent review of clinical trials treating anxiety disorder with comorbid AUD found no beneficial effect of pharmacotherapies on alcohol outcomes, even when anxiety symptoms were shown to improve (Ipser et al., 2015). This highlights the complex nature of this comorbidity – while anxiety may precede (Merikangas et al., 1998) and exacerbate

(Burns et al., 2005) AUD, decreasing anxiety responses alone does not alter problem drinking behavior. Importantly, comorbid AD increases relapse drinking after treatment for AUD, an effect that persists even with concomitant treatment for anxiety (Vorspan, Mehtelli, Dupuy, Bloch, & Lepine, 2015). This suggests that treating both AD and AUD separately does not have the therapeutic impact that one would hope for, and underlines the critical need for better understanding the shared pathophysiology of these disorders in order to identify common targets for more effective treatments.

1.2 Etiology of Anxiety Disorder and Alcohol Use Disorder

Although these two disorders have been extensively studied, there is very little consensus as to why AD and AUD tend to co-occur at such a high frequency. One issue complicating the matter is the variability in characteristics exhibited by individuals with comorbid AD and AUD, which seems to be partially dependent on which is the primary and which is the secondary diagnosis. The Netherlands Study of Depression and Anxiety (NESDA) found that individuals with primary alcohol dependence tended to be male and extroverted, while secondary alcohol dependence was associated with neuroticism, loneliness, and being single (Boschloo et al., 2011). Clearly these two groups, while both exhibiting comorbid AD and AUD, have distinguishing characteristics that will impact treatment.

Studies examining whether AD or AUD precedes the other in comorbid cases have produced mixed results, though. In one group, the onset of AD preceded the occurrence of alcohol problems and dependence, suggesting that anxiety may actually drive alcohol-related problems (Merikangas et al., 1998). Importantly, however, AD did not precede alcohol use, so while it is possible that AD increases the risk for AUD, it is equally possible that non-dependent alcohol use increases the risk for AD. Further, AD was strongly associated with alcohol

problems and dependence, but did not associate with alcohol use, suggesting a critical period in the shift between recreational use and dependence in the intercept of anxiety and alcohol. Existing hypotheses on the etiology of comorbid AD and AUD therefore vary across three central themes (reviewed extensively by Kushner et al, 2000): (1) that anxiety drives drinking, (2) that the neurobiological adaptations occurring with AUD cause anxiety, and (3) that the underlying biological vulnerability to AD and AUD is shared, and therefore they co-occur independently (Kushner, Abrams, & Borchardt, 2000).

1.2.1 Anxiety drives drinking behavior

In addition to its rewarding and sedative properties, alcohol is anxiolytic under acute conditions, so individuals may escalate alcohol intake under anxiogenic circumstances. Indeed, these anti-anxiety effects are akin to those found with anxiolytic drugs. This is perhaps not all that surprising when considering that alcohol, among its many neurobiological targets, acts as a positive allosteric modulator at GABAA receptors, in much the same way as do benzodiazepines. Patients with anxiety disorders are generally thought to have a dysregulated GABA system, and benzodiazepines are considered to be effective at treating anxiety symptoms primarily because of their ability to enhance the actions of GABA in the brain. Indeed, trait anxiety has been shown to correlate with GABA content in the brain (Delli Pizzi et al., 2016). Alcohol and other GABAA agonists not only produce similar anxiolytic behaviors, they likely do so through the same mechanism, at least partially. This is borne out in the additive effects produced when both drugs are taken simultaneously – GABAA agonists exacerbate the behavioral effects of alcohol and clinicians are urged to warn patients taking benzodiazepines to limit alcohol intake (Linnoila, 1990).

The anxiolytic effects of alcohol have been conserved across species, as evidenced by ethanol-induced anxiolysis observed in various animal laboratory models. Rats exhibit a dose-dependent increase in open arm time on the elevated plus maze with both diazepam (a benzodiazepine) and alcohol (Wilson, Burghardt, Ford, Wilkinson, & Primeaux, 2004). This anxiolytic effect is also in evidence at the earliest stages of development – infant rats administered a low dose of alcohol exhibit attenuation of conditioned aversion and increased time spent in the light side of the light/dark box (Miranda-Morales, Nizhnikov, Waters, & Spear, 2014). The acute anxiolytic effects of alcohol have also been documented in mice in a variety of tasks measuring anxiety-like behavior, including the light/dark box (Costall, Kelly, & Naylor, 1988), elevated plus maze (Lister, 1987), and escape task digging (Dudek, Maio, Phillips, & Perrone, 1986).

Further, when evaluating natural individual variation in anxiety levels of outbred mice, mice with higher levels of anxiety will show a greater preference for alcohol and consume more of it than their low-anxiety counterparts (Bahi, 2013). Similarly, alcohol-preferring (P) rats exhibit innately elevated anxiety relative to their non-alcohol-preferring (NP) rat counterparts (Stewart, Gatto, Lumeng, Li, & Murphy, 1993) and there is evidence that correcting this elevated anxiety can reduce drinking (Moonat, Sakharkar, Zhang, Tang, & Pandey, 2013). Moving beyond the rodent, drinking to cope can even be observed in non-human primate models – rhesus monkeys exhibiting high rates of fear-related behaviors after an early-life stressor consume significantly more alcohol than their peers (Higley, Hasert, Suomi, & Linnoila, 1991). Finally, the acute anxiolytic effect of alcohol can also be observed in humans in a laboratory setting. Patients with panic disorder who were administered alcohol reported fewer anxiety symptoms

before and after a panic challenge than those patients who did not consume alcohol (Kushner et al., 1996).

Outside of the laboratory, drinking during stressful life events or to relieve anxiety is a widely reported phenomenon and occurs in non-clinical samples as well as in patients with diagnosed anxiety disorder. Healthy college students report higher rates of drinking on days characterized by elevated anxiety (O'Hara, Armeli, & Tennen, 2014), and individuals with elevated social anxiety engage in heavier alcohol consumption that is directly mediated by their self-reported desire to cope (Terlecki & Buckner, 2015).

Unfortunately, this pattern of self-medication with alcohol results in negative long-term consequences that may exacerbate both anxiety symptoms and alcohol use. In college students, drinking to cope (rather than drinking for social motives or positive reinforcement) is uniquely predictive of higher self-reported anxiety and emotional dysregulation (Armeli, Sullivan, & Tennen, 2015) as well as lifetime alcohol-related problems (Carey & Correia, 1997). Individuals who report drinking to self-medicate anxiety symptoms were at an increased risk for developing alcohol dependence at a 3-year follow-up, but, interestingly, this effect was independent of an anxiety disorder diagnosis, suggesting that the co-occurrence of anxiety and alcohol use is predictive of alcohol-related problems in a manner that is independent of clinically relevant anxiety (Crum et al., 2013).

Additional evidence supporting the theory that anxiety drives alcohol use is that children with early symptoms of anxiety are at an increased risk for initiation of alcohol use (Kaplow, Curran, Angold, & Costello, 2001). This is especially problematic, because early onset drinking (before the age of 15) is strongly associated with drinking to cope behaviors, suggesting that early exposure to alcohol use can predispose an individual to self-medicating behavior (Young-

Wolff, Kendler, & Prescott, 2012). Further, early anxiety onset is an independent risk factor for the development of alcohol dependence (Boschloo et al., 2011). Additionally, the presence of a comorbid anxiety disorder predicts the persistency of an AUD (Tuithof, Ten Have, van den Brink, Vollebergh, & de Graaf, 2013), suggesting that anxiety is involved in both the development and maintenance of alcohol dependence. Indeed, in individuals with an AUD, anxiety is a known risk factor for relapse (Silberman et al., 2009). Finally, in a large prospective and retrospective study in the Netherlands of comorbid AD and AUD, alcohol dependence was not found to precede the onset of AD, but AD did precede the onset of alcohol dependence – lending additional support to the hypothesis that anxiety drives alcohol consumption (Marquenie et al., 2007).

1.2.2 Alcohol use leads to anxiety

While there is strong evidence that high anxiety states can drive drinking behavior, there is equally compelling evidence that excessive alcohol intake increases anxiety. Although alcohol acts as an anxiolytic under acute conditions, chronic alcohol use has been shown to have the reverse effect on anxiety. This is the basic premise outlined in George Koob's negative reinforcement model, or the "dark side of addiction" (Koob & Le Moal, 2005). An individual may start drinking out of positive reinforcement, but alcohol consumption (and subsequent bouts of withdrawal) will then create long-term neuroadaptations in cortical and limbic areas that will increase anxiety over time, and these elevations in anxiety will drive continued alcohol use, and so on. It is important to note that this pattern of behavior is not merely a product of social pressures (i.e., excessive alcohol consumption is associated with job loss, marital problems, etc., and it is the occurrence of those problems that leads to anxiety), because alcohol use can elevate anxiety even in rodents. Chronic intermittent alcohol exposure in rats produces immediate and

long-lasting anxiogenic behavior in the elevated plus maze (Van Skike, Diaz-Granados, & Matthews, 2015). Interestingly, this effect of drinking on anxiety is directly related to the amount of alcohol consumed, with consumption of higher concentrations of alcohol positively correlating with anxiety-like behavior; a liquid diet of 6.2% alcohol for 12 weeks produced no detectable changes in anxiety-like behavior in rats, while rats that were exposed to a 10% alcohol liquid diet for 12 weeks did display increased anxiety-like behavior (Rylkova, Shah, Small, & Bruijnzeel, 2009).

There is compelling evidence that chronic alcohol exposure induces anxiety via epigenetic mechanisms. Increased acetylation of histones leads to an opening of the chromatin and increased transcriptional activity. This increased acetylation has been associated with the acute anxiolytic effect of alcohol exposure; after a single dose of 1 g/kg alcohol, rats show decreased anxiety-like behavior, increased histone acetylation, decreased histone deacetylase (HDAC) activity, and increased transcription of the anxiolytic-like proteins CREB-binding protein and neuropeptide Y (NPY) (S. C. Pandey, Ugale, Zhang, Tang, & Prakash, 2008). Likewise, the emergence of anxiety-like behavior during withdrawal from chronic ethanol exposure is associated with the reverse epigenetic signature: decreased histone acetylation, increased HDAC activity, and decreased expression of CREB-binding protein and NPY. Supporting a causal role of these histone modifications in the anxiety phenotype, it has further been shown that inhibiting HDAC activity can reverse the elevated anxiety observed after ethanol withdrawal (S. C. Pandey et al., 2008). Further, alcohol exposure in adolescence can lead to long-term alterations in anxiety-like behavior via these same epigenetic modifications. Adolescent intermittent ethanol (AIE) exposure leads to increased HDAC activity and decreased histone acetylation, as well as increased anxiety-like behavior and voluntary alcohol

consumption in adulthood, suggesting that alcohol can directly alter ones susceptibility to anxiety in a persistent, long-term manner, via epigenetic modifications (S. C. Pandey, Sakharkar, Tang, & Zhang, 2015).

Similarly, in humans, the amount of alcohol consumed correlates with the risk for development of an anxiety disorder (Bellos et al., 2013). In a study examining psychiatric disorders in adolescent and adult drinkers, the risk of having an anxiety disorder increased with the severity of alcohol use, with young adult alcohol abusers having twice the odds of developing an anxiety disorder than lifetime abstainers (OR = 2.0), and those with alcohol dependence having three times the odds (OR = 3.2). This effect increased in adults over the age of 30, with non-dependent drinkers exhibiting a 50% increase in the risks of an anxiety disorder and dependent drinkers having nearly a six-fold increase in the risk of an anxiety disorder (Dawson, Grant, Stinson, & Chou, 2005). However, because these measures were collected at the same time, this study alone cannot suggest causality – alcohol use may have lead to elevated anxiety, but the reverse could be equally possible. Direct causality is nearly impossible to determine in human observational studies, but prospective studies at least allow for the measuring of timing of symptom emergence. Prospective studies of alcohol use have provided some evidence that continued and heavy drinking can alter distress levels – level of alcohol use in a sample of high school students at baseline correlated with anxiety symptoms over a year later (Friedman, Utada, Glickman, & Morrissey, 1987). In line with this, a study of monozygotic and dizygotic twins found that anxiety disorders were more common in dependent probands and in twins who also exhibited alcohol dependence than in twins who consumed moderate levels of alcohol (Mullan, Gurling, Oppenheim, & Murray, 1986), suggesting that in those individuals with genetic vulnerability to this comorbidity, alcohol dependence is necessary for the emergence of AD.

Yet alcohol does not only elevate anxiety under chronic conditions. The immediate withdrawal from even a single high dose of alcohol can produce an anxiogenic response (Doremus, Brunell, Varlinskaya, & Spear, 2003). This highlights an important consideration of the neurobiological effects of alcohol – when alcohol is active in the brain, its effects are largely anxiolytic, but after its metabolism the brain produces a withdrawal state associated with anxiety. What changes with chronic versus acute alcohol use is the persistence of these effects. While a single high dose of alcohol will create a short-term increase in anxiety-like behavior, repeated injections of high alcohol doses will significantly extend the duration of this anxiogenic effect (Z. Zhang, Morse, Koob, & Schulteis, 2007). This suggests that the neuroadaptations occurring after repeated bouts of binge and withdrawal from alcohol will lead to long-lasting changes in the networks underlying anxiety.

1.2.3 Shared vulnerability for anxiety disorder and alcohol use disorder

Because alcohol can be both anxiolytic and anxiogenic, determining the causal relationship of comorbid AUD and AD is extremely complicated. Rather than one disorder driving the appearance of the other, there may instead exist a reciprocal causal relationship between alcohol use and anxiety, with elevated anxiety driving drinking behaviors and vice versa (Kushner et al., 2000). There is compelling evidence in support of this reciprocal relationship – having an anxiety diagnosis increases the odds of developing an alcohol use disorder, but having an alcohol use disorder also increases the odds of later developing an anxiety disorder (Kushner, Sher, & Erickson, 1999). Reviews of epidemiologic and family studies have found that anxiety disorders are as equally likely to pre-date as to post-date alcohol dependence (Kushner et al., 1990; Merikangas et al., 1996), suggesting that there is a common third factor that is increasing vulnerability to both anxiety and alcohol dependence.

Not all people who experience anxiety and drink alcohol go on to drink excessively or experience anxiety that interferes with daily functioning. There appears to be a vulnerability in certain individuals that increases their risk for developing comorbid anxiety and alcohol use disorders. Indeed, there is evidence of just such a genetic vulnerability in family history studies. Several studies report an increased risk of AD in family members of individuals with AUD (Mathew, Wilson, Blazer, & George, 1993; Raucher-Chene et al., 2012; Reich, Earls, Frankel, & Shayka, 1993), and others report an increased risk for AUD in family members of individuals with AD (Goodwin, Lipsitz, Keyes, Galea, & Fyer, 2011; Noyes et al., 1986; Schuckit et al., 1995).

1.3 Mechanisms of interaction between anxiety disorder & alcohol use disorder

Altered neurobiological function is likely one of the mechanisms underlying this increased susceptibility to AD and AUD in vulnerable individuals. Deficits in prefrontal cortical function are associated with both AD (Shiba, Santangelo, & Roberts, 2016) and AUD (Chocyk, Majcher-Maslanka, Dudys, Przyborowska, & Wedzony, 2013; Heilig et al., 2017; Nixon & McClain, 2010). One of the primary functions of the prefrontal cortex is exerting top-down inhibitory control over subcortical areas, and deficits in prefrontal functioning can therefore lead to hyperactivation of subcortical regions, including areas that are key to regulating alcohol use and anxiety, such as the amgydala (Gilpin, Herman, & Roberto, 2015; Nuss, 2015). Indeed, imaging studies have shown that patients with AD have elevated amygdala responses to threatening stimuli (Craske et al., 2009). Weaker functional amygdala-cortex connectivity has also been observed in high drinking adolescents (Muller-Oehring et al., 2017) and predicts anxiety symptoms in childhood and adolescence (Pagliaccio et al., 2015), suggesting that the amygdala is a key regulator of susceptibility to both AD and AUD.

1.3.1 Amygdala circuitry in anxiety and alcohol use

The amygdala, like most brain areas, is not a homogeneous structure, but is composed of separate nuclei with distinct cell types, characteristics, and connections (LeDoux, 2007). To simplify an incredibly complex system, the basolateral nucleus (BLA, in this context, referring to both the basal and lateral nuclei together) is composed of primarily glutamatergic cells and serves as the primary input region of the amygdala, argued by some to be an extension of the cortex due to its dense cortical inputs. The central nucleus of the amygdala (CeA), by contrast, is primarily GABAergic and serves as a critical output region. Importantly, the BLA also has significant outputs to the cortex, ventral striatum, and extended amygdala (i.e., CeA and bed nucleus of the stria terminalis [BNST]), forming reciprocal connections with many of its target regions. In this way, the BLA is in a prime position to regulate both anxiety-related behavior (by activating areas of the brain involved in fear response and fear learning and feeding back to forebrain regions involved in assessing future risk) and alcohol use (by activating areas of the brain involved in reward, like the nucleus accumbens, and enhancing drug-associated memories by imparting emotional salience to stimuli), making it an ideal nexus for comorbid AD and AUD.

In fact, the BLA has shown to play a role in both anxiety and alcohol-related phenotypes. A model of early life stress in rodents is associated with both elevated anxiety-like behaviors in adulthood as well as increased voluntary alcohol intake, behaviors that are directly attributable to increased excitability of BLA neurons (Rau, Chappell, Butler, Ariwodola, & Weiner, 2015). The BLA has also been shown to regulate context-dependent alcohol seeking (Sciascia, Reese, Janak, & Chaudhri, 2015) and reinstatement of alcohol seeking (Marinelli, Funk, Juzytsch, & Le, 2010), suggesting a role for the BLA in susceptibility to relapse drinking. Neuroimaging studies using

positron emission tomography (PET) found a significant attenuation in BLA activity after a 6-8 week course of SSRI treatment relative to placebo, but this was only evident in those patients who showed a reduction in anxiety symptoms with treatment ("responders"), suggesting that the BLA is critical to anxiety symptoms in patients with AD and SSRIs are only effective at reducing anxiety when they produce changes in BLA signaling (Faria et al., 2012).

Further, the BLA undergoes drastic and long-lasting changes with alcohol exposure. Rats given chronic intermittent access to alcohol were found to have altered expression levels of several genes in the BLA, including genes regulating GABA and corticotropin releasing factor (CRF) (Falco, Bergstrom, Bachus, & Smith, 2009). Postnatal exposure to alcohol produces alterations in glutamatergic signaling in the BLA and this hyperactivity is associated with elevated anxiety in adolescence, further supporting evidence that the BLA is both a target of alcohol exposure and a regulator of anxiety (Baculis, Diaz, & Valenzuela, 2015).

BLA projections to the CeA may be particularly critical for its role in regulating anxiety and behavioral responses to alcohol. Stimulating direct BLA-to-CeA projections produces an acute anxiolytic effect, while inhibiting these same projections increased anxiety-like behavior (Tye et al., 2011). Further, in mice selectively bred for high anxiety (HAB) and low anxiety (LAB), neural activity in the circuitry connecting lateral amygdala to CeA correlated with anxiety-like behavior, and environmental factors that were able to normalize anxiety levels (i.e., environmental enrichment in the HAB and chronic mild stress in the LAB) could also "normalize" the neural signaling in the amygdala (Avrabos et al., 2013).

While there is mounting evidence that the BLA regulates both behavioral responses to alcohol and anxiety-like behavior, the manner in which it does so is still not well delineated. BLA projection neurons can target a number of cells, both in and out of the amygdala, making

the study of their effects highly complicated. Just within the amygdala circuitry, the BLA sends glutamatergic connections to both the lateral and medial portions of the CeA (CeL and CeM, respectively) and to the intercalated cells (ITC) located between the BLA and CeA. Altogether, this network of connections allows for precise control of amygdala output. Although the BLA projections are glutamatergic, the ITC and both portions of the CeA contain mostly GABAergic cells, so that the ITC inhibits the CeL and the CeL inhibits the CeM. In one scenario, BLA activation can activate the ITC, which would inhibit the CeL and disinhibit the CeM, leading to greater CeA output. But because the BLA can also activate the CeL or CeM directly (or for that matter, CeA output targets such as the BNST as well), activation of a subset of neurons of the BLA can produce varied and potentially opposing effects. To complicate matters further, a single neuron in the BLA can innervate multiple cells, either within the BLA, other amygdala nuclei, and/or extra-amgydaloid targets (Pitkanen, Savander, Nurminen, & Ylinen, 2003). For instance, optogenetic stimulation of BLA terminals in the CeL produces a rapid anxiolytic effect, but stimulation of those same BLA cell bodies does not, suggesting that the BLA cells projecting to the CeL likely have other targets that can attenuate the net effect of CeL stimulation (Tye et al., 2011). Clearly, BLA output is carefully regulated and incredibly complex.

One of the ways in which BLA activity is regulated is via a subset of interneurons expressing parvalbumin. Parvalbumin (Pv+) interneurons are fast-spiking interneurons whose unique firing characteristics allow for exquisite spatio-temporal control of large cell networks (Hu, Gan, & Jonas, 2014). They have long dendrites and extensive axonal arborizations, so they are able to gain input from a large population of principal cells and quickly turn that excitatory input into massive divergent inhibitory output. Pv+ interneurons also preferentially target the perisomatic region of cells, allowing for even tighter control of action potential initiation.

Because of their vast connections and powerful inhibitory potential, Pv+ neurons are critical for network oscillations and show bidirectional control of learning behavior. The action of Pv+ interneurons in the BLA have been shown to be critical for the acquisition of fear conditioning (Wolff et al., 2014), while Pv+ interneurons in the PFC regulate extinction of reward seeking behavior (Sparta et al., 2014).

1.3.2 Gamma-aminobutyric acid (GABA)

The specialized roles played by Pv+ interneurons in regulating signaling highlights the critical role that GABA signaling has in modulating excitatory tone throughout the brain. Although GABAergic interneurons only comprise about 20% of neurons in the brain, GABA signaling is necessary for proper neurodevelopment (Ben-Ari, 2002; Cellot & Cherubini, 2013), regulating neuroinflammation (Crowley, Cryan, Downer, & O'Leary, 2016), whole-brain tuning of network oscillations (Lee & Maguire, 2014), glial communication with neurons (Yoon & Lee, 2014), and its dysregulation is implicated in a range of psychiatric disorders (Coghlan et al., 2012; Lydiard, 2003; Stan & Lewis, 2012).

GABA binds to a number of receptors, one of which is the group of heteropentameric ligand-gated ion channels termed $GABA_A$ receptors. These receptors are extremely heterogeneous, with 19 possible subunits so far identified (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3); the α1β2γ2 is the most common formation in the brain. The specific composition of receptor subunits can have profound effects on receptor functionality, including altering agonist affinity, desensitization rate, and proximity to the synapse (Ferando & Mody, 2014), and as such, differential expression is observed across brain regions (Nuss, 2015).

Alcohol acts at a number of sites in the brain, modulates both neurotransmitter and neuropeptide function, and has been shown to cross the membrane of cells, making it a “messy

drug" in terms of its neurophysiological impact. That said, the effects of alcohol on GABA signaling are well characterized. An acute application of alcohol has been shown to increase both spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs, respectively), indicating increased GABA release. There is evidence that alcohol increases GABA release via neuropeptide receptors at the presynaptic site (Gilpin et al., 2011; Kelm, Criswell, & Breese, 2011; Z. Nie et al., 2004). GABA signaling is also altered with chronic alcohol exposure – individuals with AUD exhibit decreased plasma GABA levels (Coffman & Petty, 1985), a phenomenon that may also be driving the elevated anxiety observed after chronic alcohol use.

Not only does alcohol stimulate GABA release, but it also acts directly at the $GABA_A$ receptor. Examining the effects of alcohol on $GABA_A$ receptor channels has shown that alcohol increases both the frequency of GABA-mediated opening and the open time for these channels (Tatebayashi, Motomura, & Narahashi, 1998). Chronic alcohol exposure also produces a cross-tolerance to benzodiazepines, supporting the role for $GABA_A$ receptor involvement in both alcohol use and anxiety (Liang, Spigelman, & Olsen, 2009). The extent to which alcohol can alter $GABA_A$ receptor activity is dependent on the subunit composition of the receptor, and alcohol itself can alter subunit expression – after an acute high dose of alcohol exposure, the expression of $GABA_A$ α4 and δ subunits are decreased at the surface, but after 48-hours of withdrawal, α4 is actually increased, δ continues to be decreased, and α1 and α2 also show decreases in surface expression (Lindemeyer et al., 2014). This suggests that alcohol not only alters $GABA_A$ receptor plasticity while it is metabolically active, but also produces changes in a state of withdrawal. Importantly, these effects on $GABA_A$ receptor plasticity were observed in the BLA, further highlighting the critical role the BLA serves in regulating responses to alcohol. Further, changes in $GABA_A$ receptor subunit surface expression were also observed in adult rats

that were subjected to chronic intermittent ethanol exposure in adolescence (Centanni et al., 2014), suggesting that alcohol withdrawal effects on $GABA_A$ receptor plasticity are both acute and long lasting.

Changes in subunit composition can lead to significant alterations in cell signaling. The δ subunit of the $GABA_A$ receptor is localized extrasynaptically and is known for modulating tonic inhibition (Lee & Maguire, 2014). Acutely, alcohol will increase firing of cells expressing the α1 subunit, but decrease firing of cells expressing the extrasynaptic δ subunit (Herman, Contet, Justice, Vale, & Roberto, 2013). Chronic alcohol exposure induces alterations in δ-subunit expression in the amygdala that results in a shift of cell types receiving tonic inhibition, and subsequently, increased amygdala output (Herman, Contet, & Roberto, 2016). Similarly, chronic alcohol exposure also induces changes in the way that alcohol acts on $GABA_A$ receptors in the hippocampus, with a gain in responsiveness to alcohol in synaptic receptors and a loss of responsiveness in extrasynaptic receptors (Liang et al., 2006). These neuroadaptations that occur with chronic alcohol use likely contribute to continued alcohol intake.

While alcohol can alter $GABA_A$ receptor subunit expression, specific subunits can also alter alcohol-related phenotypes. $GABA_A$ agonists will suppress binge-like alcohol intake, but agonists specifically targeting the δ-subunit can produce a more robust suppression (Quoilin & Boehm, 2016). Adolescents were particularly sensitive to this suppression, suggesting a differential expression pattern of δ-subunit-containing $GABA_A$ receptors in this age group. In contrast, targeting δ-subunits specifically in the nucleus accumbens will have the reverse effect on alcohol consumption – knocking down the δ-subunit in the medial shell of the accumbens via viral-mediated RNA interference reduced alcohol intake (H. Nie, Rewal, Gill, Ron, & Janak, 2011). One potential explanation for the opposing effects of the δ-subunit on alcohol intake is

that its effect on alcohol-related behaviors is dependent on which brain region and/or cell type it is expressed. Similarly, the α4 subunit of the $GABA_A$ receptor has been implicated in the reinforcing aspect of alcohol, but its effects are also region-specific (H. Nie et al., 2011; Rewal et al., 2012).

GABA signaling is associated with anxiety particularly via its actions in the amygdala. Infusions of GABA agonists into the amygdala produce an anxiolytic response, while GABA antagonist infusion is anxiogenic (Barbalho, Nunes-de-Souza, & Canto-de-Souza, 2009; Sanders & Shekhar, 1995). The specific composition of $GABA_A$ receptor subunits also influences anxiety. Activating δ-containing $GABA_A$ receptors in the BLA is necessary for fear learning (Liu et al., 2016). Genetic manipulation of $GABA_A$ receptor subunit expression early in the postnatal developmental period increases anxiety-like behavior in adulthood (Q. Shen, Fuchs, Sahir, & Luscher, 2012). Benzodiazepines exert their influence by binding at the interface between the α and γ subunits, with a particularly affinity for receptors containing the α1 subunit. Several other subunits have also been associated with anxiety, including the α2, α3, α4, α5, β2, γ2, and δ subunits (Botta et al., 2015; Chandra, Korpi, Miralles, De Blas, & Homanics, 2005; Dias et al., 2005; Gulinello, Orman, & Smith, 2003; Skorzewska et al., 2014; Sundstrom-Poromaa et al., 2002; Yoshimura et al., 2014). While much work remains to be done on the exact nature of subunit-specificity in regulating behavior, GABA remains a significant regulator of both anxiety and alcohol-related behaviors.

1.3.3 Corticotropin releasing factor (CRF)

Corticotropin releasing factor (CRF) is most commonly known for its role in initiating the hypothalamic-pituitary-adrenal (HPA) axis, and indeed, the highest concentration of CRF-containing cell bodies in the brain is found in the paraventricular nucleus of the hypothalamus.

However, CRF-containing interneurons are expressed throughout the brain (Koob, 2009), and CRF and its receptors have been shown to modulate a wide array of behaviors, including anxiety (Dunn & Berridge, 1990; Stenzel-Poore, Heinrichs, Rivest, Koob, & Vale, 1994), substance use (Giardino et al., 2011; Kaur, Li, Stenzel-Poore, & Ryabinin, 2012), selection of partner preference (Lim et al., 2007), feeding (Stengel & Tache, 2014), maternal behavior (Klampfl, Brunton, Bayerl, & Bosch, 2014; Klampfl et al., 2016), and fear learning (Abiri et al., 2014; G. M. Gafford & Ressler, 2015) and memory (G. Gafford, Jasnow, & Ressler, 2014).

Given its critical role in the stress response, perhaps it's not surprising that CRF also regulates anxiety. Administering CRF systemically (Dunn & Berridge, 1990) or over-expressing it in transgenic mice (Stenzel-Poore et al., 1994) will increase anxiety-like behavior. These effects are likely driven by the actions of CRF acting at its type 1 receptor (CRF1R) – administering an antagonist specific for CRF1R blocks the anxiogenic effects of CRF in the rat (Zorrilla, Valdez, Nozulak, Koob, & Markou, 2002) and mice lacking the CRF1R show anxiolytic behavior (Contarino et al., 1999). The association between CRF and anxiety has also been observed in humans – patients with obsessive-compulsive disorder have elevated cerebrospinal fluid CRF levels relative to controls (Altemus et al., 1992), and some alleviation of anxiety symptoms has been observed with CRF1R antagonist treatment in small clinical trials (Ising & Holsboer, 2007).

CRF has also increasingly emerged as a target for alcohol abuse. Elevated levels of extracellular CRF in the brain have been observed in animals dependent on alcohol (Olive, Koenig, Nannini, & Hodge, 2002), and binge drinking has been shown to increase CRF mRNA in extrahypothalamic areas of the brain (Rinker et al., 2017). Once again, the CRF1R emerges as the mediator of CRF's effects. CRF1R knockout mice exhibit decreased binge-like alcohol

consumption (Kaur et al., 2012) and show no abstinence-induced escalation in drinking (Chu, Koob, Cole, Zorrilla, & Roberts, 2007). Pharmacologically antagonizing the CRF1R reduces binge drinking (Lowery et al., 2010) and prevents withdrawal-induced escalation in drinking (Chu et al., 2007; Funk, Zorrilla, Lee, Rice, & Koob, 2007). Additionally, rats selectively bred for high alcohol preference show up-regulation of the CRF1R (Ciccocioppo et al., 2006), and access to alcohol normalizes CRF1R expression (Hansson, Cippitelli, Sommer, Ciccocioppo, & Heilig, 2007). The *Crhr1* gene in humans has also been associated with binge drinking and lifetime prevalence of alcohol intake (Treutlein et al., 2006). Although the bulk of evidence points to CRF1R as the mediator of CRF's effects in alcohol use, there is some evidence that CRF2R may be critical in this exchange – inhibiting the CRF1R in the ventral tegmental area (VTA) reduces binge drinking, but only if the CRF2R is available (Rinker et al., 2017), suggesting that activation of CRF2R may be actively opposing the effects of the CRF1R on binge drinking.

Importantly, CRF becomes increasingly relevant at the crossover between alcohol use and anxiety. As noted before, withdrawal from alcohol induces an anxiogenic state, and CRF may be critical for the emergence of this alcohol-withdrawal-induced anxiety. Blocking CRF1Rs systemically (Knapp, Overstreet, Moy, & Breese, 2004; Valdez et al., 2002) will prevent withdrawal-induced anxiety, even after a protracted abstinence period (Breese, Overstreet, Knapp, & Navarro, 2005).

The amygdala is an especially important site of action for CRF regulation of anxiety and alcohol-related behaviors. *Crhr1* gene expression in the BLA is elevated in mice selectively bred for high-anxiety (HAB), and administering a CRF1R antagonist into the BLA attenuates anxiety behavior to that of low-anxiety (LAB) mice (Sotnikov et al., 2014). This effect can be replicated

by genetically manipulating CRF1R levels in the BLA, as well - knocking down *Crhr1* gene expression in the BLA of C57BL6/J mice produces a robust anxiolytic phenotype (Sztainberg, Kuperman, Tsoory, Lebow, & Chen, 2010). Further, CRF1R expression in the amygdala dynamically changes with alcohol dependence (Roberto et al., 2010), and with immediate and protracted alcohol withdrawal (Eisenhardt, Hansson, Spanagel, & Bilbao, 2015; Zorrilla, Valdez, & Weiss, 2001). Elevating CRF levels pharmacologically throughout the extended amygdala prior to exposing rats to alcohol will elevate their withdrawal-induced anxiety, an effect that can be reversed by administration of a CRF1R antagonist (Huang et al., 2010). Finally, administration of CRF1R antagonists directly into the amygdala will reduce binge drinking in both non-dependent (Lowery-Gionta et al., 2012) and dependent animals (Heilig & Koob, 2007) and prevent alcohol-withdrawal-induced anxiety (Rassnick, Heinrichs, Britton, & Koob, 1993). What's more, CRF1R colocalizes with specific $GABA_A$ receptor subunits to alter signaling in the amygdala (Herman et al., 2013) – while tonic GABAergic inhibition is mediated by the δ-subunit in cells lacking the CRF1R, tonic inhibition in CRF1R-containing neurons is mediated by receptors containing the α1 subunit. Importantly, alcohol only enhances tonic inhibition in the CRF1-lacking neurons containing the δ-subunit, highlighting a complex interplay between CRF1R and $GABA_A$ receptors and cell signaling in the amygdala.

There is clearly a shared neurobiology between alcohol use and anxiety, and yet there does not exist a single treatment effective for both AD and AUD (Helton & Lohoff, 2015). Given the critical role of the amygdala in regulating alcohol-related phenotypes and anxiety, identifying genes that regulate signaling in the amygdala, particularly involving GABA or CRF systems, may provide novel targets for effective treatments of comorbid AD and AUD.

1.4 LMO proteins

Recently, a family of proteins involved in cell differentiation and specification, the Lim-domain-only (LMO) proteins, have emerged as candidate genes for the regulation of substance abuse (Lasek, Giorgetti, Berger, Tayor, & Heberlein, 2011; Lasek et al., 2010) fear learning (Maiya, Kharazia, Lasek, & Heberlein, 2012), and anxiety (Qin et al., 2015). Each of the four proteins in this family (LMO1-4) have only two tandem LIM domains, and they can function as transcriptional regulators via protein-protein interactions (Zheng & Zhao, 2007). These LIM domains are comprised of 50-60 amino acids with eight highly conserved residues (mostly cysteine and histidine) that function as zinc-finger motifs. Unlike transcription factors, LMO proteins do not directly bind DNA – they instead function as scaffolding proteins by binding to co-factors and transcription factors to form large multi-protein complexes at the transcriptional start site. Alternatively, they can decrease transcription by sequestering cofactors away from the transcriptional complex. In addition to their role in regulating transcription, there is mounting evidence that at least two of these proteins can also function in the cytosol to alter intracellular signaling cascades (Arber & Caroni, 1996; Baron et al., 2015; N. R. Pandey et al., 2013; Qin et al., 2015).

Although efforts to identify a protein sequence preference for binding of a LIM domain have proven unsuccessful, all LMO proteins interact with the nuclear LIM-domain binding protein-1 (LDB1). LDB1 binds specifically to the related LMO and LIM-homeodomain (Lhx) protein families (Matthews et al., 2008). These two families of proteins are expressed in a unique combinatorial pattern throughout development (forming the “LIM code”) to promote cell differentiation and cell type specification (Gadd et al., 2011). LDB1’s LIM interaction domain (LID) can bind a single LIM domain, but binds with higher affinity to two tandem LIM domains

(Deane et al., 2004). Competitive binding of LDB1 by LMO proteins may be a key mechanism of LMO functioning in cell fate specification and development – upon binding, LMO proteins sequester LDB1 from binding to Lhx proteins, thereby attenuating the activation of transcription by Lhx (Matthews et al., 2008). LMO proteins have limited solubility and stability (Deane et al., 2001), and may in fact be obligate binding proteins (in the absence of true binding partners they may be indiscriminate in their binding) (Matthews, Lester, Joseph, & Curtis, 2013). As nuclear transcriptional co-regulators, LMO proteins can have either positive or negative effects on gene transcription, influencing cellular processes as important as cell cycle regulation, differentiation, and proliferation. As such, these proteins tend to be critical for survival – the deletion of LMO2 or LMO4 in mice is lethal, as is the combined null mutation of LMO1 and LMO3 (although mice lacking only one of the two genes are viable) (Tse et al., 2004). LMO proteins have important roles in development, but they continue to be expressed in adulthood, suggesting that these proteins may also be involved in the maintenance of specific cell types (Hinks et al., 1997). Indeed, evidence that LMO proteins can associate with epigenetic factors, such as histone deacetylase 2 (HDAC2), suggest a mechanism by which they can function to dynamically regulate cell specificity (Wang et al., 2007).

The expression patterns of the LMO family tend to be tissue restricted (except in the most divergent member, LMO4, which is widely expressed), with LMO3 being highly expressed in the brain and vestibular ganglion cells (Deng, Pan, Xie, & Gan, 2006). Because of its expression patterns throughout the brain and its involvement in the development of the central nervous system (Tse et al., 2004), LMO3 is of particular interest as a potential regulator of psychiatric functioning. LMO3 was originally isolated by use of an LMO1 probe (Boehm, Spillantini, Sofroniew, Surani, & Rabbitts, 1991) and the two proteins were found to be highly related

(Foroni et al., 1992). LMO1 and LMO3 have overlapping but distinct profiles of expression in the developing mouse brain – with LMO1 being expressed early in development and LMO3 gaining expression after embryonic day 19 and persisting into adulthood (Tse et al., 2004). While these proteins may have complementary functions (their sequence homology, timing of expression, and their combined null mutation being lethal, suggest this), LMO3 has much greater and more ubiquitous expression throughout the brain, particularly in postnatal brains, suggesting a unique role for this protein in both brain development and function.

1.4.1 LMO3

Lmo3 is a transcriptional target of the ARX protein, which is involved in cortical interneuron generation, migration, and differentiation (Friocourt & Parnavelas, 2011). More critically, LMO3 itself has been shown to regulate cortical interneuron differentiation, specifically by promoting the parvalbumin subtype, with LMO3 null mice displaying a significant reduction in parvalbumin-positive cortical interneurons (Au et al., 2013). Given the critical role that parvalbumin has in regulating network signaling, as described above, LMO3 is therefore in a position to have profound effects on brain functioning. In addition to its role in GABAergic cell differentiation, *Lmo3* has also been shown to be a dopamine-responsive gene. In a study evaluating the molecular events following dopamine receptor activation in glial cells, it was found that LMO3 was upregulated in response to dopamine treatment (Shi et al., 2001).

Few of LMO3's targets and/or interacting proteins are known. LMO3 interacts with HEN2 (Aoyama et al., 2005; Isogai et al., 2015), a neuron-specific transcription factor, to induce transcription of Mash1 (Isogai et al., 2011). HEN2 (a.k.a. NHLH2) and Mash1 are both expressed in neuroblastoma and the developing nervous system (L. Brown, Espinosa, Le Beau, Siciliano, & Baer, 1992). In addition to HEN2, LMO3 has also been shown to bind to the

calcium-and integrin-binding protein (CIB) and translocate from the nucleus to the cytoplasm, an effect that resulted in an inhibition of cell proliferation (Hui et al., 2009). CIB is involved in a variety of cellular processes, but notably supports both the PI3K/AKT and MEK/ERK pathways (Leisner, Freeman, Black, & Parise, 2016). LMO3 also directly interacts with the tumor suppressor p53 and reduces expression of its target genes, potentially explaining why p53 levels are so high in neuroblastoma patients despite the presence of cancer (Larsen et al., 2010). Perhaps most intriguingly for alcohol research, LMO3 has been shown to be expressed in midbrain dopaminergic neurons and to associate with Pitx3, a dopaminergic cell-type-specific transcription factor (Bifsha, Balsalobre, & Drouin, 2016).

There is even less known about the regulation of LMO3 transcription, but a few studies have resulted in some intriguing findings. The microRNA miR-101 has been shown to suppress LMO3 expression via DNA methylation of the promoter region of the *Lmo3* gene, a process that becomes disrupted in glioma cells (X. Liu et al., 2015). Similarly, miR-630 inhibits LMO3 expression, and over-expressing LMO3 in vitro blocks the ability of miR-630 to suppress cell proliferation (Song et al., 2015). Several transcription factors have also been identified that regulate *Lmo3* expression. FOXA1, a transcription factor critical for the formation of midbrain dopaminergic neurons (Ang, 2009), promotes *Lmo3* expression (H. Watanabe et al., 2013), while Gbx2, a transcription factor required for proper striatal cholinergic interneurons (L. Chen, Chatterjee, & Li, 2010), represses *Lmo3* expression (Chatterjee et al., 2012). Two additional transcription factors have been identified that bind to the *Lmo3* promoter and regulate its transcription – USF (upstream stimulatory factor) and MZF1 (myeloid zinc finger 1) (X. Liu et al., 2015). MZF1 has been shown to regulate transcription of neuroimmune markers, like TGF-β1, and its activity is decreased by alcohol exposure (Driver et al., 2015). USF transcription

factors regulate activity-dependent transcription (W. G. Chen et al., 2003), are responsive to cellular stress, and have also been shown to regulate immune responses (Corre & Galibert, 2005). Notably, USF cooperates with cAMP response element binding (CREB) protein to regulate transcription of brain derived neurotrophic factor (BDNF) (W. G. Chen et al., 2003; Pruunsild, Sepp, Orav, Koppel, & Timmusk, 2011; Tabuchi, Sakaya, Kisukeda, Fushiki, & Tsuda, 2002) and several subunits of the GABA-B receptor (Steiger, Bandyopadhyay, Farb, & Russek, 2004). Finally, USF has also been found to regulate gene expression changes in the rat amygdala after chronic alcohol exposure, suggesting a direct role in LMO3-related regulatory pathways in neuroadaptations associated with alcohol abuse (Freeman et al., 2013).

1.4.2 LMO proteins regulating behavior

Beyond their role in development, until recently there was no evidence that LMO proteins had relevant functional roles in adult behavior. However, emerging work has suggested a unique role for these proteins in regulating behavioral responses to drugs of abuse. Reduced expression of the Drosophila *dLmo* (the homolog to the mammalian LMO family) is associated with increased acute cocaine sensitivity (Tsai, Bainton, Blau, & Heberlein, 2004) and increased sensitivity to the sedative effects of alcohol (Lasek, Giorgetti, et al., 2011). Similarly, reduced expression of *Lmo4* in mice was associated with increased cocaine sensitization, an effect driven by the actions of *Lmo4* in the nucleus accumbens (Lasek et al., 2010). In addition to regulating cocaine phenotypes, *Lmo4* also regulates cue reward learning (Maiya, Mangieri, Morrisett, Heberlein, & Messing, 2015) and fear learning (Maiya et al., 2012) through its actions in the BLA. Interestingly, while Maiya et al did not find an effect of LMO4 on anxiety-like behavior through knockdown of its expression in the BLA, knockout of *Lmo4* specifically in glutamatergic neurons *did* induce an anxiogenic phenotype that could be reversed through

inhibition of a downstream target of LMO4 specifically in the BLA (Qin et al., 2015). These data suggest that LMO4 may have opposing effects in glutamatergic and non-glutamatergic cells.

As for alcohol-specific effects of LMO proteins, LMO3 has been observed to regulate several alcohol-related phenotypes. As with *dLmo* in Drosophila, reducing expression levels of *Lmo3* via RNA interference increased sedation in response to alcohol in mice. The same animals were then tested in a two-bottle choice drinking experiment and positive correlations between *Lmo3* expression and alcohol consumption were found, suggesting that LMO3 may also play a role in alcohol preference (Lasek, Giorgetti, et al., 2011). Interestingly, LMO1 (the family member most closely related to LMO3) was also found to be associated with the maximum number of alcoholic drinks consumed in a 24-hour period in a genome-wide association study of alcoholics (Kapoor et al., 2013). Given the ubiquitous expression of LMO3 throughout the brain, including the amygdala, and its continued expression throughout adulthood, its role in interneuron and dopaminergic cell differentiation, and its regulation of alcohol-specific phenotypes, the *Lmo3* gene is a particularly attractive candidate gene for comorbid AD and AUD.

1.5 Summary and project overview

In summary, the high comorbidity of alcohol use disorders (AUD) and anxiety disorders (AD) suggests a common genetic and neurobiological vulnerability (Kushner et al., 1990). The comorbidity of AD and AUD have significant impacts on society, yet not a single pharmacological treatment is efficacious at treating both disorders (Ipser et al., 2015). This is especially problematic since the co-occurrence of AD and AUD is associated with greater impairment and poorer response to treatment (Boschloo et al., 2012; Burns et al., 2005). This highlights the great need for identifying genes that are associated with both alcohol use and

anxiety that could be targeted for more effective treatments. The basolateral amygdala has emerged as a brain area critical for the regulation of anxiety and alcohol-related phenotypes (Faria et al., 2012; Marinelli et al., 2010; Rau et al., 2015; Sciascia et al., 2015; Tye et al., 2011). Both $GABA_A$ receptors and CRF and its receptor type 1 (CRF1R) regulate BLA signaling (Ehrlich, Ryan, Hazra, Guo, & Rainnie, 2013; Lin, Tseng, Mao, Chen, & Gean, 2011; Ugolini, Sokal, Arban, & Large, 2008), and manipulating these receptors can alter both alcohol and anxiety phenotypes (Liu et al., 2016; Lowery et al., 2010; Quoilin & Boehm, 2016; Zorrilla et al., 2002). Given these lines of evidence, finding genes that can regulate $GABA_A$ receptor or CRF1R expression in the BLA could provide insight into the molecular mechanisms of anxiety and alcohol abuse. The LMO family of proteins has emerged as regulators of both anxiety-like behavior (Qin et al., 2015) and substance abuse (Lasek et al., 2010). *Lmo3* is an intriguing target given its expression throughout the brain (Tse et al., 2004), its potential role in both GABAergic and dopaminergic cell development and maintenance (Ang, 2009; Au et al., 2013; Bifsha et al., 2016), and its regulation of several alcohol-related phenotypes (Lasek, Giorgetti, et al., 2011).

This dissertation focuses on the role of the *Lmo3* gene in modulating anxiety and alcohol phenotypes. The primary hypothesis is that *Lmo3* will regulate anxiety-like behavior and alcohol consumption via transcriptional regulation of genes that modulate the GABA and CRF systems. The aims of this dissertation are therefore to (1) characterize the role of *Lmo3* in regulating behavioral responses to alcohol and anxiety-like behavior, (2) determine transcriptional target genes of *Lmo3* that alter behavior, and (3) establish which brain regions are critical for the regulation of behavior by *Lmo3*. The second chapter will therefore focus on the role of *Lmo3* in regulating anxiety-like behavior – putative *Lmo3* expression was first examined in the brains of heterozygous mice (which express the *LacZ* gene, encoding β-galactosidase, from the *Lmo3*

promoter and functions as a reporter for *Lmo3* expression) to elucidate which brain areas showed expression of *Lmo3*. Then *Lmo3* knockout mice were utilized for behavioral testing and gene expression analyses. Finally, viral-mediated knockdown of *Lmo3* was employed in an attempt to replicate the behavioral phenotypes observed in the knockout mouse model in a region-specific manner in adult mice. The third chapter then characterizes the role of LMO3 in regulating binge drinking, a hazardous form of alcohol consumption that increases the risk for alcohol dependence – *Lmo3* knockout mice were once again utilized for behavioral testing and gene expression analyses, and knockdown of *Lmo3* was performed in two different brain areas to attempt to define the area in which *Lmo3* may modulate binge drinking. Finally, the fourth chapter will describe unique ways in which *Lmo3* can function in a sex-specific manner to regulate alcohol-related behaviors, namely ethanol conditioned place preference (a measure of drug reward) and ethanol-induced anxiolysis.

2. LMO3 DRIVES ANXIETY-LIKE BEHAVIOR IN A MOUSE MODEL VIA ITS ACTIONS IN THE BASOLATERAL AMYGDALA

2.1 Introduction

Anxiety disorders carry a heavy cost, both at the personal (Murray et al., 2012) and at the societal level (DuPont et al., 1996), and their occurrence is increasing at an alarming rate (Twenge, 2000). Unfortunately, although several therapeutics are available for treatment, very few show long-lasting efficacy and a significant proportion of individuals fail to respond altogether (Koen & Stein, 2011). One of the limitations in targeting new treatment for anxiety disorders is the lack of understanding of the etiology of these disorders. Although anxiety disorders are highly heritable and several genes have been associated with their occurrence (Lacerda-Pinheiro et al., 2014), few genes have been identified that have proven to aid in treatment outcomes (Serretti et al., 2009). There is, therefore, a great need to identify genes that confer either susceptibility to or resilience from the development of anxiety disorders.

Research into the neurobiology of anxiety has provided insight into its mechanisms. For years it's been known that increasing GABAergic signaling in the brain produces an anxiolytic response (Kalueff & Nutt, 2007), and this effect is especially pronounced in the amygdala (Sanders & Shekhar, 1995), an area of the brain that is primarily known for its regulation of fear conditioning and processing of emotional salience. Specifically, the circuitry connecting the basolateral amygdala (BLA) to the central nucleus of the amygdala (CeA) is particularly crucial to anxiety regulation (Tye et al., 2011). Neuropeptides can play a significant role in regulating the firing patterns of cells throughout the brain, and the neuropeptide corticotropin releasing factor (CRF) has emerged as a key regulator of amygdalar signaling (Silberman & Winder,

2013) and anxiety (Dunn & Berridge, 1990; Stenzel-Poore et al., 1994). CRF is most commonly known for its role in initiating the hypothalamic-pituitary-adrenal (HPA) stress response, but it is also involved in local signaling within the brain and can potentiate the formation of fear learning (Abiri et al., 2014) and anxiety. CRF binds to two receptors, and its actions at the CRF1 receptor (CRF1R) appear to be particularly critical to its effects on anxiety. Mice lacking the CRF1R show an anxiolytic phenotype (Contarino et al., 1999), and CRF1R antagonists administered systemically (Zorrilla et al., 2002) or directly into the amygdala (Sotnikov et al., 2014) reduce anxiety-like behavior. Finding proteins that regulate gene expression of CRF and its receptor in the amygdala may then provide avenues for manipulation of amygdalar signaling and modulation of anxiety symptoms.

LMO3 is one of four LMO proteins that are unique transcriptional regulators involved in cell differentiation and behavior (Zheng & Zhao, 2007). LMO3 is important for central nervous system development (Tse et al., 2004) and plays a role in both interneuron and dopaminergic cell development and maintenance (Ang, 2009; Au et al., 2013; Bifsha et al., 2016). Several LMO proteins have also been associated with drug- and alcohol-related phenotypes, in *Drosophila* (Lasek, Giorgetti, et al., 2011), rodents (Lasek et al., 2010), and humans (Kapoor et al., 2013). LMO3, specifically, has been shown to regulate alcohol consumption and sensitivity to the sedative effects of alcohol (Lasek, Giorgetti, et al., 2011). Recent research has also implicated a closely related member of LMO3, LMO4, in regulation of anxiety (Qin et al., 2015) and fear learning (Maiya et al., 2012) via its actions in the basolateral amygdala. Given the high comorbidity of alcohol abuse and anxiety (Kushner et al., 1990; Merikangas et al., 1998; Regier et al., 1990), the ubiquitous expression of LMO3 throughout the central nervous system (Deng et al., 2006; Tse et al., 2004) and its unique role in regulating interneuron migration and

differentiation (Au et al., 2013; Friocourt & Parnavelas, 2011), LMO3 may be a novel target for regulation of anxiety through its actions in the amygdala.

Here, we sought to identify whether LMO3 regulates anxiety-like behavior utilizing the *Lmo3* null (*Lmo3*^{Z}) mouse, which is viable, fertile, and shows no gross abnormalities (Tse et al., 2004). *Lmo3*^{Z} mice were tested alongside their wild type littermates in two well-validated measures of anxiety-like behavior: the elevated plus maze task and the novelty-induced hypophagia task. In order to elucidate where in the brain *Lmo3* may be acting to influence behavior, we measured reporter gene expression in *Lmo3* heterozygous mice that contain an insertion of the *lacZ* (β-galactosidase) gene in the *Lmo3* locus driven by the endogenous *Lmo3* promoter. Additionally, we examined whether *Lmo3* may regulate expression of genes that are known to be critical for anxiety, namely CRF and its receptor type 1. Finally, to elucidate whether the actions of *Lmo3* are critical to its effects on anxiety-like behavior, we knocked down *Lmo3* in the amygdala of wild type mice using a lentiviral vector expressing a short hairpin RNA (shRNA) targeting *Lmo3*, and tested them in the elevated plus maze. Our results indicate a role for *Lmo3* regulating anxiety-like behavior, specifically via its actions in the basolateral amygdala (BLA) and its regulation of *Crhr1* gene expression.

2.2 Materials and methods

2.2.1 Subjects

Lmo3^{Z} mice containing an *IRES-LacZ* insertion in exon 2 of *Lmo3* have been described previously (Tse et al., 2004). Mice were re-derived from embryonic stem cells and backcrossed 2 generations into the C57BL/6J background for behavioral testing. Adult (10-16 weeks old) male and female homozygous *Lmo3*^{Z} and wild-type littermates were used for behavioral testing and

gene expression experiments. *Lmo3* heterozygous mice were used for β-galactosidase detection. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were group housed with same-sex cage mates (except during the novelty-induced hypophagia task) in a temperature- and humidity-controlled environment under a 14-hour light/dark cycle (lights on at 6 am and off at 8 pm). All behavioral testing took place in the morning, approximately four hours into the light phase. Mice had access to food and water *ad libitum* for the duration of the study and were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures with mice were approved by the University of Illinois at Chicago (UIC) Institutional Animal Care and Use Committee.

2.2.2 Elevated plus maze

The elevated plus maze is widely used as a measure of anxiety-like behavior in rodents. It exploits the natural tendency of rodents to avoid open, exposed spaces as well as heights. The amount of time the animal spends in the open arm is an indirect measure of anxiety, with anxiolytic agents increasing open arm activity. The maze itself consists of four arms (two open and two enclosed by 12 cm high walls) that are 45 cm long and 10 cm wide, elevated approximately 50 cm above the ground. At the start of a trial, the mouse was placed into the center of the maze with its nose directed at the same closed arm, and allowed to freely explore for 10 minutes. The total distance traveled, as well as the amount of time spent in each arm and the number of entries into each arm were digitally tracked and recorded. Additional parameters determined in data analysis included percent entries into each arm relative to total entries.

2.2.3 Novelty-induced hypophagia

Novelty-induced hypophagia is a well-documented phenomenon occurring in rodents and it can be observed without the use of food restriction (Dulawa, 2007). Importantly, when utilized as a behavioral measure in the laboratory, novelty-induced hypophagia exhibits strong predictive validity as a measure of anxiety. Although not as widely utilized as the elevated plus maze task, the novelty-induced hypophagia task has the advantage of allowing for within-subject comparisons for the primary outcome measure, removing the difficulty of between-groups differences that could alter the primary outcome measure (i.e. locomotor activity). Mice were singly housed for 3 days prior to and for the duration of testing in this task. Water bottles were removed from cages and mice receive sipper tubes of diluted sweetened condensed milk (Eagle Brand, diluted in water 1:3) for 30 minutes across 3 training days. The amount of milk consumed in each session was recorded and used to ensure that groups did not differ in consumption levels by the end of training. On the fourth day (home-cage testing), testing was nearly identical to training days, except that the latency to the first lick of milk was measured, and consumption was measured every 5 minutes throughout the 30-minute period. On the fifth day, mice were placed in a "novel cage" – a new, clean cage with bedding removed – for testing. The cage was placed on white paper and a light was shone overhead to enhance the anxiogenic environment. Once again, mice received the sipper tubes containing the sweetened milk solution for 30 minutes and latency and consumption were recorded as in the home cage test environment.

2.2.4 Beta-galactosidase (β- gal) detection

Heterozygous mice were anesthetized with pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) and then 4% paraformaldehyde (PFA). Brains were post-fixed

for 60 minutes in 4% PFA and then placed in PBS-30% sucrose overnight. Sections were cut on a sliding microtome to a thickness of 60 μm and placed into a 12-well plate. Sections were incubated overnight in X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) at 37°C. Sections were then mounted on slides and coverslipped with xylene.

2.2.5 Quantitative polymerase chain reaction (qPCR)

For gene expression experiments, *Lmo3*^{Z} and wild type mice were euthanized by CO_2 inhalation and rapidly decapitated. Brains were removed, rinsed in cold PBS, and sectioned on ice into 1 mm-thick coronal sections using an adult mouse brain matrix (Zivic Instruments, Pittsburgh, PA, USA), from which individual brain areas were punched from the tissue sections using disposable glass Pasteur pipettes. Tissue was immediately frozen on dry ice in 1.5 mL centrifuge tubes and stored at -80°C. RNA was isolated using the GeneJET RNA Purification kit (Thermo Fisher Scientific) and cDNA was synthesized using the Maxima First Strand cDNA Synthesis kit for RT-qPCR (Thermo Fisher Scientific). Quantitative real-time PCR was performed using Maxima Probe qPCR Master Mix (Thermo Fisher Scientific), 20X *Crh* and *Crhr1* probe/primer mixes, and 20X mouse Actin probe/primer mix from Life Technologies (Carlsbad, CA, USA).

2.2.6 Western blotting

Tissue was collected in the same manner as described for qPCR analysis (above). Tissue was homogenized in 100 μl of lysis buffer (1X RIPA) containing protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo Scientific). Protein concentrations were determined using the BCA Protein Assay Kit (Fisher Scientific, Pittsburgh, PA, USA). Equal amounts of protein (30 μg) were subjected to SDS-PAGE in polyacrylamide gels (Novex WedgeWell 10% Tris-Glycine

Gel, Invitrogen, Thermo Fisher Scientific) and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk (for β-Actin) or 5% BSA (CRF1R) in TBST (25 mM Tris-HCl, 137 mM NaCl and 0.1% Tween 20) and incubated with primary antibodies overnight at 4°C (goat polyclonal anti-CRF1, 1:500, Abcam, ab59023; mouse monoclonal anti-β-Actin, Sigma Aldrich A5441, 1:10,000). The membranes were then incubated with HRP-conjugated secondary antibody (BioRad goat anti-mouse, 1:3000; BioRad rabbit anti-goat, 1:1000) at room temperature for 90 minutes and developed with enhanced chemiluminescence (ECL) detection reagents (Pierce ECL Western Blotting Substrate, Thermo Scientific). Band intensities were quantified using NIH Image J software and protein levels were normalized to β-Actin protein for each sample.

2.2.7 Lentiviral construct

Lentivirus expressing short hairpin RNAs (shRNAs) targeting *Lmo3* (shLmo3, 5'-373_GGCUAACCUUAUCCUUUGU-3') or a nonspecific sequence not known to target any gene in the mouse genome (shScr) in the pLL3.7 vector were created and utilized previously by Lasek et al (Lasek, Giorgetti, et al., 2011). Knockdown efficiency was determined by infection of Neuro2A cells. The shLmo3 construct was able to reduce expression of Lmo3 by ~30% compared with the shScr construct (Lasek, Giorgetti, et al., 2011).

2.2.8 Surgical craniotomy procedure

8-10 week old male and female mice were anesthetized with xylazine (8 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.) and placed in a digital stereotaxic alignment apparatus (Model 1900, David Kopf Instruments, Tujunga, CA, USA). After bregma alignment and skull leveling, 0.28 mm diameter holes were drilled bilaterally (A/P: -1.6, M/L: ±3.1) for microinjections of virus.

Mice were randomized to receive either shLmo3 or shScr. A dual cannulae insertion system (33 gauge) was then utilized to target the basolateral amygdala (D/V: -4.8) and 2 µl of virus was infused bilaterally at a rate of 0.2 µl per minute (see Lasek et al for more in-depth methods (Lasek & Azouaou, 2010)). Mice received meloxicam (2 mg/kg) post-surgery and were monitored for two weeks. Behavioral testing began 3 weeks after transfection. A separate group of mice did not undergo behavioral testing, but were instead used to verify in vivo knockdown of the lentivirus. These mice were again allowed to recover for 3 weeks after surgery before they were sacrificed according to the protocol listed above for qPCR analysis using primers to *Lmo3*.

2.2.9 Diaminobenzidine (DAB) staining

In order to verify surgical placements, mice were transcardially perfused with PBS and then 4% PFA. Brains were removed and post-fixed overnight at 4°C in 4% PFA, and were then transferred to PBS-30% sucrose for an additional 24 hours. Brains were mounted with OCT for sectioning on the cryostat and were cut to 50 µm free-floating sections in PBS. The sections were treated with 3% H_2O_2 for 10 minutes followed by 50% ethanol twice, each time for 10 minutes. 10% normal donkey serum in 0.25% Triton was used for blocking (30 minutes) and sections were then incubated with diluted leftover blocking buffer (1:5) in PBS with mouse anti-GFP monoclonal antibody (Life Technologies, A11120) diluted 1:1000 overnight at 4°C. Sections were then incubated with biotin-conjugated horse anti-mouse secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA, BA-2000) for 1 hour at room temperature followed by ABC-Peroxidase solution (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA, PK-6100) for an hour. DAB peroxidase substrate (Vector Laboratories, Burlingame, CA, USA) was then applied for ~1 minute for brown color detection of the GFP immunostaining. Sections were

mounted on gelatin-coated slides and allowed to dry. Counterstaining with cresyl violet was performed and slides were coverslipped with Permount.

2.2.10 Statistical analysis

Unpaired student's t-tests were employed to evaluate elevated plus maze and gene expression (qPCR and Western Blot) data between genotypes. Two-way repeated measures (RM) analysis of variance (ANOVA) was utilized for evaluation of novelty-induced hypophagia data (genotype x cage/day). Post hoc comparisons were performed using the Holm-Sidak test. All statistical analyses were performed using GraphPad Prism software version 6.05 (GraphPad, La Jolla, CA, USA).

2.3 Results

2.3.1 $Lmo3^{Z}$ mice exhibit increased open arm activity in the elevated plus maze

To determine whether *Lmo3* may regulate anxiety-like behavior, we compared $Lmo3^{Z}$ mice to their wild type littermates in the elevated plus maze task. Analysis revealed an increase in open arm activity in the $Lmo3^{Z}$ mice, as evidenced by increased open arm time (Figure 1a, $t(44) = 2.198$, $p < .05$) and a greater percentage of entries into the open arm relative to total entries (Figure 1b, $t(44) = 3.161$, $p < .005$). Because mice will avoid the open arms in this maze, due to an innate fear of open spaces and heights, an increase in open arm activity is interpreted as a reduction in anxiety-like behavior. However, open arm activity is dependent on overall locomotor activity, and $Lmo3^{Z}$ showed an overall reduction of activity relative to wild type mice, in both total entries and distance traveled (Figure 1c, $t(44) = 11.61$, $p < .0001$; Figure 1d, $t(44) = 15.65$, $p < .0001$, respectively). While $Lmo3^{Z}$ mice exhibit increased open arm activity, an effect

that is consistent with reduced anxiety, the results of this task are difficult to interpret alone as there was a pronounced reduction in overall locomotor activity in the *Lmo3*^{Z} mice.

2.3.2 Lmo3^{Z} *mice fail to develop novelty-induced hypophagia*

We next sought an anxiety measure that was independent of locomotor activity in order to determine whether the increased open arm activity of the *Lmo3*^{Z} mice was truly due to an anxiolytic phenotype or just a byproduct of altered locomotion. Most of the traditional anxiety measures for mice are dependent on locomotion (i.e., the open field and light-dark box tasks), so we chose instead to utilize the novelty-induced hypophagia task. While this task still depends on locomotion to some extent, the comparisons are within-subjects for each outcome measure, allowing for any differences in activity to be controlled (i.e., if there is reduced locomotion that is independent of anxiety-like behavior, it will be observed in both the home cage and the novel cage and will not impact primary outcome measures).

Baseline consumption of the sweetened condensed milk solution did not differ between genotypes across the training days, but there was a significant effect of time, with both *Lmo3*^{Z} and wild type mice consuming more sweetened solution across the training period (Figure 2a, Two-way RM ANOVA, Time: $F_{2,60} = 40.54$, $p < .0001$). Genotypes also did not differ in the latency to first lick measure – while both *Lmo3*^{Z} and wild type mice exhibited an increased latency to first lick in the novel cage relative to the home cage, they did so to the same extent (Figure 2b, Time/Cage: $F_{1,30} = 41.75$, $p < .0001$). This change in latency within the novel cage suggests that both groups recognize that they are in a novel environment and spend time exploring before approaching the sipper tubes. However, while the wild type mice showed the expected reduction of feeding in the novel cage relative to the home cage in the first 15 minutes

(Figure 2c, $t(30) = 8.655$, $p < .0001$), *Lmo3*^{Z} mice did not (Figure 2c, $t(30) = 1.978$, *ns*; Time: $F_{1,30} = 51.96$, $p < .0001$; Genotype: $F_{1,30} = 13.74$, $p < .001$; Interaction: $F_{1,30} = 17.99$, $p < .0005$).

The failure to reduce feeding in the novel, anxiogenic environment exhibited by the *Lmo3*^{Z} mice suggests that they are resistant to induction of anxiety. These results, together with the elevated plus maze results, suggest that *Lmo3*^{Z} mice exhibit a reduction in anxiety-like behavior relative to wild type littermates.

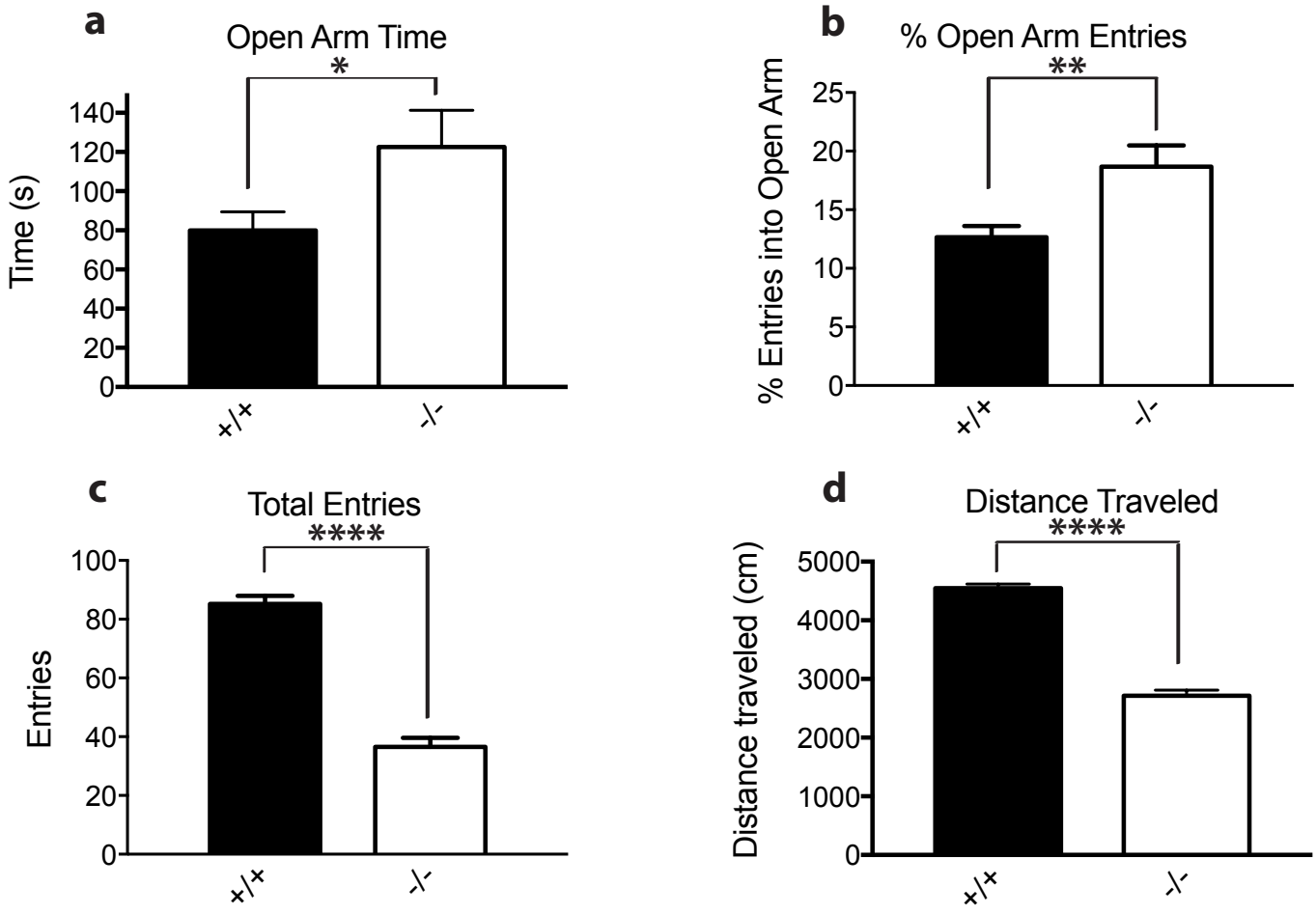


Figure 1. Increased open arm activity and reduced locomotion in *Lmo3*^{Z} mice

Lmo3^{Z} (-/-, white bars, n = 19) and wild type littermates (+/+, black bars, n = 28) were tested in the elevated plus maze. (a) *Lmo3*^{Z} mice spent significantly more time in the open arms than wild type mice, $p = .03$, and (b) had a greater number of entries into open arms relative to total entries (portrayed as percent open arm entries, $p = .0002$). However, *Lmo3*^{Z} mice also exhibited a significant reduction in locomotor activity compared to wild type mice, as measured by (c) total entries ($p < .0001$) and (d) total distance traveled ($p < .0001$).

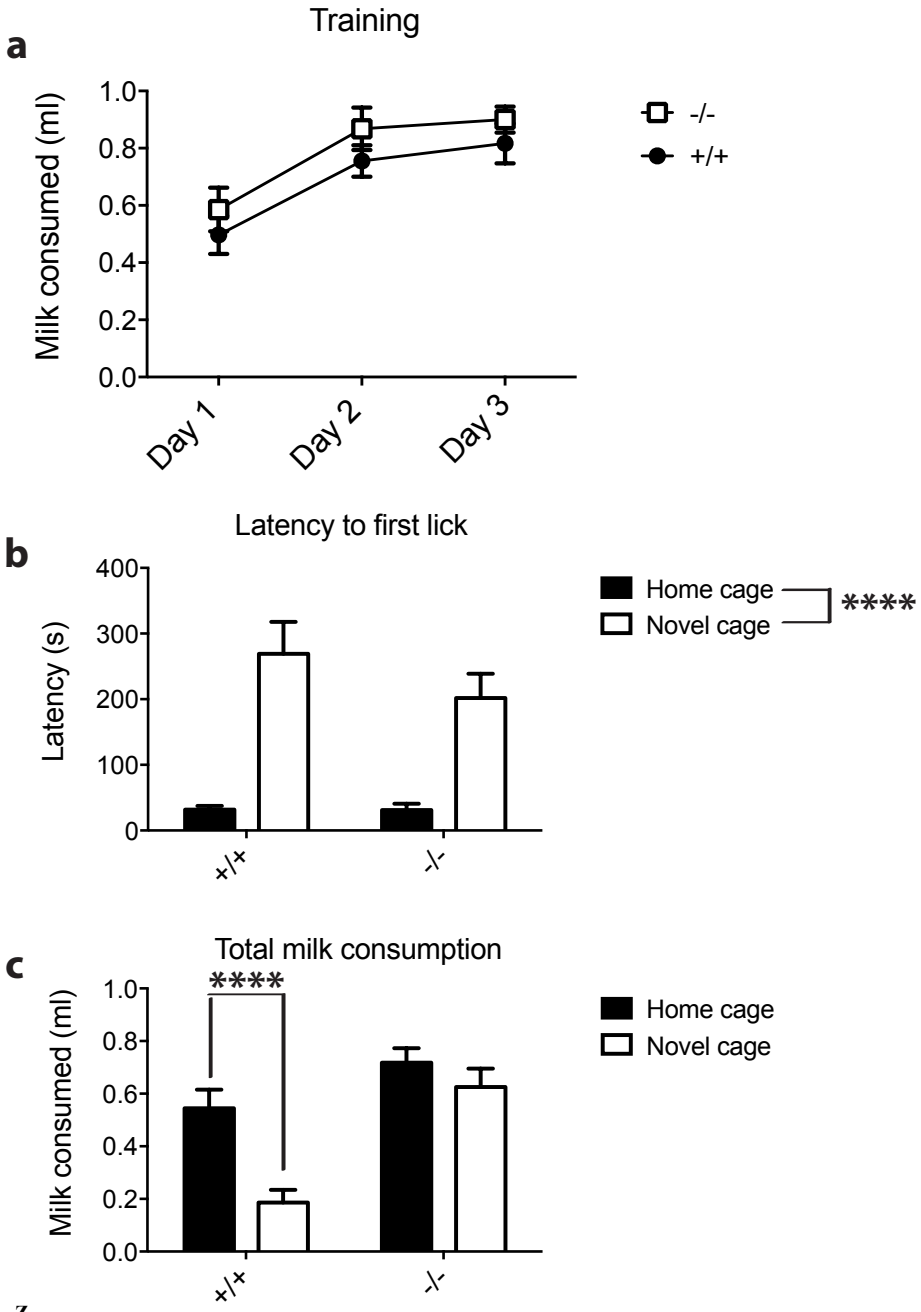


Figure 2. *Lmo3*^{Z} mice exhibit reduced anxiety-like behavior in the novelty-induced hypophagia task

Lmo3^{Z} (-/-, n = 14) and wild type littermates (+/+, n = 18) were tested in the novelty-induced hypophagia task. (a) No differences were observed in baseline milk solution consumption between genotypes. (b) All mice exhibited a greater latency to consume milk in the novel cage relative to the home cage ($p < .0001$), but no difference was observed between genotypes. (c) Although wild type mice exhibited the exhibited hypophagia behavior in the novel cage relative to the home cage ($p < .0001$), *Lmo3*^{Z} mice showed no reduction of feeding in the novel environment.

2.3.3 Lmo3 shows dense expression patterns in subcortical areas

In order to determine where *Lmo3* may be acting to produce this anxiolytic phenotype, we sought to characterize in which brain regions *Lmo3* is expressed. *Lmo3*^{Z} mice contain an insertion of the *LacZ* gene in exon 2 of *Lmo3* and therefore express β-gal from the endogenous *Lmo3* promoter. To characterize the putative expression of *Lmo3* in mouse brain, adult heterozygous mice were examined for β-gal reporter expression. β-gal was widely detected throughout the brain, predominantly in subcortical areas. Expression was strongest in the nucleus accumbens, caudate putamen, piriform cortex, hippocampus, hypothalamus, and most notably, specific nuclei of the amygdala (Figure 3). Amygdala staining was particularly strong in the lateral olfactory tract and basolateral nucleus, consistent with *Lmo3* expression in the embryonic mouse amygdala (Remedios, Subramanian, & Tole, 2004). In the hippocampus, we observed intense staining in the CA1 and dentate gyrus regions, with lighter staining in the CA3 region. Hypothalamic staining was most prominent in the ventromedial hypothalamus and paraventricular nucleus. β-gal was also detected in the septum, habenula, superior colliculus, interpeduncular nucleus, cortex, olfactory tubercle, ventral pallidum, and substantia nigra (data not shown). These results are consistent with prior published work examining *Lmo3* mRNA expression in the brain using *in situ* hybridization (Hinks et al., 1997).

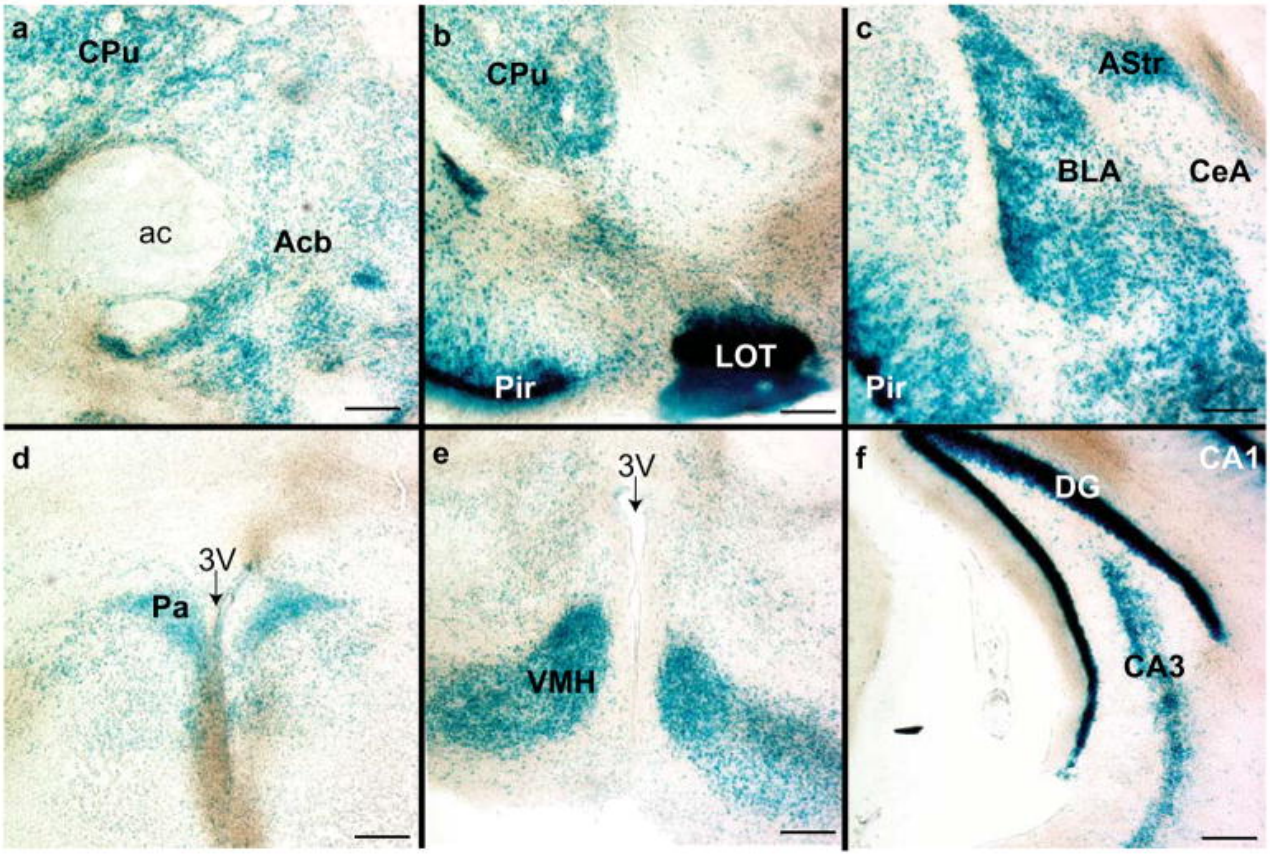


Figure 3. β-gal reporter expression in the brains of heterozygous *Lmo3*^{Z} mice

X-Gal staining of coronal brain sections reveals robust β-gal expression in the (a) caudate putamen (CPu) and nucleus accumbens (Acb), (b) piriform cortex (Pir) and lateral olfactory tract (LOT), (c) basolateral amygdala (BLA) and amygdalostriatal transition area (AStr), (d) paraventricular hypothalamic nucleus (Pa), (e) ventromedial hypothalamic nucleus (VMH), and (f) hippocampus, dentate gyrus (DG), CA1 and CA3 regions. Abbreviations: ac, anterior commissure; 3V, 3rd ventricle, CeA, central nucleus of the amygdala. Scale bar, 200 microns.

2.3.4 Lmo3^{Z} mice express reduced Crhr1 mRNA and protein in the BLA

Due to the dense β-gal staining that was observed in the BLA, we postulated that LMO3 may have an important function in this brain region. Because the BLA plays such a prominent role in regulating anxiety, we sought to determine whether LMO3 may be regulating transcription of genes in the BLA that underlie anxiety-like behavior – i.e., *Crh* (encoding CRF) and *Crhr1* (encoding the CRF receptor CRF1R). To examine this question, we collected amygdala tissue from the brains of *Lmo3*^{Z} and wild type mice for analysis of mRNA (by qPCR) and protein levels (by Western blotting). In addition to the BLA, the central nucleus of the amygdala (CeA) was collected (*Lmo3* expression was sparse in the CeA, but sparse expression does not preclude regulation of relevant signaling).

We observed a significant reduction of *Crhr1* mRNA in *Lmo3*^{Z} mice relative to wild type mice, in both the BLA (Figure 4a, $t(42) = 3.059$, $p < .005$) and the CeA (Figure 4c, $t(33) = 2.827$, $p < .01$). No difference was observed in *Crh* mRNA in the BLA (Figure 4b, $t(44) = 1.648$, *ns*) or CeA (Figure 4d, $t(34) = 0.2547$, *ns*). We next sought to determine whether these changes in transcriptional activity were indicative of functional changes in CRF1R by examining protein expression in the *Lmo3*^{Z} and wild type mice via Western blotting. In line with the transcriptional changes observed, protein expression of CRF1R was also reduced in the BLA of *Lmo3*^{Z} mice relative to wild type mice (Figure 5a, $t(20) = 2.259$, $p < .05$), but there was no significant reduction of CRF1R protein expression observed in the CeA in *Lmo3*^{Z} mice (Figure 5b, $t(23) = 0.8272$, *ns*). These results suggest that *Lmo3* regulates transcription of the *Crhr1* gene in the BLA that has direct effects on functional output of the CRF1R protein in this brain region.

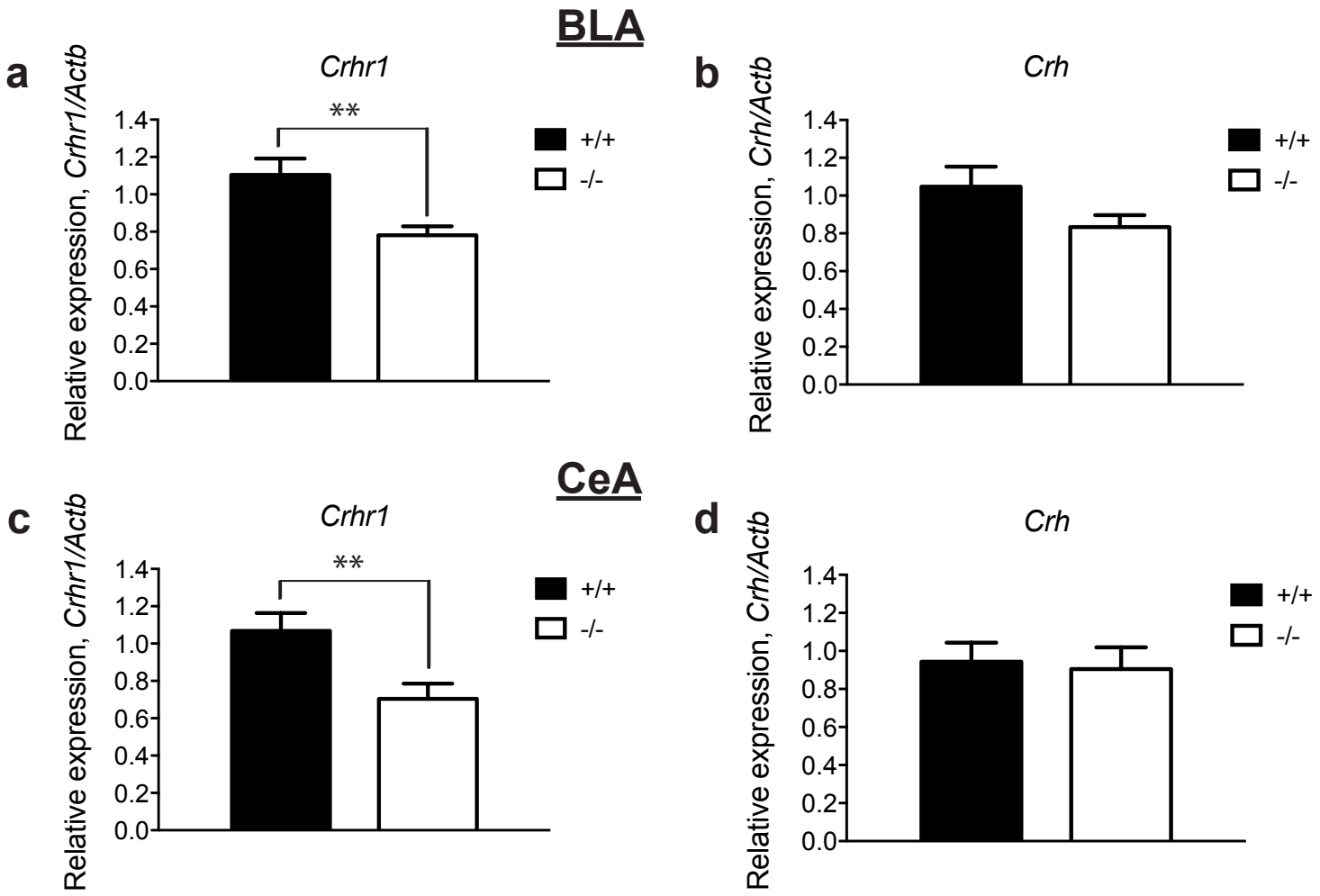


Figure 4. Alterations in *Crhr1* mRNA expression in amygdala of *Lmo3^{Z}* mice

Amygdala (BLA and CeA) tissue was collected from *Lmo3^{Z}* (-/-, white bars) and wild type (+/+, black bars) mice for qPCR analysis. *Lmo3^{Z}* mice have reduced *Crhr1* mRNA expression in both the (a) BLA (p = .003, n = 20) and (c) CeA (p = .007, n = 16) relative to wild type mice (n = 24 and 19, respectively). There was no difference in *Crh* mRNA in (b) the BLA of *Lmo3^{Z}* mice (p = .11, n = 21) or (d) in the CeA (p = 80, n = 17) relative to wild type mice (n = 25 and 19, respectively).

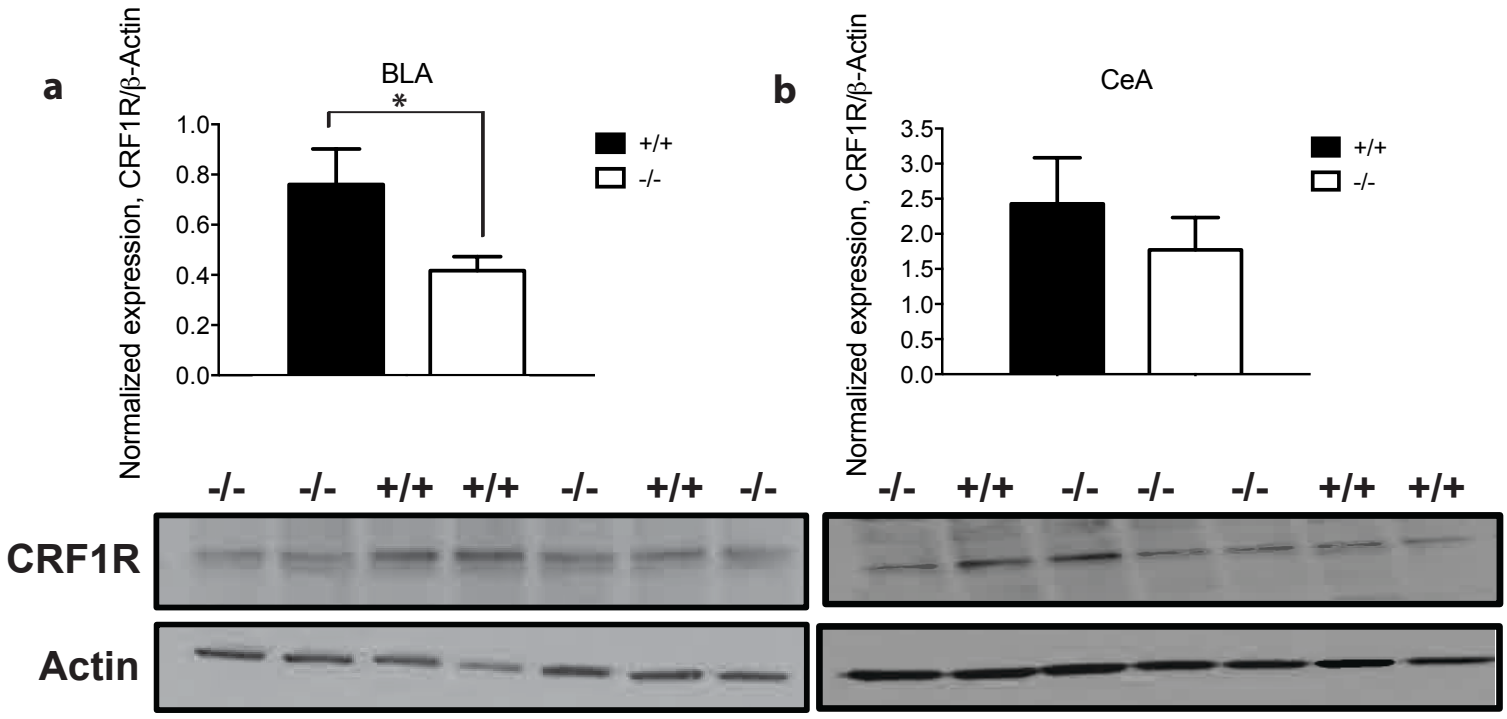


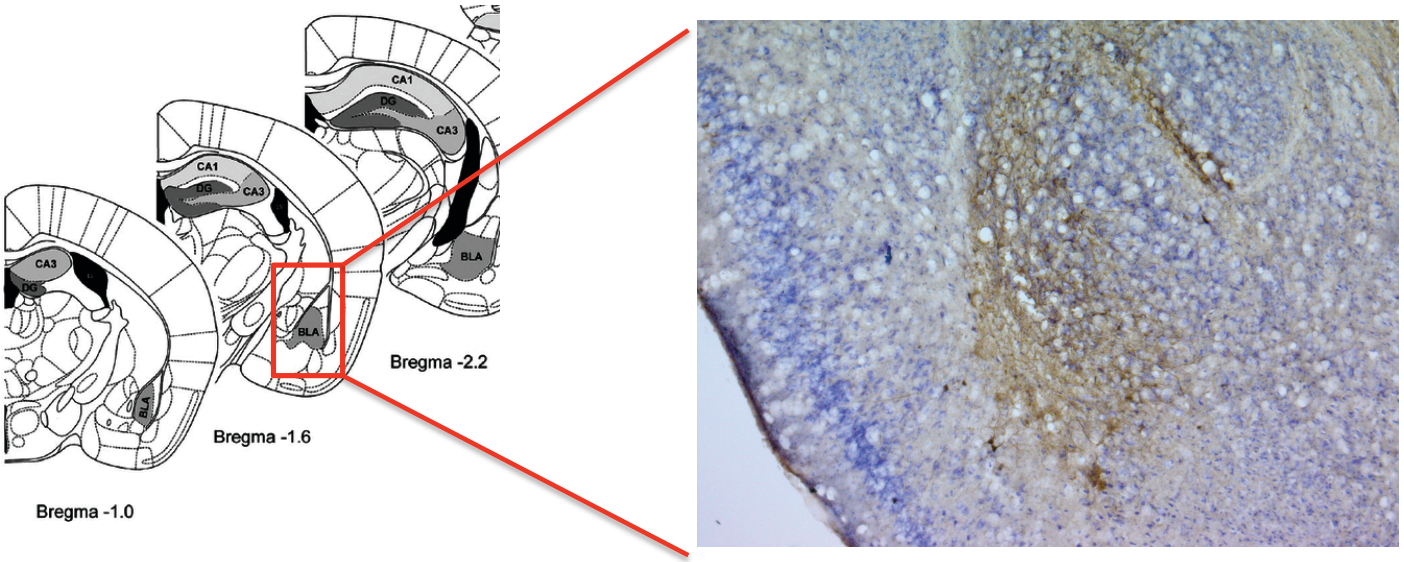
Figure 5. Reduction of CRF1R protein in the BLA of *Lmo3^{Z}* mice

Amygdala (BLA and CeA) tissue was collected from *Lmo3^{Z}* (-/-, white bars) and wild type (+/+, black bars) mice for Western Blot analysis. (a) *Lmo3^{Z}* mice have reduced CRF1R protein expression in the BLA (p = .04, n = 9) relative to wild type mice (n = 13), (b) but there was no significant difference between *Lmo3^{Z}* mice (n = 13) and wild type mice (n = 12) in CRF1R protein expression in the CeA (p = .42).

2.3.5 Knockdown of Lmo3 in the basolateral amygdala reduces anxiety-like behavior

To determine whether the anxiolytic phenotype observed in *Lmo3*^{Z} mice was due to the actions of *Lmo3* in the BLA, we utilized viral-mediated RNA interference to knock down expression of *Lmo3* in the BLA of adult C57BL/6 mice. After three weeks of recovery, mice were tested in the elevated plus maze. Increased open arm time (Figure 6a, $t(21) = 2.099$, $p < .05$) and percent open arm entries (Figure 6b, $t(21) = 2.201$, $p < .05$) were observed in the mice that received shLmo3 relative to those who received the control virus, replicating the anxiolytic phenotype observed in *Lmo3*^{Z} mice. Contrary to *Lmo3*^{Z} mice, however, knockdown of *Lmo3* in the BLA of adult mice had no effect on locomotor activity (Figure 6c, $t(21) = 0.298$, ns; Figure 6d, $t(21) = 0.0516$, *ns*), suggesting that the locomotor deficit observed in *Lmo3*^{Z} mice was either due to actions of *Lmo3* in a different brain region or an effect of *Lmo3* during development. Additionally, the anxiolytic phenotype produced via *Lmo3* knockdown was specific to the BLA – mice with knockdown of *Lmo3* in the nearby CeA showed no significant alteration in anxiety-like behavior (Figure 7a, $t(4) = 0.7173$, *ns*; Figure 7b, $t(4) = 0.194$, *ns*).

In vivo knockdown was verified in a separate group of mice that did not undergo behavioral testing. Transfection with shLmo3 led to ~25% reduction of *Lmo3* mRNA in the BLA (Figure 6e, $t(7) = 2.128$, $p < .10$), similar to the 30% reduction observed in vitro (Lasek, Giorgetti, et al., 2011). To test whether the anxiolytic effects of *Lmo3* knockdown in the BLA were due to transcriptional changes in CRF1R, we also measured *Crhr1* mRNA expression in the same tissue used to verify in vivo knockdown. Contrary to our expectations, no differences in *Crhr1* mRNA were observed between shLmo3 and shScr mice (Figure 6f, $t(7) = 0.3756$, *ns*).


CA1
DG
CA3
BLA
Bregma -2.2
Bregma -1.6
Bregma -1.0

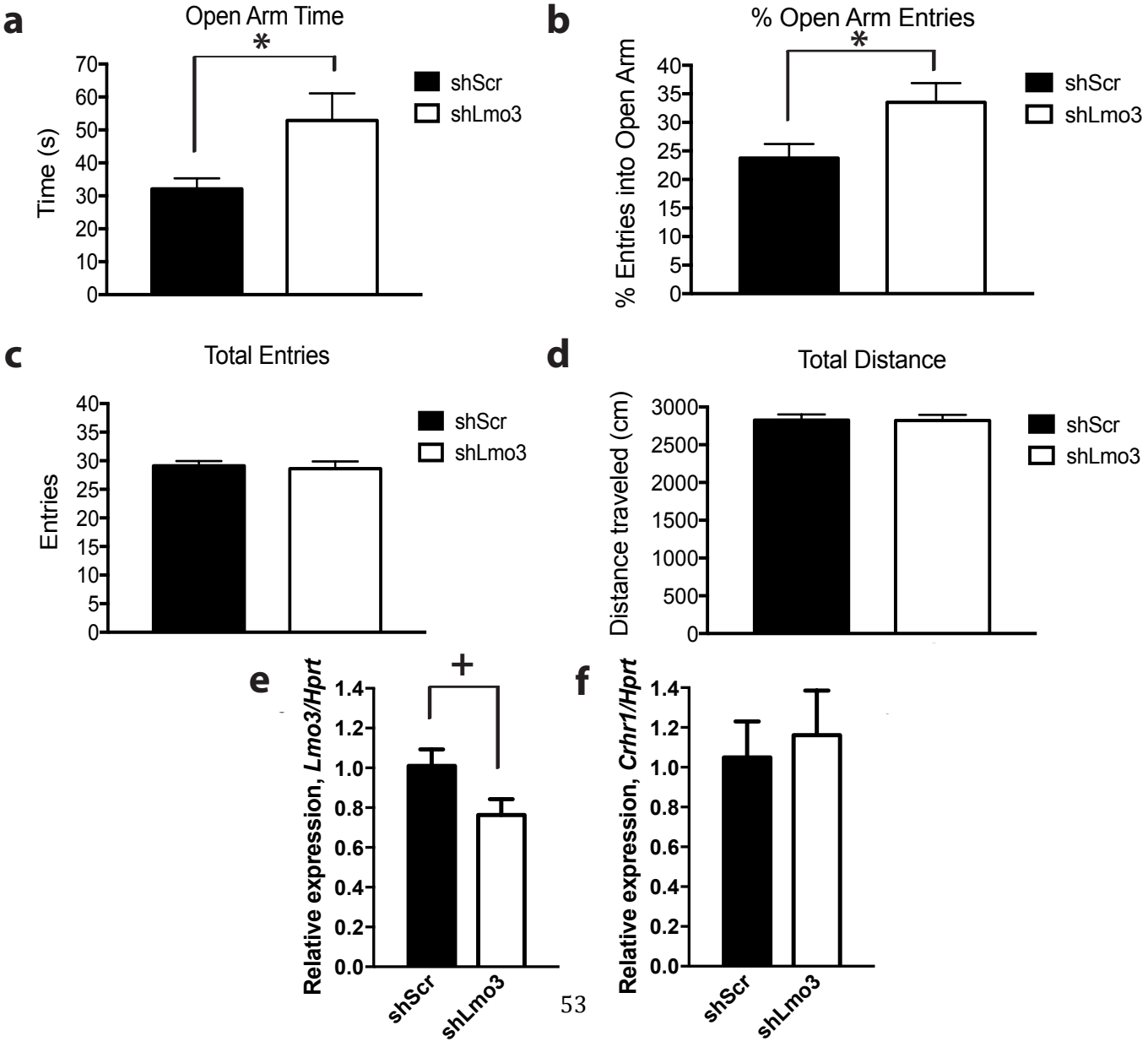

a
Open Arm Time
Time (s)
shScr
shLmo3
b
% Open Arm Entries
% Entries into Open Arm
c
Total Entries
Entries
d
Total Distance
Distance traveled (cm)
e
Relative expression, Lmo3/Hprt
f
Relative expression, Crhr1/Hprt

Figure 6. Knockdown of *Lmo3* in the BLA reduces anxiety-like behavior in the elevated plus maze

Lentiviral-mediated RNA interference was utilized to knockdown *Lmo3* in the BLA of adult C57BL/6J mice and mice were subsequently tested in the elevated plus maze. Mice who received the shLmo3 virus (n = 13) exhibited increased open arm activity, as measured by (a) open arm time (p = .03) and (b) percent entries into the open arm (p = .03), relative to mice who received a control virus (n = 10). No difference was observed between groups in either the (c) total number of entries or (d) distance traveled. (e) qPCR analysis revealed a 25% knockdown of *Lmo3* mRNA in BLA tissue of mice who received the shLmo3 virus (n = 5) compared to those who received the control virus (n = 4, p = .07). No change was observed in *Crhr1* mRNA expression upon transfection with shLmo3.

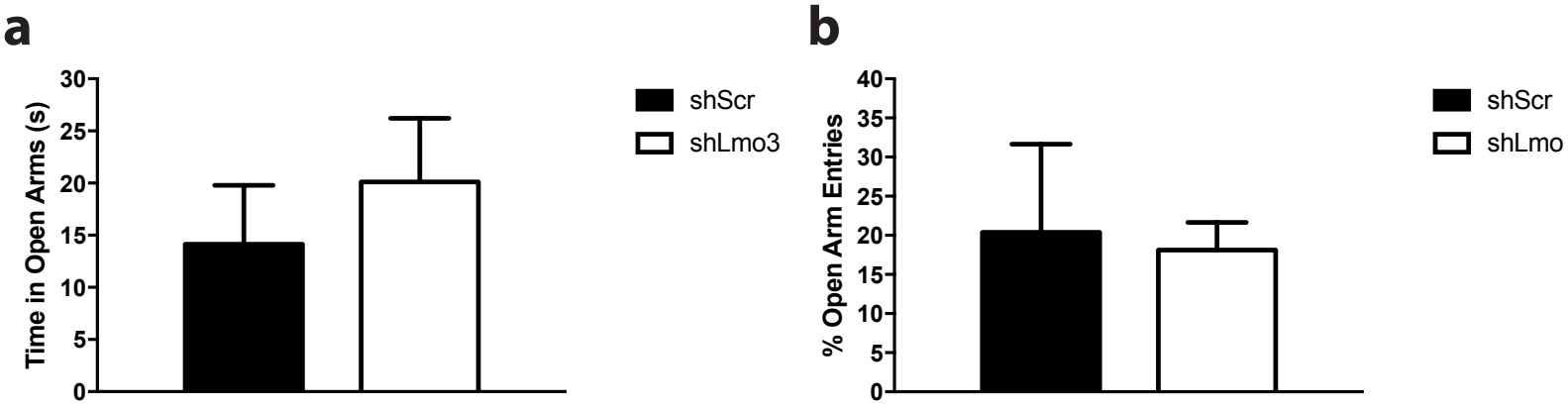


Figure 7. Knockdown of *Lmo3* in the CeA has no effect on anxiety-like behavior in the elevated plus maze

Lentiviral-mediated RNA interference was utilized to knockdown *Lmo3* in the CeA of adult C57BL/6J mice and mice were subsequently tested in the elevated plus maze. No difference in either (a) open arm time or (b) percent entries into the open arm were observed between groups (n = 6).

2.4 Discussion

These results suggest a novel role for the transcriptional regulator *Lmo3* in the control of anxiety-like behavior, specifically via its actions in the basolateral amygdala (BLA). Previous work has shown that *Lmo4* regulates anxiety-like behavior through its actions in BLA glutamatergic neurons (Qin et al., 2015), but no one had yet examined whether other LMO proteins were also implicated in anxiety. Herein, we demonstrate that $Lmo3^{Z}$ mice display an anxiolytic-like phenotype that can be replicated by knocking down *Lmo3* in the BLA of adult C57BL/6J mice. $Lmo3^{Z}$ mice also have a reduction of CRF1R mRNA and protein in the BLA that may be partially responsible for the effects of *Lmo3* on regulation of anxiety-like behavior, although this has yet to be demonstrated conclusively. Collectively, these data shed new light on the role of *Lmo3* in regulating behavior in the adult mouse and suggest a novel genetic target for modulation of anxiety-like behavior.

It is well established that LMO3 regulates cell differentiation in the central nervous system, but not much is known about how it functions in the adult brain. One limitation of working with a global knockout mouse model is the inability to differentiate behavioral changes that are due to the lack of protein acutely in the adult mouse and the lack of protein throughout development. One possible explanation for the reduced anxiety phenotype observed in the $Lmo3^{Z}$ mice is that *Lmo3* regulates differentiation of neuronal cells that creates structural abnormalities in the adult mouse that subsequently alter behavior. Indeed, the decrease in parvalbumin-positive interneurons that has been documented in the $Lmo3^{Z}$ mouse lends support to this hypothesis (Au et al., 2013). An advantage then to utilizing RNAi is the ability to distinguish between loss of protein in the adult mouse and the lack of protein through development. Another important advantage of RNAi is the targeting of a single brain area – knockout mice lack *Lmo3* expression globally, so no conclusions

can be drawn about which brain regions it operates in to influence anxiety-like behavior. However, here we were able to demonstrate that in mice lacking the LMO3 protein from conception, an anxiolytic phenotype emerges in adulthood that can be replicated by knocking down *Lmo3* expression solely in the adult mouse BLA.

Evidence that CRF1R regulates anxiety is robust. The reduction of CRF1R mRNA and protein expression in *Lmo3*^{Z} mice relative to wild type mice, specifically in the BLA where CRF1R activation has been shown to induce anxiety, is suggestive of a transcriptional mechanism by which *Lmo3* could regulate anxiety-like behavior. However, knocking down *Lmo3* in the BLA had no effect on CRF1R mRNA expression. At this time, it is unknown how *Lmo3* is regulating transcription of the *Crhr1* gene. As a transcriptional regulator, *Lmo3* may be promoting transcription of the *Crhr1* gene by binding co-activator proteins and shuttling them to the transcriptional complex, or alternatively, *Lmo3* may be binding co-repressor proteins and sequestering them away from the transcriptional start site to allow transcription to proceed unimpeded. In our model, the shLmo3 virus only produced about a 25% reduction in *Lmo3* mRNA expression. It is possible that this knockdown was not robust enough for us to observe alterations in *Crhr1* transcription given the signal-to-noise ratio in this analysis. Alternatively, it may be that the *Lmo3* reduction produced an initial decrease in *Crhr1* transcription (and a subsequent decrease in CRF1R protein expression) that then caused a compensatory increase in transcriptional activity in the cells' remaining *Lmo3*. This would result in decreased CRF1R protein (that could then decrease anxiety-like behavior) without producing a coincident decrease in *Crhr1* mRNA. Due to the limitations in collecting tissue (namely that we are only able to detect expression at a single time point), we were unable to measure both mRNA and protein expression in the mice that underwent surgery. We chose to measure mRNA levels rather than protein expression in our

samples, but future work should examine the effect of *Lmo3* knockdown in the BLA on CRF1R protein to explore this possible mechanism.

An alternative explanation for the lack of *Crhr1* mRNA expression change with shLmo3 transfection is that *Lmo3* may also be acting via a different mechanism to impact behavior in the BLA. This does not preclude a direct role for *Lmo3* in regulating anxiety via *Crhr1*, but rather suggests that the effects of *Lmo3* may not be restricted to its transcriptional regulation of *Crhr1*. Indeed, the majority of parvalbumin-positive interneurons in the BLA express CRF1R (Calakos, Blackman, Schulz, & Bauer, 2017), so the reduction of CRF1R in *Lmo3*^{Z} mice could potentially be due to reductions in the number of parvalbumin-positive interneurons observed in these mice, and may have little bearing on their anxiolytic phenotype. Instead, given the role for *Lmo3* in cortical interneuron migration and differentiation, *Lmo3* may additionally be altering the balance between glutamatergic and GABAergic signaling in the BLA. Activation of BLA neurons has been shown to induce both anxiogenic and anxiolytic responses, a seemingly contradictory effect that is likely due to which neurons are being activated and to which regions these neurons project. Activation of BLA outputs to the ventral hippocampus has been shown to be anxiogenic (Felix-Ortiz et al., 2013), while activation of BLA outputs to the CeA produces an anxiolytic response (Tye et al., 2011), and both of these responses were bidirectional (with inhibition of the BLA outputs producing the reverse phenotype). Further, while the BLA is composed of primarily glutamatergic projection neurons, modulation of the GABAergic cells also bidirectionally alters anxiety. Inhibiting only GABAergic cells within the BLA will increase anxiety-related behaviors (Diaz, Chappell, Christian, Anderson, & McCool, 2011), while activating these cells produces an anxiolytic response (Bi et al., 2015). Up-regulation of $GABA_A$ receptors and down-regulation of the GluR1 subunit in AMPA and NMDA receptors in the BLA has been associated with reduced

anxiety (S. B. Liu et al., 2015), so it is possible that *Lmo3* could be acting to regulate transcription of $GABA_A$ receptors in BLA projection neurons, making them less susceptible to inhibition by GABAergic interneurons and subsequently increase BLA output. In this framework, it is also conceivable that the shLmo3 virus preferentially targeted GABAergic neurons to reduce *Lmo3* mRNA in a subset of cells. The reduction of *Lmo3* may, in fact, have decreased *Crhr1* expression significantly in these cells to alter inhibition, but the effect was masked by the greater number of projection neurons in the BLA that did not show a reduction of *Lmo3* expression.

The CeA also plays a critical role in anxiety and is under tight regulation by BLA input. In addition to reductions of CRF1R in the BLA, $Lmo3^Z$ mice had reduced *Crhr1* mRNA expression in the CeA. Although we did not observe a corresponding decrease in CRF1R protein expression in the CeA of $Lmo3^Z$ mice, the reduction in transcription could be indicative of an impairment of the CRF1R protein function in this region (i.e., deficient recycling or trafficking of the receptor). This could in fact be evidence of impaired input from the BLA. If $Lmo3^Z$ mice have reductions in BLA output via GABAergic manipulation, the CeA may be receiving fewer excitatory inputs that would trigger an anxiolytic response (Tye et al., 2011).

Taken together, the current data highlight a novel role for *Lmo3* in regulating anxiety-like behavior via its actions in the BLA, although the mechanism by which it does so remains unclear. $Lmo3^Z$ mice show a reduction of CRF1R expression in the BLA that may be indicative of altered signaling in this brain region that could change behavior. These data reinforce the role of the BLA in regulating anxiety, but suggest that CRF1R may not, on its own, be critical for this anxiety regulation. *Lmo3* may be a novel contributor to the etiology of anxiety disorders, and better understanding its role in the regulation of amygdalar signaling and stress-related networks could provide new discoveries for effective therapeutics.

3. LMO3 INHIBITS EXCESSIVE ALCOHOL INTAKE IN A DRINKING-IN-THE-DARK PARADIGM (ADAPTED FROM SAVARESE ET AL, GENES BRAIN BEHAVIOR 2014)

3.1 Introduction

LMO3 is highly expressed in the mammalian central nervous system during development and in the adult (Bulchand, Subramanian, & Tole, 2003; Hinks et al., 1997; Remedios et al., 2004; Tse et al., 2004), yet the specific role that LMO3 plays in adult brain function remains largely unknown. Recent work has suggested a role for *Lmo3* in regulating anxiety-like behavior – mice with null mutations for *Lmo3* exhibit increased open arm activity in the elevated plus maze and fail to show reduced feeding in novel environments, measured by the novelty-induced hypophagia task. Further, these mice show dysregulation of the CRF system in the basolateral amygdala (BLA) and knocking down *Lmo3* expression in the BLA of wild type C57BL/6J mice replicates the anxiolytic phenotype observed in the *Lmo3* null ($Lmo3^{Z}$) mice.

Alcohol and anxiety have similar neurobiological bases and are often comorbid. In addition to its role in regulating anxiety, *Lmo3* has previously been associated with two behavioral responses to alcohol – the loss of righting reflex (LORR) task, a measure of sensitivity to the sedative effects of alcohol, and alcohol consumption in the 2-bottle choice task (Lasek, Giorgetti, et al., 2011; Savarese, Zou, Kharazia, Maiya, & Lasek, 2014). The *Drosophila* homolog of the *Lmo3* gene, *dLmo*, has been shown to regulate alcohol sedation sensitivity (Lasek, Giorgetti, et al., 2011), as well as acute sensitivity to cocaine (Tsai et al., 2004), suggesting an evolutionarily conserved role for LMO proteins in regulating behavioral responses to drugs of abuse. Additionally, two transcription factors that regulate *Lmo3* expression, USF and MZF1, have

altered activity after alcohol exposure (Driver et al., 2015; Freeman et al., 2013), supporting a role for *Lmo3*-related regulatory pathways in the neuroadaptations associated with alcohol use.

Binge drinking, a pattern of drinking that brings an individual's blood alcohol concentration (BEC) to 0.08 g/dl, is both prevalent and dangerous – this pattern of drinking has been associated with an increased risk of experiencing alcohol-related problems and of developing an AUD (Jennison, 2004) and is a necessary component of the addiction cycle (Koob & Volkow, 2010). Understanding the genetic vulnerability to binge drinking behavior could aid in the understanding of alcohol use disorder etiology, yet the molecular mechanisms that drive this particular pattern of excessive alcohol consumption are just beginning to be elucidated. CRF, acting through CRF receptor 1 (CRF1R), has been shown to be critical in regulating binge-drinking behavior (Kaur et al., 2012; Lowery et al., 2010; Treutlein et al., 2006), and $Lmo3^{Z}$ mice show reductions of CRF1R protein expression, suggesting a potential role for *Lmo3* in the regulation of binge drinking via its transcriptional control of *Crhr1* expression.

In addition to its role in regulating anxiety, the BLA is also important for binge drinking (Marshall et al., 2016). The BLA sends glutamatergic input to the CeA, and inhibition of glutamate receptors in the CeA reduces binge alcohol intake (Cozzoli et al., 2014). Although CRF1R regulates binge drinking in the CeA, it does not appear to do so in the BLA (Lowery-Gionta et al., 2012), suggesting that the CRF inputs from the BLA to the CeA are critical for this phenotype. Interestingly, while knocking down *Lmo3* in the BLA replicated the phenotype observed in $Lmo3^{Z}$ mice, knockdown did not replicate the reduction of *Crhr1* transcription observed in $Lmo3^{Z}$ mice, suggesting that *Lmo3* regulated anxiety in a manner at least partially independent of CRF1R expression. Beyond CRF1R activation, an additional molecular mechanism underlying both anxiety and alcohol-related behaviors is altered GABAergic

signaling (Centanni et al., 2014; Chandra et al., 2005; Coffman & Petty, 1985; Dias et al., 2005; Liang et al., 2009; Q. Shen et al., 2012), and *Lmo3* has been shown to regulate GABAergic cell differentiation (Au et al., 2013). One of the primary receptors for GABA, the $GABA_A$ receptor, is composed of five subunits, the specific combination of which allows for remarkable heterogeneity in function and distribution (Ferando & Mody, 2014; Nuss, 2015). Regulation of $GABA_A$ receptors within the BLA has been associated with both anxiety (S. B. Liu et al., 2015) and alcohol exposure (Herman et al., 2016; Liang et al., 2006; Lindemeyer et al., 2014).

A region rich in GABAergic neurons, the nucleus accumbens (NAc) is also critical to binge drinking (Cozzoli et al., 2012). As may be expected, GABA signaling in the NAc has been shown to be especially critical for modulation of binge-drinking behavior (Kasten & Boehm, 2014). Chronic exposure to alcohol increases extracellular GABA in the NAc, which is associated with a sensitized glutamate response to alcohol (Szumlinski et al., 2007). Intriguingly, in addition to its dense expression in the BLA, *Lmo3* expression is also robust in the NAc, and C57BL/6J mice exhibit a negative correlation between binge-like alcohol consumption and *Lmo3* expression in the NAc 24-hours after the last drinking session (Savarese et al., 2014).

Given its role in regulating both CRF1R expression and GABAergic cell development (Au et al., 2013; Friocourt & Parnavelas, 2011), its dense expression in areas of the brain critical to binge drinking (the BLA and NAc), and its regulation of several alcohol-related phenotypes, *Lmo3* is a prime candidate for regulation of binge drinking behavior. To examine directly whether *Lmo3* regulates excessive alcohol consumption, we tested $Lmo3^Z$ mice in drinking in the dark (DID), a model of binge drinking behavior. We also measured sucrose consumption under the same parameters of the DID task to examine whether any altered consummatory behavior in $Lmo3^Z$ mice would extend to naturally rewarding substances, or whether the effects are specific

to alcohol. To begin to understand how LMO3 might function in the brain to regulate binge drinking behavior, we examined $GABA_A$ receptor subunit expression in *Lmo3*^{Z} mice in the amygdala and NAc, areas of the brain that show dense expression of *Lmo3* as well as areas important for binge drinking (Cassataro et al., 2014; Marshall et al., 2016). Lastly, we sought to determine where in the brain *Lmo3* is acting to regulate binge drinking by knocking down *Lmo3* in the NAc and in the BLA and testing these mice in the DID task.

3.2 Materials and Methods

3.2.1 Subjects

Adult (10-16 weeks old) male and female homozygous *Lmo3*^{Z} and wild-type littermates were used for behavioral testing and gene expression experiments. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were group housed with same-sex cage mates in a temperature- and humidity-controlled environment under a 14-hour light/dark cycle (lights on at 6 am and off at 8 pm) and tested during the light phase, unless they underwent the drinking-in-the-dark procedure. Mice had access to food and water *ad libitum* for the duration of the study and were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the University of Illinois at Chicago (UIC) Institutional Animal Care and Use Committee.

3.2.2 Drinking in the Dark (DID)

The DID model of alcohol consumption is a limited-access paradigm wherein mice are allowed ethanol access only during the dark cycle, when they are most active. The combination of limited access to ethanol (water bottles are removed and mice have only 2-4 hours of access to ethanol per day) and access during the dark cycle reliably produces pharmacologically

meaningful (i.e. intoxicating) blood ethanol concentrations, making this model a particularly good model for binge-like, excessive alcohol consumption.

Mice were individually housed in a reverse dark cycle room (lights off at 10 am and on at 10 pm) for at least two weeks prior to testing in order to acclimate to changes in circadian rhythm. Mice were then tested for 4-day DID as described in Rhodes et al (Rhodes, Best, Belknap, Finn, & Crabbe, 2005) using a 20% ethanol solution. Sipper tubes containing ethanol were given 3 hours into the dark cycle for 2 hours on Monday, Tuesday, and Wednesday and 4 hours on Thursday. A separate group of mice underwent a similar protocol with a 10% sucrose solution rather than ethanol to examine sucrose consumption. These mice had 2-hour access to sucrose across all four testing days.

3.2.3 Measurement of blood ethanol concentrations (BECs)

Immediately after the final session of the DID, blood (20 mcl) was collected in heparinized capillary tubes via tail vein puncture. Blood samples were stored at -80^{o}C until BECs were determined using an NAD-ADH enzymatic assay as described in Zapata et al (Zapata, Gonzales, & Shippenberg, 2006).

3.2.4 Quantitative polymerase chain reaction (qPCR)

For gene expression experiments, *Lmo3*^{Z} and wild type mice were euthanized by CO_2 inhalation and rapidly decapitated. Brains were removed, rinsed in cold PBS, and sectioned on ice into 1 mm-thick coronal sections using an adult mouse brain matrix (Zivic Instruments, Pittsburgh, PA, USA), from which individual brain areas were collected using disposable glass Pasteur pipettes. Tissue was immediately frozen on dry ice in 1.5 mL centrifuge tubes for storage at -80°C. RNA was isolated using the GeneJET RNA Purification kit (Thermo Fisher Scientific)

and cDNA was synthesized using the Maxima First Strand cDNA Synthesis kit for RT-qPCR (Thermo Fisher Scientific). Quantitative real-time PCR was performed using Maxima Probe qPCR Master Mix (Thermo Fisher Scientific), *Gabra1*, *Gabra4*, and *Gabrd* primers, and 20X mouse Actin probe/primer mix from Life Technologies (Carlsbad, CA, USA).

3.2.5 Western blotting

Tissue was collected in the same manner as for qPCR analysis (above). Tissue was homogenized in 100 µl of lysis buffer (1X RIPA) containing protease inhibitors (Halt Protease Inhibitor Cocktail, Thermo Scientific). Protein concentrations were determined using the BCA Protein Assay Kit (Fisher Scientific, Pittsburgh, PA, USA). Equal amounts of protein (30 µg) were subjected to SDS-PAGE in polyacrylamide gels (Novex WedgeWell 10% Tris-Glycine Gel, Invitrogen, Thermo Fisher Scientific) and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk (for β-Actin) or 5% BSA (for $GABA_A$ α4 and $GABA_A$ δ subunits) in TBST (25 mM Tris-HCl, 137 mM NaCl and 0.1% Tween 20) and incubated with primary antibodies overnight at 4°C (rabbit polyclonal anti- $GABA_A$ receptor alpha4, 1:1000, Millipore, AB5457; rabbit polyclonal anti- $GABA_A$ receptor delta, 1:2000, Millipore, AB9752; mouse monoclonal anti-β-Actin, Sigma Aldrich A5441, 1:10,000). The membranes were then incubated with HRP-conjugated secondary antibody (BioRad goat anti-mouse, 1:3000 [Actin]; BioRad goat anti-rabbit, 1:1000 [$GABA_A$ α4] and 1:2000 [$GABA_A$ δ]) at room temperature for 90 minutes and developed with enhanced chemiluminescence (ECL) detection reagents (Pierce ECL Western Blotting Substrate, Thermo Scientific). Band intensities were quantified using NIH Image J software and protein levels were normalized to β-Actin protein for each sample.

3.2.6 Surgical craniotomy procedure

Lentivirus containing short hairpin RNAs (shRNAs) targeting *Lmo3* (shLmo3) or a nonspecific sequence not known to target any gene in the mouse genome (shScr) were created and tested for efficacy previously by Lasek et al (Lasek, Giorgetti, et al., 2011). 8-10 week old male and female mice were anesthetized with xylazine (8 mg/kg, i.p.) and ketamine (100 mg/kg, i.p.) and placed in a digital stereotaxic alignment apparatus (Model 1900, David Kopf Instruments, Tujunga, CA, USA). After bregma alignment and skull leveling, 0.28 mm diameter holes were drilled bilaterally for microinjections of virus. Mice were randomized to receive either shLmo3 or shScr. A dual cannulae insertion system (33 gauge) was then utilized to target either the BLA (A/P: -1.6, M/L: ±3.1, D/V: -4.8) or NAc (A/P: +1.7, M/L: ±0.9, D/V: -4.6) and 2 µl of virus was infused bilaterally at a rate of 0.2 µl per minute (see Lasek et al for more in-depth methods (Lasek & Azouaou, 2010)). Mice received meloxicam (2 mg/kg) post-surgery and were monitored for two weeks. One week post-surgery, mice were singly housed and moved to the dark room, and DID began two weeks later.

3.2.7 Diaminobenzidine (DAB) staining

In order to verify surgical placements, mice were transcardially perfused after behavioral testing with PBS and then 4% PFA. Brains were removed and post-fixed overnight at 4°C in 4% PFA, and were then transferred to PBS-30% sucrose for an additional 24 hours. Brains were mounted with OCT for sectioning on the cryostat and were cut to 50 µm free-floating sections in PBS. The sections were treated with 3% H_2O_2 for 10 minutes followed by 50% ethanol twice, each time for 10 minutes. 10% normal donkey serum in 0.25% Triton was used for blocking (30 minutes) and sections were then incubated with diluted leftover blocking buffer (1:5) in PBS

with anti-GFP antibody (mouse, monoclonal, Life Technologies, A11120) diluted 1:1000 overnight at 4°C. Sections were then incubated with biotin-conjugated horse anti-mouse secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA, BA-2000) for 1 hour at room temperature followed by ABC-Peroxidase solution (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA, PK-6100) for an hour. DAB peroxidase substrate (Vector Laboratories, Burlingame, CA, USA) was then applied for ~1 minute for brown color detection of the GFP immunostaining. Sections were mounted on gelatin-coated slides and allowed to dry. Counterstaining with cresyl violet was performed and slides were coverslipped with Permount.

3.2.8 Statistical analysis

All data were analyzed using Prism software version 6.05 (GraphPad, La Jolla, CA, USA). DID data was analyzed using two-way repeated measures (RM) analysis of variance (ANOVA) for genotype and time. Four-hour DID, BEC, and gene expression data were analyzed using two-way ANOVA for genotype and sex. DID data for mice that underwent surgical knockdown was analyzed via two-way RM ANOVA for the 2-hour sessions (virus x time) and via Student's t-test for the 4-hour drinking data. Post hoc comparisons were performed using the Holm-Sidak test.

3.3 Results

3.3.1 Lmo3^{Z} mice engage in elevated binge drinking behavior in the DID task

Previous work has demonstrated a positive correlation between levels of *Lmo3* in the brain and alcohol intake in the 2-bottle choice consumption test (Lasek, Giorgetti, et al., 2011). Additionally, *Lmo3* has been shown to be associated with several alcohol-related phenotypes, including sensitivity to the sedative effects of alcohol and moderate alcohol consumption. In order to evaluate whether *Lmo3* may also be regulating more high-risk excessive alcohol

consumption, we tested *Lmo3*^{Z} mice in a 4 day DID binge-like procedure. During the 2-hour drinking sessions on days 1-4, *Lmo3*^{Z} mice drank significantly more alcohol than their wild type littermates (Two-way RM ANOVA, Genotype: $F_{1,29} = 13.90$, $p < .001$, Time: $F_{3,87} = 7.09$, $p < .0005$, Interaction: $F_{3,87} = 2.37$, $p < .10$). This effect was significant in both males (Figure 8a, Genotype: $F_{1,15} = 7.98$, $p < .05$, Time: $F_{3,45} = 4.17$, $p < .05$, Interaction: $F_{3,45} = 0.836$, *ns*) and females (Figure 8b, Genotype: $F_{1,12} = 7.39$, $p < .05$, Time: $F_{3,36} = 3.38$, $p < .05$, Interaction: $F_{3,36} = 1.47$, *ns*). Analysis of alcohol consumption during the final 4-hour drinking session on day 4 also revealed a significant genotype effect (*Lmo3*^{Z} mice consumed more than wild type mice), a sex effect (females consumed more than males) and a significant sex by genotype interaction (male and female *Lmo3*^{Z} and female wild type mice consumed more than male wild type mice, Figure 8c, Genotype: $F_{1,27} = 16.02$, $p < .0005$, Sex: $F_{1,27} = 9.88$, $p < .005$, Interaction: $F_{1,27} = 5.60$, $p < .05$). In agreement with the alcohol consumption data during the final 4-hour drinking session, blood alcohol levels were elevated in *Lmo3*^{Z} mice compared with wild type mice (Figure 8d, Genotype: $F_{1,27} = 10.87$, $p < .005$, Sex: $F_{1,27} = 7.77$, $p < .05$, Interaction: $F_{1,27} = 0.02$, *ns*). These data suggest that *Lmo3* serves to limit excessive alcohol consumption, and its loss leads to higher levels of binge-like drinking.

3.3.2 Lmo3^{Z} *mice do not differ from wild type mice in sucrose consumption*

We next wanted to examine whether the elevated binge drinking observed in the *Lmo3*^{Z} mice was indicative of increased general consummatory behavior that would extend to any rewarding substance, or whether this was an effect that was specific to alcohol. In order to answer this question, we tested *Lmo3*^{Z} mice in the same DID procedural protocol, but instead of administering 20% ethanol in the sipper tubes, we administered a 10% sucrose solution. No difference was observed in consumption of the sucrose solution between *Lmo3*^{Z} and wild type

mice, an effect that was true in both males (Figure 9a, Two-way RM ANOVA, Genotype: $F_{1,14}$ = 0.1043, *ns*, Time: $F_{3,42}$ = 21.61, $p < .0001$, Interaction: $F_{3,42}$ = 0.491, *ns*) and in females (Figure 9b, Genotype: $F_{1,16}$ = 1.212, *ns*, Time: $F_{3,48}$ = $p < .0001$, Interaction: $F_{3,48}$ = 0.2246, *ns*). These data then suggest that *Lmo3* does not affect consumption of naturally rewarding substances, but promotes specific consumption of alcohol.

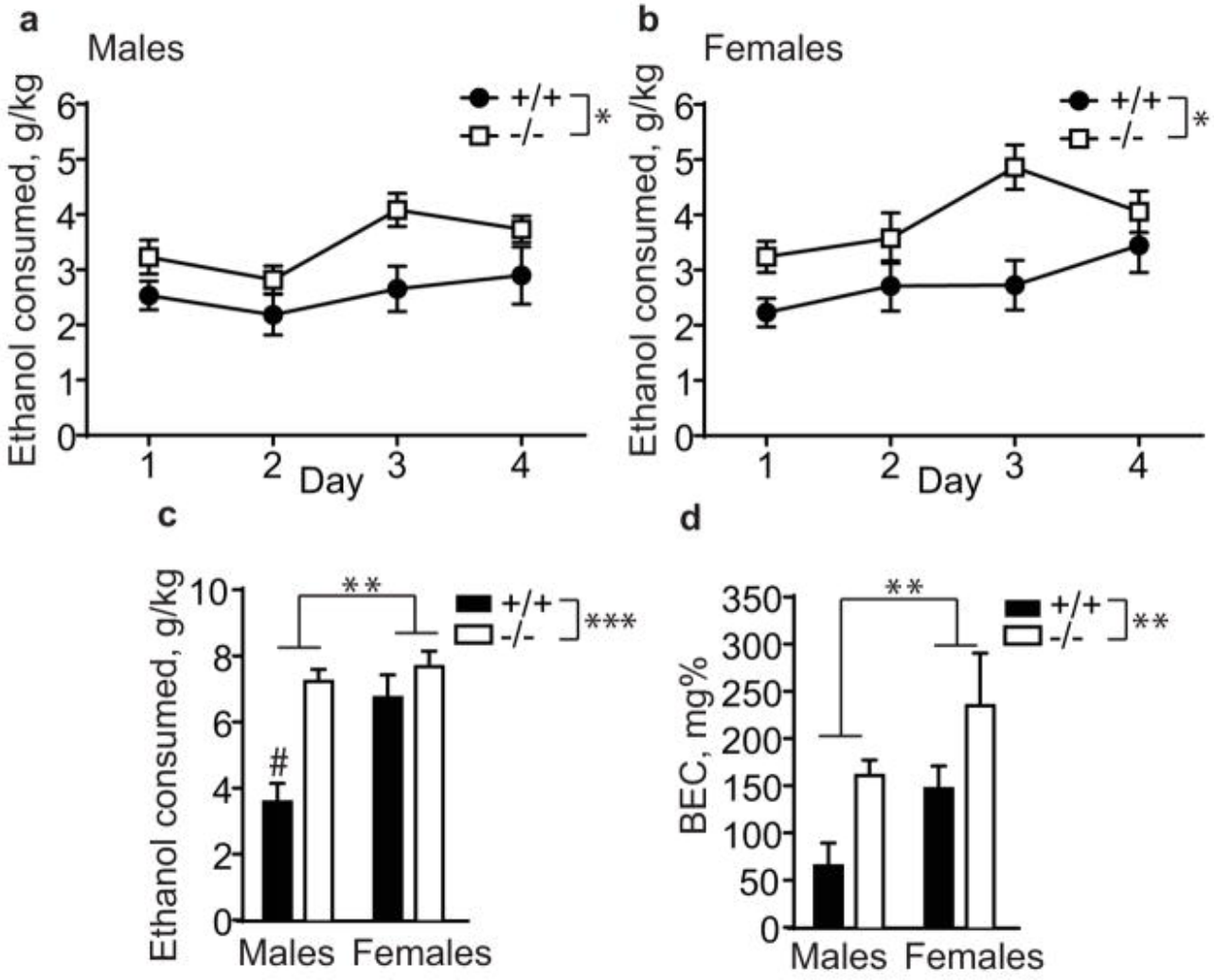


Figure 8. Increased binge-like ethanol consumption in *Lmo3*^{Z} mice

Lmo3^{Z} mice (-/-, open squares, n = 15) drank more than wild type mice (+/+, filled circles, n = 16) in the 2-hour drinking sessions in the DID task. This was true in both (a) males (p = .013) and in (b) females (p = .019). (c) *Lmo3*^{Z} (−/−, white bars) mice also consumed more alcohol in the 4-hour drinking session on day 4 than wild type mice (+/+, black bars), p = .0004. There were also significant effects of sex (p = .004) and a genotype by sex interaction (p = .03) with all groups consuming more than male wild type mice. (d) Blood ethanol concentrations (BEC, mg%) corresponded to 4-hour drinking levels, with *Lmo3*^{Z} mice exhibiting significantly greater BECs than wild type mice (p = .003) and females having higher BECs than males (p < .01).

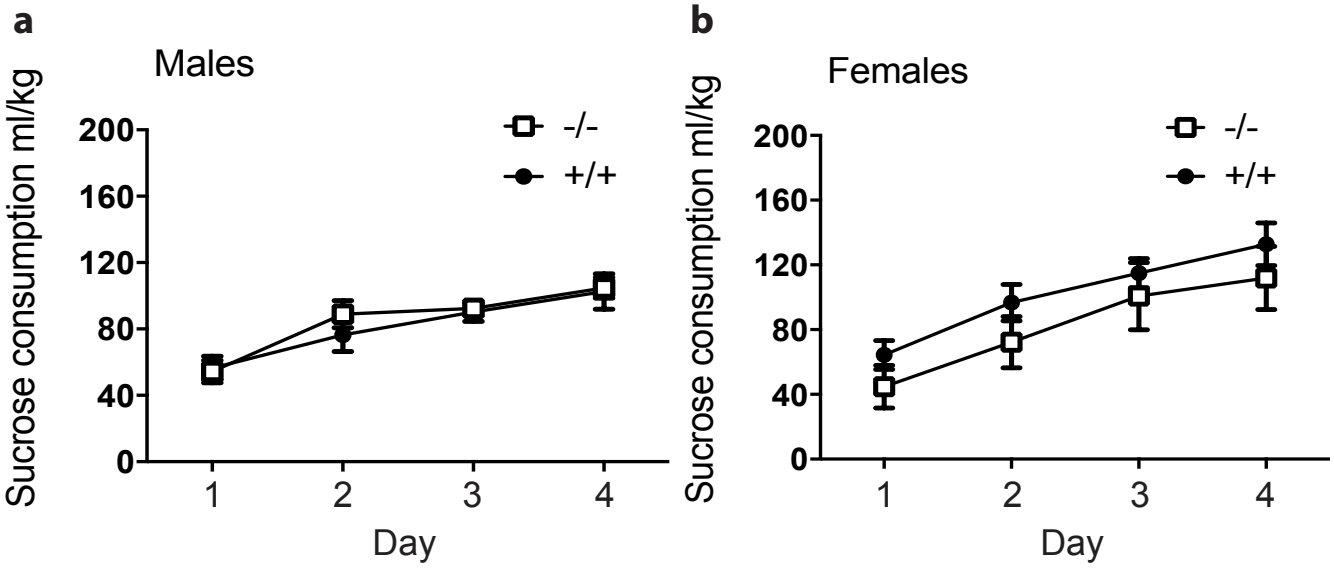


Figure 9. No alteration of sucrose consumption in *Lmo3*^{Z} mice

Lmo3^{Z} mice (-/-, open squares, n = 11) did not differ from wild type mice (+/+, filled circles, n = 23) in sucrose consumption in a limited access paradigm similar to the DID binge drinking protocol. This was true in both (a) males ($p = .75$) and in (b) females ($p = .29$), although all groups exhibited an increase in sucrose consumption across drinking sessions (Time: $p < .0001$, for both sexes).

3.3.3 Lmo3^{Z} mice have altered GABA$_A$ receptor subunit mRNA expression in the basolateral amygdala but no change in protein expression

Lmo3^{Z} mice have reduced levels of CRF1R mRNA and protein in the BLA, and a strong body of evidence supports a role for CRF1R activation in the promotion of binge drinking (Kaur et al., 2012; Lowery et al., 2010). These data would suggest that *Lmo3^{Z}* mice would exhibit reduced binge drinking, and yet in the DID task, they binge drink more. We wanted to examine how *Lmo3* may be regulating binge drinking by examining its potential downstream transcriptional targets. We focused on two areas of the amgydala (the BLA and CeA) and the NAc, regions of the brain in which *Lmo3* is known to be expressed, and which play important roles in regulating alcohol consumption (Cozzoli et al., 2012; Cozzoli et al., 2014; Marshall et al., 2016). Interestingly, modulation of GABAergic signaling in the accumbens is associated with binge drinking, but not with saccharin consumption (Kasten & Boehm, 2014), suggestive of an alcohol-specific modulatory circuit. We investigated whether *Lmo3* may be regulating expression of GABA$_A$ receptor subunits in these regions because *Lmo3* is known to influence the GABA system, and GABA$_A$ receptor subunit composition can have profound effects on alcohol consumption, particularly the GABA$_A$ δ and α4 subunits (H. Nie et al., 2011; Quoilin & Boehm, 2016; Rewal et al., 2012). Given these lines of evidence, we specifically focused on expression of the δ (*Gabrd*) and α4 (*Gabra4*) subunits, and the α1 (*Gabra1*) subunit, as it has the most widespread expression throughout the brain.

We first examined transcriptional changes in these genes by measuring mRNA via qPCR. We observed a sex effect in *Gabra1* mRNA expression in the NAc, with females showing greater expression than males (Figure 10a, Two-way ANOVA, $F_{1,35} = 4.39$, $p < .05$), but no genotype effect ($F_{1,35} = 2.702$, *ns*) or interaction ($F_{1,35} = 0.0499$, *ns*) was observed. No further

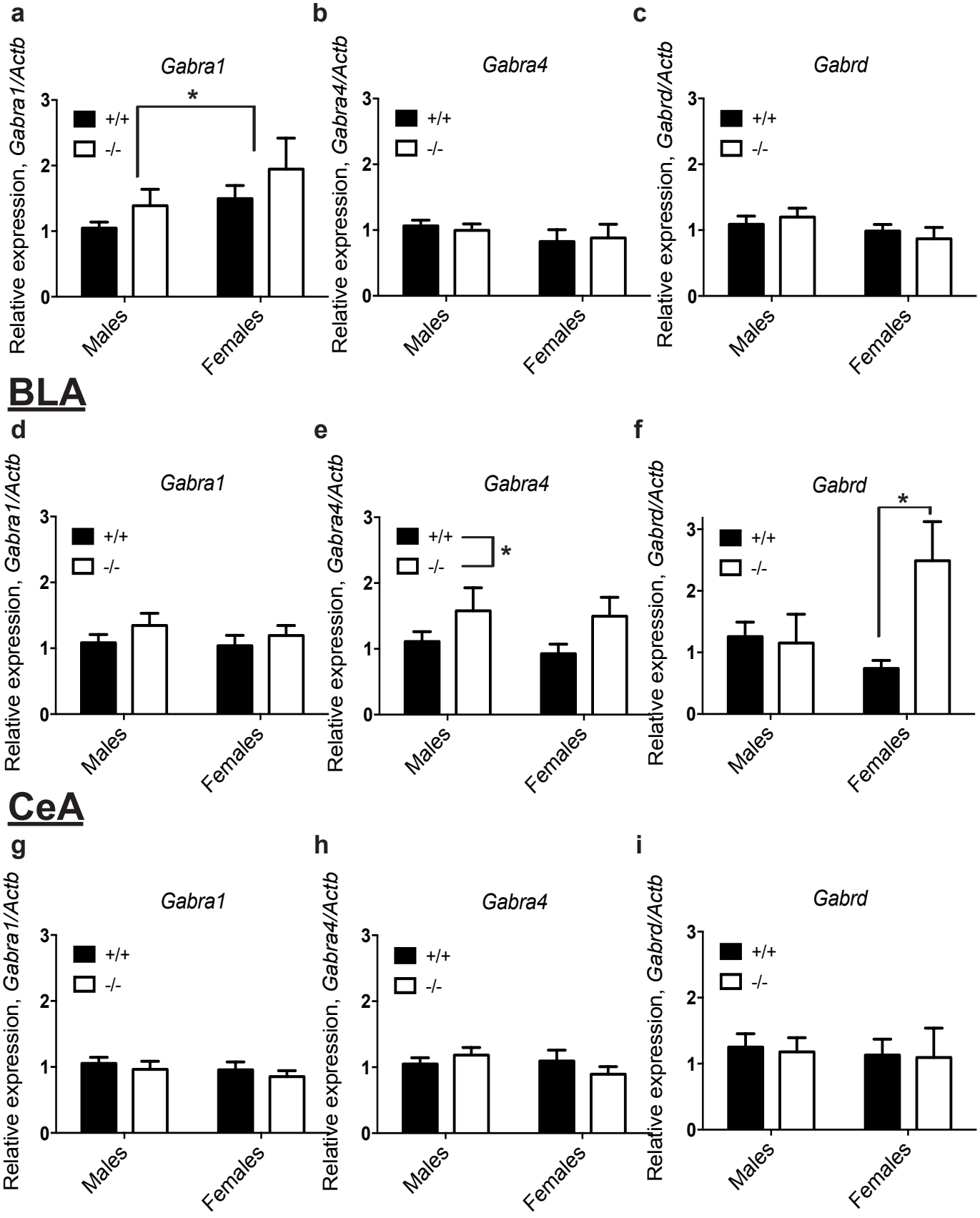
significant results were obtained in the NAc for either *Gabra4* or *Gabrd* expression (Figure 10b, Sex: $F_{1,37}$ = 1.507, *ns*, Genotype: $F_{1,37}$ = 0.0011, *ns*, Interaction: $F_{1,37}$ = 0.1834, *ns*; Figure 10c, Sex: $F_{1,37}$ = 2.547, *ns*, Genotype: $F_{1,37}$ = 0.0007, *ns*, Interaction: $F_{1,37}$ = 0.7247, *ns*; respectively). In the BLA, we detected no change in *Gabra1* expression (Figure 10d, Sex: $F_{1,39}$ = 0.3989, *ns*, Genotype: $F_{1,39}$ = 1.79, *ns*, Interaction: $F_{1,39}$ = 0.1168, *ns*), but there was a significant difference in *Gabra4* expression between genotypes, with *Lmo3*^{Z} mice exhibiting elevated *Gabra4* expression relative to wild type mice (Figure 10e, Sex: $F_{1,34}$ = 0.29, *ns*, Genotype: $F_{1,34}$ = 4.339, $p < .05$, Interaction: $F_{1,34}$ = 0.0413, *ns*). Additionally, we detected a sex by genotype interaction in *Gabrd* expression in the BLA, with female *Lmo3*^{Z} mice showing enhanced *Gabrd* expression relative to female wild type mice and male *Lmo3*^{Z} mice (Figure 10f, Sex: $F_{1,33}$ = 0.8525, *ns*, Genotype: $F_{1,33}$ = 3.435, $p < .10$, Interaction: $F_{1,33}$ = 4.349, $p < .05$). No expression changes were observed in any of the genes in the CeA (Figure 10g [Gabra1], Sex: $F_{1,39}$ = 0.8589, *ns*, Genotype: $F_{1,39}$ = 0.7489, *ns*, Interaction: $F_{1,39}$ = 0.0035, *ns*; Figure 10h [Gabra4], Sex: $F_{1,37}$ = 0.8837, *ns*, Genotype: $F_{1,37}$ = 0.0626, *ns*, Interaction: $F_{1,37}$ = 1.67, *ns*; Figure 10i [Gabrd], Sex: $F_{1,37}$ = 0.1397, *ns*, Genotype: $F_{1,37}$ = 0.0395, *ns*, Interaction: $F_{1,37}$ = 0.0042, *ns*).

Next, we examined whether the changes observed in *Gabra4* and *Gabrd* mRNA expression in the BLA would also extend to alterations in corresponding protein levels. With new tissue samples from *Lmo3*^{Z} and wild type mice, we examined protein expression of the $GABA_A$ δ and α4 subunits utilizing Western blotting. Contrary to our mRNA findings, there were no changes in $GABA_A$ α4 (Figure 11a, Sex: $F_{1,33}$ = 3.281, $p < .10$, Genotype: $F_{1,33}$ = 0.0733, *ns*, Interaction: $F_{1,33}$ = 0.2135, *ns*) or $GABA_A$ δ (Figure 11b, Sex: $F_{1,33}$ = 0.5758, *ns*, Genotype: $F_{1,33}$ = 0.8137, *ns*, Interaction: $F_{1,33}$ = 0.5723, *ns*) protein expression observed in the BLA of *Lmo3*^{Z} mice.

These data support a role for *Lmo3* transcriptional regulation of $GABA_A$ receptor subunits, particularly in the BLA, but suggest that the transcriptional changes are not indicative of alterations in expression of these proteins.

Figure 10. Elevated *Gabra4* mRNA expression in the BLA of *Lmo3^Z* mice and enhanced *Gabrd* mRNA expression in the BLA of female *Lmo3^Z* mice

Amygdala (BLA and CeA) and NAc tissue was collected from *Lmo3^Z* (-/-, white bars) and wild type (+/+, black bars) mice for qPCR analysis. Two-way ANOVA was performed for sex and genotype. (a) In the NAc, there was a sex effect of *Gabra1* expression, with females expressing more *Gabra1* than males ($p = .04$), but there was no significant genotype effect ($p = .11$). No significant effects were observed for either sex or genotype in (b) *Gabra4* ($p = .22$, $p = .97$, respectively) expression or in (c) *Gabrd* ($p = .12$, $p = .98$, respectively) expression in the NAc. (d) In the BLA, no significant effects were observed in *Gabra1* expression for genotype ($p = .19$) or sex ($p = .53$). (e) *Gabra4* expression was significantly elevated in *Lmo3^Z* mice relative to wild type mice in the BLA ($p = .04$) with no differences in sex observed ($p = .59$). (f) A sex by genotype interaction emerged for *Gabrd* expression in the BLA (Sex: $p = .36$, Genotype: $p = .07$, Interaction: $p = .04$), with female *Lmo3^Z* mice showing greater expression than female wild type mice ($p = .02$). (g-i) No gene expression changes were observed in the CeA for *Gabra1* (Sex: $p = .36$, Genotype: $p = .39$), *Gabra4* (Sex: $p = .35$, Genotype: $p = .80$), or *Gabrd* (Sex: $p = .71$, Genotype: $p = .84$).


NAc
a
Gabra1
Relative expression, Gabra1/Actb
*
+/+
-/-
Males
Females
b
Gabra4
Relative expression, Gabra4/Actb
c
Gabrd
Relative expression, Gabrd/Actb
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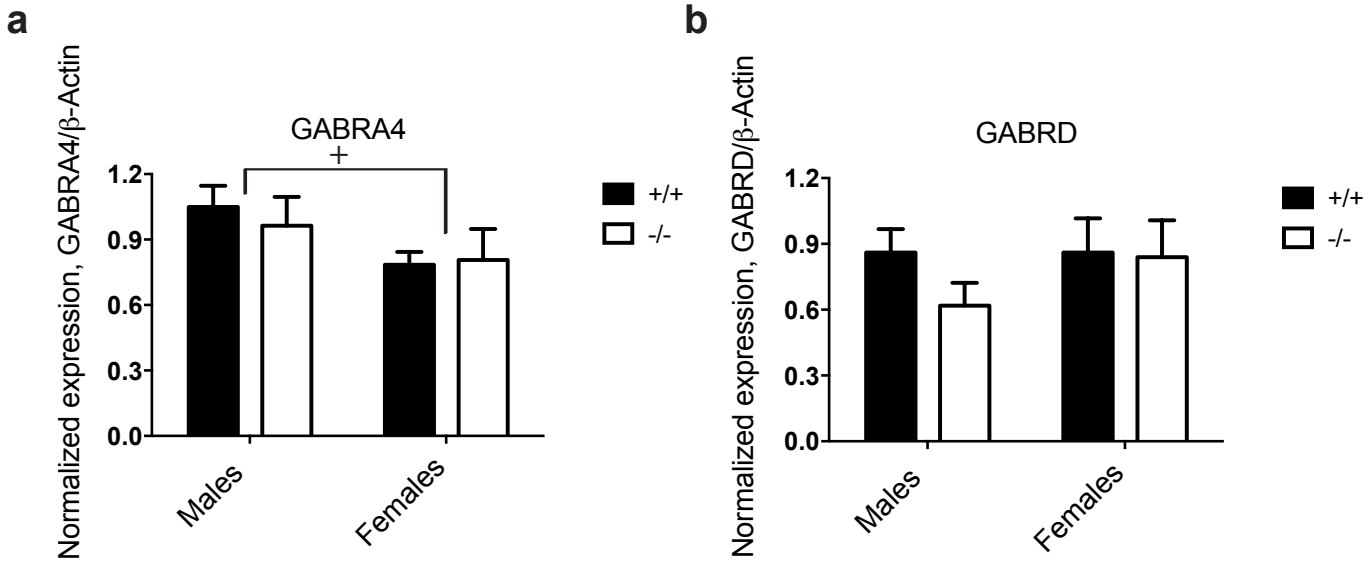


Figure 11. No change detected in $GABA_A$ α4 or $GABA_A$ δ protein expression in the BLA of *Lmo3*^{Z} mice

BLA tissue was collected from *Lmo3*^{Z} (-/-, white bars) and wild type (+/+, black bars) mice for Western Blot analysis. Two-way ANOVA was performed for sex and genotype analysis. (a) No significant genotype effect was detected for $GABA_A$ α4 protein expression, although there was a trend for a sex effect ($p = .07$), with females showing reduced $GABA_A$ α4 protein relative to males. (b) No significant effects were observed in $GABA_A$ δ protein expression in the BLA, for any of the groups.

3.3.4 *Knockdown of Lmo3 in the basolateral amygdala or in the nucleus accumbens does not alter binge drinking*

Next, we sought to identify a brain region in which *Lmo3* is acting to regulate binge drinking. Given the transcriptional changes observed in the BLA of $Lmo3^{Z}$ mice, we targeted the BLA for viral-mediated knockdown of *Lmo3* expression. Mice underwent the surgical knockdown procedure and were tested in the DID task three weeks later. Contrary to our expectations, we did not observe any change in binge-drinking behavior in the mice with knockdown of *Lmo3* in the BLA. Mice that received the shLmo3 virus drank similar amounts of alcohol in both the 2-hour drinking sessions (Figure 12a, Group: $F_{1,12} = 0.6414$, *ns*, Time: $F_{3,36} = 11.17$, $p < .0001$, Interaction: $F_{3,36} = 0.4401$, *ns*) and the 4-hour drinking session (Figure 12b, $t(12) = 1.021$, *ns*) as the mice that received the control virus.

We next targeted the NAc for viral-mediated knockdown of *Lmo3* to see whether it might regulate the binge drinking behavior observed in $Lmo3^{Z}$ mice. Once again, mice underwent the surgical knockdown procedure and were tested in the DID three weeks later. Again, we did not observe any change in binge-drinking behavior in the mice with knockdown of *Lmo3*. Mice that received the shLmo3 virus in the NAc drank similar amounts of alcohol in both the 2-hour drinking sessions (Figure 12c, Group: $F_{1,18} = 0.9506$, *ns*, Time: $F_{3,54} = 5.63$, $p < .005$, Interaction: $F_{3,54} = 1.183$, *ns*) and the 4-hour drinking session (Figure 12d, $t(17) = 1.286$, *ns*) as the mice that received the control virus.

These data suggest that neither the BLA nor the NAc is the primary site of the regulation of binge drinking in $Lmo3^{Z}$ mice. The brain region underlying the elevated binge drinking observed in these mice is still unknown.

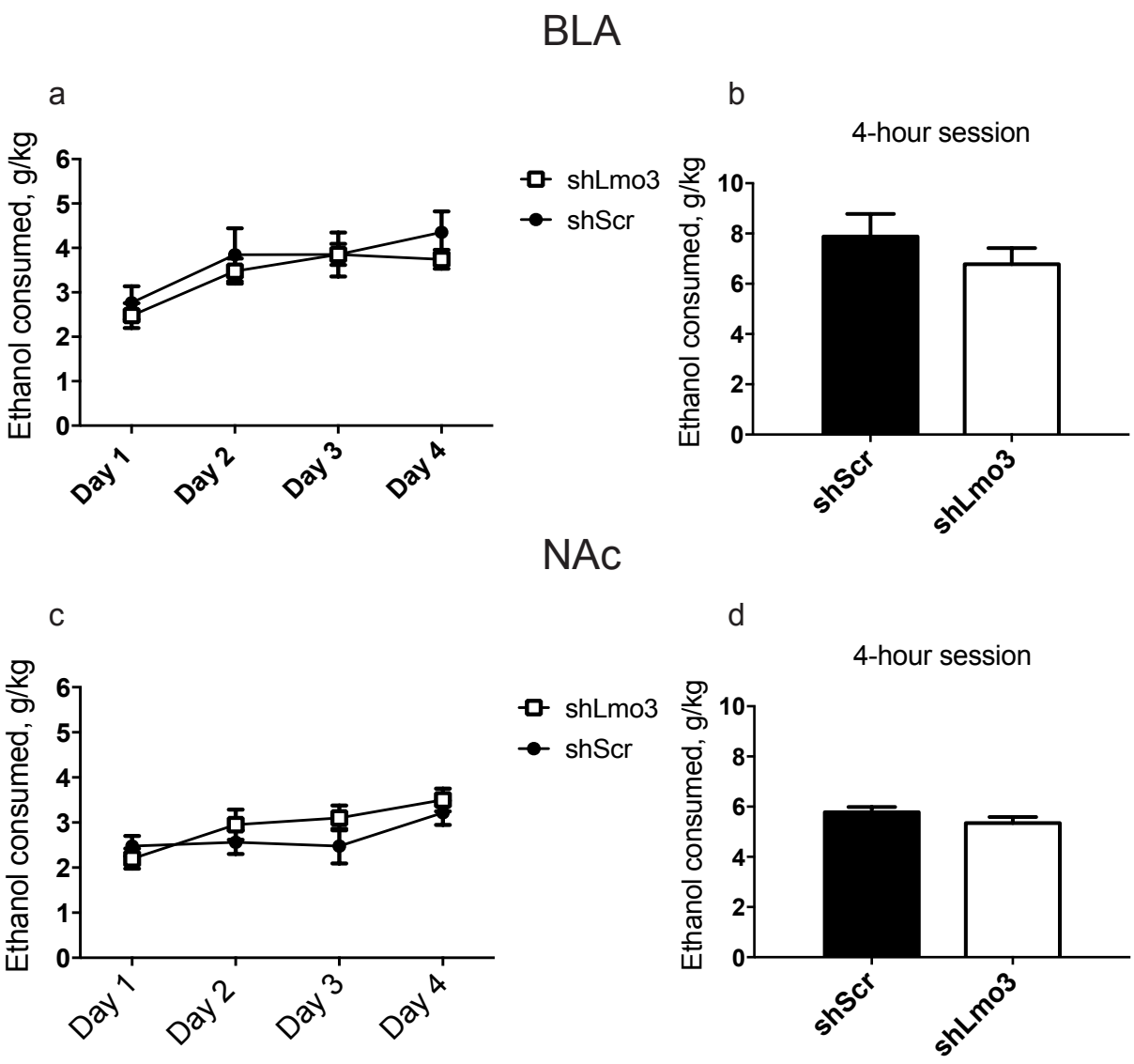


Figure 12. Knockdown of *Lmo3* in the BLA and in the NAc had no effect on binge drinking

Lentiviral-mediated RNA interference was utilized to knockdown *Lmo3* in the BLA and in the NAc of adult C57BL/6J mice and mice were subsequently tested in the DID task. No differences were observed in binge drinking behavior in either the (a) 2-hour drinking sessions or the (b) 4-hour drinking session in mice that received the shLmo3 virus in the BLA. Similarly, no differences were observed in alcohol consumption in either the (c) 2-hour drinking sessions or the (d) 4-hour drinking session in mice that received the shLmo3 virus in the NAc.

3.4 Discussion

Here, we examined the role of *Lmo3* in modulating excessive alcohol intake by testing *Lmo3*^{Z} mice in the binge drinking model Drinking-in-the-Dark, a task that capitalizes on the increased activity of mice during the dark cycle and reliably stimulates pharmacologically relevant (a.k.a. intoxicating) blood ethanol concentrations (above 100 g/dl). We observed a significant difference in alcohol intake in this model, with *Lmo3*^{Z} mice consuming more alcohol in both the 2-hour and 4-hour drinking sessions than their wild type counterparts, suggesting a protective role of *Lmo3* in limiting excessive alcohol consumption. Importantly, this difference in ethanol consumption is not due to alterations in ethanol metabolism; *Lmo3*^{Z} mice and wild type littermates clear ethanol at the same rate (Savarese et al., 2014).

These results were contrary to our expectations in that *Lmo3*^{Z} mice have also been shown to exhibit reduced anxiety-like behavior. There is robust evidence that anxiety drives drinking behavior, in both otherwise healthy individuals (O'Hara et al., 2014) and in clinical populations (Boschloo et al., 2011; Silberman et al., 2009; Tuithof et al., 2013). In rodent models, the connection between anxiety and alcohol use is less established. Two rat lines which have been selectively bred for high alcohol consumption, the alcohol-preferring (P) rat and the Sardinian alcohol-preferring (sP) rat, both show high levels of basal anxiety (Ciccocioppo et al., 2006; Colombo et al., 1995). However, several other rat lines which have been selectively bred for alcohol consumption show either no difference in anxiety-like behavior or a reduced anxiety phenotype (Acewicz et al., 2014; Hwang, Stewart, Zhang, Lumeng, & Li, 2004; Myers, Robinson, West, Biggs, & McMillen, 1998; Tuominen, Hilakivi, Paivarinta, & Korpi, 1990). Similarly, while mice with high trait anxiety show a greater anxiolytic response to alcohol than mice with lower anxiety levels, the two groups do not differ in alcohol consumption (Correia,

Ribeiro, Brunialti Godard, & Boerngen-Lacerda, 2009). Genetic background may be the mediating factor in whether alcohol and anxiety are associated – i.e., Swiss mice show no correlation between anxiety and alcohol intake (Correia et al., 2009), yet in outbred Tuck-Ordinary ("TO") mice anxiety levels are predictive of alcohol consumption and preference (Bahi, 2013). Lending credence to this theory, mutant mice lacking the RIIbeta subunit of protein kinase A show reduced baseline anxiety when maintained on a pure C57BL/6J background, but elevated anxiety when maintained on a 129/SvEv x C57BL/6J background; yet on both backgrounds, these mutant mice consume more ethanol than wild type littermates. This study suggests that not only can a given gene have opposing effects on behavior when expressed within a different genetic context, but also that some behaviors (in this case, alcohol consumption) are more resistant to the influence of genetic context than others (in this case, anxiety) so the association between the two behaviors is not a fixed phenomenon.

Additionally, when there does exist an association between alcohol consumption and anxiety, it is not always a positive correlation. One line of mice that has been selectively bred specifically for high alcohol consumption in the DID task (the high drinking in the dark, or HDID, mice) show reduced, not elevated, anxiety-like behavior (Barkley-Levenson & Crabbe, 2015), a phenotype similar to *Lmo3*^{Z} mice. Similarly, rats bred for high anxiety-related behavior (HAB) show reduced alcohol intake relative to the low-anxiety (LAB) counterparts (Henniger, Spanagel, Wigger, Landgraf, & Holter, 2002). There is some evidence that rodent models of low anxiety may also be representative of a high sensation or novelty-seeking profile, a factor that has been associated with increased alcohol consumption and alcohol problems (Kabbaj, Devine, Savage, & Akil, 2000; Kampov-Polevoy et al., 2014; Stead et al., 2006) and could potentially

explain elevated drinking in a reduced anxiety state. Whether *Lmo3*^{Z} mice also have a high novelty-seeking phenotype is not currently known.

The elevated binge drinking observed in *Lmo3*^{Z} mice was also surprising in that these mice have reductions of *Crhr1* mRNA and CRF1R protein expression in the BLA. The evidence for CRF1R promoting alcohol abuse is robust – CRF1R antagonism has been shown to decrease binge drinking in rodent models (Lowery et al., 2010) and *Crhr1* has also been associated with alcohol intake in humans (Treutlein et al., 2006). That said, CRF1R activation in the BLA has been shown to not have any effect on binge drinking (Lowery-Gionta et al., 2012), so it is possible that the reduced CRF1R expression in the BLA of *Lmo3*^{Z} mice is irrelevant to alcohol consumption. Further, there is some evidence that the effect of CRF1R antagonism on binge drinking requires available CRF2R (Rinker et al., 2017), suggesting that both CRF receptors work together to modulate binge drinking. It is not known if *Lmo3*^{Z} mice have altered levels of CRF2R that may explain the unexpected results observed in drinking behavior.

In pursuit of the underlying neurobiological mechanisms driving the binge-like alcohol consumption in the *Lmo3*^{Z} mice, we analyzed gene expression of several $GABA_A$ receptor subunits in *Lmo3*^{Z} and wild type mice. We focused on three areas of the brain: the BLA, the CeA, and the NAc. Our reasons for focusing on these regions were that (1) transcriptional alterations have been found in the BLA and CeA of *Lmo3*^{Z} mice, (2) *Lmo3* is densely expressed in the BLA and NAc, and (3) expression levels of *Lmo3* correlated with binge drinking behavior in the NAc of wild type C57BL/6J mice who underwent the DID task. The $GABA_A$ receptor subunits that we analyzed were δ (*Gabrd*), α4 (*Gabra4*), and α1 (*Gabra1*). Both the δ and α4 subunits have been shown to regulate alcohol consumption, and the α1 subunit is widely expressed throughout the brain and has a prominent role in regulating GABAergic activity.

While we did not observe relevant differences in $GABA_A$ receptor subunit expression in the NAc or the CeA, we did observe several changes within the BLA. *Gabra4* mRNA was elevated in *Lmo3*^{Z} mice relative to wild type mice, and *Gabrd* was elevated specifically in female *Lmo3*^{Z} mice. Intriguingly, δ and α4 subunits are often located extra-synaptically (Chandra et al., 2006) and when receptors containing these subunits are located in the intercalated cells surrounding the BLA they help to control information flow into and out of the BLA via tonic inhibition (Marowsky & Vogt, 2014). These receptors have also been shown to modulate binge drinking – Ro15-4513, an inverse agonist of $GABA_A$ δ-containing receptors, reduces binge alcohol consumption in female mice (males were not tested) (Melon & Boehm, 2011) and limited access alcohol intake in male Wistar rats (females were not tested) (Buczek, Tomkins, Le, & Sellers, 1997; June, Hughes, Spurlock, & Lewis, 1994). Additionally, $GABA_A$ receptors containing the α4 subunit have been shown to mediate the reinforcing effects of alcohol (Rewal et al., 2012), with knockdown of *Gabra4* causing a reduction of alcohol responding, as well as alcohol consumption and preference (Rewal et al., 2009). In fact, there is evidence that $GABA_A$ receptors containing both δ and α4 subunits are especially sensitive to alcohol and mediate many of the behavioral effects observed with relevant physiological doses (Glowa, Crawley, Suzdak, & Paul, 1988; Hanchar et al., 2006).

However, when we examined whether the transcriptional changes observed in $GABA_A$ receptor subunit expression were indicative of altered protein expression in these mice, we were unable to detect significant differences in either the δ and α4 subunits in the BLA of *Lmo3*^{Z} mice. Importantly, though, failure to observe differences in protein expression does not mean that activity levels of proteins are unaltered. Indeed, the alterations in transcription of the *Gabra4* and *Gabrd* genes suggest that there may be aberrant protein function (i.e., deficient trafficking of

these receptors to the membrane or accelerated protein degradation) that could induce elevated transcription as a compensatory mechanism. $GABA_A$ receptor subunits are dynamically expressed at the membrane, and internalization of the extrasynaptic δ and α4 subunits occurs in hyperactivated states (as in alcohol withdrawal) to switch from a state of extrasynaptic to synaptic inhibition (Liang, Cagetti, Olsen, & Spigelman, 2004). This internalization of the δ and α4 subunits also occurs with acute high dose ethanol exposure and is associated with transcriptional changes of the α4 subunit (Chandra et al., 2006). Taken together, these data suggest that the elevated α4 subunit mRNA expressed in *Lmo3*^{Z} mice could be suggestive of altered $GABA_A$ receptor function in the BLA that could potentially mediate the increased binge drinking observed in these mice.

Our efforts to target the brain region underlying the binge drinking behavior determined that *Lmo3* expression in neither the BLA nor NAc is critical for this phenotype; knockdown of *Lmo3* in either brain region produced no significant alterations in binge-like consumption. However, our work has shown that the knockdown of *Lmo3* produced by this particular shLmo3 virus is modest – only about 25% knockdown was observed in vivo. Perhaps the knockdown of *Lmo3* produced by this virus was not sufficient in the BLA to reproduce the elevated binge drinking phenotype observed in *Lmo3*^{Z} mice. Future work is needed to determine where in the brain *Lmo3* regulates alcohol consumption.

Altogether, these experiments are the first to investigate the role of LMO3 in modulating binge-like alcohol consumption. Our results support a protective role for LMO3 in excessive alcohol intake. Further, these data suggest that this phenotype is not just due to an exaggerated reward response in these mice that drives increased consumption of all rewarding substances – sucrose consumption was unchanged in *Lmo3*^{Z} mice, lending support to an alcohol-specific

effect of LMO3. One limitation to this study is the single concentration of sucrose used to test consumption. It is possible that the concentration we used was too high to detect differences between the genotypes – perhaps with a lower concentration of sucrose, differences in consumption would be observed. *Lmo3* knockdown in the NAc, a critical region in the reward network, failed to alter binge-drinking behavior, but it is possible that the knockdown was not robust enough to produce a significant behavioral effect. Alternatively, the NAc may be relevant to the elevated binge drinking in the $Lmo3^{Z}$ mice, but it requires *Lmo3* actions in other brain regions as well, dampening the effect that exclusive NAc *Lmo3* knockdown would produce. Finally, it is possible that *Lmo3* is not acting in the adult brain to modulate binge drinking, but rather, $Lmo3^{Z}$ mice have structural alterations in the brain from lacking *Lmo3* throughout development that predisposes them to elevated binge drinking. Future work should be done to evaluate alterations in the reward system of $Lmo3^{Z}$ mice. These experiments also reinforce a potential transcriptional role for *Lmo3* in the BLA, previously shown with *Crhr1* regulation, with $Lmo3^{Z}$ mice exhibiting alterations in $GABA_A$ receptor subunit mRNA expression only in the BLA. *Lmo3* may prove to be a critical regulator of both anxiety and alcohol-related behaviors, specifically via its actions in the BLA.

4. THE REWARDING AND ANXIOLYTIC PROPERTIES OF ALCOHOL ARE REGULATED BY LMO3 IN A SEX-SPECIFIC MANNER

4.1 Introduction

Consuming high levels of alcohol is the fourth leading preventable cause of death in the United States and costs the U.S. billions of dollars each year (Bouchery et al., 2011). Binge drinking, a pattern of drinking that leads to intoxication, is associated with future alcohol-related problems (Jennison, 2004) and is very common, with 30% of adults under the age of 35 in the U.S. engaging in this pattern of drinking (Kanny et al., 2013). Yet in a large prospective study of young men, only 11% of those who abused alcohol went on to develop alcohol dependence (Schuckit, Smith, & Landi, 2000). The factors relaying vulnerability or resilience to the development of an alcohol use disorder (AUD) remain unclear.

Acute behavioral responses to alcohol can be predictive of future risk for alcohol-related problems. Level of response (LR) to an acute alcohol challenge (a dose equivalent to 3-5 drinks) can be calculated through a number of measures, including cortisol release, subjective feelings of intoxication, and body sway. A low LR to alcohol is observed at a higher frequency in sons of alcoholics than their age-matched peers without a family history of alcoholism, is uniquely predictive of future AUD (Schuckit, 1994), is associated with an earlier age of onset of AUD (Schuckit & Smith, 2001), and may actually mediate family history as a risk factor for AUD (Schuckit & Smith, 1996).

An additional acute response to alcohol that can influence alcohol consumption is the perception of alcohol's rewarding properties. Heavy drinkers report a greater stimulant and

rewarding response to alcohol than do light drinkers, and among heavy drinkers greater positive effects of alcohol consumption predict future increased binge-like consumption (King, de Wit, McNamara, & Cao, 2011). In animals, the perception of alcohol's rewarding properties can be measured through the use of a conditioned place preference (CPP) task. Ethanol CPP has been found to be associated with alcohol consumption, specifically sweetened ethanol intake, in mice (Cunningham, 2014), and a number of agents that reduce alcohol consumption will also reduce ethanol CPP (Al Ameri, Al Mansouri, Al Maamari, & Bahi, 2014; Al Maamari, Al Ameri, Al Mansouri, & Bahi, 2014; Dutton, Chen, You, Brodie, & Lasek, 2017). Highlighting the role of this task in measuring reward, the formation of ethanol CPP has been shown to be dependent on the mesolimbic dopaminergic system, the brain's central reward hub (Bahi & Dreyer, 2014; Pina & Cunningham, 2014). Dopamine release positively reinforces the use of alcohol and can lead to continued and escalated use.

Rather than drinking for positive reinforcement (reward), negative reinforcement (the removal of a negative outcome, like anxiety, when alcohol is introduced) can also drive alcohol consumption (Koob & Le Moal, 2005). The anxiolytic properties of alcohol can lead to a "drinking to cope" behavior in both non-clinical (O'Hara et al., 2014) and clinical populations (Crum et al., 2013), that is predictive of future alcohol problems. Indeed, one of the factors that has been found to mediate the connection of low LR to alcohol with later alcohol problems is drinking to cope behavior (Schuckit et al., 2011). The Sardinian alcohol-preferring (sP) rat, which has been selectively bred for high alcohol intake, exhibit increased anxiety-like behavior on the elevated plus maze (EPM) compared to their associated non-preferring line (Colombo et al., 1995). Similarly, Wistar rats with high anxiety also had higher alcohol intake and preference than low-anxiety animals (Spanagel et al., 1995). One of the ways in which researchers measure

the anxiolytic properties of alcohol is through the use of an acute injection of alcohol preceding a standard measure of anxiety (i.e. the elevated plus maze). Alcohol-preferring (P) rats, that have higher levels of alcohol consumption than non-alcohol preferring (NP) rats, also show a greater anxiolytic response to alcohol (Stewart et al., 1993). The same effect can be observed in high drinking-in-the-dark mice (HDID) that have been bred for high binge-like alcohol consumption (Barkley-Levenson & Crabbe, 2015).

Determining genetic factors that underlie sensitivity to both the rewarding and anxiolytic effects of alcohol may provide insight into the mechanisms that induce vulnerability to alcohol abuse. One gene that has recently emerged at the intersection of both alcohol and anxiety is *Lmo3*. *Lmo3* null (*Lmo3*^{Z}) mice engage in elevated binge drinking, but show reduced baseline anxiety-like behavior. LMO3 is a transcriptional regulator that is highly expressed throughout the central nervous system. It has a role in GABAergic cell development, promoting a parvalbumin-subtype of interneuron (Au et al., 2013), and it is also expressed in dopaminergic cells (Bifsha et al., 2016) and its expression may be regulated by dopamine (Shi et al., 2001). Few of the transcriptional targets of *Lmo3* have been identified, but it has been shown to regulate *Crhr1* mRNA and CRF1R protein expression in the BLA that may underlie its effect on anxiety-like behavior. Knockdown of *Lmo3* in the BLA replicated the anxiety phenotype observed in *Lmo3*^{Z} mice. *Lmo3*^{Z} mice also show enhanced transcriptional activity of $GABA_A$ receptor subunit genes *Gabra4* and *Gabrd* in the BLA. *Gabra4* and *Gabrd* are unique among $GABA_A$ receptor subunits for being highly sensitive to low doses of alcohol (Glowa et al., 1988; Hanchar et al., 2006) and have been shown to regulate consumption (Buczek et al., 1997; Melon & Boehm, 2011). Although *Lmo3*^{Z} mice show elevated binge drinking behavior, the mechanisms

driving this excessive consumption are unknown. To date, no one has studied whether *Lmo3* regulates the rewarding or anxiolytic effects of alcohol.

In this project, we examine the role of *Lmo3* in modulating two different acute behavioral responses to alcohol: alcohol reward and alcohol-induced anxiolysis. To measure these behavioral responses, we tested $Lmo3^{Z}$ mice along with their wild type littermates in the ethanol CPP task and elevated plus maze task after an acute injection of ethanol (ethanol-induced anxiolysis). Additionally, we evaluated whether $Lmo3^{Z}$ mice exhibit alterations in perception of the rewarding effects of cocaine using cocaine CPP, to see whether the regulation of alcohol-related behaviors extends to other drugs of abuse. Results from this project will provide insight into the mechanisms underlying the binge drinking phenotype observed in $Lmo3^{Z}$ mice and may suggest a novel gene target for the modulation of acute behavioral responses to alcohol.

4.2 Material and methods

4.2.1 Subjects

Adult (10-16 weeks old) male and female homozygous $Lmo3^{Z}$ and wild type littermates were used for behavioral testing and gene expression experiments. Mice were group housed with same-sex cage mates in a temperature- and humidity-controlled environment under a 14-hour light/dark cycle (lights on at 6 am and off at 8 pm) and tested during the light phase. Mice had access to food and water *ad libitum* for the duration of the study and were maintained and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the University of Illinois at Chicago (UIC) Institutional Animal Care and Use Committee.

4.2.2 Ethanol conditioned place preference (CPP)

Conditioned place preference is a classical conditioning task, wherein a rewarding substance becomes paired with a neutral environment, and over time that neutral environment represents that rewarding substance. It is utilized as an indirect method of measuring the rewarding properties of a drug; the more "rewarding" an animal finds a drug, the more time it will spend in the drug-paired side of a chamber post-conditioning compared to pre-conditioning.

A modified 48-channel infrared photobeam detector open field apparatus (27.3 cm L x 27.3 cm W x 20.3 cm H) and Activity Monitor software (Med Associates, St. Albans, VT) were utilized for all training and test days in the ethanol CPP task. The open field was divided into two chambers through the use of clear acrylic vertical dividing panels (custom cut by the UIC Scientific Instrument Shop). Each chamber was identical except for two texturally distinct floor inserts (either "prismatic" or "grid" textured, also created by the UIC Scientific Instrument Shop). Behavior was conducted at the same time each day for all training and test sessions.

On the first test day, mice were exposed to the two-chambered open field and allowed to freely explore both chambers and floors for a 30-minute period. No drug was administered on this day. The amount of time the mouse spent in each chamber was used to determine a baseline preference for floor inserts. Following the first test session, animals had 8 conditioning days of alternating ethanol (2 g/kg, i.p.) or saline injections (4 session for ethanol and 4 for saline). Ethanol injections were paired with the animal's least preferred floor insert from the initial test session, in a biased assignment design (a biased design has been shown to be critical to the formation of CPP in female, but not male, mice, Lasek et al, unpublished results). Immediately following the injection of either ethanol or saline, mice were placed into the assigned chamber for 5 minutes and promptly removed. At the conclusion of the 8 training sessions, animals were

again placed into the open field with access to both chambers and floor in a manner that mirrored their first test exposure. Once again, no drug was administered and mice were allowed to freely explore for 30 minutes and the amount of time spent on each side of the box was calculated.

4.2.3 Cocaine conditioned place preference

The cocaine CPP protocol occurred in the same open field apparatus as ethanol CPP, and first and last test sessions were identical. Conditioning sessions differed in both duration (6 training days, 3 cocaine and 3 saline) and in duration (15 minutes spent in the chamber). Mice were once again assigned to receive cocaine (5 mg/kg, i.p.) on their least preferred side of the chamber in a biased design.

4.2.4 Ethanol-induced anxiolysis

Ethanol-induced anxiolysis was measured on the elevated plus maze. The maze consists of four arms (two open and two enclosed by 12 cm high walls) that are 45 cm long and 10 cm wide, elevated approximately 50 cm above the ground. At the start of a trial, each mouse was injected with either ethanol (1 g/kg, i.p.) or saline and immediately placed into the center of the maze with its nose directed at the closed arm. Mice were left to freely explore for 10 minutes. The total distance traveled, as well as the amount of time spent in each arm and the number of entries into each arm was digitally tracked and recorded. Additional parameters determined in data analysis included percent entries into each arm relative to total entries.

4.2.5 Statistical analysis

All data were analyzed using Prism software version 6.05 (GraphPad, La Jolla, CA, USA). CPP data was analyzed using two-way repeated measures (RM) analysis of variance (ANOVA)

for genotype and time. Two-way ANOVA was also utilized for the analysis of ethanol-induced anxiolysis (genotype x condition). All post hoc comparisons were performed using the Holm-Sidak test.

4.3 Results

4.3.1 Female Lmo3^{Z} mice fail to develop ethanol conditioned place preference

In order to examine whether *Lmo3* may regulate the rewarding effects of alcohol, we tested *Lmo3*^{Z} and wild type mice in ethanol CPP. In this task, mice are considered to have developed preference if they spend considerably more time on the ethanol-paired side of the box after conditioning trials than they did prior to the conditioning trials. In male mice, there was a significant effect of time observed, with both *Lmo3*^{Z} and wild type mice spending more time on the ethanol-paired side of the box post-conditioning than pre-conditioning, but there was no effect of genotype and no interaction (Figure 13a, Genotype: $F_{1,23} = 0.383$, *ns*, Time: $F_{1,23} = 25.27$, $p < .0001$, Interaction: $F_{1,23} = 0.2026$, *ns*). Male *Lmo3*^{Z} mice developed ethanol CPP to the same extent as wild type mice (Figure 13b, $t(23) = 0.4501$, *ns*). However, in female mice, we observed a genotype by time interaction (Figure 13c, Genotype: $F_{1,22} = 5.747$, $p < .05$, Time: $F_{1,22} = 13.59$, $p < .005$, Interaction: $F_{1,22} = 4.728$, $p < .05$). While female wild type spent significantly more time on the ethanol-paired side of the box post-conditioning than pre-conditioning ($t(22)$: 4.539, $p < .0005$), the female *Lmo3*^{Z} mice showed no preference for the ethanol-paired side of the box post-conditioning ($t(22)$: 0.9895, *ns*). Importantly, female *Lmo3*^{Z} and wild type mice did not differ in the amount of time spent on the ethanol-paired side of the box prior to conditioning ($t(44)$: 0.4875, *ns*), but they did differ after conditioning ($t(44)$: 3.232, $p < .005$), with female *Lmo3*^{Z} mice spending considerably less time on the ethanol-paired side of

the box in the final test than wild type mice. This was also evident in their preference scores (Figure 13d, $t(22)$: 2.174, $p < .05$), preference being calculated by subtracting the time spent on the ethanol-paired side of the box pre-conditioning from the time spent there post-conditioning.

Contrary to our expectations, *Lmo3* does not appear to enhance the rewarding effects of alcohol. In fact, while *Lmo3* does regulate alcohol reward, it does so in a sex-specific manner and in the opposite direction that we anticipated – female $Lmo3^{Z}$ mice do not show preference for alcohol, even though they engage in elevated binge drinking relative to wild type mice.

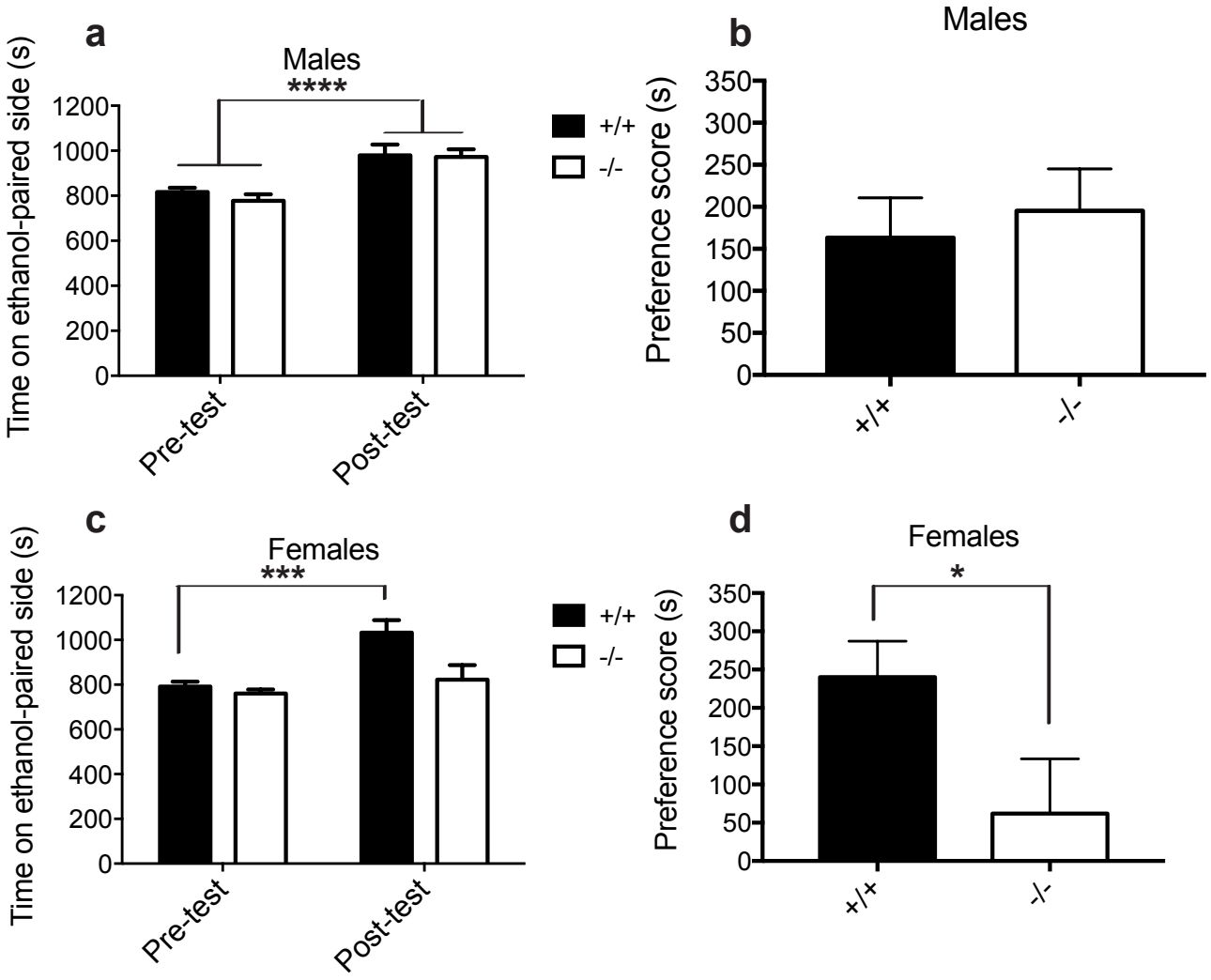


Figure 13. Female *Lmo3^{Z}* mice do not respond to the rewarding effects of alcohol

Lmo3^{Z} (-/-, white bars, n = 20) and wild type littermates (+/+, black bars, n = 30) were tested in the ethanol conditioned place preference task. (a) Male *Lmo3^{Z}* and wild type mice spent significantly more time in the ethanol-paired side of the box post-conditioning than at baseline ($p < .0001$). (b) In male mice, there was no genotype effect in ethanol preference scores ($p = .66$). (c) Females exhibited a genotype by time interaction (Genotype: $p = .03$, Time: $p = .001$, Interaction: $p = .04$), wherein female wild type mice spent significantly more time on the ethanol-paired side of the box post-conditioning than they did at baseline ($p = .0003$), but female *Lmo3^{Z}* mice did not ($p = .33$). (d) There was a genotype effect in female preference scores, with female wild type mice having significantly greater scores than female *Lmo3^{Z}* mice ($p = .04$).

4.3.2 Female Lmo3^{Z} mice exhibit enhanced cocaine conditioned place preference

Next we sought to determine whether *Lmo3*^{Z} mice would be differentially responsive to a drug of abuse other than alcohol. Because *Lmo3* has dense expression throughout the mesolimbic dopaminergic system (the brain's "reward" network) and it is a dopamine-responsive gene (Shi et al., 2001), we chose a drug that is known to act primarily by modulating dopamine transmission – cocaine. Therefore, we tested *Lmo3*^{Z} and wild type mice in the cocaine CPP task to measure cocaine reward.

All groups of mice formed preference for cocaine. In males, there was a significant effect of time, with more time spent on the cocaine-paired side of the box post-conditioning than at baseline, no main effect of genotype, and a trend towards an interaction (Figure 14a, Genotype: $F_{1,13} = 0.046$, *ns*, Time: $F_{1,13} = 64.94$, $p < .0001$, Interaction: $F_{1,13} = 4.119$, $p < .10$). However, in females, while both genotypes developed preference, a genotype by time interaction emerged (Figure 14c, Genotype: $F_{1,20} = 1.134$, *ns*, Time: $F_{1,20} = 68.06$, $p < .0001$, Interaction: $F_{1,20} = 5.177$, $p < .05$). Female mice did not differ in time spent on the cocaine-paired side of the box at baseline ($t(40) = 0.8647$, *ns*), but *Lmo3*^{Z} mice spent considerably more time on the cocaine-paired side of the box post-conditioning than did wild type mice ($t(40) = 2.365$, $p < .05$). Interestingly, when looking at the preference scores, there was a significant difference in preference score between genotypes in females (Figure 14d, $t(20) = 2.275$, $p < .05$) and there was a trend towards significance in males (Figure 14b, $t(13) = 2.03$, $p < .10$) , suggesting enhanced cocaine CPP in both sexes of *Lmo3*^{Z} mice.

Once again, a sex by genotype interaction has emerged in drug reward, with female *Lmo3*^{Z} mice exhibiting a distinct phenotype. Contrary to ethanol CPP, where female *Lmo3*^{Z} mice failed

to develop any preference, female *Lmo3*^{Z} mice showed an exaggerated reward response to cocaine relative to wild type mice. Here, male *Lmo3*^{Z} mice also exhibited a trend towards an enhanced preference for cocaine, suggesting that *Lmo3* may be regulating cocaine reward in such a way that females are more impacted by its loss than males.

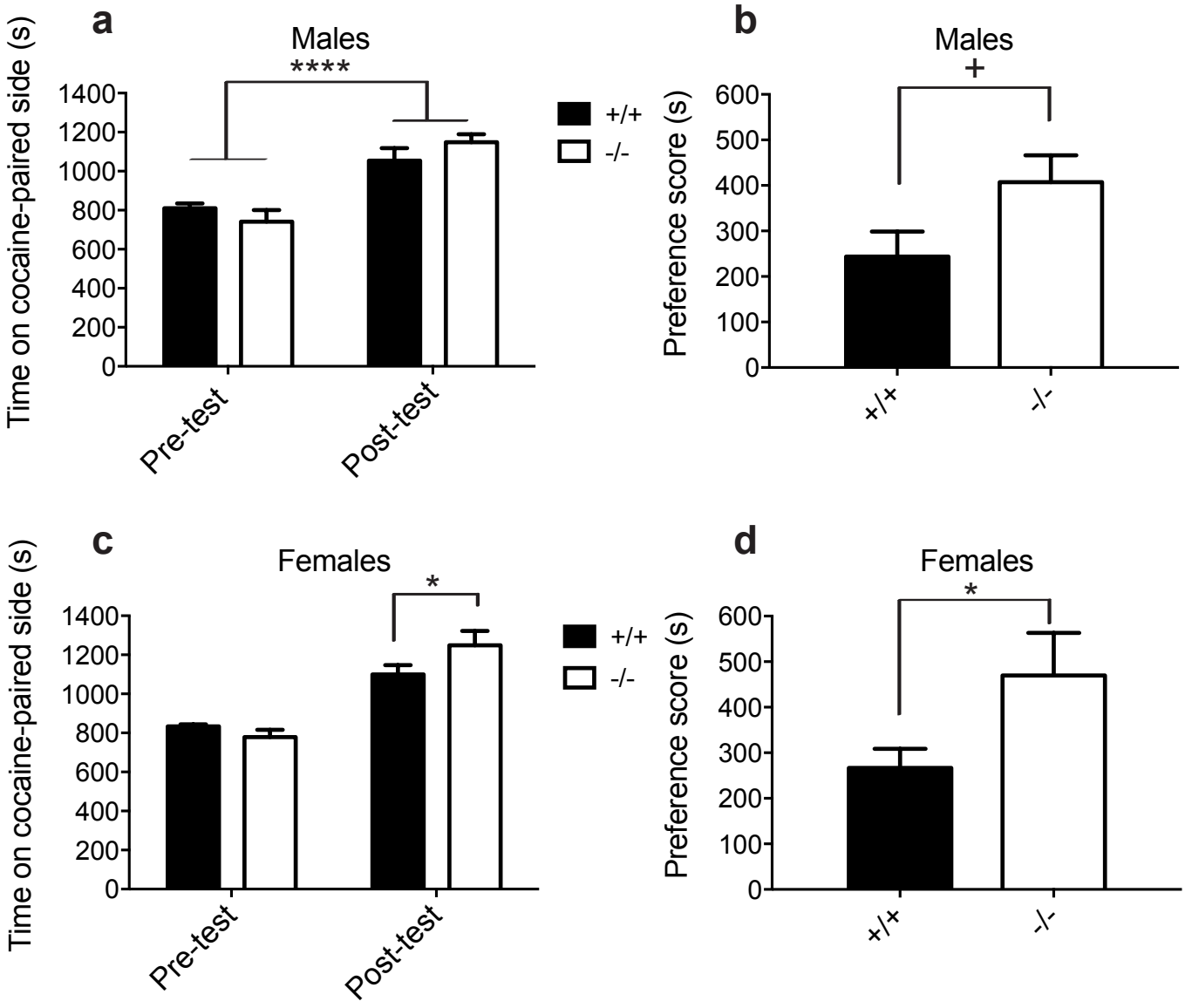


Figure 14. Female *Lmo3^Z* mice show enhanced cocaine conditioned place preference

Lmo3^Z (-/-, white bars, n = 15) and wild type littermates (+/+, black bars, n = 22) were tested in the cocaine conditioned place preference task. (a) Male *Lmo3^Z* and wild type mice spent significantly more time in the cocaine-paired side of the box post-conditioning than at baseline ($p < .0001$). (b) There was a trend toward a genotype effect in cocaine preference scores in males, with *Lmo3^Z* mice showing increased preference relative to wild types ($p = .06$). (c) There was a genotype by time interaction in female mice (Genotype: $p = .30$, Time: $p < .0001$, Interaction: $p = .03$), wherein female *Lmo3^Z* mice spent significantly more time on the cocaine-paired side of the box post-conditioning than wild type mice ($p = .05$), with no difference observed at baseline ($p = .39$). (d) Female *Lmo3^Z* mice exhibited increased preference relative to wild types ($p = .03$).

4.3.3 Female Lmo3^{Z} mice fail to develop ethanol-induced anxiolysis

In previous work, *Lmo3*^{Z} mice were shown to have a reduced baseline anxiety phenotype relative to their wild type littermates, and yet they engage in elevated binge drinking. In order to determine whether *Lmo3*^{Z} mice exhibited any alterations in the anxiolytic effect of acute alcohol exposure, we tested *Lmo3*^{Z} and wild type mice in the elevated plus maze after an acute injection of either saline or a low dose of ethanol. Given the significant locomotor differences observed between wild type and *Lmo3*^{Z} mice, all comparisons are within-groups, i.e. a comparison of arm time between treatment (saline vs. ethanol) groups within male *Lmo3*^{Z} mice, female *Lmo3*^{Z} mice, etc.

We found a robust anxiolytic effect of ethanol in wild type mice. In male wild type mice, an interaction emerged whereby male wild type mice showed a strong preference for the closed arm over the open arm that was abolished with a saline injection (Figure 15a, Arm: $F_{1,40} = 48.62$, $p < .0001$, Treatment: $F_{1,40} = 0.1629$, *ns*, Interaction: $F_{1,40} = 29.74$, $p < .0001$; Saline: $t(40) = 8.787$, $p < .0001$, Ethanol: $t(40) = 1.074$, *ns*). Female wild type mice exhibited the same phenotype (Figure 15b, Arm: $F_{1,30} = 54.05$, $p < .0001$, Treatment: $F_{1,30} = 0.0136$, *ns*, Interaction: $F_{1,30} = 24.98$, $p < .0001$; Saline: $t(30) = 9.002$, $p < .0001$, Ethanol: $t(30) = 1.617$, *ns*). Male *Lmo3*^{Z} mice also exhibited an anxiolytic response to the ethanol injection, but it is not as robust as that seen in wild type mice (Figure 15c, Arm: $F_{1,28} = 45$, $p < .0001$, Treatment: $F_{1,28} = 0.1633$, *ns*, Interaction: $F_{1,28} = 7.964$, $p < .01$). Male *Lmo3*^{Z} mice show preference for the closed arm with a saline injection ($t(28) = 6.739$, $p < .0001$) that is attenuated, but still significant, when ethanol is on board ($t(28) = 2.748$, $p < .05$). Female *Lmo3*^{Z} mice, on the other hand, exhibited no anxiolytic response to ethanol. They prefer the closed arm to the open arm, regardless of treatment (Figure 15d, Arm: $F_{1,32} = 59.06$, $p < .0001$, Treatment: $F_{1,32} = 0.0430$, *ns*, Interaction: $F_{1,32} = 1.773$, *ns*).

These data would suggest that *Lmo3* promotes the anxiolytic response to alcohol, with $Lmo3^{Z}$ mice showing deficits in the perception of alcohol's anxiolytic effects. Further, *Lmo3* seems to be regulating ethanol-induced anxiolysis in a sex-specific manner such that male $Lmo3^{Z}$ mice have an attenuated anxiolytic response and female $Lmo3^{Z}$ mice lack an anxiolytic response altogether.

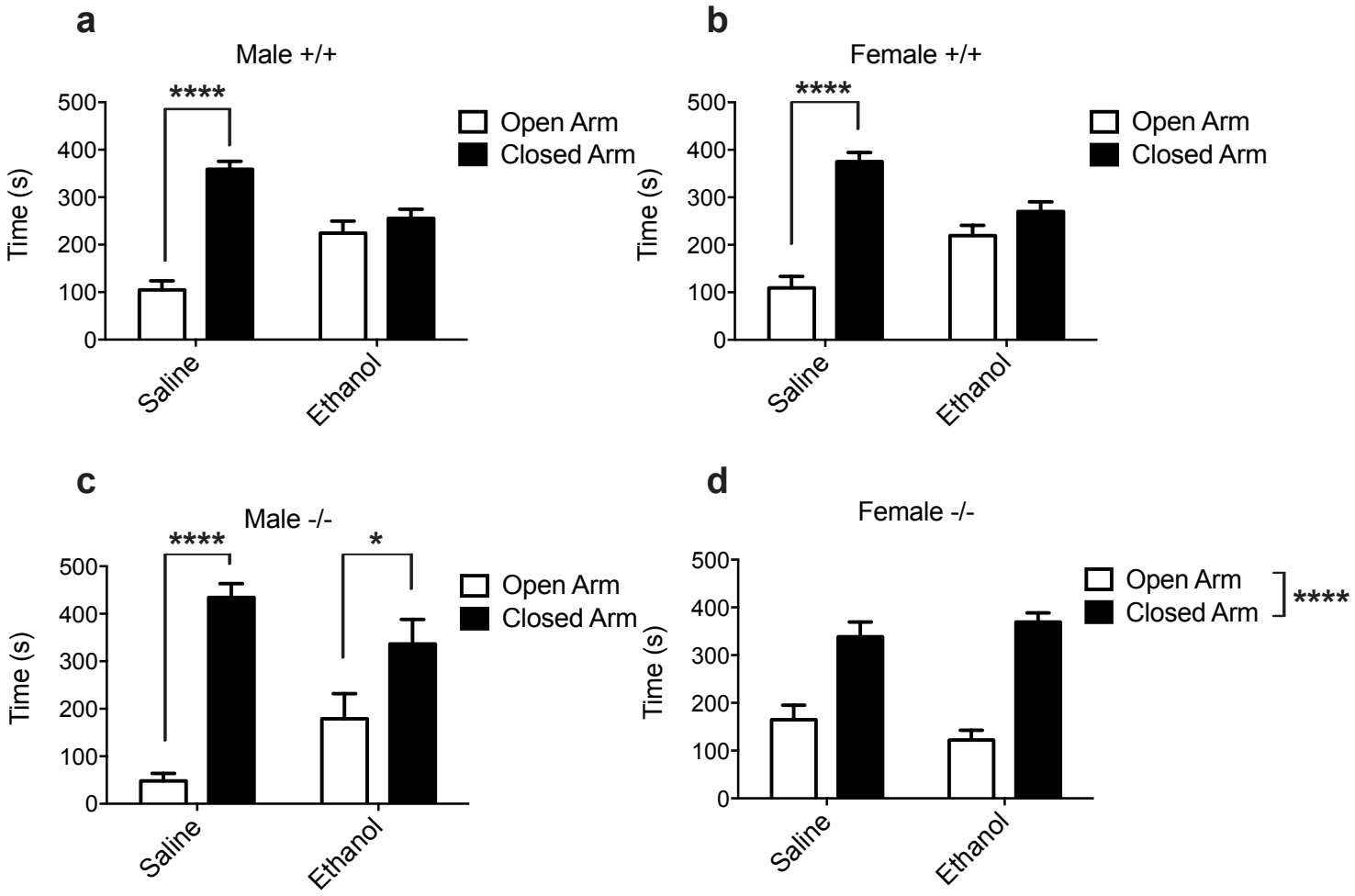


Figure 15. Female *Lmo3^{Z}* mice fail to exhibit ethanol-induced anxiolysis

Lmo3^{Z} (-/-) and wild type littermates (+/+) were tested in the elevated plus maze following a saline or ethanol injection (1 g/kg, i.p.). (a) Male wild type mice (n = 22) showed a robust anxiolytic response to ethanol, with no preference for the closed arm following an ethanol injection (Arm: $p < .0001$, Treatment: $p = .69$, Interaction: $p < .0001$, Saline: $p < .0001$, Ethanol: $p = .29$). (b) Female wild type mice (n = 17) also showed a robust anxiolytic response to ethanol (Arm: $p < .0001$, Treatment: $p = .91$, Interaction: $p < .0001$, Saline: $p < .0001$, Ethanol: $p = .12$). (c) Male *Lmo3^{Z}* mice (n = 16) showed an attenuated anxiolytic response to ethanol (Arm: $p < .0001$, Treatment: $p = .69$, Interaction: $p = .009$); they still had a preference for the closed arm relative to the open arm after an ethanol injection ($p = .01$), but it was attenuated relative to saline ($p < .0001$). (d) Female *Lmo3^{Z}* mice showed no anxiolytic response to ethanol (Arm: $p < .0001$, Treatment: $p = .84$, Interaction: $p = .19$).

4.4 Discussion

These experiments were conducted in order to investigate the role of *Lmo3* in modulating acute behavioral responses to alcohol. Contrary to its effects on binge drinking and basal anxiety-like behavior, *Lmo3* seems to be regulating drug rewards and ethanol-induced anxiolysis in a sex-specific manner. Female $Lmo3^{Z}$ mice fail to form ethanol CPP (an indication that they do not respond to the rewarding effects of alcohol) and fail to experience ethanol-induced anxiolysis in the elevated plus maze task. Interestingly, these behavioral phenotypes in female $Lmo3^{Z}$ mice are likely not attributable to merely a deficit in learned behavior, as evidenced by the cocaine CPP task. Not only do female $Lmo3^{Z}$ mice form cocaine CPP, they actually exhibit enhanced preference for this drug relative to wild type mice.

It should be noted that while clear sex differences emerged in all three experiments, male $Lmo3^{Z}$ mice do show alterations in both cocaine CPP and ethanol-induced anxiolysis. The cocaine preference score failed to reach a statistically significant difference in males, but $Lmo3^{Z}$ mice showed a trend for increased preference relative to wild type mice. Similarly, while male $Lmo3^{Z}$ mice did exhibit ethanol-induced anxiolysis, it was an attenuated response relative to that which was seen in wild type mice. Clearly, male $Lmo3^{Z}$ mice responded to the anxiolytic effect of alcohol, but they still showed an anxiety-like response on the maze after an ethanol injection that was completely abolished in wild type mice.

There is very little known about how *Lmo3* could be operating to regulate behavior in a sex-specific manner. A related member of the LMO family of proteins, *Lmo4*, has been known to associate with estrogen receptor alpha (ERα) to alter transcription (Lasek, Gesch, Giorgetti, Kharazia, & Heberlein, 2011; Singh, Barnes, Talukder, Fuqua, & Kumar, 2005), and the *Lmo3*

cofactor protein CLIM has also been shown to inhibit ERα transcriptional activity (Johnsen et al., 2009). It is possible that *Lmo3* also interacts with ERα (or perhaps a different estrogen receptor) to repress its activity. In this case, the loss of *Lmo3* would lead to enhanced ERα activity, which could impact female $Lmo3^{Z}$ mice (with higher levels of the ERα ligand, estrogen) to a greater extent than male $Lmo3^{Z}$ mice.

Additionally, parvalbumin is known to be sexually dimorphic, particularly in the basolateral amygdala, with females showing greater numbers of parvalbumin-positive neurons than males (Rowniak, Bogus-Nowakowska, & Robak, 2015). Given that $Lmo3^{Z}$ mice have been shown to have deficits in parvalbumin-positive interneuron expression (Au et al., 2013), it is possible that the loss of *Lmo3* may hit females harder than males.

Intriguingly, female $Lmo3^{Z}$ mice show elevations in transcription of the delta subunit of the $GABA_A$ receptor (*Gabrd*). This subunit is regulated by allopregnanolone (ALLO), a derivative of the sex hormone progesterone and allosteric modulator of the $GABA_A$ receptor (with a preference for receptors containing the delta subunit) (H. Shen & Smith, 2009). ALLO levels are higher in females than in males (Quinones-Jenab et al., 2008), and males and females show differences in ALLO release after both cocaine and ethanol exposure (Finn et al., 2004; Quinones-Jenab et al., 2008). Interestingly, ALLO has been shown to have no effect on the formation of ethanol preference (Gabriel, Cunningham, & Finn, 2004; Murphy, Sakoori, & Okabe, 2006), but this may be due in part to the dose used, with low doses producing no effect on preference and high doses actually inducing an aversion (Beauchamp, Ormerod, Jhamandas, Boegman, & Beninger, 2000). It is unknown whether ALLO levels are altered in female $Lmo3^{Z}$ mice and whether this may explain the enhanced transcription of *Gabrd* in these mice or, indeed,

the altered drug reward observed for both alcohol and cocaine. ALLO levels may in fact be negatively regulated by *Lmo3*; future work would need to determine whether this is the case.

At this point it is unclear whether *Lmo3* influences transcription differently between males and females (perhaps through association with sexually dimorphic receptors, like estrogen receptors), or whether it creates structural changes in development that promote sexually dimorphic behavior (via parvalbumin expression). Additionally, although no sex difference was observed in binge drinking or basal anxiety, the sexually dimorphic results documented here in $Lmo3^{Z}$ mice may only be a subset of behaviors that are altered in a sex-specific manner. Future work should examine additional behavioral measures in male and female $Lmo3^{Z}$ mice to fully answer this question.

5. CONCLUSIONS

5.1. Introduction

Anxiety disorders (AD) and alcohol use disorders (AUD) are highly prevalent and often comorbid. To date, no effective treatment exists for comorbid AD and AUD, highlighting the current lack of understanding of the etiology of these disorders. Genetic vulnerability accounts for much of the heritability risk, highlighting the great need to identify genes that contribute to both anxiety and alcohol use. Therefore, the purpose of this dissertation was to examine the role of a gene involved in central nervous system development, Lim-domain-only 3, or *Lmo3*, in the regulation of anxiety-like behavior and alcohol-related phenotypes. We further sought to characterize the regions of the brain in which *Lmo3* acted to regulate behavior, as well as potential downstream targets of *Lmo3* that could account for any alterations in behavioral outcomes.

5.2. *Lmo3* promotes anxiety-like behavior and inhibits excessive alcohol consumption

Utilizing the $Lmo3^{Z}$ mice, we identified *Lmo3* as a novel regulator of both anxiety (Figures 1 and 2) and binge-like alcohol consumption (Figure 8), although these results were not in the direction that we anticipated. Anxiety and alcohol use often co-occur, and the direction of this comorbidity is typically positive (i.e., high anxiety drives drinking behavior or excessive alcohol use leads to anxiety). Here however, $Lmo3^{Z}$ mice exhibit a reduced anxiety phenotype, yet they engaged in elevated binge drinking. Three rat lines that have been selectively bred for high alcohol consumption, the alcohol-preferring (P) rat, the Sardinian alcohol-preferring rat (sP), and its closely related Marchigian Sardinian alcohol-preferring (msP) rat, all exhibit high anxiety

phenotypes that are thought to at least partially account for their elevated alcohol consumption (Ciccocioppo et al., 2006; Colombo et al., 1995; Colombo et al., 2014; Hwang et al., 2004). However, this association between high anxiety and high alcohol consumption in rodent lines is far from ubiquitous. A number of additional rat lines that have been selectively bred for high alcohol consumption either fail to show an association with anxiety, or exhibit an anxiolytic-like phenotype. For instance, the high-alcohol-drinking (HAD) rat displays similar phenotypes on the elevated plus maze as its low-alcohol-drinking (LAD) counterpart (Hwang et al., 2004). Similarly, the ALKO alcohol-preferring AA rat line exhibits no difference in performance on the elevated plus maze when compared to its alcohol-avoiding ANA line (Tuominen et al., 1990). Further, the Warsaw Alcohol High-Preferring (WHP) line actually exhibits a reduced anxiety phenotype (Acewicz et al., 2014) and the high-ethanol-preferring (HEP) rat shows a sexually dimorphic effect of anxiety and alcohol consumption, with the females consuming more alcohol than the males while exhibiting less anxiety (Myers et al., 1998). Additionally, in mice, the high-drinking-in-the-dark (HDID) mouse lines exhibit either no alteration in anxiety-like behavior or a slight reduction in basal anxiety levels (Barkley-Levenson & Crabbe, 2015), and the high-alcohol-preferring (HAP) mouse lines either exhibit reduced anxiety or no alterations in anxiety, depending on the task (Can, Grahame, & Gould, 2012).

One explanation for the reduced-anxiety/high-alcohol-consumption phenomenon could be an increase in risk-taking or exploratory behavior. Indeed, the sP rat line that exhibits a high anxiety phenotype also shows reduced risk-taking behavior in the multivariate concentric square field (MCSF) test (Roman & Colombo, 2009). AA rats that exhibit reduced anxiety also display an elevation in risk-taking in this task (Roman, Meyerson, Hyytia, & Nylander, 2007). In this model, rather than the "drinking to reduce anxiety" concept, an anxiolytic phenotype is thought

to result in a concomitant increase in risky behaviors that drive drinking. It is possible that the *Lmo3*^{Z} mice engage in elevated binge drinking because of their reduced anxiety phenotype; reduced anxiety in these mice may be paired with increased novelty-seeking or risk-taking that is driving alcohol consumption.

There are also behavioral measures that correlate with high alcohol consumption that are completely independent of anxiety-like behavior. One such behavioral measure is conditioned taste aversion. The HDID mouse lines and the WHP rat line exhibit a reduced sensitivity to taste aversion relative to their control lines (HS and WLP, respectively) (Barkley-Levenson, Cunningham, Smitasin, & Crabbe, 2015; Dyr et al., 2016). Indeed, the resistance to conditioned taste aversion in the HDID lines was one of the only behavioral measures that distinguished HDID mice from their heterogeneous control stock population (HS) – no difference between lines has been observed for behavioral inhibition (Tipps, Moschak, & Mitchell, 2014), ethanol reward (Barkley-Levenson et al., 2015), ethanol-induced anxiolysis (Barkley-Levenson & Crabbe, 2015), low-to-moderate two-bottle choice consumption (Crabbe, Spence, Brown, & Metten, 2011), or fear conditioning (Crabbe et al., 2016). Conditioned taste aversion may then be a reliable predictor for binge-like alcohol consumption.

To date, very little is known about how *Lmo3* alters behavior. Beyond the scope of this dissertation, only a few projects have even attempted to evaluate *Lmo3* as a regulator of behavior. Our lab has previously explored the role of *Lmo3* in regulating sensitivity to the sedative effects of alcohol as well as low-to-moderate alcohol consumption and preference in a two-bottle choice task. Interestingly, *Lmo3* reduced sensitivity to the sedative effects of alcohol and its expression was positively correlated with low-to-moderate alcohol consumption (Lasek, Giorgetti, et al., 2011). This latter result is a particularly intriguing finding in that it suggests that

Lmo3 drives low-to-moderate alcohol consumption, while this dissertation provided evidence that *Lmo3* serves a protective role in inhibiting binge-like alcohol consumption. The opposing results obtained in low-to-moderate alcohol consumption and binge-like alcohol consumption could be due to several factors. First, manipulation of *Lmo3* varied between these two experiments, with low-to-moderate alcohol consumption being measured in mice with global knockdown of *Lmo3* (≤50% reduction of *Lmo3* observed) and binge drinking in the drinking-in-the-dark (DID) task being conducted in complete knockout mice. It is possible that the binge drinking phenotype we observed in $Lmo3^{Z}$ mice was due to structural alterations in brain connectivity that were a result of a complete lack of *Lmo3* throughout critical periods of development. Future work is needed to parse out the contributions of *Lmo3* in the adult mouse versus the developing mouse in this binge drinking phenotype. Further, genetic background could also account for discrepancies observed in these two behaviors – mice with knockdown of *Lmo3* were created with C57BL/6J embryos, while $Lmo3^{Z}$ mice had approximately 25% 129 background (the remainder being C57BL/6J). Finally, it is important to acknowledge that this discrepancy between low-to-moderate alcohol consumption and binge-like alcohol consumption is not an aberration. HDID mice that exhibit increased binge-like consumption show no alteration in two bottle choice consumption (Crabbe et al., 2011), an effect that is also observed in $Lmo3^{Z}$ mice (Savarese et al., 2014). The two tasks are distinct, with differences in frequency of alcohol access (limited access in DID, continuous access in two-bottle choice), ethanol concentrations (20% in DID, increasing gradations from 3-20% in two-bottle choice), and typical blood ethanol concentrations (BECs) observed (> 100 mg/dl in DID, < 100 mg/dl in two-bottle choice), that can contribute to differences in patterns of drinking and overall consumption.

Beyond this limited work on the role of *Lmo3* in regulating behavior, several other LMO proteins have been shown to regulate similar behavioral phenotypes. Drosophila carry a homolog to the LMO family of proteins found in mammals, *dLmo*, that has been associated with increased sensitivity to cocaine (Tsai et al., 2004) and alcohol (Lasek, Giorgetti, et al., 2011), suggesting an evolutionarily conserved role for these proteins in the regulation of behavioral responses to drugs of abuse. Although these proteins have been largely ignored in human behavioral research, one genome-wide association study found an association between the *Lmo1* gene and the maximum number of alcohol drinks consumed in a 24-hour period (Kapoor et al., 2013), highlighting the significance this family of proteins can have in translational research of alcohol abuse. In rodents, other LMO proteins also regulate relevant behavioral phenotypes, most notably LMO4. Heterozygous *Lmo4* knockout mice and mice with *Lmo4* knockdown in the nucleus accumbens (NAc) show increased cocaine sensitization (Lasek et al., 2010), although no effects of *Lmo4* regulation of alcohol have yet emerged. Interestingly, *Lmo4* has also been shown to regulate anxiety-like behavior; mice lacking LMO4 exhibit an anxiogenic phenotype (Qin et al., 2015). This result is particularly intriguing in that it suggests potential complementary roles of *Lmo3* and *Lmo4* in the regulation of anxiety, with *Lmo3* promoting, and *Lmo4* inhibiting, anxiety-like behavior.

To summarize, while $Lmo3^{Z}$ mice are likely not drinking more in order to reduce anxiety, future work should investigate other behavioral phenotypes (i.e., elevated risk-taking or novelty-seeking, or reduced conditioned taste aversion and/or behavioral inhibition) in these mice to shed light on the underlying mechanism of their excessive alcohol consumption. Taken together, these results suggest that the alteration in anxiety-like behavior and excessive alcohol consumption observed in $Lmo3^{Z}$ mice is likely due to independent mechanisms or pathways of *Lmo3*. We next

sought to observe whether manipulating *Lmo3* expression in various brain regions could alter anxiety-like behavior or binge drinking in $Lmo3^{Z}$ mice.

5.3. *Lmo3* promotes anxiety-like behavior via its actions in the basolateral amygdala

Anxiety and alcohol use share common neural networks that could underlie much of the comorbidity observed between these phenotypes. The amygdala, in particular, has emerged as a critical regulator of both alcohol abuse and anxiety (Craske et al., 2009; Gilpin et al., 2015; Muller-Oehring et al., 2017; Nuss, 2015). When examining putative *Lmo3* expression in the brains of heterozygous mice, we observed dense staining of the reporter β-galactosidase in the basolateral amygdala (BLA) and weaker staining in the central nucleus of the amygdala (CeA), (Figure 3). While density of staining is not always indicative of the impact a protein has on functional activity, nonetheless we first chose to focus on the potential role of the BLA as *Lmo3*'s site of action for regulation of anxiety-like behavior and binge drinking.

One of the disadvantages to the knockout mouse model is the inability to distinguish between behavioral phenotypes that are due to (1) an active role of that gene in the adult mouse and (2) structural changes that are induced by the lack of gene expression throughout development. This is an especially important consideration when examining genes that have a primary role in developmental neural differentiation and migration, as does *Lmo3* (Deng et al., 2006; Hinks et al., 1997; Tse et al., 2004). To circumvent this limitation of the knockout mouse, viral-mediated RNA interference (RNAi) allows for greater temporal control of gene expression by leaving developmental expression intact and reducing gene expression only in adult mice. RNAi has the added advantage of spatial control. Another disadvantage to a knockout mouse model is the inability to distinguish where in the brain a given gene is acting to influence behavior, since the

entire brain lacks gene expression. RNAi, however, involves viral delivery to a discrete brain region, allowing for the observance of behavioral changes induced by gene manipulations within a single brain area.

Therefore, to investigate the role of *Lmo3* in the BLA, we utilized RNAi by delivering a lentivirus containing a short hairpin RNA (shRNA) targeting *Lmo3* (shLmo3) or a nonspecific control sequence (shScr) into the BLA of wild type C57BL/6J mice. We found that reducing *Lmo3* expression specifically in the BLA of these mice replicated the behavioral phenotype observed in $Lmo3^{Z}$ mice (Figure 6), suggesting a direct role for *Lmo3* in regulating anxiety-like behavior in this brain region. Interestingly, *Lmo4* has also been shown to regulate anxiety, likely via its actions in the BLA (Qin et al., 2015). Knocking out *Lmo4* specifically in glutamatergic neurons induces an anxiogenic phenotype that can be reversed by inhibiting a downstream target of LMO4 in the BLA. Localizing the actions of both LMO3 and LMO4 in anxiety regulation to the BLA lends further support to the idea that these proteins may be working in a complementary fashion to regulate anxiety. Intriguingly, targeting the BLA for *Lmo4* knockdown produces no alteration in anxiety-like behavior (Maiya et al., 2012). This suggests either that a 50% knockdown of *Lmo4* is not sufficient to alter anxiety-like behavior, or that it is specifically the actions of LMO4 in glutamatergic neurons that produce the anxiety phenotype. The latter explanation would then suggest that LMO4 has opposing roles in glutamatergic versus non-glutamatergic cells.

It is possible that *Lmo4* and *Lmo3* are expressed in different cell types of the BLA. To date, no one has examined the expression of *Lmo3* within distinct subpopulations of cells in the BLA. There is evidence that *Lmo3* is expressed in GABAergic cells (Friocourt & Parnavelas, 2011) and in glia (Shi et al., 2001), so it is possible that it is opposing the actions of *Lmo4* via its role in

these cell subtypes. Alternatively, *Lmo3* and *Lmo4* could be expressed in the same cells in a dynamic fashion to regulate BLA output. Indeed, the fact that the BLA is primarily glutamatergic and *Lmo3* expression appears to be so dense in this area, suggests that *Lmo3* is likely expressed in glutamatergic cells. In support of this same-cell theory, evidence of cooperation between *Lmo3* and *Lmo4* has been shown in the regulation of the glycoprotein hormone alpha-subunit (αGSU) promoter in pituitary cell lines. *Lmo3* was shown to activate this promoter region while *Lmo4* inhibited it (Susa et al., 2009). Whether *Lmo3* and *Lmo4* function in a similar manner in the BLA to regulate anxiety remains to be determined.

These data suggest a novel role for *Lmo3* in regulating anxiety-like behavior, specifically via its actions in the adult mouse BLA. Importantly, knockdown of *Lmo3* in the neighboring CeA produced no alterations in anxiety-like behavior (Figure 7), further highlighting the importance of the BLA as the site of action for the regulation of anxiety-like behavior by *Lmo3*. Future work is needed to determine in which cell types *Lmo3* is expressed in the BLA to influence behavior. Stimulation of BLA projection neurons targeting the lateral portion of the CeA (CeL) produces a rapid anxiolytic response, so it may be that *Lmo3* is acting to decrease BLA output to this region of the CeL and therefore promote an anxiety-like phenotype. Whether *Lmo3* is decreasing BLA output to the CeL via its actions in these glutamatergic cells or whether it is acting in the GABAergic neurons of the BLA to inhibit these projection neurons, remains to be delineated.

5.4. Knockdown of *Lmo3* in neither the basolateral amygdala nor the nucleus accumbens alters binge-like alcohol consumption

After identifying an *Lmo3* locus of control for anxiety-like behavior in the brain, we next sought to identify which brain regions might contribute to the elevated binge-drinking phenotype observed by $Lmo3^{Z}$ mice. Unfortunately, attempts at replicating this behavioral phenotype in

mice with knockdown of *Lmo3* in the BLA or in the NAc proved unsuccessful (Figure 12). There could be many reasons for this failure to detect binge-drinking alterations in these mice. The simplest explanation is that *Lmo3* does not regulate excessive alcohol consumption through its actions in these brain regions. In addition to the BLA and NAc, other areas of the mesolimbic dopaminergic system like the ventral tegmental area (VTA) have also been implicated in binge drinking (Albrechet-Souza et al., 2015). *Lmo3* has been shown to be a dopamine-responsive gene (Shi et al., 2001), but it has also been found in dopaminergic cells (Bifsha et al., 2016), suggesting a potential role for its action in dopaminergic-cell-dense brain regions like the VTA. Interestingly, another protein involved in cell differentiation and survival, glial cell line-derived neurotrophic factor (GDNF) has been shown to regulate binge drinking via its actions in the VTA (Ahmadiantehrani, Barak, & Ron, 2014). Specifically, it acts in much the same manner as *Lmo3* by inhibiting excessive alcohol consumption. Further, CRF1R antagonism in the VTA reduces binge-like alcohol consumption (Rinker et al., 2017), suggesting a link between anxiety-related neuropeptides and drinking in this brain region.

Additionally, it is equally plausible that *Lmo3* does regulate binge drinking via its actions in either the BLA or the NAc, but the knockdown we observed was not sufficient to produce a behavioral phenotype. We observed only about 25% reduction of *Lmo3* mRNA after shLmo3 transfection. This was sufficient to alter anxiety-like behavior, but it is possible that the anxiety phenotype is more sensitive to alterations in *Lmo3* than binge drinking. Perhaps a more robust knockout of *Lmo3* expression in the BLA or NAc would have produced changes in binge drinking. Additionally, it is possible that *Lmo3* regulation of binge drinking requires its coordinated actions in more than one brain area. BLA projections to the NAc core bidirectionally control reinforcement and reward behavior (Stuber et al., 2011), an effect that is specific to BLA

excitatory input (mPFC inputs do not produce the same effect). It is possible that *Lmo3* acts both in the BLA to attenuate excitatory output to the NAc, as well as in the NAc (as a dopamine-responsive gene) to dampen the effect of VTA dopamine release on NAc activation. This type of model would require either (1) a more robust knockdown of *Lmo3* in either the BLA or NAc, or (2) simultaneous knockdown of *Lmo3* in both regions in order to alter binge drinking.

Finally, the possibility that *Lmo3* does not acutely modulate binge drinking at all, but rather $Lmo3^Z$ mice exhibit structural changes in the brain that lead to a drinking phenotype in adulthood, cannot be ruled out. $Lmo3^Z$ mice show decreases in the number of cortical parvalbumin-positive (Pv+) interneurons, and Pv+ interneurons in the prefrontal cortex have been shown to regulate extinction of reward seeking (Sparta et al., 2014). Therefore, the deficit in Pv+ cortical interneurons may suggest an exaggerated reward seeking phenotype in $Lmo3^Z$ mice that could induce elevated binge drinking. Further, inhibition of Pv+ interneurons also produces an elevated novelty-seeking phenotype (J. A. Brown et al., 2015). As mentioned previously, elevated novelty-seeking is often associated with a reduced anxiety phenotype, and novelty-seeking has not yet been examined in $Lmo3^Z$ mice.

Although the mechanism by which *Lmo3* regulates binge drinking is yet unknown, experiments in $Lmo3^Z$ mice suggest that *Lmo3* serves to inhibit excessive alcohol consumption. This phenotype is unique to a binge-drinking model, since $Lmo3^Z$ mice do not differ in low-to-moderate alcohol consumption. These data suggest that *Lmo3* may serve a protective role in the development of alcohol abuse, perhaps acting to promote resilience to escalated alcohol intake in individuals who drink moderately. Future studies should investigate whether $Lmo3^Z$ mice develop alcohol dependence at the same rate as wild type mice, or whether they become dependent at a faster rate.

5.5. *Lmo3* regulates transcription of CRF and GABA systems in the amygdala

LMO proteins can function in a number of ways, but perhaps they are best characterized for their role in transcriptional regulation. Instead of binding to DNA directly (like bona fide transcription factors), LMO proteins can act as scaffolding proteins to form multiprotein complexes, recruiting both transcription factors and co-activator/co-repressor proteins to the transcriptional start site. While this method of transcriptional regulation is well known, there is nearly nothing known about the target genes for *Lmo3*. LMO3 has been shown to transactivate transcription of *Mash1* (Isogai et al., 2011), a transcription factor involved in regulating neurogenesis, and likely does so early in embryonic development to promote proper neural differentiation. Likewise, overexpression of *Lmo3* activates αGSU promoter activity, while knocking down *Lmo3* has the reverse effect (Susa et al., 2009). Beyond these genes, no other transcriptional targets of *Lmo3* have been identified, to the best of our knowledge.

Because of the acute effect of *Lmo3* knockdown in the BLA on anxiety-like behavior, we attempted to identify downstream targets of *Lmo3* that may regulate anxiety. We focused on CRF and its receptor type 1 (CRF1R) given its robust role in regulation of anxiety (Contarino et al., 1999; Ising & Holsboer, 2007; Zorrilla et al., 2002), and we targeted the BLA given the results of *Lmo3* knockdown observed there. We detected a significant reduction in *Crhr1* mRNA expression (via qPCR) and CRF1R protein expression (via Western blotting) in the BLA of $Lmo3^{Z}$ mice relative to wild type mice (Figures 4 and 5), suggesting that *Lmo3* may promote transcription of the *Crhr1* gene in the BLA.

It is possible that this reduction of transcription and translation of the *Crhr1* gene is responsible for the anxiolytic phenotype we observed in $Lmo3^{Z}$ mice. However, we did not find

that *Lmo3* knockdown in the BLA produced a subsequent reduction in *Crhr1* transcription as one would expect if this were true. The knockdown may not have been robust enough to alter transcriptional activity of *Lmo3* targets, but it *was* robust enough to alter anxiety-like behavior, suggesting that *Lmo3* may be mediating anxiety through a CRF1R-independent mechanism. Alternatively, the techniques used to detect mRNA in the BLA of shLmo3 mice were just not sensitive enough to detect transcriptional changes in *Crhr1*, even though those transcriptional changes were significant enough to produce relevant behavioral alterations. CRF1R has been found to localize almost exclusively on the glutamatergic projection neurons of the BLA (Rajbhandari, Baldo, & Bakshi, 2015), so a reduction of CRF1R in the BLA of $Lmo3^{Z}$ mice would presumably lead to alterations in BLA output. CRF1Rs are G-protein coupled receptors that have been shown to bind up to 5 different G-proteins in the cortex alone, leading to either activation or inhibition of the cells in which they are expressed (Grammatopoulos, Randeva, Levine, Kanellopoulou, & Hillhouse, 2001). For this reason, it is not clear whether reduced CRF1R expression in the BLA would lead to increased or decreased BLA output. As mentioned above, activation of BLA projections to the CeL is anxiolytic so it may be that $Lmo3^{Z}$ mice have enhanced BLA-CeL output. If CRF1R expression was reduced in these specific projection neurons, CRF1R may be linked to a G_i protein in these cells, such that a reduction of CRF1R expression would produce increased cellular activity and result in elevated BLA-CeL activation.

Interestingly, we did not observe significant alterations of CRF1R in the CeA of $Lmo3^{Z}$ mice, although we did detect reductions of *Crhr1* mRNA (Figures 4 and 5). These data potentially hint at altered CRF1R protein activity in the CeA (hyperactivity) that would lead to a compensatory downregulation of gene transcription. LMO proteins have been shown to operate in the cytosol as well as functioning as transcriptional regulators (Baron et al., 2015; Hui et al., 2009; Qin et

al., 2015), so it is conceivable that *Lmo3* could regulate transcription of the *Crhr1* gene and, separately, the activity level of its protein CRF1R. If this were the case, enhanced CRF1R protein activity in the CeA could very well underlie the elevated binge drinking observed in these mice, as there is a rich literature surrounding the role of CRF1R in the CeA promoting alcohol intake (Gilpin & Roberto, 2012; Haass-Koffler et al., 2016; Lowery-Gionta et al., 2012). Unfortunately, we did not choose the CeA as one of our neural targets for *Lmo3* knockdown in the binge drinking studies, so this question remains unanswered. Future work should examine whether CRF1R protein activity is altered in *Lmo3*^{Z} mice and whether this alteration underlies their elevated binge-drinking phenotype.

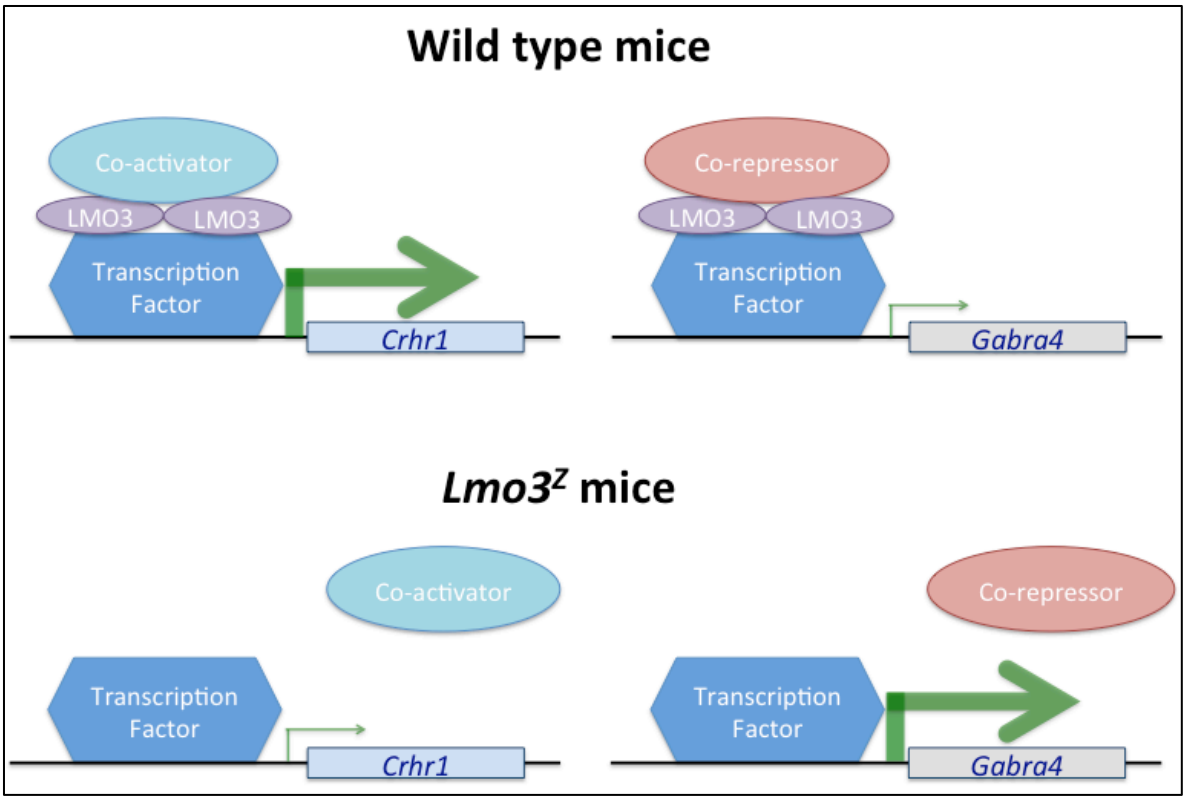


Figure 16. Potential mechanism for transcriptional regulation of the *Crhr1* and *Gabra4* genes by LMO3

In addition to examining transcription of CRF and CRF1R in the brains of *Lmo3*^{Z} mice, we examined expression levels of $GABA_A$ receptor subunits α1 (*Gabra1*), α4 (*Gabra4*), and δ (*Gabrd*). We detected no genotype differences in the mRNA expression of these 3 subunits in either the NAc or the CeA, but in the BLA we saw enhanced *Gabra4* mRNA expression in *Lmo3*^{Z} mice as well as enhanced *Gabrd* mRNA expression in female *Lmo3*^{Z} mice (Figure 10). However, once again the results we obtained for mRNA levels did not coincide with protein expression changes measured via Western blotting. No differences were seen in either $GABA_A$ α4 subunit or the $GABA_A$ δ subunit in the BLA of *Lmo3*^{Z} mice (Figure 11). While these results were not what we anticipated, as with CRF1R in the CeA, the dissociation between mRNA and protein expression could very well indicate altered function of the $GABA_A$ receptor subunits in *Lmo3*^{Z} mice. In this case, enhanced mRNA expression could be a compensatory change indicative of hypoactive $GABA_A$ receptors containing the α4 or δ subunits. These receptors may undergo posttranslational modifications that inhibit their trafficking to the membrane, or increase their rate of internalization, or potentially even enhance degradation. There is evidence of dynamic changes in surface expression of the α4 subunit that occurs with ethanol exposure and withdrawal that is independent of total expression of this subunit (Lindemeyer et al., 2014), suggesting that transcriptional activity of the *Gabra4* gene is not always indicative of protein function. $GABA_A$ α4 and δ subunits are unique among $GABA_A$ receptor subunits in that they predominantly localize extrasynaptically and mediate tonic inhibition (Chandra et al., 2006; Marowsky & Vogt, 2014). Alterations in their function that could induce transcriptional changes could include a switch from extrasynaptic to synaptic receptor localization, as is seen with ethanol withdrawal (Liang et al., 2004). The effects of these alterations on signaling could be varied, depending on the cell types on which they are expressed, but there is evidence of

alterations in the $GABA_A$ α4 subunit in at least one rat one selectively bred for high alcohol consumption (Stankiewicz et al., 2015), suggesting a potential role for this protein in the regulation of binge drinking.

The sex-specific effect of *Lmo3* on *Gabrd* transcription is intriguing. Delta subunits of the $GABA_A$ receptor are unique in that they are regulated by the neurosteroid allopregnanolone (ALLO), a derivative of the sex hormone progesterone (H. Shen & Smith, 2009). It may be that *Lmo3* regulates ALLO synthesis, and because ALLO expression has been shown to be higher in females than in males (Quinones-Jenab et al., 2008), the effects of the loss of *Lmo3* are felt more acutely in female $Lmo3^Z$ mice than in male $Lmo3^Z$ mice. Because we failed to observe a difference in protein expression of the δ subunit but an elevation of *Gabrd* mRNA was detected in female $Lmo3^Z$ mice, it may be that $Lmo3^Z$ mice show a deficit in δ-containing $GABA_A$ receptor function that then leads to an increase in *Gabrd* transcription as a compensatory response.

It is important to note here that these results do not show a direct role for *Lmo3* in regulating transcription of the above-mentioned genes. While $Lmo3^Z$ mice show alterations in mRNA expression, these may in fact be secondary effects of the loss of *Lmo3* induced by other neural alterations in these mice. In order to examine a direct effect of *Lmo3* on transcriptional regulation, additional experiments would need to be conducted. One such potential experiment is chromatin immunoprecipitation (ChIP), whereby a direct examination of *Lmo3* forming a complex at the promoter region of a gene could be conducted. The main technical limitation is we have yet to identify an antibody that is specific to *Lmo3*. We tested several LMO3 antibodies that identified bands at the approximate weight of LMO3 in both $Lmo3^Z$ and wild type mice in Western blotting experiments, suggesting a non-specific binding of the antibodies.

Taken together, these results show altered transcription of genes key to anxiety-like behavior and binge drinking in the *Lmo3*^{Z} mice that may indicate a role for *Lmo3* in their transcriptional regulation. LMO proteins do not only function as transcriptional regulators, though. Mounting evidence suggests roles for them in posttranslational modifications of proteins (Qin et al., 2015) and LMO3 specifically has been shown to translocate into the cytosol when bound to calcium-and-integrin-binding protein (CIB) (Hui et al., 2009). Future work is needed to determine whether *Lmo3* acts as a transcriptional regulator to alter *Crhr1* and $GABA_A$ receptor subunit genes, or whether its primary role is in the cytosol. Additionally, in order to better understand how alterations in *Lmo3* could affect signaling pathways, it is critical that the cell types in which *Lmo3* is expressed be identified.

5.6. *Lmo3* inhibits the perception of the rewarding effects of cocaine in females

Given the role of Drosophila *dLmo* in regulating sensitivity to both ethanol and cocaine, and the role of *Lmo4* in regulating cocaine phenotypes, we asked whether the effects of *Lmo3* on alcohol-related phenotypes also extend to cocaine. To examine this possibility, *Lmo3*^{Z} and wild type mice were tested in the cocaine conditioned place preference (CPP) task as a measure of cocaine reward. We hypothesized that we would see alterations in cocaine reward because *Lmo3* expression levels in the NAc correlate with drug intake (Savarese et al., 2014), the primary neurobiological target of cocaine is the dopamine transporter, *Lmo3* is a dopamine-responsive gene (Shi et al., 2001), and *Lmo3* has a role in dopaminergic cell development (Bifsha et al., 2016).

Female *Lmo3*^{Z} mice exhibited a significant enhancement in cocaine CPP relative to female wild type mice, and there was also a trend toward enhanced cocaine CPP in male *Lmo3*^{Z} mice

(Figure 14). One mechanism that may be driving this change in cocaine reward is alterations in ALLO in *Lmo3*^{Z} mice. In addition to being a positive allosteric modulator of δ-containing $GABA_A$ receptors, ALLO has also been shown to negatively regulate cocaine-related behaviors. In cocaine dependent individuals, administration of ALLO has been shown to reduce craving for cocaine (Milivojevic, Fox, Sofuoglu, Covault, & Sinha, 2016). Additionally, female rats that receive ALLO do not escalate cocaine self-administration (Anker, Zlebnik, & Carroll, 2010) and are resistant to stress-induced reinstatement of cocaine seeking (Anker & Carroll, 2010). Importantly, this latter effect of ALLO was only found in female rats – ALLO produced no alteration in stress-induced reinstatement of cocaine seeking in male rats (Anker & Carroll, 2010). These data suggest that ALLO regulates cocaine-related behaviors in a sex-specific manner and lend support for a role of ALLO in the cocaine reward enhancement exhibited by female *Lmo3*^{Z} mice.

Importantly, this enhancement of cocaine CPP was not due to a hypersensitive reward system. *Lmo3*^{Z} mice show no alterations in consumption of a naturally rewarding substance, sucrose. This effect could be indicative of a role for ALLO as well – administration of ALLO to female rats does not alter sucrose consumption and the escalation in sucrose intake observed after yohimbine is not blocked by ALLO (Anker et al., 2010), suggesting distinct mechanisms in the regulation of sucrose and cocaine intake by ALLO. Future work is needed to determine whether ALLO is regulated by *Lmo3*, or whether it is altered in *Lmo3*^{Z} mice to account for differences in their sensitivity to rewarding substances.

5.7. *Lmo3* regulates ethanol reward and ethanol anxiolysis in a sex-specific manner

Acute behavioral responses to alcohol may shed light on the mechanisms driving binge-drinking behavior. Because we failed to identify a pathway for regulation of binge drinking by *Lmo3*, we sought to further characterize the responses of $Lmo3^{Z}$ mice to acute alcohol exposure. We examined the rewarding effects of alcohol with the ethanol CPP task (Figure 13) and the anxiolytic effects of alcohol with the elevated plus maze task following a single low dose of ethanol (Figure 15). Female $Lmo3^{Z}$ mice once again showed a unique phenotype in that they failed to develop ethanol CPP (suggesting a failure to detect ethanol's rewarding properties) and ethanol-induced anxiolysis (suggesting a failure to detect the acute anxiolytic effects of ethanol).

These results are intriguing in that female $Lmo3^{Z}$ mice show no indication that they find alcohol rewarding, yet they still engage in elevated binge drinking relative to wild type mice. This dissociation could be indicative of a high reward threshold for female $Lmo3^{Z}$ mice – they have to drink more to reach the same level of reinforcement of wild type mice. This explanation is flawed, though, in that female $Lmo3^{Z}$ mice show enhanced reward for cocaine, suggesting that the reward system in these mice is not hypoactive. There is evidence that ALLO produces a dose-dependent effect on ethanol reward, promoting the rewarding effects of alcohol at lower doses and producing an aversive response at high doses (Beauchamp et al., 2000), more evidence that supports an investigation of ALLO alterations in $Lmo3^{Z}$ mice.

An alternative explanation is that female $Lmo3^{Z}$ mice are not able to perceive alcohol's rewarding properties when they first receive it in a passive, post-ingestive manner (via intraperitoneal injection in the CPP task). This could also account for the failure of female $Lmo3^{Z}$ mice to experience ethanol-induced anxiolysis since the method of ethanol exposure is the same

as in ethanol CPP. A similar effect has been documented in the UChB rat line that has been selectively bred for high alcohol consumption. UChB rats will develop conditioned place aversion to ethanol if they have never been exposed to it before, but voluntary consumption of alcohol for two months prior to undergoing the CPP task will produce an enhanced ethanol CPP response in these rats (Quintanilla & Tampier, 2011).

Mice show initial signs of pain after an i.p. injection of ethanol, so it may be that female *Lmo3*^{Z} mice have an enhanced pain sensitivity that overrides any pleasurable intoxicating or anxiolytic effects of an acute dose of ethanol. Future experiments should pursue this possibility of altered pain sensitivity in *Lmo3*^{Z} mice. Once again, ALLO emerges as a potential regulator of this sex-specific behavioral phenotype. ALLO levels are negatively correlated with pain sensitivity in humans (Mechlin, Morrow, Maixner, & Girdler, 2007; Naylor et al., 2016) and in rats (M. Zhang et al., 2016) and mice (Sasso et al., 2012).

It is worth mentioning too that while female *Lmo3*^{Z} mice showed deficits in both ethanol CPP formation and ethanol-induced anxiolysis, this is likely not due to an alteration in learning in these mice. While no direct test of learning was administered, female *Lmo3*^{Z} mice actually exhibited an enhanced CPP to cocaine, an effect that would not have been observed had they not been able to learn to associate the drug with the environment. It is also important to note that while female *Lmo3*^{Z} mice showed reduced basal anxiety, this is not sufficient to explain the lack of ethanol-induced anxiolysis. They still exhibited a clear preference for the closed arm, even though they spent more time in the open arm than wild type mice. Wild type mice exhibit such a robust anxiolytic response to an acute dose of ethanol that they spend equal amounts of time in both the open and closed arm, while female *Lmo3*^{Z} mice exhibit preference for the closed arm under basal and post-ethanol conditions.

It is also important to note that while the genotype effect was only observed in females, there was a trend towards similar responses in male *Lmo3*^{Z} mice for ethanol-induced anxiolysis. This is intriguing in that it suggests an intermediate phenotype in males. *Lmo3* may be regulating alcohol anxiolysis in both sexes, but females are more sensitive to the loss of *Lmo3* than males. Whether this is due to differences in network connectivity produced throughout development or whether sex hormones may be acutely modulating these responses remains to be explored.

Table 1. Sex-specific behavioral phenotypes regulated by *Lmo3*

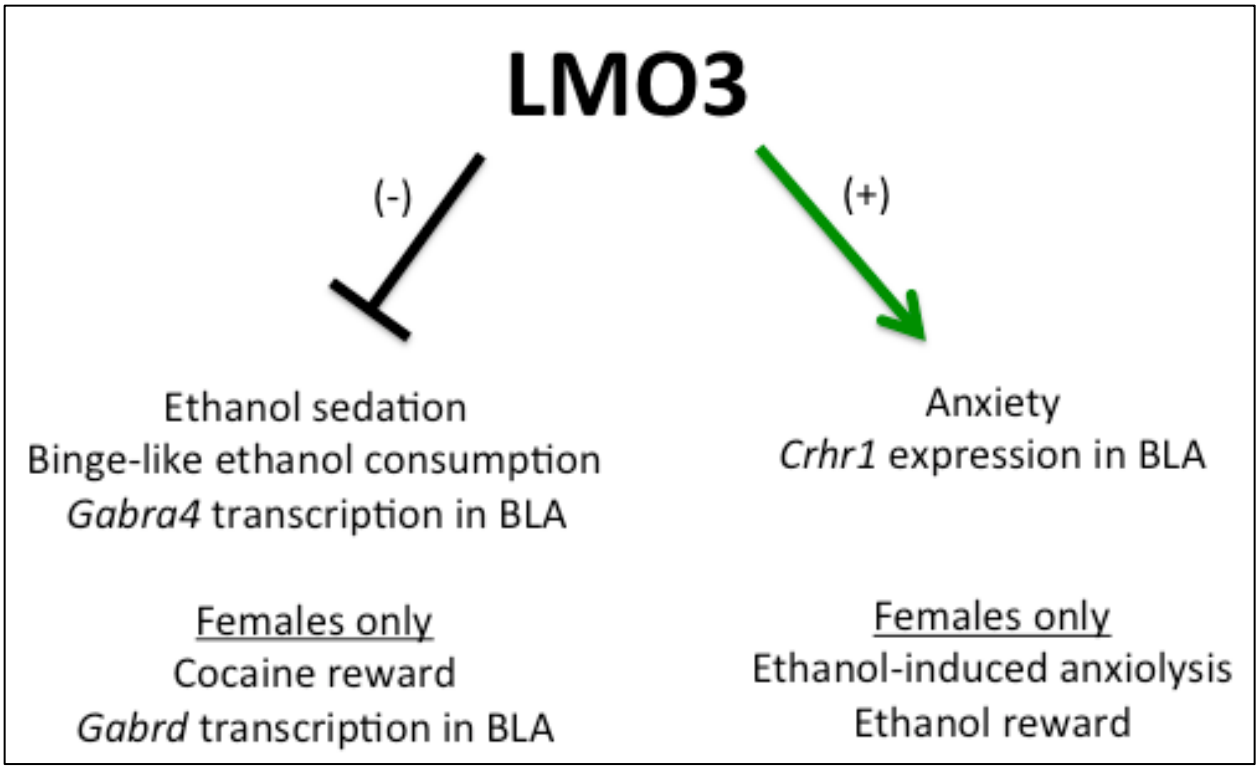
	Females	Males
Cocaine CPP	Enhanced in *Lmo3*^{Z} mice	No genotype effect
Ethanol CPP	Absent in *Lmo3*^{Z} mice	No genotype effect
Ethanol-induced anxiolysis	Absent in *Lmo3*^{Z} mice	No genotype effect
***Gabrd* expression**	Enhanced in *Lmo3*^{Z} mice	No genotype effect

5.8. Summary

The results of this dissertation support a role for *Lmo3* in the regulation of both anxiety and binge-like alcohol consumption. Specifically, *Lmo3* promotes anxiety-like behavior and inhibits excessive alcohol intake. The actions of *Lmo3* in the BLA drive its promotion of anxiety-like behavior, although the neural mechanisms underlying its inhibition of binge drinking are less clear. Intriguingly, the closely related gene *Lmo4* has previously been identified as a protective factor against anxiety. This work paves the way for a more in-depth investigation into the coordinated actions of *Lmo3* and *Lmo4* in dynamic regulation of anxiety.

These results also suggest new targets of *Lmo3* – namely, CRF1R and the $GABA_A$ receptor subunits α4 and δ. Whether *Lmo3* is directly regulating transcription of these targets or is modulating their expression via an indirect mechanism remains to be delineated. Additionally, these targets are likely only a small subset of the genes that can be altered by *Lmo3* – a more extensive exploration of downstream targets of *Lmo3* is justified. Finally, *Lmo3* operates in a sex-specific manner to regulate several drug-related phenotypes, including ethanol and cocaine reward, and ethanol-induced anxiolysis. The elevated sensitivity to the actions of *Lmo3* in female mice is an intriguing effect that should be further explored.

Figure 17. Summary of findings of transcriptional and behavioral regulation by *Lmo3*



5.9. Future directions

Although many questions were answered in the course of this dissertation, much remains unknown about how LMO3 is functioning in the adult brain to influence behavior, specifically alcohol-related behavior. Future work should particularly focus on (1) the mechanisms by which *Lmo3* is regulating binge drinking, (2) how *Lmo3* operates differently in the male and female brain to produce sex-specific behavioral phenotypes, and (3) whether *Lmo3* is involved in the development of alcohol dependence.

Identifying which cell types express *Lmo3* will provide a better understanding of how *Lmo3* may be regulating binge drinking. Although there is no good antibody for *Lmo3*, heterozygous *Lmo3* mouse brain tissue could be stained for beta-galactosidase as a marker for putative *Lmo3* expression. Staining for GABAergic (GAD67), glutamatergic (CaMKII), and glial (GFAP) cell markers will then provide greater insight into the cellular location of *Lmo3* in the brain. Further, this dissertation has provided evidence that *Lmo3* regulates transcription of GABA-A receptor subunits, but it remains unknown whether these receptors are functioning differently in $Lmo3^{Z}$ mice. Although protein expression of the GABRA4 subunit remained unchanged in $Lmo3^{Z}$ mice, there may exist alterations in receptor localization and/or function in these mice. To examine this question, surface levels of GABRA4 and GABRD should be measured in $Lmo3^{Z}$ and wild type mice, at baseline and after an acute dose of ethanol. Results from this experiment will lend insight into whether $Lmo3^{Z}$ mice have deficits in trafficking or internalization of these GABA-A receptor subunits. It is conceivable, however, that alterations in the GABA-A receptor are not responsible for the altered binge drinking phenotype observed in $Lmo3^{Z}$ mice. In order to investigate other potential downstream targets of *Lmo3* that may be important for binge drinking, an unbiased approach could be utilized, such as RNA-Seq (RNA-sequencing). Novel targets of

Lmo3 may then be identified that can inform future mechanistic studies. Lastly, to investigate whether the reduced baseline anxiety observed in *Lmo3*^{Z} mice promotes binge-like alcohol consumption by increasing novelty seeking or risk-taking, *Lmo3*^{Z} and wild type mice can be tested in a novel object exploration task or a predator odor risk-taking task. The former task measures the amount of time animals spend with novel and familiar objects, and could determine whether *Lmo3*^{Z} mice have an enhanced interest in novelty. The predator odor risk-taking task, in contrast, evaluates risk-taking behavior where mice have to weigh the benefit of receiving a highly palatable food with the risk of being exposed to predator odor. Taken together, these experiments will help determine whether *Lmo3*^{Z} mice have alterations in inhibitory-like behaviors that underlie their propensity to binge drink more.

In order to explore the sex-specific effects of *Lmo3* in regulating drug reward and ethanol-induced anxiolysis, allopregnanolone (ALLO) levels should be measured in female *Lmo3*^{Z} and wild type mice. ALLO has been shown to negatively regulate cocaine craving (Milivojevic et al., 2016) and pain sensitivity (Mechlin et al., 2007; Naylor et al., 2016; Sasso et al., 2012; M. Zhang et al., 2016). Female *Lmo3*^{Z} mice may have decreased ALLO levels and increased pain sensitivity, whereby the pain associated with an intraperitoneal injection of ethanol overrides the rewarding or anxiolytic effects of the drug. Low levels of ALLO in female *Lmo3*^{Z} mice could then explain the enhanced cocaine reward, as well as the lack of alcohol reward or ethanol-induced anxiolysis, exhibited by these mice. Measuring ALLO levels and pain sensitivity (utilizing Von Frey microfilaments for mechanical pain evaluation) in *Lmo3*^{Z} and wild type mice may provide a mechanism to explain the sex-specific behaviors observed by these mice.

Finally, the work completed thus far on characterizing the role of *Lmo3* in regulating alcohol-related behaviors has been under acute or short-term alcohol exposure. No work has yet been

done to determine whether *Lmo3* is involved in the development of alcohol dependence. *Lmo3*^{Z} mice engage in elevated binge drinking which may mean that they are more susceptible to the formation of alcohol dependence. Alternatively, the elevated binge drinking could be a product of increased novelty-seeking in *Lmo3*^{Z} mice and may normalize after a single week of alcohol exposure. In this case, *Lmo3*^{Z} mice may actually be resistant to the development of dependence. Testing the *Lmo3*^{Z} and wild type mice in an extended 6- or 12-week DID could determine whether Lmo3 continues to regulate binge-like alcohol consumption after chronic alcohol exposure. Testing these mice in an anxiety measure, like the elevated plus maze task, after the extended DID would then also provide a measure of ethanol-withdrawal-induced anxiety. *Lmo3*^{Z} mice have reduced baseline anxiety relative to wild types, but exhibit either attenuated ethanol-induced anxiolysis (male *Lmo3*^{Z} mice) or fail to exhibit any ethanol-induced anxiolysis (female *Lmo3*^{Z} mice). It is possible therefore, that *Lmo3*^{Z} mice may also show altered susceptibility to ethanol-withdrawal-induced anxiety as well.

Taken together, these experiments could provide greater insight into whether *Lmo3* serves as a resilience factor for alcohol abuse and dependence, and how it may operate differently between males and females.

5.10. Overall conclusions

This body of work is the first to comprehensively explore the role of the *Lmo3* gene in regulating behavior, and particularly how this regulation of behavior could be driven by the actions of *Lmo3* in distinct brain regions and via regulation of specific genes. Importantly, this work is the first to identify *Lmo3* as a genetic risk factor for anxiety. While *Lmo3* is likely not a viable target for drug discovery currently, that is not to say that with the advent of enhanced drug

delivery systems that could target specific brain regions discriminately that *Lmo3* would not serve to be an effective target for anxiolytic therapies. Reducing *Lmo3* expression specifically in the BLA could prove to be therapeutic for individuals with anxiety disorder. In the meantime, determining SNPs in the *Lmo3* gene that are indicative of high *Lmo3* expression could serve to identify certain individuals most at-risk for developing anxiety. Results from this dissertation also suggest a novel role for *Lmo3* as a protective factor against alcohol abuse and could serve to increase understanding of the mechanisms underlying resilience to alcohol dependence. Taken together, these data suggest that LMO3 is a novel regulator of anxiety-like behavior and behavioral responses to alcohol that may serve to shed light on distinct neural mechanisms that underlie anxiety and alcohol abuse.

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APPENDICES

APPENDIX A

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APPENDIX B

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (OACIB) (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

4/21/2017

Amy Lasek
Psychiatry
M/C 912

Dear Dr. Lasek:

The protocol indicated below was reviewed in accordance with the Animal Care Policies and Procedures of the University of Illinois at Chicago and **renewed on 4/21/2017.**

Title of Application: Regulation of Excessive Alcohol Consumption by the LMO-ALK Axis
ACC NO: 15-060
Original Protocol Approval: 4/30/2015 **(3 year approval with annual continuation required).**
Current Approval Period: 4/21/2017 **to** 4/21/2018

Funding: ***Portions of this protocol are supported by the funding sources indicated in the table below.***
Number of funding sources: 2

Funding Agency	**Funding Title**			**Portion of Funding Matched**
NIH/NIAAA- National Inst. on Alcohol Abuse and Alcoholism	*Regulation of Excessive Alcohol Consumption by the LMO-ALK Axis*			*Protocol is linked to form G 15-062*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
U01AA020912	*Expired*	*201206875*	*UIC*	*Amy Lasek*
Funding Agency	**Funding Title**			**Portion of Funding Matched**
Departmental	*N/A*			*Other: N/A*
Funding Number	**Current Status**	**UIC PAF NO.**	**Performance Site**	**Funding PI**
N/A	*Funded*		*UIC*	*Amy Lasek*

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of the UIC.

Sincerely,

John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO/*kg*
cc: BRL, ACC File

Phone (312) 996-1972 • Fax (312) 996-9088

VITA

NAME: Antonia Marie Savarese

EDUCATION: B.S., Psychology, Loyola University Chicago, Chicago, Illinois, 2008

Ph.D., Neuroscience, University of Illinois at Chicago, Chicago, Illinois, 2017

HONORS: Damen Scholarship, Loyola University Chicago, Chicago, Illinois, 2004-2006

Presidential Scholarship, Loyola University Chicago, Chicago, Illinois, 2006-2008

University Fellowship, Graduate College of the University of Illinois at Chicago, Chicago, Illinois, 2011-2012, 2014-2015

Travel Award, Graduate Student Council at University of Illinois at Chicago, Chicago, Illinois, 2013

National Institute on Drug Abuse Travel Scholarship, Short Course on the Genetics of Addiction, Bar Harbor, Maine, 2014

Pre-doctoral Education for Clinical & Translational Scientists Supplemental Program Award, Center for Clinical and Translational Science at University of Illinois at Chicago, Chicago, Illinois, 2014-2015

Chancellor's Graduate Research Fellowship, Graduate College of the University of Illinois at Chicago, Chicago, Illinois, 2014-2015, 2015-2016

Invited Talk, "Characterization of behavioral phenotypes and gene expression changes related to alcohol use disorders in mice deficient in the Lim-only protein, LMO3", Psychiatric Institute Neuroscience Seminar Series at University of Illinois at Chicago, Chicago, Illinois, 2016

2nd Place Poster Award, Annual Neuroscience Day at University of Illinois at Chicago, Chicago, Illinois, 2016

1st Place Poster Award, Annual Research Forum of the Department of Psychiatry of University of Illinois at Chicago, Chicago, Illinois, 2016

National Institute on Alcohol Abuse and Alcoholism Travel Award, Fourth International Congress on Alcoholism and Stress, Volterra, Italy, 2017

Travel Award, Graduate Student Council at University of Illinois at Chicago, Chicago, Illinois, 2017

2nd Place Poster Award, Annual Retreat of the Center for Alcohol Research in Epigenetics at University of Illinois at Chicago, Chicago, Illinois, 2017

Invited Talk, "The Lmo3 gene: a regulator of anxiety and behavioral responses to alcohol", Trainee Data Blitz, Annual Retreat of the Center for Alcohol Research in Epigenetics at University of Illinois at Chicago, Chicago, Illinois 2017

National Institute on Alcohol Abuse and Alcoholism Student Merit Award, 40th Annual Research Society on Alcoholism Meeting, Denver, Colorado, 2017

Exceptional Poster Award, Fourth International Congress on Alcoholism and Stress, Volterra, Italy, 2017

Invited Talk, "The Lmo3 gene: a regulator of anxiety and behavioral responses to alcohol", Junior Scholars Neuroscience Seminar Series, University of Illinois at Chicago, Chicago, Illinois, 2017

National Institute on Alcohol Abuse and Alcoholism Post-doctoral fellowship, "Biological Bases of Alcoholism" (T32 AA007468-30S1 – PI: Andrey Ryabinin), 2017-2018

PUBLICATIONS:

Savarese A., Zou M.E., Kharazia V., Maiya R., Lasek A.W. (2014). Increased behavioral responses to ethanol in Lmo3 knockout mice. *Genes, Brain & Behavior*, 13(8): 777-83.

Maki P.M., Rubin L.H., Mordecai K.L., Sundermann E., **Savarese A.**, Eatough E., Drogos L.L. (2015). Menstrual cycle effects on cortisol responsivity and emotional retrieval following a psychosocial stressor. *Hormones and Behavior,* 74: 201-8.

Maki P.M., Rubin L.H., **Savarese A.**, Drogos L., Shulman L.P., Banuvar S., Walega D.R. (2016). Stellate ganglion blockade and verbal memory in midlife women: Evidence from a randomized trial. *Maturitas*, 92: 123-9.

Mordecai, K. L., Rubin, L. H., Eatough, E., Sundermann, E., Drogos, L., **Savarese, A.** Maki, P. M. (2017). Cortisol reactivity and emotional memory after psychosocial stress in oral contraceptive users. *Journal of Neuroscience Research*, 95(1-2): 126-35.

SELECTED ABSTRACTS:

Sundermann, E., Rubin, L.H., Mordecai, K.L., Eatough, E., **Savarese, A.**, & Maki, P.M. The effects of menstrual cycle phase and stress on cognitive flexibility. *14th World Congress of Gynecological Endocrinology. Florence, Italy, March 2010*.

Savarese, A., Rubin, L.H., Mordecai, K., Eatough, E., Drogos, L., Sundermann, E., & Maki, P.M. Personality and the stress response in women. *40th Annual Meeting of the Society for Neuroscience (SFN). San Diego, CA, November 2010.*

Maki, P.M., Rubin, L.H., Mordecai, K.L., **Savarese, A.**, Eatough, E., & Drogos, L. Cortisol responsivity to social stress varies across the menstrual cycle. *49th Annual Meeting of the American College of Neuropsychopharmacology. Miami, FL, December 2010.*

Rubin, L.H., Carter, S.C., Drogos, L., Pournajafi-Nazarloo, H., **Savarese, A.**, Sweeney, J.A., & Maki, P.M. Sex-specific associations between peripheral oxytocin and positive emotion perception in schizophrenia. *International Congress of Schizophrenia Research. Colorado Springs, CO, April 2011.*

Maki, P.M., **Savarese, A.**, Drogos, L., Rubin, L.H., Banuvar, S., Shulman, L.P., & Walega, D. Cognitive function in relation to hot flashes in women undergoing stellate ganglion block: baseline findings. *15th World Congress of Gynecological Endocrinology. Florence, Italy, March 2012.*

Wisslead, L.B., Drogos, L.L., Rubin, L.H., **Savarese, A.**, Eatough, E., Mordecai, K., & Maki, P.M. Effect of oral contraceptive use on heart rate variability during laboratory-induced stress. *94th Annual Meeting of the Endocrine Society (ENDO 2012). Houston, Texas, June 2012.*

Savarese, A.M., Zou, M., Kharazia, V., Heberlein, U., & Lasek, A.W. Behavioral responses to ethanol are regulated by the LIM only protein LMO3. *43rd Annual Meeting of the Society for Neuroscience (SFN). San Diego, CA, November 2013.*

Savarese, A.M., Zou M.E., Kharazia V., Maiya R., & Lasek A.W. Increased behavioral responses to ethanol in *Lmo3* knockout mice. *16th Annual Genes, Brain & Behavior Meeting (IBANGS). Chicago, IL, May 2014.*

Savarese, A.M. & Lasek A.W. Modulation of ethanol reward, anxiety, and gene expression by the transcriptional regulator Lim-domain only 3 (Lmo3). *39th Annual Research Society on Alcoholism Meeting. New Orleans, LA, June 2016.*

Savarese, A.M. & Lasek, A.W. Lmo3 differentially modulates drug reward, anxiety, and amygdalar gene expression in a sex-specific manner. *46th Annual Meeting of the Society for Neuroscience (SFN). San Diego, CA, November 2016.*

Savarese, A.M. & Lasek, A.W. Regulation of anxiety-like behavior and Crhr1 gene expression by the transcriptional regulator Lim-domain-only 3 (*Lmo3*). *4th International Congress on Alcoholism and Stress. Volterra, Italy, May 2017.*

Savarese, A.M. & Lasek, A.W. Regulation of anxiety-like behavior and Crhr1 gene expression by the transcriptional regulator Lim-domain-only 3 (*Lmo3*). *40th Annual Research Society on Alcoholism Meeting. Denver, CO, June 2017.*

TEACHING: Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: BIOS 286, Biology of the Brain, 2014

Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois: BIOS 386, Seminar on Neurobiology, 2014-2017

PROFESSIONAL MEMBERSHIP: Research Society on Alcoholism
International Behavioral and Neural Genetics Society
Society for Neuroscience