ALCOHOL SELF-ADMINISTRATION AND RELAPSE-LIKE BEHAVIOR: A FUNCTIONAL ROLE FOR ENHANCED ACTIVITY AT AMPA RECEPTORS

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ABSTRACT

Reginald D. Cannady: Alcohol Self-administration and Relapse-like Behavior: A Functional Role for Enhanced Activity at AMPA Receptors
"Under the direction of Drs. Clyde Hodge and Joyce Besheer"

Mechanisms underlying excessive alcohol drinking behavior and relapse are not fully understood and are critical for mapping the pathological course of alcohol use disorders (AUD). Long-term ethanol consumption results in strengthened excitatory neurotransmission and increased AMPA receptor signaling in animal models. However, the mechanistic role of enhanced AMPA receptor activity in ethanol-reinforcement and alcohol-seeking behavior remains unclear. Thus, the experiments in this dissertation sought to elucidate the behavioral and molecular mechanisms that underlie AMPA receptor-mediated ethanol reinforcement processes and relapse to ethanol-seeking behavior using a preclinical model of high alcohol preference, the alcohol-preferring (P-) rat. Enhancement of AMPA receptor signaling by systemically administered aniracetam (AMPA receptor positive allosteric modulator) significantly increased ethanol self-administration in a reinforcer-specific manner. Moreover, aniracetam potentiated cue-induced reinstatement in P-rats, which suggests that enhanced activity at AMPA receptors promotes reinforcement and ethanol-seeking behavior. Experiments further characterized enhanced AMPA receptor signaling in modulating operant self-administration and relapse-like behavior by examining neuroanatomical contributions to AMPA receptor-mediated alterations in ethanol reinforcement. Since AMPA receptor activity is potentiated by post-translational modification (e.g. phosphorylation of GluA1

subunits), immunohistochemistry was used to examine neuroadaptive changes in pGluA1 in limbic brain regions after a history of ethanol self-administration. Increased pGluA1 immunoreactivity was observed in sub-nuclei of the amygdala and nucleus accumbens of ethanol self-administering P-rats relative to the sucrose controls. Guided by immunohistochemistry results, the effects of aniracetam on ethanol self-administration were examined via site-specific microinjections in the amygdala and nucleus accumbens. Intra-amygdala, but not intra-accumbens, aniracetam increased ethanol self-administration in a reinforcer-specific manner. Furthermore, co-administration of intra-amygdala aniracetam and myristolated AIP (CaMKII peptide inhibitor) blocked aniracetam-induced increased ethanol self-administration; which demonstrates a critical role for amygdala CaMKII activity in AMPA receptor-mediated potentiation of ethanol reinforcement. These data suggest that enhanced amygdala AMPA receptor activity promotes drinking and ultimately could contribute to alcohol use disorders. In contrast, intra-amygdala aniracetam did not significantly alter cue-induced reinstatement; which suggest that enhanced AMPA receptor activity in this region may not significantly contribute to promoting cue-induced ethanol-seeking. Collectively, key experimental results provide novel insight into AMPA receptor-related mechanisms in excessive alcohol drinking behavior and vulnerability to relapse.

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TABLE OF CONTENTS

LIST OF FIGURES XI

LIST OF TABLES XIII

LIST OF ABBREVIATIONS XIV

CHAPTER 1: INTRODUCTION 1

An Overview of Alcoholism 1

Impact, Prevalence, and Risk Factors Associated with Alcohol Abuse in Humans 2

Excessive Drinking in Humans: Neurological and Behavioral Maladaptations 4

Value of Preclinical Animal Drinking Models 5

Rodent Alcohol Drinking Models 6

Alcohol preferring (P-) rats 8

Measuring Ethanol Reinforcement 9

Modeling Relapse to Ethanol-Seeking 10

The Actions of Alcohol in the Central Nervous System 11

GABA Receptors 11

Serotonin Receptors 12

Dopamine Receptors 12

Glutamate 13

Metabotropic Glutamate Receptors 14

Group I Receptors: mGluR1 and mGluR5 14

Group II Receptors: mGluR2 and mGluR3 15

Group III Receptors: mGluR4 and mGluR6-8 15
Ionotropic Glutamate Receptors 15
NMDA Receptors 15
Kainate Receptors 16
AMPA Receptors 17
Rationale 18
Specific Aim 1: 19
Specific Aim 2: 20
Specific Aim 3: 20
CHAPTER 2: ENHANCED AMPA RECEPTOR ACTIVITY INCREASES OPERANT ALCOHOL SELF-ADMINISTRATION AND CUE-INDUCED REINSTATEMENT 22
Introduction 22
Materials and Methods 25
Animals 25
Apparatus 25
Operant Self-administration 26
Examination of positive modulation of glutamate activity at AMPA receptors on ethanol self-administration. 27
Test for aniracetam-induced alterations in locomotor behavior 28
Confirmation of AMPA receptor involvement in modulating increased alcohol self-administration. 29
Test for aniracetam-induced alterations in ethanol pharmacokinetics. 29
Test for reinforcer specificity via sucrose-self-administration. 29
Examination of positive modulation of glutamate activity at AMPA receptors on regulating response-contingent cue-induced reinstatement. 30
Drugs. 31

Data Analysis 31

Results 32

Examination of positive modulation of glutamate activity at AMPA receptors on ethanol self-administration. 32

Test for aniracetam-induced alterations in locomotor behavior. 33

Confirmation of AMPA receptor involvement in modulating increased ethanol self-administration. 33

Test for aniracetam-induced alterations in ethanol pharmacokinetics. 34

Test for reinforcer specificity via sucrose self-administration. 35

Examination of positive modulation of glutamate activity at AMPA receptors on regulating response-contingent cue-induced reinstatement. 35

Discussion 37

CHAPTER 3: POTENTIATION OF AMYGDALA AMPA RECEPTOR ACTIVITY SELECTIVELY PROMOTES ETHANOL SELF-ADMINISTRATION IN A CAMKII-DEPENDENT MANNER 51

Introduction 51

Materials and Methods 54

Animals 54

Apparatus 54

Operant Self-administration 55

Experiment 1: Examination of GluA1 phosphorylation (pGluA1) after chronic ethanol self-administration 56

Experiment 2: Examination of site-specific positive modulation of glutamate activity at AMPA receptors on ethanol self-administration. 58

Experiment 3: Test for intra-amygdala aniracetam-induced alterations in locomotor behavior. 59

Experiment 4: Test for reinforcer specificity via sucrose-self-administration. 60

Experiment 5: Examination of CaMKII involvement in aniracetam-induced potentiation of ethanol self-administration 60

Drugs. 60

Data Analysis 61

Results 61

Experiment 1: Examination of GluA1 phosphorylation (pGluA1) after chronic ethanol self-administration. 61

Experiment 2: Examination of site-specific positive modulation of glutamate activity at AMPA receptors on ethanol self-administration 63

Experiment 3: Test for intra-amygdala aniracetam-induced alterations in locomotor behavior. 64

Experiment 4: Test for reinforcer specificity via sucrose-self-administration. 64

Experiment 5: Examination of CaMKII involvement in aniracetam-induced potentiation of ethanol self-administration 65

Discussion 66

CHAPTER 4: ROLE OF ENHANCED ACTIVITY AT AMYGDALA AMPA RECEPTORS IN MODUALTING RELAPSE-LIKE BEHAVIOR 84

Introduction 84

Materials and Methods 86

Animals 86

Apparatus 87

Operant Self-administration 87

Examination of ethanol-associated cue exposure on expression of pGluA1 88

Western Blots 89

Examination of altered modulation of glutamate activity at amygdala AMPA receptors on regulating response-contingent cue-induced reinstatement. 90

Drugs 91

Data analysis 91

Results 92

Examination of ethanol-associated cue exposure on expression of pGluA1. 92

Examination of altered modulation of glutamate activity at amygdala AMPA receptors on regulating response-contingent cue-induced reinstatement. 93

Discussion 94

CHAPTER 5: GENERAL DISCUSSION 103

REFERENCES 112

LIST OF FIGURES

Figure 1: Positive modulation of AMPA receptors potentiates alcohol self-administration 46

Figure 2: Antagonism of AMPA receptors by DNQX pre-treatment blocks aniracetam-induced increased alcohol self-administration..... 47

Figure 3: Alcohol clearance is not altered by aniracetam pre-treatment. 48

Figure 4: Positive modulation of AMPA receptors does not alter sucrose self-administration. 49

Figure 5: Positive modulation of glutamate activity at AMPA receptors potentiates cue-induced reinstatement of alcohol-seeking behavior in P-rats. 50

Figure 6: Chronic ethanol and sucrose self-administration prior to immunohistochemistry.. 75

Figure 7: Ethanol self-administration upregulates phosphorylation of the GluA1 AMPA receptor subunit in subregions of the amygdala. 76

Figure 8: Ethanol self-administration upregulates phosphorylation of the GluA1 AMPA receptor subunit in subregions of the nucleus accumbens. 77

Figure 9: Positive modulation of amygdala AMPA receptors potentiates ethanol self-administration.. 78

Figure 10: Positive modulation of nucleus accumbens AMPA receptors does not alter ethanol self-administration. 79

Figure 11: Positive modulation of amygdala AMPA receptors does not alter locomotor activity 80

Figure 12: Figure 7. Positive modulation of amygdala AMPA receptors does not alter sucrose self-administration. 81

Figure 13: Inhibition of amygdala CaMKII activity attenuates ethanol self-administration. 82

Figure 14: Intra-amygdala inhibition of CaMKII activity blocks aniracetam-induced increased ethanol self-administration. 83

Figure 15: Cue-induced reinstatement of ethanol-seeking behavior in P-rats prior to western blot analysis.. 98

Figure 16: Amygdala and nucleus accumbens pGluA1 expression after response-contingent reinstatement. 99

Figure 17: Amygdala and nucleus accumbens pGluA1 expression in high and low reinstatement group responders 100

Figure 18: Potentiation or inhibition of glutamate activity at amygdala AMPA receptors does not alter cue-induced reinstatement of ethanol-seeking behavior. 101

Figure 19: Potentiation or inhibition of glutamate activity at amygdala AMPA receptors does not alter cue-induced reinstatement in high or low reinstatement group responders. 102

LIST OF TABLES

Table 1: Baseline self-administration parameters (mean ± SEM) prior to test sessions 44

Table 2: Alcohol clearance parameters (mean ± SEM) after aniracetam pretreatment 45

Table 3: pGluA1 immunoreactivity (mean ± SEM) after ethanol or sucrose self-administration 73

Table 4: Total GluA1 immunoreactivity (mean ± SEM) after ethanol or sucrose self-administration 74

LIST OF ABBREVIATIONS

ACSF	Artificial Cerebral Spinal Fluid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate acid
ANOVA	Analysis of Variance
AUD	Alcohol Use Disorder
CB1	Type 1 cannabinoid receptor
CONC	Concurrent
CaMKII	Ca^{2+} /calmodulin-dependent Protein Kinase II
DNQX	Dinitroquinoxaline-2,3-dione
DSM-V	Diagnostic and Statistics Manual of Mental Disorders: Fifth Edition
ETOH	Ethyl Alcohol or Ethanol
EXT	Extinction
FDA	Food and Drug Administration
FR	Fixed-Ratio
GABA	gamma-Aminobutyric acid
GluA1	GluA1 AMPA Receptor Subunit
HR	High responders
IR	Immunoreactivity
I.P.	Intraperitoneal injection
LR	Low Responders
mGluR	Metabotropic Glutamate Receptor
Myr-AIP	Myristolated Autoinhibitory Peptide

NBQX	2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide
NMDA	N-Methyl-D-aspartic Acid
pCaMKII	Phosphorylated Ca^{2+} /Calmodulin-dependent Protein Kinase II
pGluA1	Phosphorylated GluA1 AMPA Receptor Subunit
REIN	Reinstatement
SEM	Standard Error of the Mean

CHAPTER 1: INTRODUCTION

An Overview of Alcoholism

The consumption of alcohol by humans has been, and still is, an integral part of everyday life for many around the world. Throughout the course of recorded history there is substantial evidence of alcohol use for a variety of purposes such as celebrations, social and sporting events, or religious ceremonies. For most consumers of alcohol, the occasional indulgence is trivial and of little long-term consequence. However, some individuals are susceptible to the deleterious ramifications of long-term alcohol consumption. These individuals often chronically misuse and abuse alcohol and may develop an alcohol use disorder (AUD). Researchers and clinicians have long-recognized the symptomology associated with alcohol use disorders; however, the etiology remains much more elusive. A fundamental understanding of the etiology of alcoholism/AUD is critically important and will provide novel insights into more efficient and effective treatment options for those suffering from this debilitating condition.

The current criteria for diagnosing AUDs are set by the American Psychiatric Association and outlined in the Diagnostic and Statistics Manual for Mental Disorders: 5th Edition (DSM-5). The DSM-5, describes AUD as, “A problematic pattern of alcohol use leading to clinically significant impairment or distress” (American Psychiatric Association, 2013). At least two of eleven established criteria must be met within a 1-year period for an alcohol use disorder diagnosis which include (but are not limited to):

excessive or uncontrollable alcohol consumption, craving, tolerance, withdrawal, and continued use despite negative consequences (American Psychiatric Association, 2013). New guidelines in the recently released DMS-V merge traditional alcohol abuse- and alcohol dependence diagnosis, while allowing a diagnosis to be classified as mild, moderate, or severe based of the number of criteria met within a 1-year period. The changes in diagnostic criteria reflect acknowledgements by clinicians and researchers that AUD is a complex disorder and that the efficacy of treatments may vary due to severity of the disease. Moreover, the 1-year window of time to meet established criteria cannot be understated because it emphasizes that 1) acute intoxication episodes alone are not the primary cause of AUD and that 2) alcoholism is a chronically relapsing disorder characterized by enduring challenges to control drinking; likely the result of lasting alcohol-induced neurobiological maladaptations (Hansson et al., 2010; Luscher and Malenka, 2011). Thus, it is essential that investigators and clinicians work to identify the neurobiological factors that underlie chronic pathological maladaptations that trigger excessive alcohol drinking that could lead to alcoholism or AUD(s).

Impact, Prevalence, and Risk Factors Associated with Alcohol Abuse in Humans

Alcoholism and AUDs have a profound impact on society. In the United States alone, the third leading cause of preventable mortality is attributed to AUDs (Mokdad et al., 2004) with many other countries experiencing similar trends (Lopez et al., 2006). In the US over 85,000 deaths per year are the result of alcohol abuse; outnumbering deaths resulting from all infectious diseases combined (Mokdad et al., 2004). The financial burden attributed to AUDs is enormous. The estimated economic cost of

alcohol abuse was $223.5 billion in 2006 alone; which was calculated by factors such as lost productivity, healthcare costs, and criminal justice costs among others (Bouchery et al., 2011).

It is estimated that the 12-month prevalence (identified symptoms in the 12 month period) for alcohol use disorder is 8.5% and 4.7% for adults and teens (ages 12-17), respectively (American Psychiatric Association, 2013; Hasin et al., 2007) Men (12.4%) typically exhibit higher 12-month prevalence of alcoholism than women (4.9%), and younger populations (ages 18-29) make up a majority (16.2%) of diagnosed AUD cases (Hasin et al., 2007) (American Psychiatric Association, 2013). Further, it is well established that individuals with early onset of heavy drinking during adolescence have increased incidence of AUD in adulthood (Dawson et al., 2008). It is no coincidence that younger individuals exhibit higher rates of AUD symptomology, as this is the point in a lifespan where individuals are most likely to experiment and consume excessive amounts of alcohol (Trim et al., 2009). Importantly, chronic excessive alcohol consumption is the leading determinant in developing an alcohol use disorder (American Psychiatric Association, 2013; Saha et al., 2006) Thus, understanding neurobiological factors contributing to excessive drinking are critical for the development of therapeutic options for alcohol abuse.

Genetics is another risk factor associated with increased rates of alcoholism. Nearly 50% of the risk for alcoholism is influenced by genetics, with family history of alcoholism as a major risk factor for developing an alcohol use disorder (Grant, 1998). Interestingly, an FDA-approved drug with efficacy for reducing heavy drinking, has exhibited limited effectiveness amongst individuals who are alcohol-dependent; as

genetic variability is proposed to account for reduced efficacy (Oroszi et al., 2009). These data emphasize the value of studies that incorporate some component of genetic influence.

Excessive Drinking in Humans: Neurological and Behavioral Maladaptations

Heavy drinking and alcohol abuse lead to profound deficits in neuronal function which regulates behavior, memory, judgment, and various other functions in humans. For example, several studies show brain shrinkage and reductions in both white and grey matter in the frontal lobes of post-mortem alcoholics (Kril et al., 1997; Kubota et al., 2001). This ethanol-induced maladaptations is key, as areas of the frontal lobe modulate high order cognitive processes such as impulse control, planning, and decision making in humans (Andersen and Cui, 2009; Fellows and Farah, 2005; Qiu et al., 2013). Indeed, studies indicate that individuals with a history of excessive ethanol consumption self-report increased impulsivity (MacKillop et al., 2007) and exhibit deficits in impulse control using various tasks that measure impulsivity (Ahmadi et al., 2013; Reed et al., 2012). Furthermore, individuals who suffer from alcohol use disorders often report high levels of alcohol craving (Anton et al., 1995); sometimes the result of exposure to alcohol-associated cues (Field et al., 2004; Lee et al., 2006). Alcohol craving is critically important because it is often associated with increased risk of relapse after a period of abstinence (Schneekloth et al., 2012). Naltrexone has recently been shown to be effective at reducing alcohol-cue-elicited craving in heavy drinkers (Miranda et al., 2013).The effect of naltrexone on craving in heavy drinkers is interesting given that excessive alcohol induces maladaptations in brain development that mediate

craving and impulsivity, two possibly related phenomena (Joos et al., 2013). Although there is substantial evidence that excessive drinking can cause neuronal deficits and dysfunctional behavior in humans, the underlying molecular mechanisms are difficult to determine as investigators are limited in the scope of studies that can be conducted in human subjects.

Value of Preclinical Animal Drinking Models

Animal drinking studies are extremely valuable because behavioral pathologies of alcoholism can be modeled while offering in-depth insights into underlying neuronal mechanisms. A vast amount of information has been gained from the use of animal models to study various aspects of alcohol consumption, including the development of pharmacotherapies for the treatment of alcohol use disorders (McKinney, 2001). Animal drinking studies provide investigators with a great deal of control over various experimental variables that might affect drinking including an animal's drinking history, genetic background, access to alcohol, and environmental stimuli. It is important to note that in some cases, voluntarily consumed drugs can produce distinct neuroadaptations as compared to investigator-administered drugs (Jacobs et al., 2005). This evidence suggest a divergence of neuroadaptations caused by pharmacological actions of drugs and neuroadaptations related to voluntary drug use (Wise, 2000), and further provides an incentive to study functional implications of ethanol-induced neuroadaptations that modulate drinking behavior.

Accordingly, in this dissertation the focus is primarily on neuroadaptations and alterations in patterns of behavior related to voluntary ethanol consumption as our goal

is to identify neuronal substrates that alter drinking. Our efforts are primarily centered on measurements of ethanol reinforcement and relapse-like behavior as both are easily modeled in animals (Cannady et al., 2013; Hodge et al., 1993a; Hodge et al., 2006; Hodge et al., 1992; Schroeder et al., 2008) and implicated in various aspects of alcohol abuse disorders. Several species are used in studies that examine aspects alcohol abuse including rats, mice, hamsters, monkeys, and drosophila, among others (Cannady et al., 2013; Chen et al., 2010; Ferris, 2003; Grant et al., 2000; Hodge et al., 2006). Each species presents its own set of advantages and challenges when conducting drinking studies. Given the scope of this dissertation, we will focus primarily on rodent models of alcohol consumption.

Rodent Alcohol Drinking Models

Rodent models are very practical for studying multiple aspects of alcohol drinking. An incredible amount of literature exists on their behaviors, physiological makeup, and genetic backgrounds. Additionally, rodents can be trained to complete complex tasks that mimic behavioral processes observed in humans, such as working for an ethanol solution, or seeking ethanol after stress or exposure to alcohol-related stimuli (Cannady et al., 2013; Hodge et al., 1993b; Sidhpura et al., 2010). One challenge of using rodents in drinking studies is that most rodents do not readily consume pharmacologically relevant amounts of alcohol. In this case, the term “pharmacologically relevant” refers to the ability for a drug, such as alcohol, to travel throughout the body and exert its actions at its target sites; ultimately producing detectable changes in behavior or physiological processes (Lange and Dietrich, 2002;

Levy, 1986). Over the years investigators have adopted several methods to surmount the challenge of inducing relevant drinking levels in rodents including: forced exposure, fluid and/or food restriction, ethanol-containing liquid diet, gradually increasing ethanol concentrations, and masking/altering the flavor of alcohol with highly palatable additives (i.e. sucrose, and saccharin) (Falk et al., 1972; Knapp et al., 1998; Lieber and Decarli, 1976; Tolliver et al., 1988). Additionally, multiple rodent lines have been established using selective breeding procedures that result in rodent strains with a high (or low) preference for ethanol. Typically, it takes minimal effort of investigators to induce and maintain pharmacologically relevant drinking levels in selectively bred lines as opposed to outbred strains (McMillen et al., 1998); making selectively bred strains a valuable asset for drinking studies (Bell et al., 2006a; Crabbe et al., 2010). Given that family history is a major factor that influences excessive drinking and alcohol use disorders, selectively bred lines also allow for the opportunity to examine genetic contributions to drinking.

In most instances, selective breeding is achieved by examining preference for alcohol (vs water) using a two-bottle choice procedure where high preference mating pairs produce offspring that are subjected to the same selective breeding process for multiple generations. The culmination of continuous mating of high- (or low-) alcohol preferring offspring results in the production of selectively bred strains with similar genetic and behavioral phenotypes (Crabbe, 2008). Some common features of selectively bred rodent drinking lines include dysregulation of serotinergic signaling systems in limbic brain regions, development of rapid tolerance, and disposition to voluntary consume ethanol until they reach significantly elevated blood alcohol levels

(Crabbe, 2008; Crabbe et al., 2010). There are several selectively bred rat and mouse lines; however, this dissertation will focus primarily on alcohol preferring (P-) rats as data was collected using this selectively bred strain in all of the studies included in this dissertation.

Alcohol preferring (P-) rats

Alcohol-preferring rats (P-rats) were selectively bred at the University of Indiana in the 1970s from a heterogeneous stock of Wistar rats using bidirectional selection procedures. Male and female rats with a high preference for a 10% ethanol solution in a two-bottle choice procedure were mated producing high preference offspring; while mating pairs with a low preference produced low alcohol-preferring offspring, later called alcohol non-preferring rats (NP-rats) (Li et al., 1979; Penn et al., 1978). P-rats are prominently used in the alcohol field and provide an excellent model that closely fits proposed criteria for a rodent model of alcoholism (Bell et al., 2006a).

P-rats frequently consume pharmacologically relevant doses of ethanol and can voluntarily obtain blood ethanol concentrations up to 200mg% under 24 hour limited access conditions (Bell et al., 2006b; Rodd-Henricks et al., 2001). They consistently consume more than 5mg/kg of ethanol per day; five times more than NP rats (Li et al., 1987). P-rats are also able to develop rapid tolerance (Lumeng and Li, 1986) and exhibit signs of physical dependence and withdrawal after prolonged free choice drinking (Kampov-Polevoy et al., 2000).

Important for the context of this dissertation, P-rats can be trained in operant conditioning procedures to self-administer pharmacologically relevant doses of ethanol

solutions (Besheer et al., 2008a; Besheer et al., 2008b; Cannady et al., 2013; Samson, 1986); even in the presence of other palatable solutions such as sucrose (Samson et al., 1998). Moreover, P-rats can model aspects of relapse as evidenced by potentiated alcohol responding after alcohol deprivation or extinction of operant responding (Cannady et al., 2013; Rodd-Henricks et al., 2001; Schroeder et al., 2008).

Measuring Ethanol Reinforcement

The concept of reinforcement is fundamental to understanding addictive processes as it has been suggested that all drugs of abuse serve as positive reinforcers that maintain drug-seeking and taking (Stolerman, 1992). Reinforcement is conceptualized as a consequence that follows an operant response that increases the likelihood of that response occurring in the future (Skinner, 1965). Indeed, alcohol consumption induces pleasurable states in humans and animals that promote or "reinforce" subsequent drinking episodes. Operant conditioning procedures are ideal for measuring complex behavioral processes such as alcohol reinforcement and provide investigators with quantitative indices of the reinforcing properties of alcohol (an appetitive measurement) while also offering the ability to measure consumatory behavior (Samson, 1996). These procedures are often preferred over traditional drinking studies, such as two-bottle choice procedures which lack precise behavioral-temporal resolution and provide limited information about alcohol reinforcement and motivational properties.

In operant alcohol self-administration procedures, access to alcohol is contingent on a conditioned response (typically responding on a lever). The response requirement

or schedule of reinforcement for an animal may be manipulated to examine various aspects of behavior. For instance, animals responding on a fixed-ratio schedule of reinforcement provide an index of the reinforcing properties of ethanol. In efforts to examine motivation to work for alcohol, an animal may be tested on a progressive ratio schedule of reinforcement where response contingency increases after successfully obtaining each alcohol reinforcer (Besheer et al., 2008b).

Modeling Relapse to Ethanol-Seeking

Alcoholism is characterized by cycles of heavy drinking periods interspersed with attempts to remain abstinent and the subsequent relapse episodes (McLellan et al., 2000). Relapse remains one of the major obstacles for clinicians seeking to treat alcoholic patients. Despite lengthy periods of abstinence, individuals that abuse alcohol remain vulnerable to relapse for the duration of their lives (Vaillant, 1996). Animal models are valuable in that multiple facets of relapse can be replicated in a relatively short amount of time (Martin-Fardon and Weiss, 2013).

Often, reinstatement procedures are used to model relapse-like episodes in animal models. The three factors that contribute most to alcohol relapse susceptibility are environmental stimuli (or cues), stress, and exposure to alcohol during abstinence (also known as priming) (Martin-Fardon and Weiss, 2013). Cue-induced reinstatement procedures mimic relapse episodes triggered by environmental stimuli (Koob, 2000). During a reinstatement procedure, a cue that had previously been paired with alcohol delivery is presented. Cues are often reintroduced in the absence (or sometimes presence) of alcohol allowing for reinstatement of previous alcohol-seeking behavior.

Animals typically exhibit significantly increased alcohol-seeking (e.g. lever responding) relative to extinction conditions (Cannady et al., 2013; Schroeder et al., 2008); which mimics compulsive alcohol-seeking often observed in humans (Koob, 2000). Stress-induced reinstatement sessions differ slightly in that alcohol relapse episodes are triggered by stressful events as alcohol-seeking behavior is elicited by exposing animals to various stressors (i.e. restraint or footshock) (Zhao et al., 2006). Priming-induced reinstatement sessions model alcohol relapse episodes as alcohol-seeking behavior is triggered by exposure to alcohol (Le and Shaham, 2002). In all three reinstatement procedures, alcohol-seeking behavior is tightly controlled by the three respective factors that contribute to relapse in humans and thus engenders considerable face validity for these procedures.

The Actions of Alcohol in the Central Nervous System

Alcohol or (Ethanol; C2H5OH) is small lipid and water soluble molecule that can easily enter the bloodstream and penetrate the blood brain barrier; acting as a central nervous system (CNS) depressant. Alcohol has actions at multiple neuronal targets which allows for a wide array of effects and has been shown to directly interact with several neurotransmitter receptors (Squire, 2008).

GABA Receptors

Alcohol potentiates neurotransmission at inhibitory gamma-aminobutyric acid (GABA) receptors with increased sensitivity to those receptors containing the $GABA_A$ subunit (Wallner et al., 2003). Activation of GABA receptors is associated with sedation

(Boehm et al., 2006) and altered sensitivity to the subjective effects of alcohol (Hodge and Aiken, 1996; Hodge and Cox, 1998). It has been suggested that prolonged exposure to alcohol downregulates GABA-mediated signaling to promote a hyperexcited state which has been linked to dependence and withdrawal in humans (Golovko et al., 2002).

Serotonin Receptors

Alcohol also has actions to potentiate 5-hydroxytryptamine 3 (serotonin) receptors (Lovinger, 1999). Many alcohol-preferring rodent lines show deficits in serotonin receptor expression which suggest that alcohol interactions at this receptor could contribute to behavioral phenotypes observed in these models (Bell et al., 2006a). Further, blocking activity at serotonin receptors reduces voluntary consumption (Hodge et al., 1993b; Zhou et al., 1998) and relapse like behavior (Rodd-Henricks et al., 2000) in rodents, which suggests that this signaling pathway is important in alcohol pathology.

Dopamine Receptors

Dopaminergic signaling systems have been well-established to modulate drug-taking and seeking behavior. Therefore it is no surprise that alcohol exerts its effects through dopamine receptors. Indeed, low doses of alcohol cause dopaminergic neurons in the ventral tegmental area to fire at a high rate (Gessa et al., 1985). Additionally, knockout mice with deletions of dopamine receptor subtypes show reduced drinking behavior (Crabbe et al., 2006). Administration of dopamine receptor antagonists reduce drinking behavior in a variety of animal models (Hodge et al., 1992; Samson et al.,

1993), but see (Rassnick et al., 1993). While alcohol can have effects on these and other neurotransmitters/ neuromodualtors, the focus of this dissertation is on glutamatergic neurotransmission and the effects of alcohol to modulate signaling and glutamate-mediated behavior.

Glutamate

Glutamate is the most abundant excitatory neurotransmitter in the CNS. It has been suggested that over half of all brain synapses release glutamate (Purves, 2001); which makes glutamate an intriguing neuronal substrate for the study of pathological function. Indeed, concerted efforts to examine the role of glutamate in alcoholism have been employed by many investigators in recent years as dysfunction of glutamate signaling has been proposed to be a major contributing factor in addictive disorders (Kalivas, 2009). Accordingly, this dissertation focuses on glutamate signaling in modulating alcohol drinking behaviors.

Glutamate is synthesized within neurons from precursor molecules such as glutamamine (from glial cells) via conversion by the enzyme, glutaminase, in presynaptic terminals. Once glutamate is packaged and released, it binds to glutamate receptors and then reuptake occurs which is modulated by high-affinity vesicular glutamate transporters (vGluTs) and sodium-dependent excitatory amino acid transporters (EAATs) located in glial cells and presynaptic terminals (Purves, 2001; Squire, 2008). Upon release, glutamate binds to a diverse set of glutamate receptor subtypes. These glutamate receptors are classified into two major classes: metabotropic and ionotropic. Metabotropic glutamate receptors (mGluRs) are G-protein

coupled receptors and modulate neuronal activity via slower intracellular signaling cascades, whereas ionotropic glutamate receptors are ligand-gated ion channels that mediate rapid glutamate neurotransmission (Purves, 2001; Squire, 2008).

Metabotropic Glutamate Receptors

mGluRs are comprised of three subgroups, Groups I-III. Each group consists of multiple mGluR receptor subtypes that are grouped based on pharmacological properties, molecular structure, and similar sequence homology (Squire, 2008).

Group I Receptors: mGluR1 and mGluR5

Evidence implicates multiple mGluR subtypes in modulating various responses to alcohol. The group 1 mGluRs are comprised of mGluR1 and mGluR5. Group I receptors are $G\alpha_q$- coupled, and activation by glutamate stimulates phospholipase C, resulting in phosphoinositol hydrolysis and the formation inositol triphosphate (IP_3) and diacylglycerol (DAG), which in turn can activate various intracellular messengers (Conn and Pin, 1997; Purves, 2001; Squire, 2008). mGluR1 antagonists attenuate ethanol-induced dopamine and glutamate release (Lominac et al., 2006). Moreover, reduction of activity at Group 1 mGluRs has been shown to modulate alcohol-induced sedation (Downing et al., 2010), alcohol self-administration (Besheer et al., 2008a; Besheer et al., 2008b; Besheer et al., 2010; Gass and Olive, 2009; Hodge et al., 2006) and relapse-like behavior (Schroeder et al., 2008; Sidhpura et al., 2010), while also modulating the subjective effects of ethanol (Besheer et al., 2009; Besheer and Hodge, 2005).

Group II Receptors: mGluR2 and mGluR3

Group Group II mGluRs are comprised of mGluR2 and mGluR3. These G_i-coupled receptors function as autoreceptors and regulate presynaptic neurotransmitter release (Baskys and Malenka, 1991; Farazifard and Wu, 2010; Liu et al., 1993; Macek et al., 1996; Molinaro et al., 2009). As such, activation of mGlu2/3 receptors decreases the synaptic availability of glutamate, which allows for ‘refinement' of glutamatergic neurotransmission (Pinheiro and Mulle, 2008; Schoepp, 2001). Group II receptors have been shown to modulate relapse-like to alcohol-seeking behavior (Sidhpura et al., 2010; Zhao et al., 2006) and the subjective or discriminative stimulus effects of alcohol (Cannady et al., 2012).

Group III Receptors: mGluR4 and mGluR6-8

Group III receptors include mGluR4 and mGluR6-8 subtypes, and similar to Group II recptors, are G_i-coupled and function to regulate glutamate release (Conn and Pin, 1997; Purves, 2001; Squire, 2008). These receptors have been studied the least or the mGluR family, but evidence suggest a role for mGluR7 and mGluR8 in modulating alcohol reinforcement (Backstrom and Hyytia, 2005; Salling et al., 2008).

Ionotropic Glutamate Receptors

NMDA Receptors

Of the glutamate receptor subtypes, N-methyl-D-aspartate (NMDA) receptors have been the most widely studied in relation to alcohol use disorders. NMDA receptors are comprised of four subunits (GluN1-4) and when activated by glutamate and glycine (co-agonist), these receptors allow for influx of cations such as calcium (Ca2+) sodium

(Na+) and potasium (K+) ions (Squire, 2008). These receptors are well known for their role in modulating plasticity related events, including LTP, a cellular correlate of learning and memory (Malinow et al., 1989; Purves, 2001; Squire, 2008). Interestingly, acute alcohol has a high affinity for NMDA receptors and inhibits activity at these receptors (with increased sensitivity at GluN2 containing receptors) (Lovinger et al., 1989). Studies show that chronic ethanol upregulates the expression of these receptors (Trevisan et al., 1994); likely as a compensatory mechanism for reduced activity through inhibition by ethanol. Moreover NMDA antagonists reduce ethanol self-administration (Rassnick et al., 1992), as well as produce ethanol-like subjective effects (Grant and Colombo, 1993; Hodge and Cox, 1998).

Kainate Receptors

Considerably less is known about Kainate receptors due to a shortage of selective biochemical and pharmacological tools. These receptors are ion heterotetrameric channel receptors consisting of four of five total subunits named, GluK1-5. Kainate receptors are thought to act more as modulators, rather than mediators of excitatory neurotransmission as they have significantly slower kinetic properties than NMDA and AMPA receptors, and are not as concentrated at excitatory post-synaptic sites (Squire, 2008). Further these receptors have a unique ability to function as traditional ion channels as well as couple to metabotropic signaling pathways (Contractor et al., 2011). In relation to alcohol, very few studies have examined the contributions of these receptors. Ethanol does inhibit activity of these receptors, and in many cases at very low doses; which suggest that these receptors

may play a role in actions of lower concentrations of ethanol (Moykkynen and Korpi, 2012).

AMPA Receptors

Unlike NMDA receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors have received considerably less attention in the alcohol addiction field. AMPA receptors are comprised of four subunits (GluA 1-4) and are heterotetrameric ligand-gated ion channels that mediate rapid glutamatergic neurotransmission. When activated by glutamate, ions such as Na^{2+} (and Ca^{2+}) are allowed to pass through the channel pore and mediate rapid excitatory signaling (Squire, 2008). AMPA receptors are important as they play a major role neuronal excitation and synaptic plasticity. Specifically, trafficking and incorporation of AMPA receptors into synapses is associated with potentiation of synaptic strength; while removal results in weakening of synaptic strength (Bredt and Nicoll, 2003; Kessels and Malinow, 2009). Indeed potentiation of synaptic strength via AMPA receptor trafficking facilitates various processes including learning and memory (Lee et al., 2003). The process by which AMPA receptors are incorporated in the synapse is regulated by subunit composition (Shi et al., 2001). Several studies indicate that the GluA1 subunit is important in facilitating trafficking of AMPA receptors to synaptic sites in an activity-dependent manner (Larsson and Broman, 2008; Zhu et al., 2000). Furthermore, intracellular signaling molecules such as Ca2+ /calmodulin-dependent protein kinase II (CaMKII) function as modulators of membrane trafficking by phosphorylating sites on the GluA1 subunit (Malinow et al., 1989; Poncer et al., 2002); which allows for dynamic variations in AMPA receptor signaling.

Rationale

The actions of ethanol to influence AMPA receptor signaling have not been fully determined. At high concentrations, acute ethanol attenuates AMPA receptor signaling (Dildy-Mayfield and Harris, 1992; Moykkynen et al., 2003). However, several studies show that AMPA receptor subunits are upregulated after long-term ethanol exposure; possibly due to a compensatory mechanism. Indeed, GluA1 subunit expression is upregulated in the nucleus accumbens (Ary et al., 2012; Neasta et al., 2010); a limbic-motor interface that mediates goal-directed and the reinforcing properties of abused drugs (Salamone et al., 2007). Furthermore, studies show that chronic ethanol potentiates synaptic strength as evidenced by increases in AMPA/NMDA ratio in brain regions that mediate reinforcement (Heikkinen et al., 2009; Stuber et al., 2008); which indicates that ethanol could be altering reinforcement processes by strengthening synaptic activity through an AMPA receptor-dependent mechanism. Indeed, administration of AMPA receptor antagonists reduce ethanol self-administration (Stephens and Brown, 1999; Wang et al., 2012), but it is not known if drinking behavior can be augmented by enhancing activity at AMPA receptors. GluA1 is also upregulated in the amygdala after chronic ethanol (Christian et al., 2012); a region well-characterized in mediating intense emotional states and associative learning processes (LeDoux, 2003) including drug-reward learning (Aston-Jones and Harris, 2004; Koob et al., 1998). This region has been implicated in modulating ethanol reinforcement and relapse-like behavior (Schroeder et al., 2003; Schroeder et al., 2008). Interestingly, antagonism of AMPA receptor signaling has been shown to reduce relapse to ethanol-seeking behavior in reinstatement procedures (Backstrom and Hyytia, 2004; Sanchis-

Segura et al., 2006). It is not clear, however, as to how ***enhanced*** AMPA receptor activity could modulate ethanol reinforcement or seeking behavior.

Therefore, the overall goal of this project is to determine how enhanced AMPA receptor activity might exacerbate ethanol reinforcement and relapse-like behavior using a preclinical model of high ethanol intake; the alcohol-preferring (P-)rat. The following three Specific Aims tested the hypothesis that enhanced activity at AMPA receptors in specific limbic brain regions facilitates ethanol reinforcement and promotes relapse to alcohol-seeking.

Specific Aim 1:

Examination of pharmacological enhancement of AMPA receptor activity to modulate alcohol self-administration and relapse-like behavior. The mechanistic role of enhanced AMPA receptor activity in alcohol-reinforcement and alcohol-seeking had not been previously determined. Therefore, we examined the role of enhanced AMPA receptor function using the selective positive allosteric modulator, aniracetam, in modulating operant alcohol self-administration and cue-induced reinstatement. Male alcohol-preferring (P-) rats trained to self-administer alcohol versus water were pre-treated with aniracetam to assess effects on maintenance of alcohol self-administration. Locomotor assessments were conducted following aniracetam treatment to assess if changes in alcohol self-administration were associated with non-specific motor effects. The effects of aniracetam on alcohol clearance were tested to rule out possible alterations in alcohol clearance. To determine reinforcer specificity, P-rats were trained to self-administer sucrose versus water, and effects of aniracetam were tested. The role of

aniracetam in modulating relapse of alcohol-seeking was assessed using a response contingent cue-induced reinstatement procedure in alcohol self-administering P-rats.

Specific Aim 2:

Investigation of brain regional involvement of enhanced AMPA receptor activity to modulate excessive alcohol self-administration. Aim 1 data showed that enhanced activity at AMPA receptor receptor by systemic aniracetam increased alcohol self-administration in a rodent model of high alcohol intake, the alcohol-preferring (P-) rat. Experiments in this Aim extended findings in Aim 1 by examining neuroanatomical substrates and the behavioral and molecular mechanisms underlying enhanced AMPA receptor-mediated increases in alcohol self-administration. First, using immunohistochemistry (IHC) we examined $pGluR1_{831}$ immunoreactivity in limbic brain regions of P-rats trained to self-administer alcohol or sucrose. We focused on the amygdala and nucleus accumbens given their roles in modulating alcohol drinking behaviors. Next, guided by the IHC results, we conducted functional studies to determine neuroanatomical control of enhanced AMPA receptor activity in modulating increased alcohol self-administration. Control experiments assessed anatomical and reinforcer specificity and evaluated non-specific changes in locomotor behavior. Finally, we determined if aniracetam-induced increases in alcohol self-administration are dependent on CaMKII activity using a CaMKII peptide inhibitor (myristolated AIP).

Specific Aim 3:

Investigation of brain regional involvement of enhanced glutamate signaling at AMPA receptors in modulating relapse-like behavior. Data from Aim 1 showed that reinstatement to alcohol seeking is potentiated by systemic aniracetam after exposure

to previously-paired alcohol-associated cues. Therefore, experiments in this Aim examined the role of enhanced AMPA receptor-mediated relapse-like behavior in P-rats. First, using western blot analysis, we evaluated changes in pGluA1 in limbic brain regions in response to exposure to alcohol-paired cues during a cue-induced reinstatement test in alcohol self-administering P-rats. We predicted changes in the amygdala given its role in modulating cue-reward associations. Next, we tested the functionality of AMPA receptor in the amygdala by site-specific pharmacological enhancement of AMPA receptor activity prior to reinstatement sessions.

Collectively, these studies provide insights into novel mechanisms for excessive drinking behavior and relapse to alcohol seeking; two prominent features of alcohol use disorders.

CHAPTER 2: ENHANCED AMPA RECEPTOR ACTIVITY INCREASES OPERANT ALCOHOL SELF-ADMINISTRATION AND CUE-INDUCED REINSTATEMENT[1]

Introduction

Glutamate signaling has been widely implicated in modulating addiction to alcohol (ethanol) and other drugs of abuse (Gass and Olive, 2008; Kalivas, 2009). The role of N-Methyl-D-aspartic acid (NMDA) receptors and metabotropic glutamate receptors in ethanol reinforcement processes and relapse have been studied extensively (Backstrom and Hyytia, 2004; Besheer et al., 2010; Bienkowski et al., 1999; Schroeder et al., 2008); however, the functional role of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors in these behaviors has received less attention. AMPA receptors are a subclass of ionotropic glutamate receptors that are ubiquitously expressed throughout the brain (Petralia and Wenthold, 1992). These receptors mediate fast excitatory glutamate neurotransmission and play a key modulatory role in major neural functions and processes, including synaptic plasticity (Kessels and Malinow, 2009). This is significant given that chronic exposure to drugs of abuse is associated with molecular maladaptations that influence synaptic plasticity events within brain reward circuitry (Kalivas, 2009; Nestler and Aghajanian, 1997). Interestingly, AMPA receptors have been implicated in modulating the addictive properties of a

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variety of drugs of abuse (Bowers et al., 2010; Choi et al., 2011; Van den Oever et al., 2008). Thus, a better understanding of how activity at AMPA receptors might influence drug-taking and seeking behaviors may provide novel insights into the etiology of the multiple phases of drug addiction.

Emerging evidence suggests that investigator-administered ethanol and chronic ethanol consumption influences AMPA receptor trafficking to excitatory synapses. Studies show that a history of ethanol exposure upregulates AMPA receptor subunit proteins in rodents (Ary et al., 2012; Chandler et al., 1999; Neasta et al., 2010) and in post-mortem brain preparations of human alcoholics (Breese et al., 1995). Moreover, these ethanol -induced neuroadaptations have been implicated in the strengthening of synapses within limbic brain regions that mediate reward, as evidenced by increases in the ratio of AMPA receptor- to NMDA receptor-mediated currents (AMPA/NMDA ratio); suggesting a role for AMPA receptor-dependent enhanced synaptic function in ethanol drinking behavior (Ary et al., 2012). For example, both investigator-administered ethanol (Heikkinen et al., 2009; Saal et al., 2003) as well as voluntary ethanol consumption (Stuber et al., 2008) have been shown to increase AMPA/NMDA ratio in the ventral tegmental area, a region well characterized for it's role in mediating the reinforcing properties of drugs of abuse and relapse-like behavior (Hodge et al., 1993a). Indeed, administration of AMPA receptor antagonists have been shown to reduce alcohol self-administration (Stephens and Brown, 1999), and block reinstatement of alcohol-seeking in rats (Backstrom and Hyytia, 2004) and mice (Sanchis-Segura et al., 2006). Collectively, these studies demonstrate that AMPA receptor signaling is required for the expression of the reinforcing effects of ethanol and is important in modulating relapse-

like behavior. However, it remains unclear how enhanced activity at AMPA receptors can influence ethanol -reinforcement processes and ethanol -seeking behavior.

Thus, the goal of the present study was to determine the effect of acute potentiation of glutamate activity at AMPA receptors on operant ethanol self-administration and cue-induced reinstatement of alcohol-seeking behavior using the positive allosteric modulator, aniracetam (Isaacson and Nicoll, 1991). To address this question, we used a genetic inbred model of high ethanol intake; the alcohol-preferring (P-) rat (Lankford et al., 1991; Murphy et al., 1989). Accordingly, P-rats were trained to self-administer ethanol (15%, v/v) vs. water using a well-characterized 2-lever operant self-administration procedure. The effects of aniracetam were evaluated on the maintenance of ethanol self-administration. Locomotor assessments were conducted following aniracetam treatment to assess if changes in ethanol self-administration were associated with nonspecific motor effects. The effects of aniracetam on ethanol clearance were tested to rule out possible alterations in ethanol clearance. To confirm AMPA receptor specificity in the modulation of ethanol self-administration, the AMPA receptor antagonist 6,7-Dinitroquinoxaline-2,3-dione (DNQX), was administered prior to aniracetam pretreatment. Further, to determine reinforcer specificity, aniracetam was tested in P-rats trained to self-administer sucrose (0.8%, w/v) prior to self-administration sessions. Finally, the effects of aniracetam on relapse-like behavior were assessed using a response-contingent cue-induced reinstatement procedure.

Materials and Methods

Animals

Adult male inbred alcohol-preferring (P-) rats (400 - 500 g prior to testing) were bred in-house. P-rats were derived from a line provided by Indiana University. This stock of inbred P-rats (5B substrain) was derived from breeders of the selected line of P-rats originally provided in 1999 by Indiana University (courtesy of Dr. T.K. Li) and has been bred on-site at the University of North Carolina at Chapel Hill. Animals were pair-housed in Plexiglas cages and handled daily prior to any training procedures. All rats had access to food and water ad libitum in the homecage between test sessions (unless mentioned otherwise). The colony room was maintained on a 12 hour light/dark cycle (lights on at 7am) and experiments were conducted approximately 3 hrs into the light portion of the cycle. All procedures used were conducted in accordance with the National Institute of Health guidelines, and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Apparatus

Self-administration Chambers. Operant conditioning chambers measuring 30.5 x 24.1 x 21.0 cm (Med Associates, Georgia, VT) were located within sound-attenuating cubicles. Each cubicle was equipped with an exhaust fan for ventilation which also functioned to mask external sounds. The left and right wall of each chamber contained a liquid receptacle in addition to a response lever (i.e. two levers per chamber). Lever press responses activated a syringe pump (Med Associates) that delivered 0.1 ml of solution into the receptacle over 1.66 seconds. A stimulus light located above each

response lever was simultaneously illuminated during pump activation. Lever responses during reinforcer delivery were recorded, but did not produce programmed consequences. The chambers were interfaced (Med Associates) to a computer programmed to control sessions and record data.

Locomotor Chambers. Clear Plexiglas chambers (43.2 cm x 43.2 cm; Med Associates) were used to assess locomotor activity. Horizontal distance traveled (cm) was determined from the number of photobeam breaks and collected via computer interface in 2 min time intervals using Activity Monitor locomotor activity software (Med Associates).

Operant Self-administration

Training. One day prior to training, rats were fluid-restricted for approximately 24 hrs. Immediately afterwards, rats were placed in the operant conditioning chambers for an initial 16-hr lever-press training session in which presentation of a 0.1 ml solution of concurrently available sucrose (10 %, w/v) and water was contingent on lever responses. Lever responses were initially maintained on a concurrent fixed-ratio 1 (CONC FR1 FR1) schedule of reinforcement and were gradually increased to CONC FR2 FR2 after delivery of 4 reinforcers, and then further increased to CONC FR4 FR4 after delivery of 10 reinforcers. All reinforcer deliveries were paired with an illumination of a light cue located above each response lever. After completing the initial 16 hr training session, rats were returned to their homecage for a period of 24 hrs in which access to water was returned and remained available *ad libitum* thereafter.

Sucrose Fading and Baseline Sessions. Next, rats commenced daily (Monday–Friday) 30-min sessions (CONC FR4 FR4) where the sucrose concentration was gradually decreased and the ethanol concentration was increased using a modified sucrose-fading procedure (Samson, 1986) as previously described (Besheer et al., 2010; Hodge et al., 1993b). Briefly, ethanol was gradually added to the 10% (w/v) sucrose solution and sucrose was gradually faded out so that ethanol (15%, v/v) alone maintained lever pressing. The exact order of mixed ethanol exposure was as follows: 10% sucrose/2% alcohol (10S/2E), 10S/5E, 10S/10E, 5S/10E, 5S/15E, 2S/15E, 0S/15E. There were 2 sessions at each concentration (i.e., 12 total sucrose fading sessions). Sucrose-trained P-rats did not receive ethanol and were faded to 0.8% (w/v) sucrose. The exact order of sucrose fading was as follows: 10S, 5S, 2S, 1S, 0.8S, 0.4S. The final sucrose concentration was readjusted to 0.8% (w/v) sucrose because this concentration produced similar lever responding as compared to the 15% alcohol-trained animals. After the sucrose fading procedure, rats had a minimum of 28 baseline operant self-administration sessions with 15% ethanol vs. water (alcohol-trained P-rats) or 0.8% sucrose vs. water (sucrose-trained P-rats).

Examination of positive modulation of glutamate activity at AMPA receptors on ethanol self-administration.

To test whether potentiation of AMPA receptor signaling might influence ethanol reinforcement processes, P-rats (n = 9) were pretreated with aniracetam (0, 1, 5, 10, 30 mg/kg, i.p.) 30 minutes prior to operant ethanol (15% v/v) self-administration sessions (30-min). Aniracetam doses within this range have been shown to be effective at

altering behavior in previous studies (Knapp et al., 2002; Lebrun et al., 2000). Testing was conducted using a within-subjects design in which each aniracetam dose was administered in a randomized order. As such, BEC measurements were not conducted after each drug test session as blood sampling has the potential to disrupt ongoing self-administration behavior. There were at least 2 self-administration sessions between injections and test sessions occurred no more than two times per week. Additionally, each liquid receptacle in the operant chambers was monitored for residual fluid after every session.

Test for aniracetam-induced alterations in locomotor behavior.

Two weeks following aniracetam testing, the P-rats were tested to examine if potentiated aniracetam-induced alcohol self-administration was the result of changes in locomotor activity. Animals received aniracetam pretreatment (0 and 5 mg/kg, i.p.) 30 minutes prior to placement in locomotor chambers where distance traveled was measured and recorded via photo beam breaks. Each rat experienced two 30-minute locomotor sessions in which aniracetam dose order (0 and 5 mg/kg) was randomized. Locomotor sessions were interspersed with self-administration sessions where appropriate with at least 3 days between tests. Self-administration sessions were withheld on days of the locomotor tests.

Confirmation of AMPA receptor involvement in modulating increased alcohol self-administration.

The AMPA receptor antagonist, DNQX (0 and 3 mg/kg, i.p) was administered immediately before aniracetam (0 and 5 mg/kg) to confirm the role of AMPA receptors in potentiating ethanol self-administration induced by aniracetam. This dose of DNQX (3 mg/kg) was chosen because it was the highest dose that did *not* alter operant ethanol self-administration when administered alone in a separate preliminary study (data not shown). On test days, each subject received each possible combination of both DNQX (0 and 3 mg/kg, i.p) immediately followed by aniracetam (0 and 5 mg/kg, i.p.) in randomized order 30 minutes before testing, such that the entire dose-response curve was generated over the course of 4 test sessions.

Test for aniracetam-induced alterations in ethanol pharmacokinetics.

To determine whether aniracetam pretreatment altered ethanol clearance, another cohort of P-rats (n=6/ treatment group) with a history of ethanol self-administration were pretreated with aniracetam (0; or 5 mg/kg; i.p.) 30 minutes prior to orally administered ethanol (1 g/kg, IG) and tail blood was collected 20, 30, 60, 120 and 240 min later. Plasma supernatant (5 µl) was analyzed for ethanol content using an Analox Alcohol Analyser (Model AM1, Analox Instruments USA Inc.,Lunenburg,MA).

Test for reinforcer specificity via sucrose-self-administration.

To test if enhanced AMPA receptor activity was selective at modulating ethanol reinforcement, behavior-matched sucrose-trained P-rats (n = 11) were pretreated with

aniracetam (0, 1, 5, 10, 30 mg/kg, i.p.) 30 minutes prior to operant sucrose (0.8%, w/v) self-administration sessions (30-min). Aniracetam testing was conducted as described for the ethanol self-administration tests sessions.

Examination of positive modulation of glutamate activity at AMPA receptors on regulating response-contingent cue-induced reinstatement.

To test the effects of aniracetam on ethanol seeking behavior after exposure to an ethanol-associated cue, self-administration-trained P-rats (15% ethanol vs. water; CONC FR4 FR4) had a minimum of 28 days of baseline self-administration sessions (see above for training procedures). After stable baseline responding, rats underwent extinction sessions (30 min) in which previously reinforced responses were extinguished over 13 consecutive days by removing response contingencies (i.e. no light cue or pump sound; no reinforcers delivered). Treatment groups were matched based on baseline response totals and extinction performance. On day 14, ethanol-trained P-rats were injected with vehicle (n=8) or aniracetam (5 mg/kg, i.p.; n=7) 30 minutes prior to a reinstatement session. During this session (30 min), lever responses (FR4) resulted in the presentation of the light cue and pump activation; however, no reinforcers (ethanol or water) were delivered. Note: 0.1 ml of the 15% ethanol (v/v) solution was added to the ethanol well prior to the reinstatement session. This amount of ethanol (0.1ml) is not pharmacologically relevant and functioned to provide additional olfactory/taste cues to maximize lever responses during reinstatement (Backstrom et al., 2004).

To test the effects of aniracetam on extinction responding, the same animals were reintroduced to ethanol 15% (v/v) for 10 baseline self-administration sessions.

Rats then underwent 12 daily extinction sessions (30 min; see above) and on the 13th extinction session, animals were pretreated with vehicle (n=7) or aniracetam (n=7). Because aniracetam did not alter sucrose self-administration (see below), the role of aniracetam in sucrose reinstatement was not tested in this study.

Drugs.

Alcohol (95% (w/v); Pharmco-AAPER, Shelbyville, KY) was diluted in distilled water to 15% (v/v). Aniracetam (Tocris, Ellisville, Missouri) was suspended in 0.5% Carboxymethyl cellulose (vehicle; Sigma Aldrich, St. Louis, Missouri) and sonicated for approximately 15-20 minutes prior to injections. Aniracetam was injected at a volume of 2 ml/kg (i.p.). DNQX (Tocris, Ellisville, Missouri) was diluted in filtered water and injected at a volume of 1 ml/kg (i.p.).

Data Analysis

Alcohol- (or sucrose-) and water-reinforced responses, cumulative responses, and cumulative distance traveled (cm) were analyzed by two-way repeated measures analysis of variance (RM ANOVA). Intake (g/kg) were analyzed by one-way RM ANOVA and calculated based on rat body weight and number of reinforcers delivered. Responding was “extinguished” when ethanol-lever responses were not statistically different than water-lever responses for at least two consecutive days (two-way ANOVA). Lever responses in the reinstatement session were analyzed by two-way RM ANOVA. Tukey post-hoc comparisons were performed to identify differences between treatments/treatment groups. Significance was determined at $p \leq 0.05$. Baseline data

(mean ± S.E.M.) for the day preceding initial testing of aniracetam is reported in **Table 1**.

Results

Examination of positive modulation of glutamate activity at AMPA receptors on ethanol self-administration.

P-rats trained to self-administer ethanol or water (CONC FR4 FR4) were pretreated with aniracetam (0, 1, 5, 10, 30 mg/kg) 30 minutes prior to self-administration sessions. Statistical analysis by two-way repeated measures analysis of variance (RM-ANOVA) indicated significant main effects of reinforcer ($F(1,8)=291.82$, $p<0.001$), aniracetam dose ($F(4,32)=6.77$, $p<0.001$), and a significant interaction of reinforcer x aniracetam dose ($F(4,32)=6.49$, $p<0.001$). Tukey post-hoc comparisons showed that pretreatment with aniracetam (1 and 5 mg/kg) resulted in a significant increase in ethanol-reinforced responses vs. vehicle pretreatment ($p<0.001$), while water-reinforced responses were not altered by aniracetam pretreatment **(Figure 1A**). The mean values (± SEM) for ethanol intake (g/kg) during self-administration after pretreatment with each aniracetam dose were as follows: 0.71 ± 0.08 (vehicle), 0.90 ± 0.1 (1 mg/kg), 1.14 ± 0.07 (5 mg/kg), 0.84 ± 0.09 (10 mg/kg), and 0.86 ± 0.06 (30 mg/kg). One-way RM ANOVA showed a significant increase in ethanol intake ($F(4,32)=5.64$, $p=0.001$) after aniracetam pretreatment, with over a 60% increase in consumption (5 mg/kg aniracetam vs. vehicle treatment, $P<0.05$). Furthermore, statistical analysis of the rate of ethanol lever responding over time (cumulative lever responses) by two-way RM ANOVA showed significant main effects of time ($F(5,40)=47.20$, $p<0.001$), and dose

(F(4,32)=4.72, p=0.004), and a significant interaction of time x dose (F(20,160)=4.04, p<0.001). Post-hoc analysis indicated that the aniracetam-induced increase in ethanol-reinforced responses emerged early (by 10 min) and persisted throughout the duration of the 30-minute test session (5 mg/kg aniracetam; **Figure 1B**). Together these data suggest that enhanced glutamate activity at AMPA receptors enhances the reinforcing effects of ethanol.

Test for aniracetam-induced alterations in locomotor behavior.

To test for non-specific changes in locomotor behavior that may have influenced lever responding during operant ethanol self-administration tests, P-rats were pretreated with aniracetam (0 or 5 mg/kg) prior to locomotor assessments in an open field. Two-way RM ANOVA showed that cumulative distance traveled (cm) increased over time (F(14,112)=151.68, p<0.001), and aniracetam pretreatment did not alter this pattern of behavior (F(1,8)=0.45, p=0.84; **Figure 1C**), suggesting that increased ethanol lever responses after aniracetam pretreatment were likely not due to non-specific aniracetam-induced changes in locomotor behavior.

Confirmation of AMPA receptor involvement in modulating increased ethanol self-administration.

Rats received double injections of DNQX and aniracetam to verify the role of AMPA receptors in potentiating ethanol-reinforced responses during operant self-administration sessions. Analysis by two-way RM ANOVA showed a main effect of DNQX dose (F(1,8)=15.36, p=0.004), but no main effect of aniracetam dose

(F(1,8)=0.15, p=0.71). There was a significant interaction of aniracetam dose x DNQX dose (F(1,8)=,18.28, p=0.003). Post-hoc analysis indicated that DNQX (3 mg/kg) was ineffective at altering ethanol self-administration when administered in combination with vehicle (Aniracetam 0 mg/kg; **Figure 2** left panel). Importantly, pretreatment with DNQX (3 mg/kg) significantly blocked aniracetam-induced increased ethanol self-administration (aniracetam 5 mg/kg; **Figure 2**). Water lever responding was not altered by DNQX and aniracetam pretreatment. Two-way ANOVA showed no main effects of DNQX dose(F(1,8)=1.82, p=0.21), aniracetam dose(F(1,8)=1.74, p=0.22) and no significant interaction of DNQX x aniracetam (F(1,8)=0.03, p=0.86). These data confirm the role of AMPA receptors in mediating the aniracetam-induced increases in ethanol-reinforced responding.

Test for aniracetam-induced alterations in ethanol pharmacokinetics.

To test if increased ethanol self-administration was the result of treatment-induced alterations in ethanol clearance, P-rats were challenged with a 1 g/kg (IG) dose of ethanol following aniracetam (0 vs. 5 mg/kg; i.p) pretreatment (n=6 per treatment group). Pretreatment with aniracetam did not alter blood-ethanol clearance over a 4-hr period relative to vehicle-treated P-rats (**Figure 3**). Analysis by two-way ANOVA showed a main effect of time (F(5,48)=38.76, p<0.001), but no effect of dose (F(1,48)=2.992, p<0.09) or interaction of time x dose (F(5,48)=0.891, p=0.49). These data suggest that increased ethanol self-administration following aniracetam pretreatment was not related to a change in the pharmacokinetics of ethanol (**Table 2)**.

Test for reinforcer specificity via sucrose self-administration.

To evaluate reinforcer specificity, aniracetam (0, 1, 5, 10, 30 mg/kg) was tested prior to self-administration sessions in sucrose-trained P-rats. Aniracetam did not alter operant sucrose or water self-administration in 30-minute test sessions (**Figure 4A**). Analysis by two-way RM ANOVA showed a significant main effect of lever ($F(1,10)=31.49$, $p=0.001$), but no aniracetam-induced changes in sucrose self-administration were observed ($F(4,40)=1.02$, $p=0.41$). Moreover, examination of the cumulative sucrose responses over time (response rate) did not show any statistical differences across treatment doses (**Figure 4B**). Two-way RM ANOVA showed a main effect of time ($F(5,50)=20.14$, $p<0.001$), however, there was no main effect of dose ($F(4,40)=0.88$, $p=0.49$) or significant interaction of time x dose ($F(20,200)=0.93$, $p=0.55$). These data suggest that enhanced glutamate activity at AMPA receptors induced by aniracetam pretreatment selectively potentiates ethanol reinforcement processes, but not sucrose reinforcement (e.g. a non-drug reward).

Examination of positive modulation of glutamate activity at AMPA receptors on regulating response-contingent cue-induced reinstatement.

To examine the role of enhanced glutamate activity at AMPA receptors in modulating ethanol-seeking behavior in P-rats a response contingent cue induced-reinstatement procedure was used. Removal of response contingencies extinguished ethanol-associated lever responding over a period of 13 consecutive days (**Figure 5A**). Statistical analysis by two-way ANOVA showed a main effect of lever ($F(1,364)=324.14$, $p<0.001$), extinction session ($F(12,364)=29.39$, $p<0.001$), and a significant interaction of

lever x extinction session ($F(12,364)=25.87$, $p<0.001$). Post-hoc comparisons showed significant differences in lever responses on the ethanol and water levers, with the exception of the last three extinction sessions ($p>0.05$), which confirms extinction. During reinstatement, both treatment groups (vehicle and aniracetam) displayed response-contingent cue-induced reinstatement of ethanol-seeking behavior, as evidenced by increased responding on the ethanol-associated lever compared to their last extinction session (**Figure 5B**). Two-way RM ANOVA (with test condition as a repeating factor) showed significant main effects of aniracetam dose ($F(1,13)=10.74$, $p=0.006$) and test condition ($F(1,13)=89.74$, $p<0.001$) and a significant interaction of aniracetam dose x test condition ($F(1,13)=11.16$, $p=0.005$). Interestingly, responding on the ethanol-associated lever was potentiated in P-rats after pretreatment with aniracetam (5 mg/kg vs. vehicle group) during reinstatement ($p\leq0.05$, Tukey post-hoc).

Responses on the water lever during reinstatement were not altered by aniracetam pretreatment (**Figure 5C**) as no main effect of dose ($F(1,13)=0.12$, $p=0.74$), test condition ($F(1,13)=1.90$, $p=0.19$), or interaction ($F(1,13)=0.02$, $p=0.88$) was evident. Moreover, an examination of operant responses during extinction did not reveal any differences between the aniracetam-treated and vehicle-treated groups. The mean values (± SEM) for lever responding during extinction after pretreatment with aniracetam were as follows: 4.286 ± 0.92 (vehicle; ethanol lever), 1.286 ± 0.75 (vehicle; water lever), 8.286 ± 3.15 (5 mg/kg; ethanol lever), 1.71 ± 0.36 (5 mg/kg; water lever). Analysis by two-way ANOVA (with test condition as a repeating factor) showed no main effects of dose ($F(1,12)=0.41$, $p=0.53$), test condition ($F(1,12)=0.70$, $p=0.42$) and no interaction of dose X test condition ($F(1,12)=2.16$, $p=0.17$). These data suggest that

potentiated glutamate activity at AMPA receptors exacerbates cue-induced ethanol-seeking behavior.

Discussion

The results of this study demonstrate for the first time that enhanced glutamate activity at AMPA receptors exacerbates ethanol self-administration and promotes reinstatement of ethanol-seeking behavior. First, systemic AMPA receptor activation by aniracetam increased ethanol-reinforced responding in P-rats; an inbred rodent strain that is considered to fit many of the criteria for an animal model of alcoholism (Bell et al., 2006a; Kampov-Polevoy et al., 2000). Additionally, the aniracetam-induced increase in ethanol-reinforced responding was absent of effects on spontaneous locomotor behavior and was not the result of alterations in ethanol clearance. Moreover, the role of AMPA receptors was confirmed because the aniracetam-induced increase in ethanol self-administration was blocked by administering the AMPA receptor antagonist, DNQX. Selective modulation of the reinforcing effects of ethanol was confirmed as aniracetam pretreatment did not alter self-administration of an alternative reinforcer (i.e. sucrose). Finally, aniracetam pretreatment potentiated responding on the ethanol-associated lever during a cue-induced reinstatement procedure. Taken together, these data suggest that enhanced AMPA receptor signaling selectively contributes to facilitate ethanol self-administration by potentiating both the reinforcing effects of ethanol and the capacity of ethanol-related cues to promote relapse of ethanol-seeking behavior.

The reinforcing effect of drugs of abuse like alcohol is a major controlling process by which subsequent drug-taking episodes are facilitated (Stolerman, 1992; White,

1996). Here, a novel neuronal mechanism is provided that could contribute, in part, to increased ethanol drinking. That is, enhanced glutamate activity at AMPA receptors alters ethanol reinforcement processes in a manner that facilitates self-administration. These data are highly relevant given that excessive ethanol consumption has been shown to increase the likelihood for the development of alcoholism and/or alcohol dependence in humans (Saha et al., 2006). Interestingly, previous work by Stephens and Brown (1999) showed that administration of NBQX (AMPA receptor antagonist) not only reduced operant ethanol self-administration on a progressive ratio schedule of reinforcement, but sucrose and saccharin self-administration as well as locomotor activity; suggesting that AMPA receptors were either not selective in modulating ethanol reinforcement or results could not be determined due to non-specific motor-impairing effects of NBQX (Stephens and Brown, 1999). In contrast, the present work demonstrates a selective role for enhanced AMPA receptor activity in modulating ethanol reinforcement since AMPA receptor activation resulted in increased ethanol self-administration on a fixed ratio schedule of reinforcement. Further, reinforcer specificity was confirmed because aniracetam pretreatment was void of effects on sucrose self-administration.

An intriguing feature of the ethanol self-administration experiments is the inverted U-shaped dose response curve following aniracetam pretreatment. Lower aniracetam doses were effective at significantly increasing operant self-administration, whereas higher doses did not alter ethanol-reinforced responses. This may reflect a response to changes in extracellular glutamate levels after chronic ethanol exposure. Specifically, low doses of aniracetam may have potentiated ethanol-induced excessive glutamate

levels and promoted ethanol self-administration, while higher aniracetam doses may have exceeded the necessary threshold to alter ethanol reinforcement or engaged other receptor signaling systems (e.g. loss of receptor specificity). Several studies show that chronic ethanol exposure increases extracellular glutamate levels as measured by in vivo microdialysis (Lallemand et al., 2011; Melendez et al., 2005; Roberto et al., 2004) in brain regions that have been shown to regulate ethanol reinforcement (Besheer et al., 2010). Future investigations measuring glutamate levels/signaling after aniracetam pretreatment might assist in elucidating the underlying mechanism of this biphasic dose-response effect.

It could be argued that positive modulation of AMPA receptors induced changes in locomotor behavior that could have augmented operant responses. However, aniracetam pretreatment did not alter water or sucrose lever responding, and did not alter locomotor behavior in an open field, suggesting that increased ethanol self-administration was not due to a treatment-induced hyperactive state. These findings are consistent with others showing that aniracetam does not alter locomotor behavior in rats (Ventra et al., 1994). Operant self-administration procedures have a critical learning and memory element (White, 1996) that could possibly have been influenced by aniracetam. Indeed, aniracetam is a nootropic compound that has cognitive-enhancing effects in rodents (Lebrun et al., 2000; Ventra et al., 1994). However, increased operant responding after aniracetam pretreatment did not generalize across reinforcers (i.e. no effect on sucrose self-administration), making an explanation of general enhancement of cognitive function less plausible. An alternative explanation is that aniracetam produced an ethanol-like effect that primed further ethanol self-administration. This is

highly unlikely due to evidence suggesting that the actions of AMPA receptor positive modulators counteract those of ethanol. Specifically, ethanol has been shown to stabilize rapid sensitization of AMPA receptors after activation by glutamate (Moykkynen et al., 2003; Moykkynen et al., 2009), whereas AMPA receptor positive modulators act as desensitization inhibitors (Tang et al., 1991); allowing for increased AMPA receptor current decay time and essentially opposing the actions of ethanol (Jones et al., 2008). Interestingly, the possibility of the opposition of the effects of ethanol by aniracetam may explain the increased ethanol self-administration. That is, aniracetam may blunt the subjective or interoceptive effects of consumed alcohol which may lead to greater ethanol self-administration (Besheer et al., 2012b; Hodge et al., 2001). Future investigation assessing the role of AMPA receptor positive modulation on the discriminative stimulus effects of ethanol could provide insight into whether AMPA receptor positive modulators alter how ethanol is perceived. Regardless of the underlying mechanism, our findings demonstrate a novel role for enhanced activity at AMPA receptors in promoting ethanol self-administration.

A prominent feature of alcoholism is excessive drinking interspersed with periods of abstinence and subsequent relapse episodes (Johnson, 2010; McLellan et al., 2000). Furthermore, exposure to ethanol-associated cues has been shown to trigger craving and relapse episodes in humans and preclinical models (Papachristou et al., 2012; Schroeder et al., 2008). The current data show that enhancement of AMPA receptor activity potentiates ethanol-seeking behavior after re-exposure to ethanol-associated cues during a response-contingent reinstatement procedure. Further, aniracetam did not alter responding during extinction, highlighting the role of the cue in driving ethanol-

seeking behavior. Due to a lack of effect of aniracetam on sucrose self-administration, the role of aniracetam in sucrose reinstatement was not tested. Examining the contributions of AMPA receptor activity in modulating non-drug seeking behavior might provide insights for a specified role of these receptors in modulating relapse to alcohol. Several studies have shown that reducing glutamate signaling at AMPA receptors attenuates relapse to ethanol-seeking behavior after exposure to ethanol cues (Backstrom and Hyytia, 2004; Sanchis-Segura et al., 2006). We extend those findings by offering a novel role for enhanced AMPA receptor signaling in modulating cue-induced ethanol-seeking. That is, enhanced AMPA receptor activity may have bolstered the ability of ethanol cues to promote ethanol-seeking behavior. Interestingly, previous studies show that a history of excessive ethanol consumption increases purported conditioned responses to ethanol cues in humans (Drummond, 2000; Papachristou et al., 2012). Accordingly, the aniracetam-induced potentiation of ethanol cue salience to promote ethanol-seeking could have been a major contributing factor in driving the observed increase in ethanol self-administration. These data are of particular importance given that the present results suggest a role for enhanced AMPA receptor activity in modulating *both* heightened ethanol self-administration *and* relapse to ethanol-seeking. The findings suggest that enhanced AMPA receptor signaling could be involved in promoting vulnerability to relapse episodes.

The present data highlight the role of increased glutamate transmission in relapse-like behavior. Indeed, glutamate levels have recently been shown to be elevated in the nucleus accumbens and amygdala during ethanol-seeking behavior using a cue-induced reinstatement procedure (Gass et al., 2011). These findings are

intriguing and suggest that aniracetam may have potentiated an already-heightened glutamatergic state during reinstatement; a possibility that will be interesting for future work to address. To date, it is not known if P-rats differentially express basal levels of AMPA receptors as compared to other strains, which could influence pharmacological response to aniracetam and affect behavioral outcomes. An assessment of AMPA receptor expression across strains could provide additional understanding of AMPA receptor sensitivity to aniracetam treatment. Future experiments using AMPA receptor positive modulators and antagonists targeting AMPA receptors in specific limbic regions that regulate reward and drug-seeking could provide critical information about the neuronal circuitry involved in modulating increased ethanol self-administration and potentiated reinstatement. Interestingly, the present data parallel other studies investigating the role of AMPA receptor neurotransmission in modulating relapse to other drugs of abuse, particularly cocaine. Indeed, microinjections of AMPA agonists in the nucleus accumbens have been shown to reinstate cocaine-seeking behavior (Cornish and Kalivas, 2000); which strongly suggest that AMPA receptor signaling may regulate drug-seeking across a variety of drugs of abuse.

In conclusion, the present work provides strong evidence for the involvement of enhanced AMPA receptor activity in modulating both increased ethanol self-administration and potentiated relapse-like behavior. These data suggest that enhanced synaptic strength (particularly enhanced AMPA receptor signaling) may be one of the key pathophysiological changes that produce a behavioral phenotype similar to that of an alcoholic (i.e, increased drinking and susceptibility to relapse). A better understanding of how enhanced glutamate activity at AMPA receptors plays a role in

ethanol abuse disorders will be critically important in elucidating the underlying mechanisms of alcoholism and for the development of glutamate-based therapeutics aimed at treating alcohol abuse disorders. Future preclinical studies examining the role of therapeutic compounds that target AMPA receptors on alcohol drinking behavior could demonstrate therapeutic promise.

Table 1

Table 1. Baseline self-administration parameters (mean ± SEM) prior to test sessions.

Experiment	Alcohol Lever Responses	Water Lever Responses	Intake (g/kg)
Alcohol Self-administration (n=9)			
Prior to aniracetam	157.78 ± 20.96	7.44 ± 1.56	0.81 ± 0.11
Sucrose Self-administration (n=11)	(*Sucrose Resp.*)		
	142.18 ± 28.39	11.73 ± 4.79	
Prior to aniracetam			
Cue-induced Reinstatement			
Vehicle Group (n=8)	172.25 ± 11.61	0.88 ± 0.39	0.77 ± 0.05
Aniracetam Group(n= 7)	174.43 ± 25.60	3.00 ± 0.90	0.82 ± 0.13

Resp, Responses

Table 2

Table 2. Alcohol Clearance Parameters After Aniracetam Pretreatment.

Clearance Parameters	Vehicle Group	Aniracetam Group
BAC at 0 min (mg/dl)	101.04 ± 11.09	92.06 ± 11.06
Clearance Rate (mg/ml/min)	0.41 ± 0.04	0.38 ± 0.04
Clearance Time (min)	245.06 ± 6.24	242.03 ± 4.95
V_d / body weight (dl/g)	0.011 ± 0.001	0.012 ± 0.001
Area Under Curve (AUC)	11736.33 ± 1215.13	9185.67 ± 1392.29

V_d, Volume of distribution

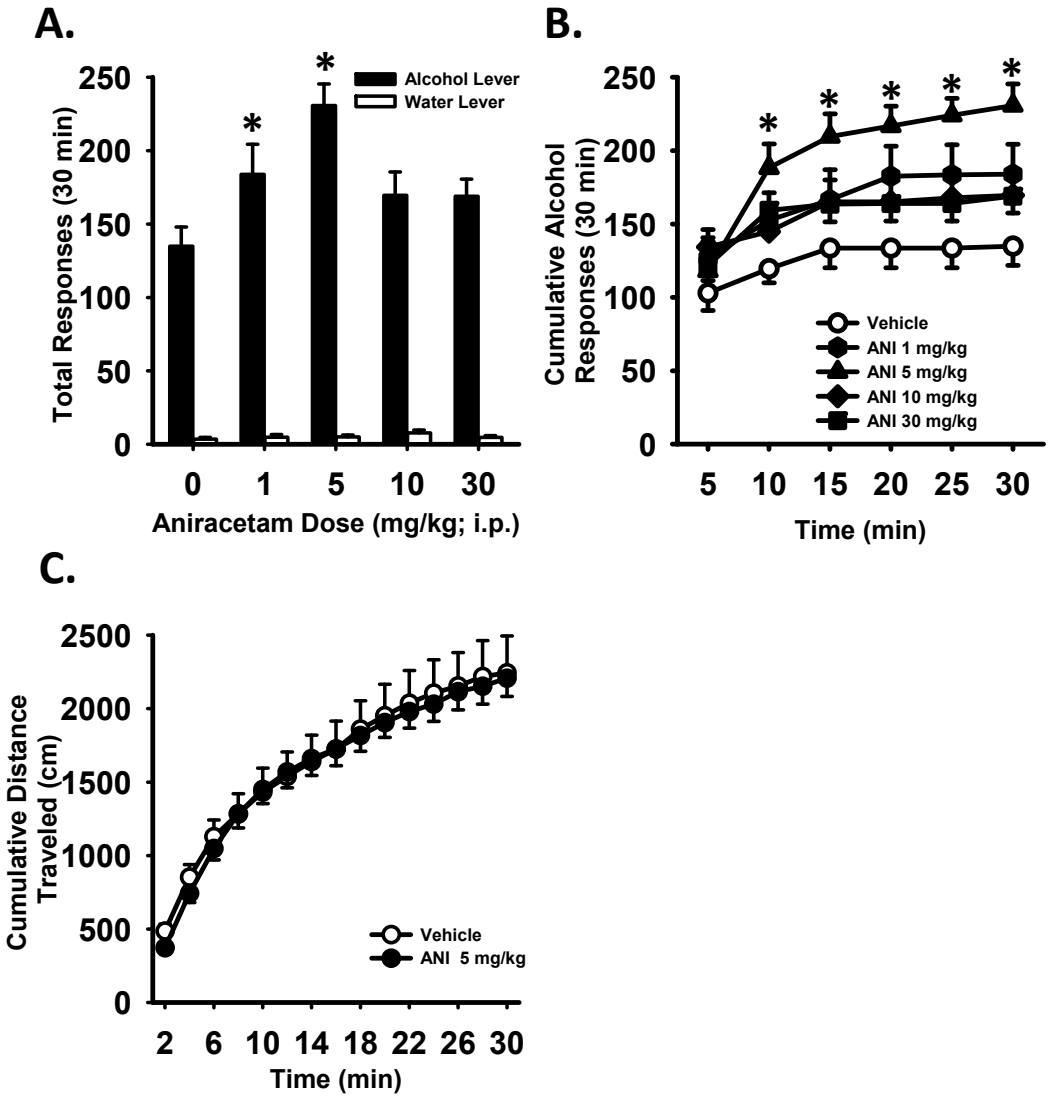


Figure 1. Positive modulation of AMPA receptors potentiates alcohol self-administration. (A) Pre-treatment with aniracetam (ANI; 1 and 5 mg/kg, i.p.) significantly increased total alcohol-, but not water-reinforced responses during operant self-administration sessions in P-rats (n = 9) trained to self-administer alcohol (15%, v/v) versus water. (B) Increased cumulative alcohol-reinforced responses became apparent early within the session and remained elevated throughout the entire session after ANI pre-treatment (5 mg/kg). (C) Spontaneous locomotor activity in an open field was not altered in P-rats pre-treated with ANI (5 mg/kg). Graphed values are expressed as mean ± standard error of the mean. *P < 0.05 versus vehicle (Tukey's post hoc)

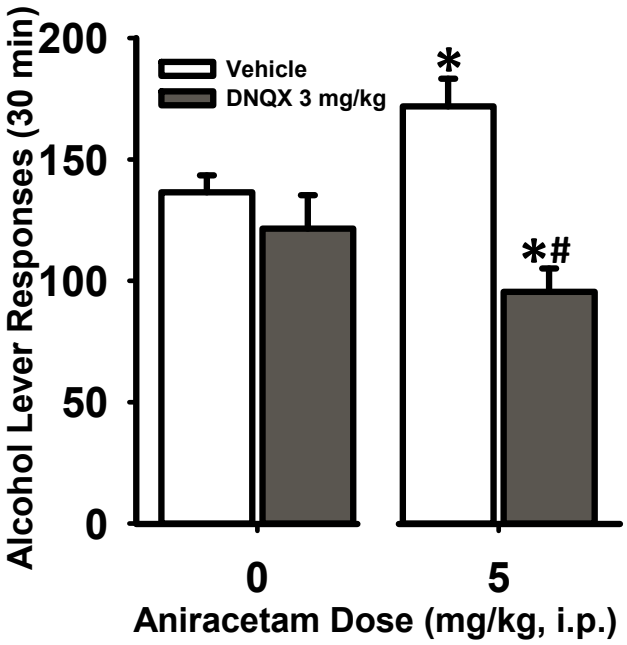


Figure 2. Antagonism of AMPA receptors by DNQX pre-treatment blocks aniracetam-induced increased alcohol self-administration. Administration of the AMPA receptor antagonist, DNQX, significantly reversed aniracetam-induced increased alcohol-lever responding in P-rats ($n = 9$) trained to self-administer alcohol (15%, v/v). Graphed values are expressed as mean ± standard error of the mean. *Indicates significant changes relative to pre-treatment with vehicle (dH2O) + vehicle (0.5% CMC). #Indicates significant changes relative to pre-treatment with vehicle (dH2O) + 5 mg/kg aniracetam (Tukey's *post hoc*).

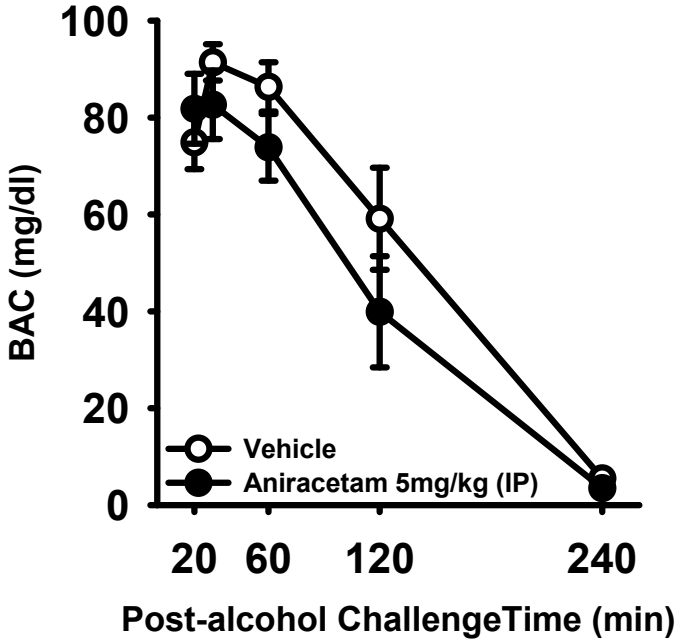


Figure 3. Alcohol clearance is not altered by aniracetam pre-treatment. Pre-treatment with aniracetam (5 mg/kg; *n* = 6) did not affect blood-alcohol content (BAC) across time after intragastric (IG) administration of alcohol (1 g/kg; IG) versus vehicle treated P-rats (*n* = 6). Graphed values are expressed as mean ± standard error of the mean.

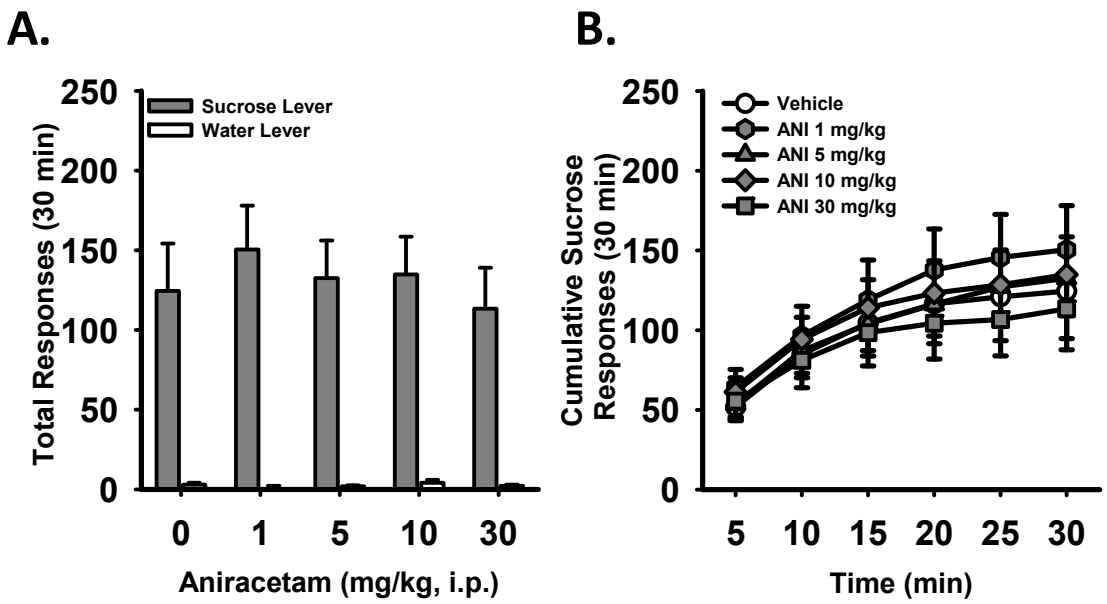


Figure 4 Positive modulation of AMPA receptors does not alter sucrose self-administration. (A) Pre-treatment with aniracetam (ANI) did not alter total sucrose- or water-reinforced responses during operant self-administration sessions in P-rats (n = 11) trained self-administer sucrose (0.8%, w/v) versus water. (B) Aniracetam pre-treatment did not alter the pattern of sucrose-reinforced responses across the course of the self-administration session. Graphed values are expressed as mean ± standard error of the mean

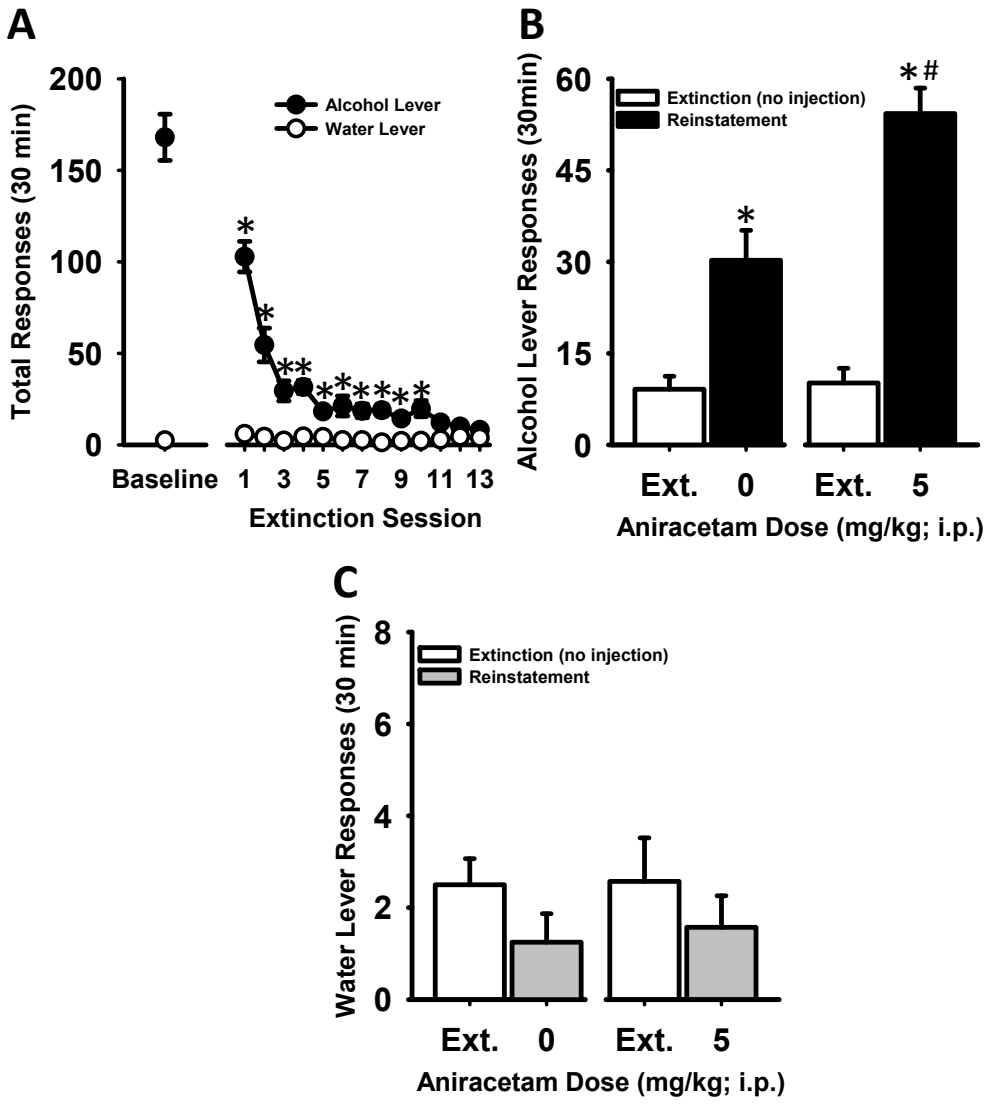


Figure 5. Positive modulation of glutamate activity at AMPA receptors potentiates cue-induced reinstatement of alcohol-seeking behavior in P-rats. (A) Removal of response contingencies significantly reduced alcohol-associated lever responding over a period of 13 consecutive days during the extinction phase (*$P < 0.05$ versus water lever). (B) Responding on the alcohol-associated lever was potentiated ($n = 7–8$ per group) after pre-treatment with aniracetam (5 mg/kg) during a cue-induced reinstatement session. [*$P < 0.05$ versus extinction; #$P < 0.05$ versus reinstatement (vehicle)]. Lever responding resulted in the illumination of an alcohol-associated cue light, but alcohol was not available during this test session. (C) Responding on the water-associated lever remained unaltered after aniracetam pre-treatment during the same reinstatement test session. Water-lever responding resulted the illumination of a water- associated cue light, but water was not available during reinstatement testing. Graphed values are expressed as mean ± standard error of the mean (Tukey's *post hoc*)

CHAPTER 3: POTENTIATION OF AMYGDALA AMPA RECEPTOR ACTIVITY SELECTIVELY PROMOTES ETHANOL SELF-ADMINISTRATION IN A CAMKII-DEPENDENT MANNER

Introduction

The reinforcing properties of drugs of abuse such as ethanol, are key contributing elements that promote subsequent drinking episodes and determine potential abuse liability (Ator and Griffiths, 2003; Griffiths et al., 2003). Elucidating the underlying neuronal mechanisms of ethanol reinforcement is critical, as novel insights have the potential to offer a greater understanding of factors that influence excessive drinking and ethanol-seeking behavior. An increasing amount of evidence suggests that AMPA-type glutamate (AMPA) receptors are important modulators of ethanol drinking behaviors (Backstrom and Hyytia, 2004; Cannady et al., 2013; Sanchis-Segura et al., 2006; Stephens and Brown, 1999; Wang et al., 2012). AMPA receptors are ligand-gated ion channel receptors comprised of four subunits (GluA1-4) and mediate rapid excitatory glutamate neurotransmission (Bredt and Nicoll, 2003; Kessels and Malinow, 2009). These receptors are involved in a variety of biological functions and dysregulation of AMPA receptors has been implicated in several neurological, psychiatric, and addictive disorders (Chourbaji et al., 2008; Kobylecki et al., 2013; Purgianto et al., 2013; Rakhade et al., 2012; Zhang and Abdullah, 2013).

Strengthening of synaptic function has been linked to increased AMPA receptor trafficking and signaling (Heynen et al., 2000; Luscher et al., 1999). Furthermore, repeated experience with ethanol leads to AMPA-receptor mediated neuroadaptations that enhance synaptic strength in brain regions known to regulate reward processes (Heikkinen et al., 2009; Stuber et al., 2008; Wang et al., 2010). Thus, ethanol-induced synaptic strengthening may functionally promote ethanol consumption through alterations in reinforcement processes. Moreover, recent studies have examined ethanol-induced alterations in AMPA receptor subunit expression within limbic regions. Specifically, GluA1 subunit expression is upregulated in both the ventral and dorsal striatum, after systemically administered ethanol (Wang et al., 2012) and chronic intermittent ethanol consumption (Neasta et al., 2010), respectively. Surface GluA1 is also upregulated in the amygdala after chronic intermittent ethanol vapor exposure (Christian et al., 2012). Collectively, these studies further highlight the ethanol-induced neuroadaptations in AMPA receptor expression and trafficking that are associated with synaptic strengthening. In addition to increased expression, AMPA receptor signaling can also be potentiated through post-translational modifications of AMPA receptor subunits. Indeed, intracellular kinases such as CaMKII have been shown to facilitate AMPA receptor signaling by phosphorylation of GluA1 subunits; consequently altering channel kinetics, function, and increased receptor trafficking (Derkach et al., 1999; Lu et al., 2010). However, no studies have examined ethanol-induced phosphorylation of AMPA receptor subunits after a history of ethanol self-administration.

Recently, systemic enhancement of glutamate activity at AMPA receptors has been shown to facilitate ethanol self-administration and seeking-behavior, which

demonstrates that heightened activity at these receptors has the potential to augment ethanol reinforcement processes (Cannady et al., 2013). Although evidence suggests that enhanced AMPA receptor activity can promote ethanol reinforcement, it is not fully clear as to which brain regions modulate AMPA receptor-mediated potentiation of ethanol reinforcement. Regions such as the amygdala and the nucleus accumbens have well-defined roles in modulating alcohol consumption, reinforcement, and seeking behaviors (Besheer et al., 2010; Chaudhri et al., 2013; Rassnick et al., 1992; Schroeder et al., 2003; Schroeder et al., 2008). Yet it is unclear if localized potentiation of AMPA receptor signaling modulates ethanol self-administration within the amygdala or nucleus accumbens.

Using a genetic rodent model of high ethanol preference, the alcohol–preferring P- rat (Bell et al., 2006a), the current study examined the functional role of enhanced glutamate activity at AMPA receptors within limbic brain regions to modulate ethanol self-administration. First, phosphorylation of the GluA1 AMPA receptor subunit was used as a maker to identify potential brain regions that might represent areas of potentiated AMPA receptor signaling as a consequence of a history of ethanol self-administration. Guided by these results, we microinjected aniracetam (AMPA receptor positive modulator) to functionally assess brain regional involvement in AMPA receptor-mediated modulation of operant ethanol self-administration. Control experiments evaluated aniracetam effects on reinforcer specificity, and alterations in locomotor behavior. Finally, we assessed whether intra-amygdala aniracetam-induced alterations in ethanol self-administration were dependent on CaMKII activity.

Materials and Methods

Animals

Adult male inbred alcohol-preferring P-rats (420 - 500 g prior to testing) were bred in-house and were derived from a line of breeders of the selected line of P-rats (5B substrain) that was originally provided in 1999 by Indiana University (courtesy of Dr. T.K. Li). This stock of inbred P-rats has been bred and maintained on-site at the University of North Carolina at Chapel Hill. Animals were pair-housed in Plexiglas cages and handled daily prior to any training procedures, and subsequently single-housed after surgeries (see below). Between sessions (unless mentioned otherwise), all rats had unlimited homecage access to food and water. The colony room was maintained on a 12 hour light/dark cycle (lights on at 7am). All procedures used were conducted in accordance with the National Institute of Health guidelines, and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Apparatus

Self-administration Chambers. Sound-attenuating cubicles enclosed the operant conditioning chambers (Med Associates, Georgia, VT) used for self-administration testing. Each cubicle was equipped with an exhaust fan for ventilation which also functioned to mask external sounds. Each operant conditioning chamber (measuring 30.5 x 24.1 x 21.0 cm) contained a liquid receptacle adjacent to a response lever on the left and right wall of each chamber. Lever press responses activated a syringe pump (Med Associates) that delivered 0.1 ml of solution into the receptacle over 1.66 seconds. A stimulus light located above each response lever was simultaneously

illuminated during pump activation and signaled reinforcer delivery. Lever responses during reinforcer delivery were recorded, but did not produce programmed consequences. All chambers were interfaced (Med Associates) to a single computer programmed to control sessions and record data.

Locomotor Chambers. Clear Plexiglas chambers (43.2 cm x 43.2 cm; Med Associates) were used to assess locomotor activity. Horizontal distance traveled (cm) was determined from the number of photobeam breaks and collected via computer interface in 2 min time intervals using Activity Monitor software (Med Associates).

Operant Self-administration

Training. Prior to the first training session, rats were fluid-restricted for approximately 24 hrs. Immediately afterwards, rats were placed in the operant conditioning chambers for an initial 16-hr lever-press training session in which presentation of a 0.1 ml solution of concurrently available sucrose (10 %, w/v) and water was contingent on lever responses. Lever responses were initially maintained on a concurrent fixed-ratio 1 (CONC FR1 FR1) schedule of reinforcement and were gradually increased to CONC FR2 FR2 after delivery of 4 reinforcers, and then further increased to CONC FR4 FR4 after delivery of 10 reinforcers. All reinforcer deliveries were paired with a cue light located above each response lever. After completing the initial 16 hr training session, rats were returned to their homecage for a period of 24 hrs in which access to water was returned and remained available *ad libitum* thereafter.

Sucrose Fading and Baseline Sessions. Next, rats began daily (Monday–Friday) 30-min sessions (CONC FR4 FR4) where the sucrose concentration was gradually

decreased and ethanol concentration was increased using a modified sucrose-fading procedure (Samson, 1986) as previously described (Besheer et al., 2010; Cannady et al., 2013; Hodge et al., 1993b). Briefly, ethanol was gradually added to the 10% (w/v) sucrose solution and sucrose was gradually faded out so that ethanol (15%, v/v) alone maintained lever pressing. The specific order of mixed ethanol exposure was as follows: 10% sucrose/2% ethanol (10S/2E), 10S/5E, 10S/10E, 5S/10E, 5S/15E, 2S/15E, 0S/15E. There were 2 sessions at each concentration (i.e., 12 total sucrose fading sessions). Sucrose-trained P-rats did not receive ethanol and were faded to 0.4% (w/v) sucrose. The exact order of sucrose fading was as follows: 10S, 5S, 2S, 1S, 0.8S, 0.6S, 0.4S. The final sucrose concentration was 0.4% (w/v) sucrose because this concentration produced similar lever responding as compared to the 15% ethanol-trained animals. After the sucrose fading procedure, rats had a minimum of 28 baseline operant self-administration sessions with 15% ethanol vs. water (ethanol-trained P-rats) or 0.8% sucrose vs. water (sucrose-trained P-rats).

Experiment 1: Examination of GluA1 phosphorylation (pGluA1) after chronic ethanol self-administration

To examine the effects of chronic ethanol self-administration on expression and post-translational modifications of the GluA1 subunit, two groups of P-rats were trained to self-administer either ethanol (15% v/v) or sucrose (0.4%, control subjects) over 28 baseline sessions. Immediately following the 28th session, P-rats were deeply anesthetized and brain tissue was fixed via transcardial perfusion by infusing sodium phosphate (0.1 M) followed by 4% paraformaldehyde at 4°C. Brains were extracted,

processed and frozen at -20°C. Next, brains were sliced into 40 micrometer sections using a freezing microtome and placed in cryoprotectant for later analysis by immunohistochemistry (IHC).

Sections were immunolabeled by rabbit polyclonal anti-phospho GluA1 (Serine 831) antibody (Phospho Solutions) or rabbit polyclonal anti-GluA1 (Millipore) antibody using DAKO Polymer HRP-Labeled anti-rabbit secondary antibody (DAKO). Phosphorylated and total GluA1 were visualized by diaminobenzidine solution (DAKO). Sections were then counterstained with toluidine blue (Fisher Scientific), mounted on glass slides and cover-slipped for analysis by light microscopy.

As previously described in (Cannady et al., 2012; Spanos et al., 2012), images were captured using a digital camera (Regita model, QImaging, Burnaby, BC) mounted on an Olympus CX41 light microscope (Olympus America, Center Valley, PA) and interfaced to a computer (Dell, Round Rock, TX). Total- and pGluA1 immunoreactivity was quantified with image analysis software (Bioquant Nova; R&M Biometric, Nashville, TN). The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition. GluA1 and pGluA1 pixel count measurements were calculated from a circumscribed field based on the brain region and divided by the area of the region, expressed as pixels/mm^2. The researcher conducting the analysis was blind to the treatment conditions. Data were acquired from subregions of the amygdala (lateral, basolateral, and central nuclei), subregions of the nucleus accumbens (core and shell), the prefrontal cortex, and the dorsal medial striatum.

Experiment 2: Examination of site-specific positive modulation of glutamate activity at AMPA receptors on ethanol self-administration.

Cannulae implantation. Stereotaxic surgery was performed in self-administration-trained rats for site-specific infusion of the AMPA receptor positive modulator, aniracetam, to test whether region-specific potentiation of AMPAR signaling might influence ethanol reinforcement processes. Bilateral guide cannulae (26-gauge; Plastics One, Roanoke, VA) were implanted into the nucleus accumbens (core) and the amygdala (CeA). The coordinates for the nucleus accumbens and amygdala were AP +1.7, ML +1.5 mm, DV −5.5 mm, and AP −1.9, ML +4.2, −6.5 DV (from dura), respectively (Paxinos and Waton, 1998). No attempt was made to functionally distinguish effects in specific sub-nuclei of the accumbens (eg, core vs shell) or amygdala (eg, CeA, BLA, or LaDL) due to the volume of fluid injected and evidence that suggests that the distance of drug diffusion after microinjection could possibly be greater than the distance between each sub-nuclei (Perez de la Mora et al., 2006). Rats were given 1 week for recovery before resuming ethanol self-administration sessions.

Ethanol self-administering P-rats were microinjected with aniracetam into the amygdala (0, 1, 3, µg/ 0.5 µl/side; n = 10) or nucleus accumbens (0, 1, 3, 6 µg/ 0.5 µl/side; n = 11) prior to operant ethanol (15% v/v) self-administration sessions (30-min). Microinjected aniracetam doses within this range have been shown to be effective at altering behavior in previous studies (Masuoka et al., 2008; Rao et al., 2001). Site-specific bilateral microinjections were made with 1.0 µl Hamilton syringes connected to 33-gauge injectors (Plastics One, Roanoke, VA) extending 2 mm below the guide cannulae. An infusion pump (Harvard Apparatus, Natick, MA) delivered a volume of

0.5 µl/side for 1 min and injectors were left in place for an additional 1.5 min to allow for diffusion. Rats began operant self-administration testing immediately following microinjections. Testing was conducted using a within-subjects design in which each aniracetam dose was administered in a randomized order. Blood-ethanol concentration measurements were not conducted after drug test sessions due to potential disruption of ongoing self-administration. There were at least 2 self-administration sessions between microinjections and test sessions occurred no more than two times per week. Additionally, each liquid receptacle in the operant chambers was monitored for residual fluid after every session.

Experiment 3: Test for intra-amygdala aniracetam-induced alterations in locomotor behavior.

Ethanol-trained P-rats (n = 9) were tested to examine if aniracetam-induced potentiated ethanol self-administration was the result of changes in locomotor activity. Animals received intra-amygdala microinjections of aniracetam (0 and 1 µg/0.5µl/side) prior to placement in locomotor chambers where distance traveled was measured and recorded via photo beam breaks. All rats experienced two 30-minute locomotor sessions in which aniracetam dose order (0 and 1 µg/0.5µl/side) was randomized. Locomotor sessions were interspersed with self-administration sessions where appropriate with at least 3 days between tests. Self-administration sessions were withheld on days of the locomotor tests. One rat was not able to be tested for locomotor behavior due to clogged guide cannulae; however, data for self-administration sessions were still included in data analysis.

Experiment 4: Test for reinforcer specificity via sucrose-self-administration.

To test if enhanced AMPAR activity within the amygdala was selective at modulating ethanol reinforcement, sucrose-trained P-rats (n = 9) were microinjected with aniracetam (0, 1, 3, 6 µg/ 0.5µL/ side,) immediately before operant sucrose (0.4%, w/v) self-administration sessions (30-min). Aniracetam testing was conducted as described for the ethanol self-administration tests sessions.

Experiment 5: Examination of CaMKII involvement in aniracetam-induced potentiation of ethanol self-administration

To test if inhibition of CaMKII phosphorylation reduced ethanol self-administration P-rats (n = 7) received intra-amygdala microinjections with Myristolated Autocamtide-2-related inhibitory peptide (mAIP; 0, 5, 10, 20 µg/ 0.5µL/ side,) immediately before operant ethanol (15%, v/v) self-administration sessions (30-min). A second group of ethanol-trained P-rats (n = 9) received intra-amygdala microinjections of vehicle, aniracetam (1 µg), AIP 10µg, and a cocktail solution consisting of aniracetam (1 µg) and AIP 10µg to test if aniracetam-induced alterations in ethanol self-administration were dependent on CaMKII activity. All microinjections were administered in a randomized order using a within subjects design.

Drugs.

Ethanol (95% (w/v); Pharmco-AAPER, Shelbyville, KY) was diluted in distilled water to 15% (v/v). Aniracetam (Tocris, Ellisville, Missouri) and myristolatred AIP (myr-

AIP); Enzo Life Sciences) was dissolved in 50% DMSO in artificial cerebral spinal fluid (ACSF).

Data Analysis

Ethanol- (or sucrose-) responses were analyzed by one-way ANOVA. Cumulative responses, and cumulative distance traveled (cm) were analyzed by two-way repeated measures analysis of variance (RM ANOVA). Tukey post-hoc comparisons were performed to identify differences between treatments/treatment groups. T-tests were performed to identify differences in pGluA1 and total GluA1 immunoreactivity between ethanol- and sucrose self-administering rats within brain regions. Statistical significance was determined at $p \leq 0.05$.

Results

Experiment 1: Examination of GluA1 phosphorylation (pGluA1) after chronic ethanol self-administration.

To examine chronic ethanol–induced neuroadaptations of AMPA receptor subunits, immunohistochemistry (IHC) was performed to assess phosphorylation and expression of the GluA1 subunit in tissue from two groups of rats that experienced 28 ethanol or sucrose self-administration sessions. Mean baseline values for ethanol- and sucrose-reinforced responses over 28 days of self-administration were 110.90 ± 11.31 and 152.20 ± 28.35, respectively (**Fig 6A and 6B**). Although sucrose-trained P-rats showed a trend for increased lever responses, a comparison of 28-day mean baseline values did not reveal statistically significant differences between ethanol- and sucrose-reinforced responses. Mean values for ethanol- and sucrose-reinforced responses on

the final (28th) day of self-administration sessions were 112.67 ± 14.85 and 119.00 ± 29.50, respectively (**Figure 6A and 6B**). A comparison of mean baseline values on the final day of self-administration sessions did not reveal statistically significant differences between ethanol- and sucrose-reinforced responses. Together, these data indicate that reinforced behavior was similar between both ethanol and sucrose self-administering P-rats, allowing for direct comparisons of GluA1 immunoreactivity between the two groups.

In the amygdala, pGluA1 immunoreactivity (IR) was significantly altered in two subregions: the central (CeA) and basolateral (BLA) nuclei. Specifically, pGluA1 IR was significantly increased in the basolateral ($t(16)= 3.671$, $p=0.002$; **Figure 7A**) and central ($t(16)= 2.173$, $p=0.045$, **Figure 7B**) nuclei of the amygdala in rats with a history of ethanol self-administration. In contrast, pGluA1 IR in the lateral nucleus of the amygdala (LA) did not vary between groups (**Figure 7C**). In the ethanol self-administration group, pGluA1 IR was significantly increased in the nucleus accumbens core ($t(15)= 2.554$, $p=0.022$, **Figure 8A**), but not the nucleus accumbens shell relative to the sucrose self-administration group (**Figure 8B**). pGluA1 immunoreactivity in the dorsal medial striatum and prefrontal cortex was also examined, however, no statistical differences were observed between groups (**Table 3**). Additionally, amygdala total GluA1 expression was measured to determine if alterations in phosphorylated GluA1 were the result of changes in subunit expression. No significant differences were observed in total GluA1 expression within the lateral, basal lateral, or central nuclei of the amygdala or the nucleus accumbens core and shell (**Table 4**). These data suggest that ethanol self-administration produces neuroadaptations (i.e. phosphorylation of GluA1 subunits)

within the amygdala and nucleus accumbens that may be associated with facilitated AMPA receptor signaling.

Experiment 2: Examination of site-specific positive modulation of glutamate activity at AMPA receptors on ethanol self-administration.

To assess whether potentiation of AMPA receptor activity within limbic brain regions would alter ethanol self-administration, aniracetam (AMPA receptor positive modulator) was microinjected into the amygdala or nucleus accumbens of P-rats trained to self-administer ethanol (15% v/v). Analysis by one-way RM ANOVA indicated that intra-amygdala infusion of aniracetam significantly increased ethanol-reinforced lever responding [$F(2, 9) = 4.2$, $p < 0.03$] (**Figure 9A**). Tukey post-hoc analysis showed a significant increase in ethanol lever responding with a low dose of aniracetam (1 µg) relative to vehicle treatment ($p=0.03$). Additionally, cumulative ethanol-reinforced responses were examined to determine the pattern of responding over time (30 min) during test sessions (**Figure 9B**). Post-hoc analysis indicated a significant main effect of intra-amygdala aniracetam dose [$F(2,18) = 3.78$, $p = .04$] at 25 minutes and 30 minutes, and a significant main effect of time [$F(5,45) = 26.88$, $p < 0.001$] as ethanol reinforced responding increased over the duration of the test sessions. However, there was no significant interaction of aniracetam dose and time.

In contrast to intra-amygdala testing, intra-accumbens infusion of aniracetam did not significantly alter ethanol-reinforced responding across the tested doses (**Figure 10A**). An analysis of the pattern of responding over time by two-way RM ANOVA showed a significant main effect of time [$F(5,50) = 30.93$, $P <0.001$], a significant main

effect of intra-accumbens aniracetam dose [F(3,30) = 3.85, P =0.02], but no significant interaction was observed (**Figure 10B**).

Experiment 3: Test for intra-amygdala aniracetam-induced alterations in locomotor behavior.

An examination of locomotor activity in an open field was performed to test if the dose of intra-amygdala aniracetam (1 µg) that caused a significant increase in ethanol reinforced-responding (**Figure 9A**) altered general locomotor behavior (**Figure 11**). Analysis of locomotor behavior after intra-amygdala aniracetam treatment by two-way ANOVA showed a significant main effect of time [F(14,112) = 169.49, P < 0.001], but no significant main effect of intra-amygdala aniracetam dose and no significant interaction. These data suggest that the observed increase in ethanol-reinforced responses was not the result of intra-amygdala aniracetam effects on locomotor behavior.

Experiment 4: Test for reinforcer specificity via sucrose-self-administration.

To determine if the observed increase in ethanol self-administration after intra-amygdala aniracetam treatment (**Figure 9**) was specific to ethanol, sucrose (0.4%) self-administering P-rats were microinjected with aniracetam into the amygdala prior to test sessions. Intra-amygdala aniracetam had no significant effect on sucrose-reinforced responses during 30-minute self-administration sessions across all tested doses (**Figure 12A**). An analysis of the cumulative sucrose-reinforced responses over time showed a main effect of time [F(5,40) = 13.53, P < 0.001], but no significant main effect of intra-amygdala aniracetam dose and no significant interaction (**Figure 12B**). These

data suggest that intra-amygdala aniracetam effects to increase ethanol self-administration are specific to ethanol and not non-drug reinforcers.

Experiment 5: Examination of CaMKII involvement in aniracetam-induced potentiation of ethanol self-administration

To determine if aniracetam-induced increased lever responding was dependent on activation of CaMKII, the CaMKII peptide inhibitor, myr-AIP, was infused into the amygdala prior to ethanol self-administration sessions. First, a dose-response curve was generated with myr-AIP alone. Analysis by one-way RM ANOVA indicated that intra-amygdala infusion of AIP significantly decreased ethanol-reinforced lever responding [$F(3, 18) = 3.92$, $p = 0.03$] as post-hoc analysis indicated that ethanol-reinforced lever responding was significantly reduced by a 20 µg dose of AIP ($p= 0.03$; **Figure 13A**). An examination of cumulative ethanol-reinforced responses over the 30-min sessions indicated a significant main effect time [$F(5,30) = 21.73$, $p < 0.001$] and a significant interaction of AIP dose and [$F(15,90) = 2.04$, $p = 0.02$]. Post-hoc analysis indicated that ethanol-reinforced responding was significantly reduced by AIP (20 µg) at 25 and 30 minutes (**Figure 13B**). Next, a cocktail of the aniracetam dose (1 µg) that was effective at potentiating ethanol self-administration (**Figure 9A**) and the highest ineffective AIP dose (10 µg; **Figure 13A**), was tested to determine if aniracetam-induced potentiation of ethanol self-administration was dependent on CaMKII activity. Significant differences in ethanol reinforced lever responding were observed after intra-amygdala administration of the aniracetam-AIP cocktail [$F(3, 24) = 12.52$, $p < 0.001$]. Post-hoc analysis indicated that intra-amygdala infusion of aniracetam (1ug) alone

significantly increased ethanol-reinforced responses ($p = 0.02$; **Figure 14**), replicating the result from the previous experiment (**Figure 9A**), whereas intra-amygdala infusion of the aniracetam-AIP cocktail significantly reduced the aniracetam-induced increase in ethanol-reinforced lever responses ($p < 0.001$; **Figure 14**). Together these data indicate that aniracetam-induced increases in ethanol self-administration are dependent on amygdala CaMKII activity.

Discussion

Previously we have shown that acute systemic enhancement of glutamate activity at AMPA receptors facilitates ethanol reinforcement (Cannady et al., 2013). Data from this study extend those findings by providing evidence that potentiated activity of amygdala AMPA receptors promotes operant ethanol self-administration in a rodent model of high ethanol preference, the alcohol preferring P-rat. First, using immunohistochemistry (IHC), subregions of the amygdala and nucleus accumbens were identified as potential areas of enhanced AMPA receptor signaling as evidence by increased phosphorylation of GluA1 subunits in P-rats trained to self-administer ethanol relative to sucrose control subjects. These findings led us to target AMPA receptors in the amygdala and nucleus accumbens to determine functional involvement of enhanced activity of these receptors in potentiating ethanol reinforcement. Region-specific facilitation of ethanol reinforcement was determined as targeted potentiation of amygdala- but not accumbens-AMPA receptor activity by aniracetam increased ethanol-self administration. Moreover, reinforcer specificity was confirmed as intra-amygdala aniracetam did not alter sucrose self-administration. Finally, enhanced AMPA receptor-

mediated facilitation of ethanol reinforcement was dependent on activation of CaMKII as co-administration of myr-AIP and aniracetam blocked aniracetam-induced increases in ethanol self-administration. Taken together, these data provide novel insights into how enhanced AMPA receptor activity may exacerbate ethanol reinforcement.

AMPA receptor activity is enhanced by several biological processes (Diaz, 2010; Partin et al., 1996; Pei et al., 2009) including phosphorylation at serine 831 of the GluA1 subunit ($pGluA1_{831}$). Here we show that a history of ethanol self-administration is associated with post-translational modifications that enhance AMPAR function within limbic brain regions. Specifically, significant up-regulation of $pGluA1_{831}$ within the amygdala (BLA and CeA) and the nucleus accumbens (core) was observed relative to sucrose-self administering controls. Up-regulation of $pGluA1_{831}$ is significant given that phosphorylation of serine 831 of the GluA1subunit is associated with long-term potentiation (LTP; a molecular correlate of learning and memory) and facilitated trafficking of AMPA receptors to synaptic sites within the plasma membrane (Barria et al., 1997; Esteban et al., 2003), and suggests that chronic ethanol self-administration may be functioning to potentiate synaptic strength within these two well-characterized brain regions that regulate drug-taking and seeking behaviors. Future experimentation directly measuring alterations in synaptic strength in the amygdala and the nucleus accumbens during or after a history of ethanol self-administration are necessary to determine if this is a possibility.

It might have been plausible that the ethanol-induced upregulation of $pGluA1_{831}$ was the result of increased GluA1 expression. However, this is unlikely as no significant changes were observed during an examination of total GluA1 immunoreactivity after a

history of operant ethanol self-administration (Table 2). These data are in contrast to recent studies that show upregulation of total GluA1 in the nucleus accumbens after ethanol exposure (Ary et al., 2012; Neasta et al., 2010). However, differences between previous findings and the current data are likely the result of methodological discrepancies as prior studies have not examined GluA1 expression using immunohistochemistry methods, or in the context of an operant behavior, or using sucrose as a control solution. It is plausible that GluA1 expression could be upregulated in sucrose-trained animals relative to subjects without exposure to a rewarding stimulus (i.e. no history of ethanol or sucrose) as evidenced by alterations in GluA1 subunit expression/trafficking after exposure to sucrose (Peng et al., 2011). An effect of up-regulation of GluA1 in sucrose-trained rats could mask differences in GluA1 between ethanol and sucrose-trained subjects. Regardless, ethanol self-administering P-rats exhibit a greater degree of pGluA1 immunoreactivity providing novel evidence of ethanol-mediated alterations in AMPA receptor signaling in the amygdala and nucleus accumbens.

Given that the amygdala and the nucleus accumbens were identified as possible areas of potentiated AMPA receptor signaling after ethanol exposure, we targeted these regions to examine the functional role of enhanced glutamate activity at AMPA receptors within these regions on the maintenance of ethanol self-administration. Potentiation of glutamate activity at amygdala AMPA receptors by a low aniracetam dose (1 μg) significantly increased ethanol-reinforced lever responses, suggesting that heightened signaling at AMPA receptors functionally modulates the reinforcing effects of ethanol. Furthermore, these data indicate that enhanced activity of amygdala AMPA

receptors contributed, at least in part, to our previous findings demonstrating that positive modulation of AMPA receptor activity facilitates ethanol self-administration (Cannady et al., 2013). Interestingly, only a low dose of intra-amygdala aniracetam potentiated ethanol-reinforced lever responses; consistent with previous findings observed when aniracetam was systemically administered prior to ethanol self-administration (Cannady et al., 2013). Indeed, other studies have demonstrated an inverted U dose-response function by aniracetam (Pizzi et al., 1993).Together these data suggest that there may be a threshold for enhanced amygdala AMPA receptor activity to facilitate ethanol reinforcement . Further experimentation is warranted to test this hypothesis.

AMPA receptors have previously been shown to modulate ethanol reinforcement and seeking behavior (Backstrom and Hyytia, 2004; Sanchis-Segura et al., 2006; Stephens and Brown, 1999). The current data support growing evidence demonstrating that the amygdala mediates operant ethanol self-administration (Economidou et al., 2008; Funk et al., 2006; Roberts et al., 1996; Schroeder et al., 2003). To our knowledge, this study is the first to potentiate ethanol reinforcement via site-specific pharmacological manipulation of the amygdala; a brain region that has been historically associated with responses to aversive stimuli and ethanol-induced negative affect (Brown et al., 2013; Ming et al., 2013). Recent evidence has shown that potentiating activity of a specific BLA-accumbens circuit via optogenetic stimulation mediates motivated behavior as evidenced by increases in nose-poke-contingent self-stimulation (Stuber et al., 2011). Given this evidence, it might have been possible that potentiation of activity at amygdala AMPA receptors could alter reinforcement of a non-drug

reinforcer. However, sucrose self-administration was not altered by intra-amygdala aniracetam; which makes such an account less plausible. Previous evidence demonstrates that enhanced glutamate activity at AMPA receptors does not alter non-drug reinforcement (Cannady et al., 2013) and is consistent with the current results as intra-amygdala aniracetam doses as high a 6 μg did not alter sucrose self-administration. Future experimentation examining amygdala afferents and efferents could provide additional insights as to which neuronal pathways are critical for ethanol and non-drug reinforcement.

In contrast to the amygdala, positive modulation of glutamate activity at nucleus accumbens AMPA receptors by aniracetam did not significantly alter ethanol-reinforced lever responding; which suggests that potentiated activity of AMPA receptors to mediate ethanol reinforcement is specific to receptors in the amygdala. A slight trend for an increase in ethanol-reinforced lever responses was observed with a 3 μg dose of intra-accumbens aniracetam, yet doses as high as 6 μg did not alter ethanol self-administration. This lack of effect in the nucleus accumbens is surprising given the well-established role for glutamate activity in this region to modulate ethanol reinforcement (Besheer et al., 2010; Rassnick et al., 1992). Recently, localized antagonism of AMPA receptors in the dorsal striatum was shown to reduce ethanol self-administration in a reinforcer specific manner (Wang et al., 2012). It would be interesting for future experimentation to examine the role of enhanced AMPA receptor signaling in the dorsal striatum, and other brain regions to map the neuroanatomical substrates that mediated increased ethanol self-administration.

Studies show that a history of ethanol exposure upregulates AMPA subunit proteins (Chandler et al., 1999; Neasta et al., 2010) in addition to increasing the expression of signaling molecules like calcium/calmodulin dependent protein kinase II (CaMKII) (Goulding et al., 2011; Lee and Messing, 2008; McBride et al., 2009). CaMKII which has been well-characterized for its role in enhancing AMPAR signaling by phosphorylating the AMPA receptor GluA1 subunit (Hayashi et al., 2000; Lee et al., 2000; Malinow et al., 1989; Poncer et al., 2002; Tan et al., 1994). Given that we observed increases in phosphorylated GluA1 after a history of ethanol self-administration and facilitated ethanol reinforcement by enhancing amygdala AMPA receptor activity, we tested whether the intra-amygdala aniracetam-induced increase in ethanol self-administration was dependent on CaMKII activity in the amygdala. Intra-amygdala co-administration of myr-AIP and aniracetam reversed aniracetam induced increases in ethanol self-administration suggesting that AMPA receptor-mediated facilitation of ethanol reinforcement functions through a CaMKII-dependent mechanism.

Along with previous findings, these data further demonstrate that enhanced activity of AMPA receptors might be involved in promoting excessive ethanol consumption; a factor linked to the risk of developing alcohol use disorder (Saha et al., 2006). These data suggest that AMPA receptors could be potential therapeutic targets to treat ethanol use disorders. Currently there are FDA-approved medications with actions to reduce activity at AMPA receptors, namely Perampanel (trade name Fycompa), an anti-epileptic compound (Hanada et al., 2011; Hsu et al., 2013). It would be interesting for future preclinical studies to examine the effects of this compound on ethanol drinking behaviors, including ethanol self-administration. Furthermore, given the

data from the present study, targeted reductions in CaMKII activity may also be of therapeutic value for treatment of ethanol use disorders. To date, there is no FDA-approved medication with primary actions at CaMKII, but further preclinical testing with CaMKII compounds is warranted and may prove to be beneficial in treating drinking disorders.

Table 3

Table 3. pGluA1 immunoreactivity (pixels/mm^2) after ethanol and sucrose self-administration.

Brain region	Ethanol Self-administration	Sucrose Self-administration
Dorsal Striatum pGluA1 IR	578061.14 ± 38087.65	512875.82 ± 41398.75
Prefrontal Cortex pGluA1 IR	90755.45 ± 11676.13	95383.20 ± 10782.16

Table 4

Table 4. Total GluA1 immunoreactivity (pixels/mm^2) after ethanol and sucrose self-administration.

Brain region	Ethanol Self-administration	Sucrose Self-administration
BLA Total GluA1 IR	278586.92 ± 15475.06	226075.41 ± 26978.66
CeA Total GluA1 IR	279894.26 ± 19990.97	231786.97 ± 22227.42
LA Total GluA1 IR	101791.44 ± 10195.63	78131.86 ± 16179.81
NAcb Core Total GluA1 IR	537871.01 ± 34170.53	496885.22 ± 31824.75
NAcb Shell Total GluA1 IR	556845.11 ± 42516.84	555220.79 ± 35620.06

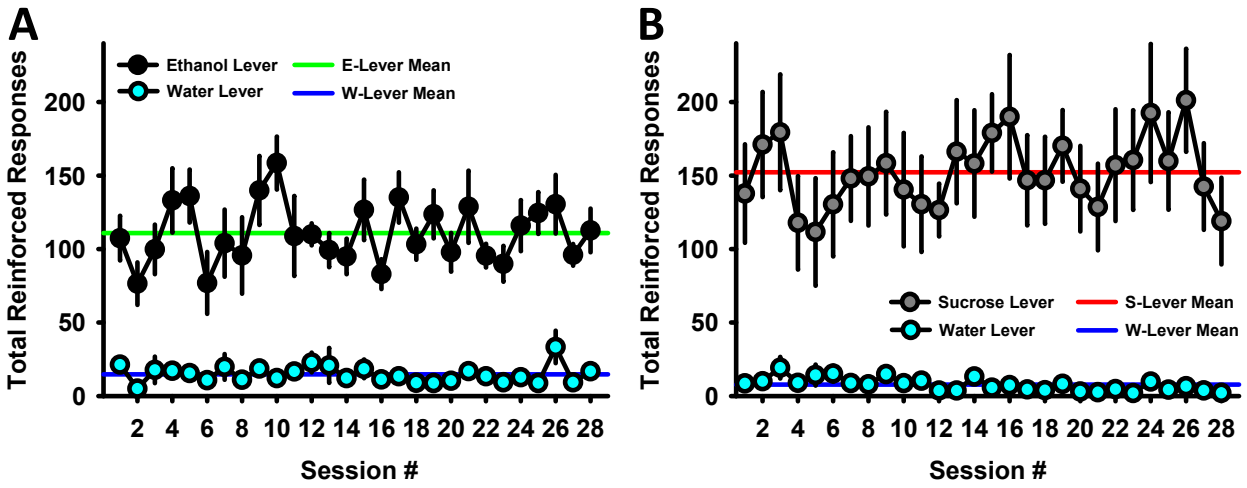


Figure 6 Chronic ethanol and sucrose self-administration prior to immunohistochemistry. Total reinforced lever responses were measured across 28 baseline (A) ethanol- or (B) sucrose self-administration sessions prior to tissue collection for immunohistochemistry (IHC).Total reinforced responses are expressed as group means ± SEM.

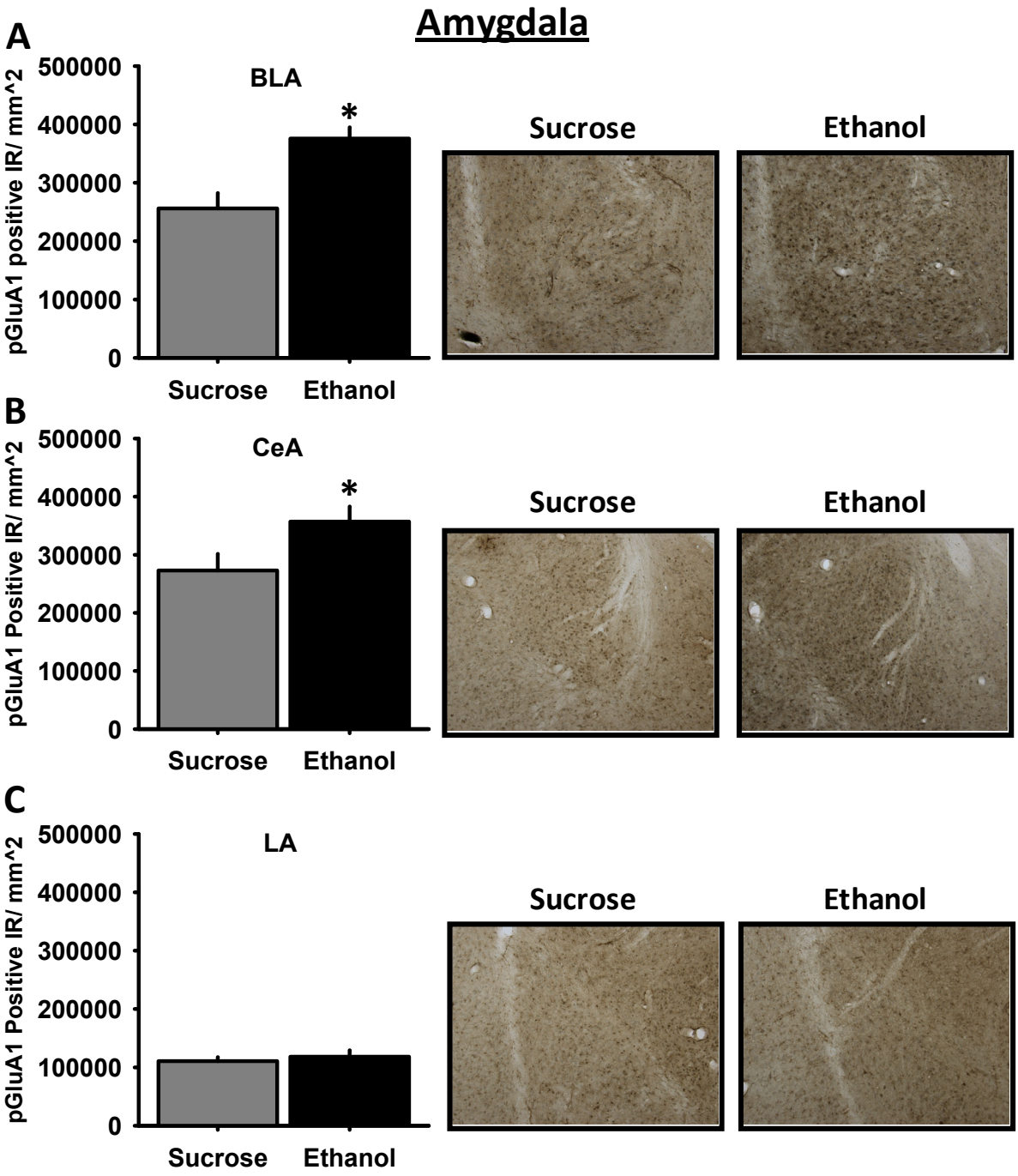


Figure 7 Ethanol self-administration upregulates phosphorylation of the GluA1 AMPA receptor subunit in subregions of the amygdala. Chronic ethanol self-administration increased phosphorylation of the GluA1 subunit (at serine 831) relative to sucrose-trained P-rats as measured by increases in positive pGluA1 immunoreactivity (IR) in the (A) basolateral (BLA) and (B) central (CeA) nuclei of the amygdala. No differences were observed in the (C) lateral nucleus of the amygdala (LA). Representative photomicrographs (10X) are of pGluA1-IR after 28 days of ethanol or sucrose self-administration. Graphed values are expressed as means ± SEM; *p<0.05 (t-test).

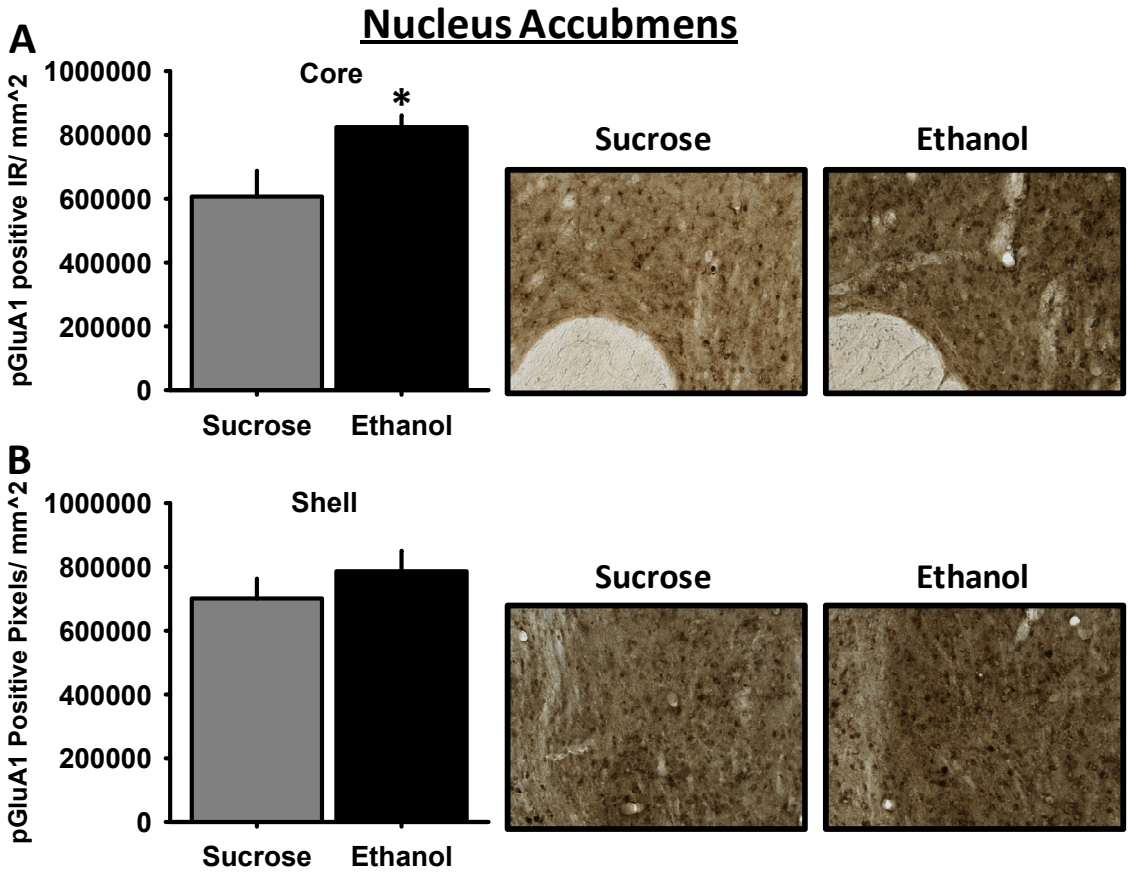


Figure 8 Ethanol self-administration upregulates phosphorylation of the GluA1 AMPA receptor subunit in subregions of the nucleus accumbens. Chronic ethanol self-administration increased phosphorylation of the GluA1 subunit (at serine 831) relative to sucrose-trained P-rats as measured by increases in positive pGluA1 immunoreactivity (IR) in the (A) core, but not the (B) shell of the nucleus accumbens. Representative photomicrographs (20X) are of pGluA1-IR after 28 days of ethanol or sucrose self-administration. Graphed values are expressed as means ± SEM; *$p<0.05$ relative to vehicle treatment.

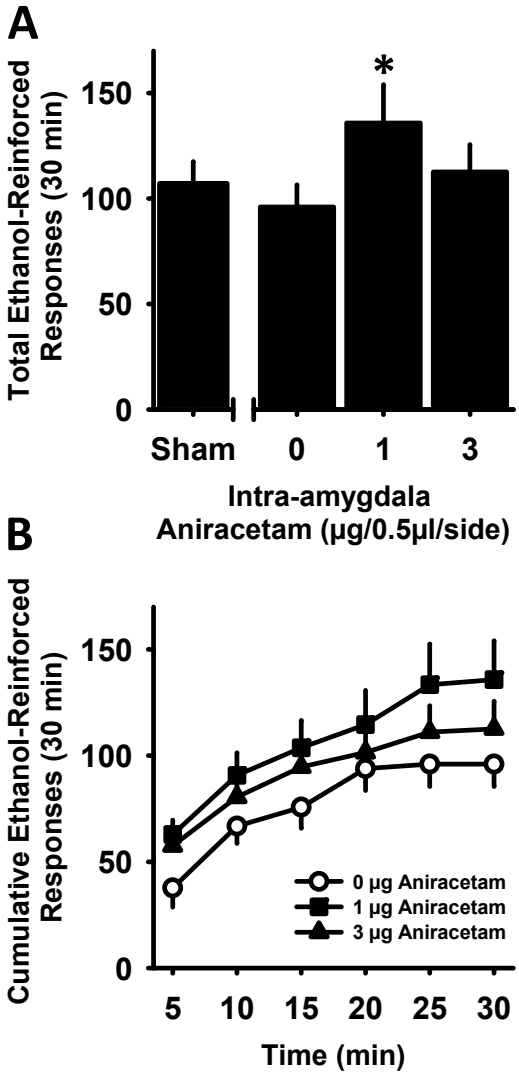


Figure 9 Positive modulation of amygdala AMPA receptors potentiates ethanol self-administration. (A) Intra-amygdala aniracetam pretreatment (1 µg) significantly increased total ethanol-reinforced responses during operant self-administration sessions in P-rats (n = 10). (B) A main effect of aniracetam dose was observed at 25 and 30 mminute time points in an anlaysis of cumulative ethanol-reinforced responses with no significant interaction of time. Graphed values are expressed as means ± SEM; *p<0.05 relative to vehicle treatment**.**

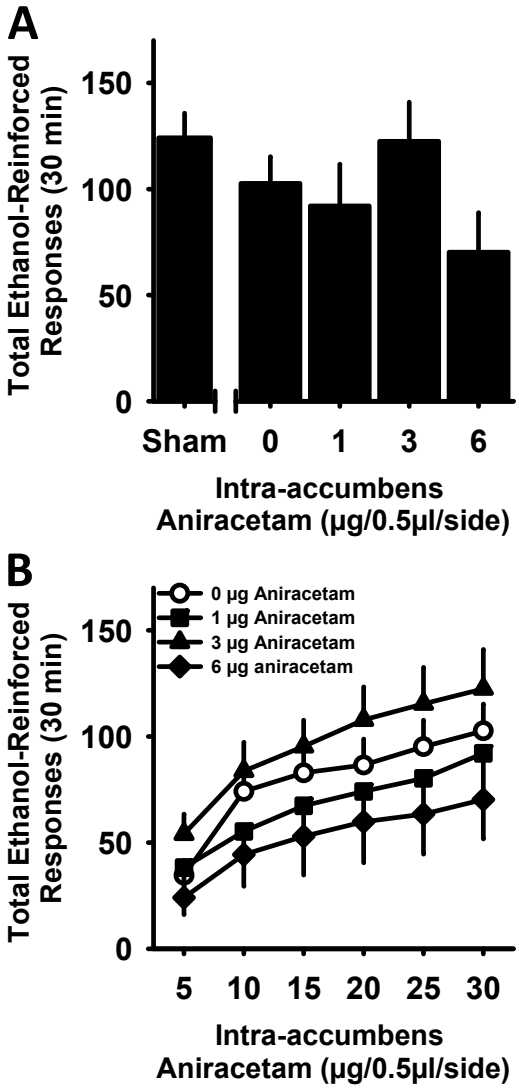


Figure 10 Positive modulation of nucleus accumbens AMPA receptors does not alter ethanol self-administration. (A) Intra-accumbens aniracetam pretreatment (0 - 6 µg) did not significantly alter total ethanol-reinforced responses during operant ethanol self-administration sessions in P-rats (n = 11). (B) Cumulative ethanol-reinforced responses were not significantly altered by intra-accumbens aniracetam pretreatment. Graphed values are expressed as means ± SEM.

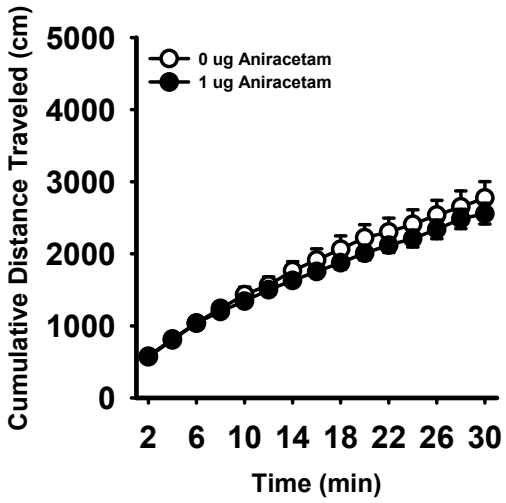


Figure 11 Positive modulation of amygdala AMPA receptors does not alter locomotor activity. Spontaneous locomotor activity in an open field was not altered in ethanol self-administration-trained P-rats (n = 9) after intra-amygdala aniracetam pretreatment (1 µg). Graphed values are expressed as means ± SEM.

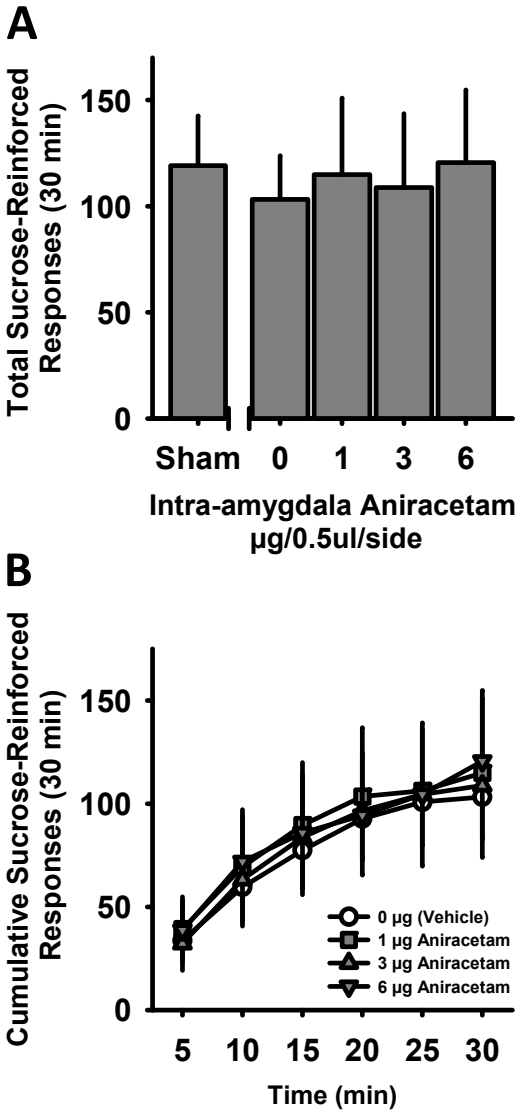


Figure 12 Figure 7. Positive modulation of amygdala AMPA receptors does not alter sucrose self-administration. (A) Intra-amygdala aniracetam pretreatment (0 - 6 µg) did not significantly alter total sucrose-reinforced responses during operant ethanol self-administration sessions in P-rats (n = 11). (B) Cumulative sucrose-reinforced responses were not significantly altered by intra-accumbens aniracetam pretreatment. Graphed values are expressed as means ± SEM.

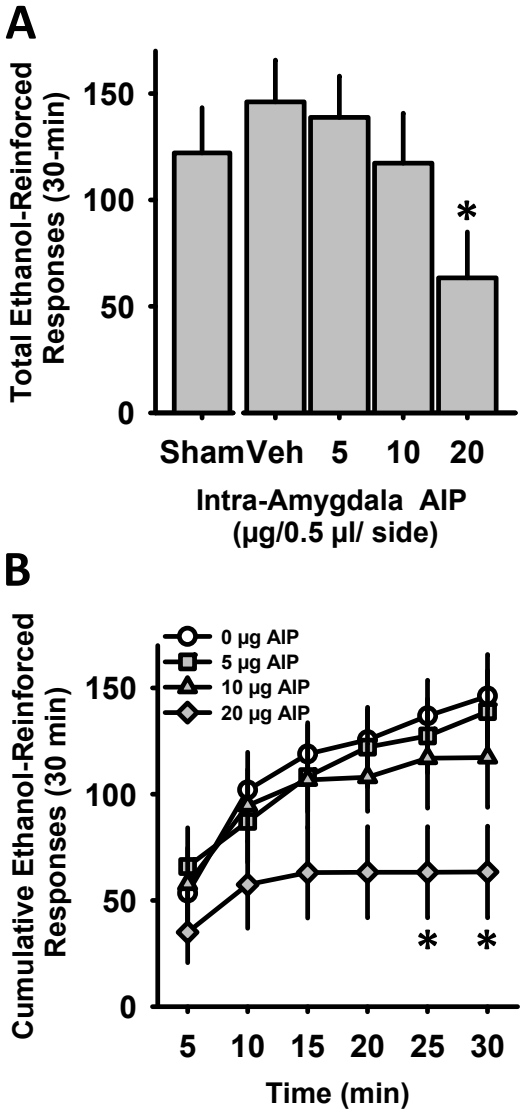


Figure 13 Inhibition of amygdala CaMKII activity attenuates ethanol self-administration. (A) Intra-amygdala pretreatment with the CaMKII peptide inhibitor, myr-AIP (20 µg), significantly decreased total ethanol-reinforced responses during operant self-administration sessions in P-rats (n = 7). (B) Cumulative ethanol-reinforced responses were significantly decreased by intra-amygdala myr-AIP pretreatment (20 µg). Graphed values are expressed as means ± SEM; *p<0.05 relative to vehicle treatment.

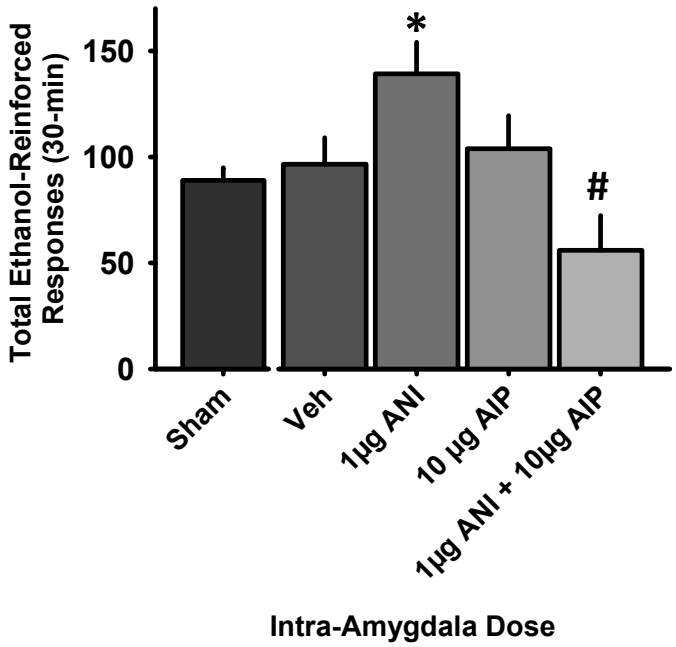


Figure 14 Intra-amygdala inhibition of CaMKII activity blocks aniracetam-induced increased ethanol self-administration. Intra-amygdala co-administration of aniracetam (1 µg) and the CaMKII peptide inhibitor, myristolated AIP (10 µg), significantly reversed aniracetam-induced increased ethanol-lever responses in P-rats (n = 9). Graphed values are expressed as means ± SEM; *p<0.05 relative to vehicle treatment. #p<0.05 relative to 1µg aniracetam (ANI).

CHAPTER 4: ROLE OF ENHANCED ACTIVITY AT AMYGDALA AMPA RECEPTORS IN MODUALTING RELAPSE-LIKE BEHAVIOR

Introduction

Relapse can be defined as “resumed binge drinking after a prolonged period of abstinence” (Becker). An enduring challenge for clinicians is identifying effective strategies and therapies that reduce the risk of relapse in patients with alcohol use disorder (Saitz et al., 2013). Indeed, risk for relapse after a period of abstinence has been estimated to be as high as 75% for individuals actively seeking treatment (Boothby and Doering, 2005). Thus, researchers are focusing intensive efforts towards identifying the underlying mechanisms that govern relapse as this may lead to better treatment outcomes for individuals with alcohol use disorders.

AMPA receptors are ionotropic glutamate receptors that allow for rapid excitatory signaling, which facilitates the firing of action potentials through cellular depolarization (LoTurco et al., 1995). These receptors are of interest as they can mediate long-term changes in the strength of neuronal signaling (Nicoll and Malenka, 1995; Nicoll and Roche, 2013); an important concept as lasting neuroadaptive changes have been proposed to underlie susceptibility to relapse (Luscher and Malenka, 2011). AMPA receptors are heterotetramers, comprised of four subunits (GluA1-4) and post-translational modifications of these subunits allows for diverse channel properties and modulation by intracellular signaling molecules (Lu et al., 2010; Lu and Roche, 2012).

Interestingly, expression of the GluA1 subunit of the AMPA receptor has been shown to be increased in limbic brain regions after extended periods of ethanol consumption (Ary et al., 2012; Neasta et al., 2010). Moreover, AMPA receptors have been shown to mediate ethanol reinforcement and relapse-like behavior (Sanchis-Segura et al., 2006; Wang et al., 2012). Indeed, we have shown that potentiation of AMPA receptor activity facilitates ethanol self-administration and relapse-like behavior; which suggest that enhanced AMPA receptor signaling may function as a maladaptive process in alcohol-drinking behavior (Cannady et al., 2013).

The amygdala is a key region of the brain known for its role in regulating associative learning processes (Cole et al., 2013; Gallagher and Holland, 1994; LeDoux, 2003). This region has been implicated in mediating drug-reward learning, as well as drug-cue associations (Cai et al., 2013; Hyman et al., 2006). Exposure to ethanol-associated cues has been shown to promote relapse in humans (Drummond, 2000; Papachristou et al., 2012) and can be modeled by increased ethanol-seeking behavior in a cue-induced reinstatement procedure (Cannady et al., 2013; Ciccocioppo et al., 2003; Schroeder et al., 2008). Our previous study indicated that AMPA receptors in the amygdala play an important role in modulating ethanol reinforcement as evidenced by increased operant self-administration after localized positive modulation of amygdala AMPA receptors (Chapter 3). However, it is not known if relapse-like behavior is modulated in the same region-specific manner. Further, it is not clear if exposure to ethanol-associated cues produce adaptations in AMPA subunit expression that may indicate modified AMPA receptor activity. Given that we have established a role for

AMPA receptors in the amygdala for modulating alcohol drinking, we hypothesized that altering glutamate activity of amygdala AMPA receptors augments ethanol-seeking.

Accordingly, we first examined alterations of AMPA receptor GluA1 subunit in the amygdala and nucleus accumbens after exposure to ethanol-associated cues following a response contingent cue-induced reinstatement procedure. Next, the effects of acute pharmacological potentiation and inhibition of glutamate activity at amygdala AMPA receptors in modulating relapse-like behavior was examined. That is, in alcohol-preferring p-rats the effects of intra-amygdala aniracetam (AMPA receptor positive modulator) or NBQX (AMPA receptor antagonist) were tested on cue-induced reinstatement after amygdala cannulae implantation.

Materials and Methods

Animals

Male alcohol preferring P-rats (380 - 460 g prior to training) were obtained from Indiana University Medical Center. Rats were quarantined for approximately 6 weeks prior to testing. Animals were pair-housed in Plexiglas cages and handled daily prior to any training procedures, and subsequently single-housed after surgeries (see below). Between sessions (unless mentioned otherwise), all rats had unlimited homecage access to food and water. The colony room was maintained on a 12 hour light/dark cycle (lights on at 7am). All procedures used were conducted in accordance with the National Institute of Health guidelines, and approved by the University of North Carolina Institutional Animal Care and Use Committee.

Apparatus

Self-administration Chambers. Operant conditioning chambers measuring 30.5 x 24.1 x 21.0 cm (Med Associates, Georgia, VT) were located within sound-attenuating cubicles. Each cubicle was equipped with an exhaust fan for ventilation which also functioned to mask external sounds. The left and right wall of each chamber contained a liquid receptacle in addition to a response lever (i.e. two levers per chamber). Lever press responses activated a syringe pump (Med Associates) that delivered 0.1 ml of solution into the receptacle over 1.66 seconds. A stimulus light located above each response lever was simultaneously illuminated during pump activation. Lever responses during reinforcer delivery were recorded, but did not produce programmed consequences. The chambers were interfaced (Med Associates) to a computer programmed to control sessions and record data.

Operant Self-administration

Training. One day prior to training, rats were fluid-restricted for approximately 24 hrs. Immediately afterwards, rats were placed in the operant conditioning chambers for an initial 16-hr lever-press training session in which presentation of a 0.1 ml solution of concurrently available sucrose (10 %, w/v) and water was contingent on lever responses. Lever responses were initially maintained on a concurrent fixed-ratio 1 (CONC FR1 FR1) schedule of reinforcement and were gradually increased to CONC FR2 FR2 after delivery of 4 reinforcers, and then further increased to CONC FR4 FR4 after delivery of 10 reinforcers. All reinforcer deliveries were paired with an illumination of a light cue located above each response lever. After completing the initial 16 hr

training session, rats were returned to their homecage for a period of 24 hrs in which access to water was returned and remained available ad libitum thereafter.

Next, rats commenced daily (Monday–Friday) 30-min sessions (CONC FR4 FR4) where the sucrose concentration was gradually decreased and the ethanol concentration was increased using a modified sucrose-fading procedure (Samson, 1986) as previously described (Besheer et al., 2010; Cannady et al., 2013; Hodge et al., 1993b). Briefly, ethanol was gradually added to the 10% (w/v) sucrose solution and sucrose was gradually faded out so that ethanol (15%, v/v) alone maintained lever pressing. The exact order of mixed ethanol exposure was as follows: 10% sucrose/2% ethanol (10S/2E), 10S/5E, 10S/10E, 5S/10E, 5S/15E, 2S/15E, 0S/15E. There were 2 sessions at each concentration (i.e., 12 total sucrose fading sessions). After the sucrose fading procedure, rats had a minimum of 28 baseline operant self-administration sessions with 15% ethanol vs. water.

Examination of ethanol-associated cue exposure on expression of pGluA1

After stable baseline responding, rats underwent extinction sessions (30 min) in which previously reinforced responses were extinguished over 12 consecutive days by removing response contingencies (i.e. no light cue or pump sound; no reinforcers delivered). On day 13, ethanol-trained P-rats were either given an additional extinction session (extinction group; n =15) or a reinstatement session (reinstatement group n = 16). For the reinstatement group, lever responses (FR4) resulted in the presentation of the light cue and pump activation; however, no reinforcers (ethanol or water) were delivered. Note: 0.1 ml of the 15% ethanol (v/v) solution was added to the ethanol well

prior to the reinstatement session. This amount of ethanol (0.1ml) is not pharmacologically relevant and functioned to provide additional olfactory/taste cues to maximize lever responses during reinstatement (Backstrom et al., 2004; Cannady et al., 2013).

Western Blots

Immediately following the reinstatement session (or extinction session for the extinction group) rats were anesthetized with isoflurane gas and brains were quickly removed and flash-frozen in an isopentane bath. Brains were stored at -80ºC for later processing by western blot analysis. As previously described (Besheer et al., 2010), brain tissue punches (1.2mm; UniCore, Sigma) of the amygdala and nucleus accumbens were taken from coronal sliced sections (thickness 1mm). Tissue was submerged in buffer (10mM Tris, 1% SDS, 1:100 Protease and Phosphatase Inhibitors) and homogenized (Branson Sonifier, Danbury, CT). After colorimetric detection and quantification (Pierce Biotechnology, Rockford, IL) of protein concentration, protein (10µg) was diluted with Sample Reducing Agent and LDS Sample Buffer (Invitrogen, Carlsbad, CA), boiled, and loaded onto a polyacrylamide gel (Biorad, Hercules CA) for separation by electrophoresis. Transfer of protein to a PVDF membrane (Invitrogen) occurred via dry blotting using iBlot (Invitrogen, Carlsbad, CA). Membranes were blocked in 3% normal goat serum (Vector Laboratories, Burlingame, CA) before overnight incubation at 4ºC in monoclonal rabbit anti-pGluA1$_{831}$ (Millipore; 1:1000) and monoclonal mouse actin (Millipore; 1:5000) in blocking solution. Following incubation, membranes were washed and incubated 1h at room temperature in hrp-conjugated goat

anti-rabbit and anti-mouse secondary antibodies, respectively (Jackson Immunoresearch Labs, Inc.; 1:10,000). Protein expression was visualized using a chemiluminescent substrate (ECL; Pierce Biotechnology, Rockford, IL) before quantification using optical density measurements (GE software). Data were converted to percent actin for each blot and expressed as percent control (Extinction group).

Examination of altered modulation of glutamate activity at amygdala AMPA receptors on regulating response-contingent cue-induced reinstatement.

*Cannulae implantation***.** As previously described (Besheer et al., 2010; Cannady et al., 2011), stereotaxic surgery was performed in self-administration-trained rats (15% ethanol vs. water; CONC FR4 FR4) for site-specific infusion of compounds to test whether pharmacological potentiation or inhibition of amygdala AMPAR signaling might alter ethanol-seeking behavior. Bilateral guide cannulae (26-gauge; Plastics One, Roanoke, VA) were aimed the the amygdala (CeA). The coordinates for amygdala were AP −1.9, ML +4.2, −6.5 DV (from dura) (Paxinos and Waton, 1998). Guides were implanted such that injectors penetrated tissue 2mm below the guide. We purposely made no attempt to functionally distinguish effects in specific sub-nuclei of the amygdala (eg, CeA, BLA, or LaDL) due to the volume of fluid injected and evidence that suggests that the distance of drug diffusion after microinjection could possibly be greater than the distance between each sub-nuclei (Perez de la Mora et al., 2006). Rats were given 1 week for recovery before resuming ethanol self-administration sessions.

Reinstatement. After stable baseline responding, rats underwent extinction sessions (30 min) in which previously reinforced responses were extinguished over 13

consecutive days (as previously described (Cannady et al., 2013). Sham microinjections were conducted after extinction sessions to habituate rats to the microinjection procedure. During Sham injections, injectors did not penetrate brain tissue and no drug was infused. On the 13th day of extinction, all rats were microinjected with vehicle after their self-administration session to habituate the animals to receiving an infusion. Treatment groups were matched based on baseline response totals and extinction performance. On day 14, ethanol-trained P-rats were microinjected with vehicle (n = 9), aniracetam (1 µg; n = 10), or NBQX (1 µg; n = 11) immediately prior to a reinstatement session. As previously mentioned, lever responses (FR4) resulted in the presentation of the light cue and pump activation; however, no reinforcers (ethanol or water) were delivered.

Drugs

Ethanol (95% (w/v); Pharmco-AAPER, Shelbyville, KY) was diluted in distilled water to 15% (v/v). Aniracetam (ABCAM Biochemicals, Cambridge, MA) and NBQX (Tocris, Ellisville, Missouri) were dissolved in 25% DMSO in aCSF. These doses were selected based on previous findings demonstrating alterations in ethanol self-administration (Chapter 3; (Wang et al., 2012).

Data analysis

Lever responses in the reinstatement sessions were analyzed by two-way RM ANOVA. T-tests were performed to identify differences in pGluA1 optical density between the reinstatement group and extinction group within brain regions. Differences

in pGluA1 optical density between the HR and LR (reinstatement group) and extinction group were analyzed by one-way ANOVA. Ethanol lever responses for reinstatement tests examining drug effects by behavioral differences were analyzed by three-way ANOVA. Tukey post-hoc comparisons were performed to identify differences between treatments/treatment groups. Statistical significance was determined at $p \leq 0.05$.

Results

Examination of ethanol-associated cue exposure on expression of pGluA1.

P-rats were exposed to cues previously paired with ethanol in a reinstatement procedure to determine if exposure to these cues could promote phosphorylation of GluA1 and CaMKII. Only the reinstatement group displayed response contingent cue-induced reinstatement of ethanol-seeking behavior, as evidenced by increased responding on the ethanol lever (**Figure 15**). Two-way RM ANOVA indicated significant main effects of cue exposure [$F(1,56) = 11.77$, $P = 0.001$] and test condition [$F(1,56) = 5.83$, $P < 0.02$] and a significant interaction of cue exposure x test condition [$F(1, 56) = 19.16$, $p < 0.001$]. Post-hoc analysis showed that the reinstatement group had significantly more lever responses on the ethanol-associated lever relative to their last extinction session ($p < 0.001$) and in comparison to the extinction group ($p < 0.001$).

Brains were extracted immediately following the cue-induced reinstatement session to examine changes in pGluA1 expression. An analysis of amygdala and nucleus accumbens pGluA1 expression did not reveal any significant differences between the reinstatement and extinction groups (**Figure 16A and 16B**). To assess whether behavioral differences during the reinstatement session could account for

alterations in phosphorylation of GluA1, the reinstatement group was divided into high responders (HR) and low responders (LR) based on their ethanol lever responses during reinstatement using a median split (30 responses). No differences were observed in GluA1 phosphorylation between HR/LR reinstatement responders relative to the extinction group (**Figure 17A and 17B**). These data suggest that cue-induced ethanol-seeking is likely not associated with rapid GluA1 phosphorylation events in the amygdala or the nucleus accumbens.

Examination of altered modulation of glutamate activity at amygdala AMPA receptors on regulating response-contingent cue-induced reinstatement.

P-rats were microinjected with vehicle, aniracetam (1 μg), or NBQX (1 μg) in the amygdala prior to a cue-induced reinstatement procedure to determine if potentiation or inhibition of AMAP receptor activity altered cue-induced ethanol-seeking behavior. All treatment groups reinstated ethanol-seeking behavior as evidenced by increases in ethanol lever responding relative to the last day of extinction (**Figure 18**). Two-way RM ANOVA indicated a significant main effects of test condition [$F(1,54) = 67.63$, $P < 0.001$] but no main effect of dose [$F(2, 54) = 1.14$, $p = 0.33$] or significant interaction of dose x test condition [$F(2, 54) = 1.65$, $p = 0.20$]. To assess whether behavioral differences during the reinstatement session could account for alterations in response to drug treatment, treatment groups were divided into high responders (HR) and low responders(HR) using a median split. Three-way ANOVA showed a main effect of test condition [$F(1,48) = 159.29$, $P < 0.001$], an interaction of dose x behavior [$(2,48) = 24.26$, $P < 0.001$], and an interaction of test condition x dose x behavior [$(2,48) = 12.64$,

P < 0.001]. Post-hoc analysis did not reveal a test condition x dose interaction at high (p = 0.13) or low level of behavior (p = 0.96). Together these data indicate that there was no significant effect of intra-amygdala aniracetam or NBQX on cue-induced reinstatement (**Figure 19**).

Discussion

Previous work showed that pharmacological enhancement of AMPA receptor activity potentiated ethanol reinforcement via actions within the amygdala (Chapter 3). This study sought to extend those findings by examining the role of ethanol-associated cues to alter expression of pGluA1; a plasticity-associated marker. Comparisons were made between rats that received a cue-induced reinstatement test relative to an extinction group. The reinstatement group exhibited robust ethanol-seeking behavior, but interestingly, no significant differences were observed in pGluA1 optical density measurements in the amygdala or nucleus accumbens of cue-exposed rats and extinction rats. Given that ethanol self-administration was increased after intra-amygdala pretreatment with aniracetam, we examined the contribution of amygdala AMPA receptors to potentiate (aniracetam) or inhibit (NBQX) the expression of cue-induced reinstatement. Although all treatment groups exhibited cue-induced reinstatement, there was no effect of intra-amygdala AMPA receptor potentiation or inhibition on cue-induced reinstatement. Together, these data do not suggest a prominent role for amygdala AMPA receptors in mediating relapse to ethanol-seeking.

Based on our previous observation of increased pGluA1 expression after chronic ethanol self-administration (Chapter 3), we predicted that GluA1 phosphorylation would

be altered after exposure to ethanol-associated cues in a reinstatement procedure. To our surprise, we observed no significant differences in pGluA1 expression after exposure to ethanol-associated cues. Given that these rats had a long history of cue-ethanol pairings during training and self-administration sessions, it seems likely that they would have developed a strong learned association with the cues; detectable by changes in plasticity markers. However, alterations in pGluA1 were not evident. One possible explanation is that the time point that brains were extracted (immediately following the reinstatement session) was too short to detect changes in phosphorylation between groups. Others have shown alterations in expression of other markers such as c-fos after a reinstatement session; however, changes in c-fos expression occurred long after the session (90 min) (Dayas et al., 2007). An alternative to examining protein expression is to examine mRNA levels as transcription is a much more rapid process than translation and protein synthesis. Future studies will incorporate longer time points or an evaluation of transcripts after reinstatement. Additionally, other brain regions have to be examined to fully determine the extent of reinstatement-induced changes in pGluA1 expression.

Our previous findings (Cannady et al., 2013) along with others (Backstrom and Hyytia, 2004; Sanchis-Segura et al., 2006) demonstrates AMPA receptor-mediated bi-directional modulation of relapse-like behavior. That is, positive modulation of AMPA receptors promotes relapse-like behavior; while antagonism has opposite effects. Therefore, we examined both potentiation and inhibition of amygdala AMPA receptors in modulating cue-induced reinstatement. This approach allowed for maximal experimental outcome during a single test trial reinstatement session as opposed to administration of

two aniracetam or two NBQX doses (high and low) which may or may not have been effective with a limited testing window of one single microinjection per subject. Surprisingly, neither intra-amygdala aniracetam nor NBQX modulated ethanol-seeking. One factor that may have contributed to the lack of effect of aniracetam and NBQX is that doses may have been insufficient to alter reinstatement. Doses used within the current study were based on previous findings that 1 µg/ 0.5 µl is sufficient to alter ethanol self-administration (Chapter 3 and (Wang et al., 2012). Testing higher doses may have proven to be effective at altering reinstatement.

Another factor that may have influenced the lack of effect of aniracetam or NBQX on reinstatement is the placement of cannulae. Indeed, cannulae were aimed at the central nucleus of the amygdala as this is the region is the site of aniracetam-induced ethanol self-administration (Chapter 3). Cannulae implantation in the BLA may have produced different results as this region has consistently been implicated modulating reinstatement of ethanol-seeking (Chaudhri et al., 2013; See et al., 2003). Indeed, recent studies have measured increased glutamate levels in this region in response to ethanol-associated cues (Gass et al., 2011). It may have been more appropriate to examine the role of the CeA in relapse to ethanol-seeking in response to stress as stress pathways are mediated by the CeA (Knapp et al., 2011; Koob, 2009). Furthermore, recent evidence demonstrates a selective role for the CeA, but not the BLA, in modulating stress-induced reinstatement as measured by a reduction in ethanol-seeking after administration of a glucocorticoid receptor antagonist (Simms et al., 2012).

The differences in IHC data (increased pGluA1) after ethanol self administration versus western blot analysis (no change in pGluA1) after reinstatement are intriguing. The divergence of pGluA1 response to ethanol or cues may suggest that pGluA1 is sensitive to ethanol experience as opposed to ethanol-associated stimuli. Replicating the changes (or lack thereof) in pGluA1 using both methodologies (IHC and western blot) will be key in determining if this is a possibility. As previously mentioned, it will be critical to test other brain regions to see if there is differential response to cues or ethanol experience.

Other brain regions are implicated in modulating relapse in response to alcohol stimuli. Evidence shows that glutamate activity in the nucleus accumbens modulates cue-induced ethanol-seeking (Sinclair et al., 2012). Further, others have shown increased c-fos expression in the nucleus accumbens after exposure to cues previously paired with ethanol (Jupp et al., 2011), which indicates that accumbens neurons are activated in response to ethanol-related cues. Although our previous findings do not indicate that enhanced glutamate activity in the accumbens modulates ethanol-self-administration (Chapter 3), there still may be a role for this region in modulating cue-induced ethanol seeking. Neuronal activation is also increased in the prefrontal cortex in response to ethanol-associated cues (Jupp et al., 2011); an intriguing finding, as the prefrontal cortex is activated in response to cues in humans and associated with increased vulnerability to subsequent relapse (Grusser et al., 2004). It would be interesting to see if enhanced AMPA receptor activity in the prefrontal cortex could promote cue-induced reinstatement. Future region-specific studies may reveal other targets that are sensitive to AMPA receptor mediated-increases in ethanol-seeking.

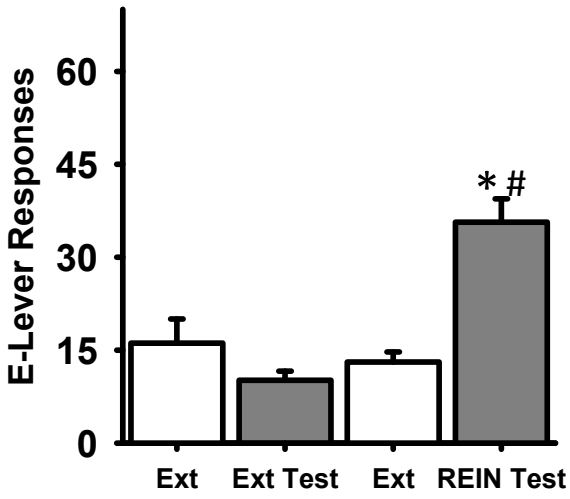


Figure 15 Cue-induced reinstatement of ethanol-seeking behavior in P-rats prior to western blot analysis. Responding on the ethanol-associated lever was potentiated in the reinstatement group (n = 16) relative to the extinction group (n = 15) after a response-contingent cue-induced reinstatement session. Lever responding resulted in the illumination of an ethanol-associated cue light, but ethanol was not available during this test session.*P < 0.05 versus extinction group; #P < 0.05 versus last day of extinction.

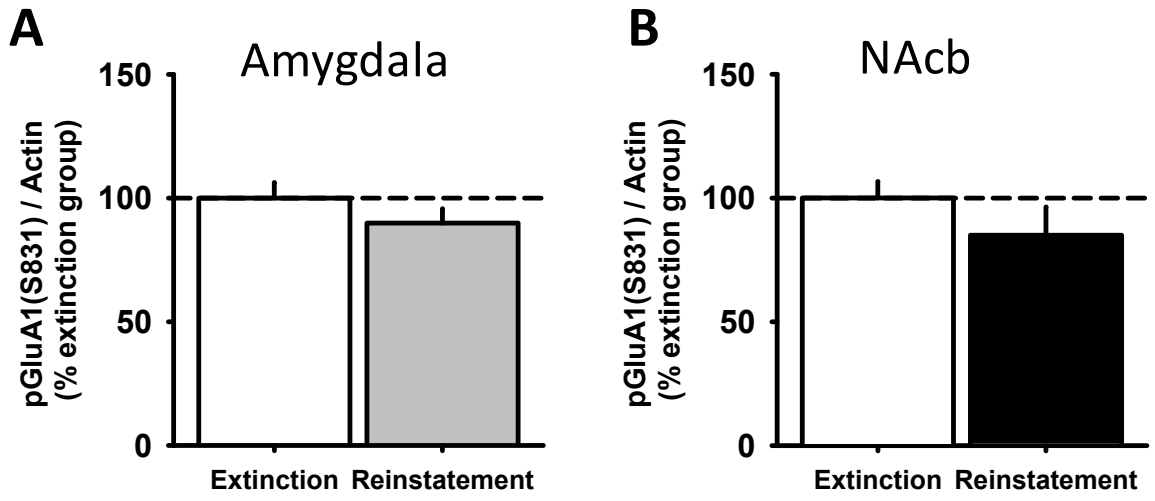


Figure 16 Amygdala and nucleus accumbens pGluA1 expression after response-contingent reinstatement. Response-contingent cue-induced reinstatement did not alter pGluA1(S831) expression in the reinstatement group (relative to extinction) as optical density(OD) measurements did not vary in the (A) amygdala or (B) nucleus accumbens (NAcb) of P-rats. Graphed values are expressed as pGluA1(S831) relative to beta actin as a percentage of the extinction group.

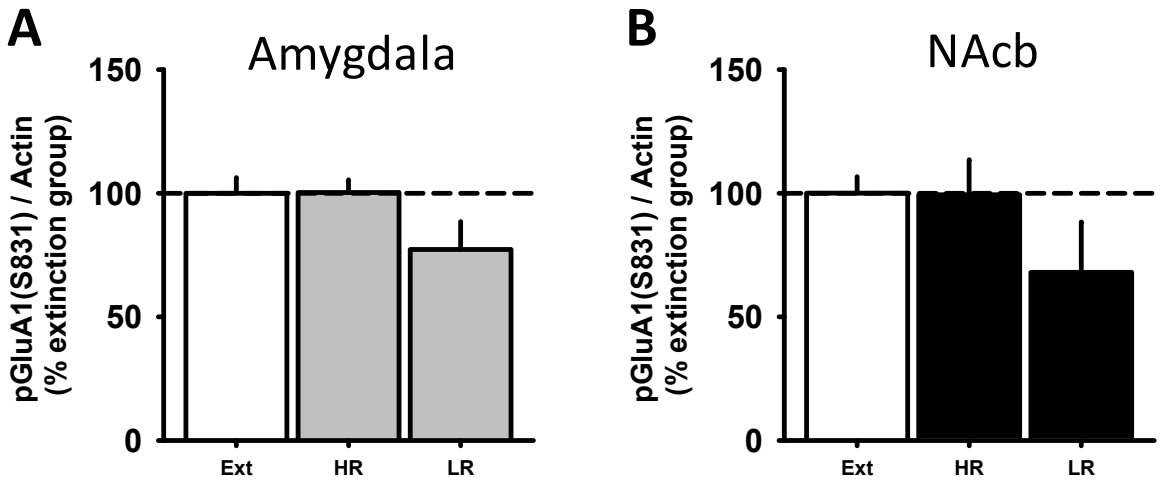


Figure 17 Amygdala and nucleus accumbens pGluA1 expression in high and low reinstatement group responders. Response-contingent cue-induced reinstatement did not significantly alter pGluA1(S831) expression in P-rats with high responses (HR) or low responses (LR) during reinstatement (relative to extinction) as optical density(OD) measurements did not vary in the (A) amygdala or (B) nucleus accumbens (NAcb). Graphed values are expressed as pGluA1(S831) relative to beta actin as a percentage of the extinction group.

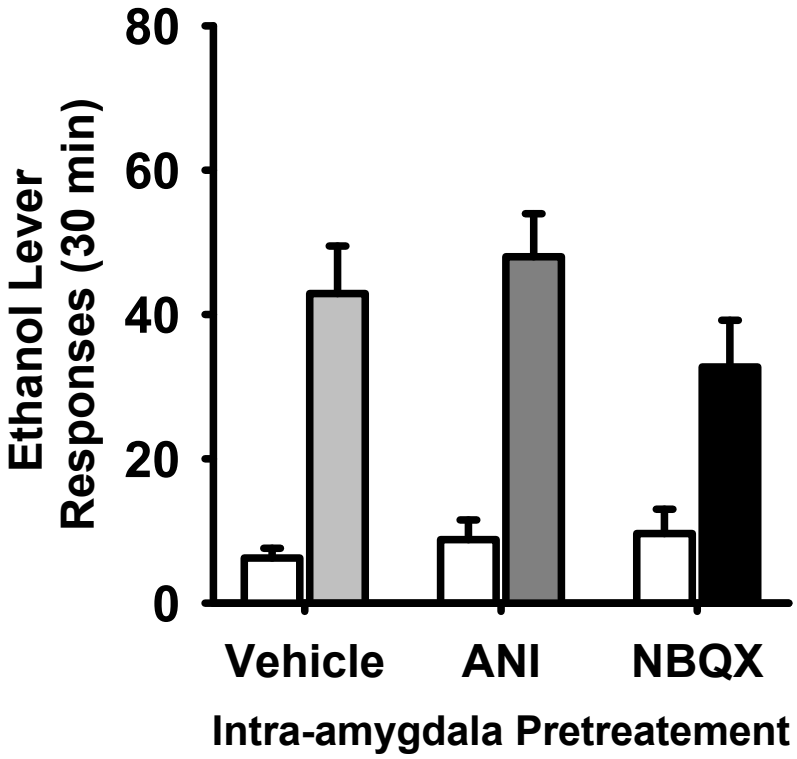


Figure 18 Potentiation or inhibition of glutamate activity at amygdala AMPA receptors does not alter cue-induced reinstatement of ethanol-seeking behavior. All treatment groups exhibited cue-induced reinstatement of ethanol seeking behavior. However, responding on the ethanol-associated lever was not altered by intra-amygdala aniracetam (1µg) or NBQX (1µg) pre-treatment (n = 9 – 11 per treatment group) during a cue-induced reinstatement session. White bars represent group ethanol lever response means on the last day of extinction. Filled bars represent group ethanol lever response means during reinstatement. Graphed values are expressed as group means ± SEM.

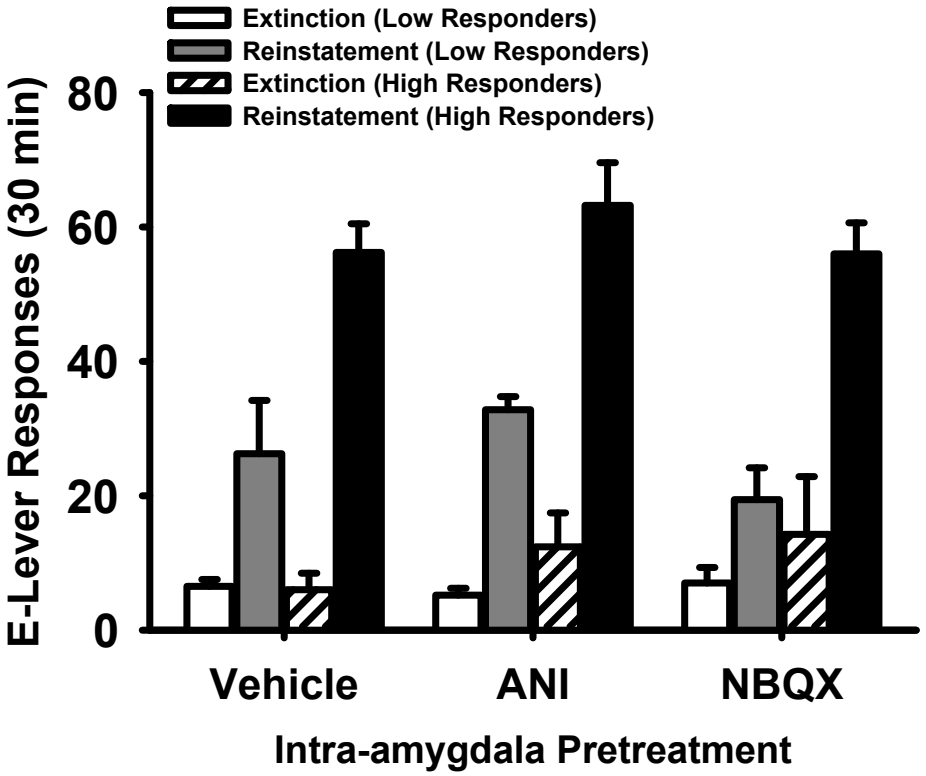


Figure 19 Potentiation or inhibition of glutamate activity at amygdala AMPA receptors does not alter cue-induced reinstatement in high or low reinstatement group responders. All treatment groups exhibited cue-induced reinstatement of ethanol-seeking behavior. However, responding on the ethanol-associated lever was not altered by intra-amygdala aniracetam (1µg) or NBQX (1µg) pre-treatment (n = 4 – 7 per group) in high or low responders during a cue-induced reinstatement session. Graphed values are expressed as group means ± SEM.

CHAPTER 5: GENERAL DISCUSSION

Repeated cycles of heavy drinking and subsequent relapse episodes are common features of alcoholism and alcohol use disorders (Johnson, 2010; McLellan et al., 2000). A challenge for researchers and clinicians is to identify novel pathways that mediate excessive drinking and vulnerability to relapse; as current medications are never effective in all patients seeking treatment for drinking problems (Miller et al., 2011). Increases in synaptic strength after chronic ethanol are proposed to modulate ethanol-related drinking behaviors (Cannady et al., 2013; McCool, 2010; Stuber et al., 2011). Therefore we sought to determine if acute enhancement of glutamate signaling, particularly at AMPA receptors, could augment ethanol self-administration and relapse-like behavior. Overall, results from the studies within this dissertation provide a novel role for enhanced glutamate activity at AMPA receptors in the facilitation of ethanol reinforcement processes and cue-induced ethanol-seeking behavior.

First, positive modulation of AMPA receptors by systemically-administered aniracetam selectively increased ethanol- but not sucrose-reinforced lever responding in an operant self-administration procedure using P-rats; a rodent genetic model with a predisposition to consume ethanol (Bell et al., 2006a). Control studies indicated that increased ethanol self-administration was not the result of aniracetam-induced alterations in spontaneous locomotor behavior or ethanol clearance. Additionally, the role of AMPA receptors was confirmed as sytemic administration of an AMPA receptor

antagonist blocked aniracetam-induced increased ethanol self-administration. Further, positive modulation of AMPA receptor activity following systemic injection increased ethanol-seeking behavior during a response contingent cue-induced reinstatement procedure. Collectively, these studies suggest that enhanced glutamate activity at this receptor has a functional role in promoting drinking and cue-induced relapse-like behavior.

Evidence that indicates that chronic ethanol potentiates synaptic strength in regions that mediate drinking behavior (Stuber et al., 2008). Therefore, we examined markers of increased synaptic plasticity associated with chronic ethanol self-administration such as GluA1 phosphorylation (pGluA1) at Ser-831; as phosphorylation of this AMPA receptor subunit is linked with activity dependent enhancement of synaptic strength (Barria et al., 1997; Lee et al., 2000). Increased pGluA1 was observed in the amygdala (BLA and CeA) and the nucleus accumbens (core); brain regions well-characterized for modulating ethanol-related behaviors. Guided by these results we examined the functional role of enhanced glutamate activity at AMPA receptors within these brain regions by site-specific infusion of aniracetam. Intra-amygdala infusion increased ethanol self-administration in a region- and reinforcer specific manner; as intra-accumbens aniracetam had no effect on ethanol self-administration and intra-amygdala aniracetam was void of effects in sucrose-trained P-rats. Importantly, we showed that the aniracetam induced increased in the amygdala in ethanol self-administration was dependent on CaMKII activity. This finding is highly relevant as it provides a mechanism to account for aniracetam induced facilitation of ethanol reinforcement through actions within the amygdala.

Finally, given that we determined a functional role of enhanced AMPA receptor activity in the amygdala and potentiated ethanol-seeking after systemic aniracetam pretreatment, we next sought to determine the role of enhanced and inhibited activity of amygdala AMPA receptors in modulating cue-induced reinstatement. We hypothesized that intra-amygdala potentiation of AMPA receptor activity would promote ethanol-seeking, while inhibition would attenuate ethanol-seeking. However, reinstatement of ethanol-seeking behavior was not altered by intra-amygdala administration of aniracetam or NBQX; suggesting that AMPA receptors in this region minimally contribute to cue-induced ethanol seeking.

The amygdala is a key limbic brain region that shares reciprocal glutamatergic connections between the hippocampus, nucleus accumbens, and prefrontal cortex, while receiving primary dopaminergic inputs from the ventral tegmental area (VTA) (LeDoux, 2003) . These glutamatergic connections to the amygdala are important as they suggest that glutamate neurotransmission from these regions may be converging to influence effects observed with aniracetam-induced ethanol self-administration. Given that chronic ethanol exposure can increase extracellular glutamate levels (Lallemand et al., 2011; Melendez et al., 2005; Roberto et al., 2004), and promote release of dopamine in the amygdala (Yoshimoto et al., 2000), conditions may be favorable for promoting sensitivity to ethanol reinforcement. Recent evidence indicates that the nucleus accumbens may be critical in modulating amygdala-dependent alterations in reinforcement (Stuber et al., 2011). Further dissection of the circuits involved in modulating increased reinforcement will provide greater specificity as to which regions are contributing to amygdala-mediated reinforcement.

Previous studies have decreased ethanol self-administration and relapse-like behavior by blocking activity at AMPA receptors (Backstrom and Hyytia, 2004; Sanchis-Segura et al., 2006; Stephens and Brown, 1999). However, we extend those findings by providing novel evidence for bi-directional modulation of ethanol reinforcement and ethanol-seeking as positive modulation of AMPA receptors *facilitated* these behaviors. Others have also shown neurotransmitter receptor bidirectional regulation of alcohol drinking behavior. Indeed, the type 1 cannabinoid (CB1) receptor agonist and antagonist bi-directionally modulate operant ethanol self-administration; with only low doses of agonist potentiating ethanol-reinforced lever-responses (Malinen and Hyytia, 2008). These data are similar to our findings as low doses of aniracetam increased ethanol self-administration. It may be interesting to determine if these two receptor signaling systems could work in concert to synergistically facilitate reinforcement. GABA agonists and antagonists have also been shown to bi-directionally regulate consumption (Boyle et al., 1993); however, bi-directional modulation of GABA receptors remains to be tested in an operant self-administration procedure. It is not known if other glutamate receptor subtypes can bi-directionally modulate ethanol reinforcement and ethanol-seeking, but it will be interesting for future studies to address this possibility. Collectively, these data highlight the use of gain-of-function studies to help fully determine mechanisms related to drinking behavior.

It is interesting that enhanced AMPA receptor activity in the amygdala, but not the nucleus accumbens modulated ethanol-reinforced lever responding. Several studies indicate that glutamate activity in the nucleus accumbens is important in modulating ethanol reinforcement. Indeed, intra-accumbens blockade of NMDA and mGluR5

receptors has been shown to reduced ethanol self-administration (Besheer et al., 2010; Gass and Olive, 2009; Rassnick et al., 1992). It may be that expression levels of AMPA receptors vary between the accumbens and amygdala and that a higher aniracetam dose in the accumbens is necessary to obtain effects on ethanol self-administration. However, this is unlikely as an intra-accumbens dose six times higher than that of the effective dose in the amygdala was ineffective at changing behavior. Based on the observed increase in pGluA1 expression as measured by immunohistochemistry in ethanol-trained rats, an alternative explanation is related to microinjection site. Several lines of evidence suggest discrete roles for each accumbens subregion (core vs shell) in mediating the rewarding properties of drugs of abuse (Chaudhri et al., 2010; Ito and Hayen, 2011). Future experimentation examining potentiated AMPA receptor signaling in the shell may produce different effects on ethanol self-administration. The same could be argued as to why intra-amygdala aniracetam or NBQX did not alter reinstatement as placements were aimed at the CeA. Multiple studies implicate the BLA in modulating cue-induced relapse (Gass et al., 2011; See et al., 2003). Thus, aiming guide cannulae in BLA has the potential to show differential effects as compared to the CeA. Moreover, by no means does our data implicate only the amygdala in mediating AMPA receptor modulation of ethanol effects. Several other brain regions express AMPA receptors (Petralia and Wenthold, 1992) and may be equally, if not more, involved in mediating ethanol reinforcement and relapse-like behavior.

Ethanol self-administration procedures allow for analysis of complex behavior, but are limited in that consumption levels are difficult to measure over extended periods of time. It would be interesting to investigate the effects of enhanced activity of AMPA

receptors in modulating chronic consumption of ethanol. Alternatives would be to use two-bottle drinking procedures, or binge models to evaluate the effects of enhanced AMPA receptor signaling on those consumatory behaviors. However; an inherent limitation is that acute dosing is often not adequate to examine extended access to ethanol. Thus, chronic administration of aniracetam would likely be necessary to determine if enhanced activity at AMPA receptors promotes increased consumption.

It is intriguing that positive modulation of AMPA receptor activity facilitates ethanol self-administration. Current data herein suggest that potentiated cue salience to ethanol-related stimuli after aniracetam pretreatment may partially explain increased self-administration. Yet, a limitation of the current study is that self-administration procedures do not fully explain mechanisms underlying behavioral changes. Other behavioral mechanisms may also account for the increase in ethanol self-administration. For example, the interoceptive or discriminative stimulus effects of ethanol are proposed to be major controlling processes that regulate drug-taking as consumed ethanol produces distinct subjective cues by which the presence of ethanol is detected (Stolerman, 1992). Multiple glutamate receptor subtypes modulate the interoceptive effects of ethanol (Besheer et al., 2010; Cannady et al., 2012; Grant and Colombo, 1993; Hodge and Cox, 1998; Hodge et al., 2001); however, no studies to date have examined functional role of AMPA receptors in the expression of ethanol subjective properties. It could be that pretreatment with aniracetam might have altered the subjective effects of ethanol, thereby promoting increased drinking. Indeed increased ethanol self-administration has recently been associated with altered ethanol discriminative stimulus effects (Besheer et al., 2012b; Besheer et al., 2013). Moreover,

the amygdala is implicated in aniracetam-induced increased ethanol self-administration. Given that this region is well-characterized for modulating ethanol subjective effects (Besheer et al., 2003; Besheer et al., 2012a; Cannady et al., 2012; Hodge and Cox, 1998), it could be interesting to see if systemic or brain region-specific enhanced activity at AMPA receptors alters the subjective cue.

Another limitation of the current methodology is that it is difficult to determine the physiological mechanism(s) by which potentiation of AMPA receptor signaling is facilitating reinforcement and relapse-like behavior. The exact amount of potentiation of glutamate activity at AMPA receptors by administered aniracetam doses is difficult to determine in behaving animals, which is crucial as we observed inverted U-shaped dose-response curves following systemic and intra-amygdala aniracetam treatment during ethanol self-administration sessions. These data suggest that the amount of potentiation of AMPA receptor activity by aniracetam is critically important in modulating behavior. Further experimentation in slice preparations after aniracetam treatment may allow for a better quantitative determination of AMPA receptor potentiation thresholds necessary to alter behavior.

Aniracetam binds to AMPA receptors and acts as a potent desensitization inhibitor allowing for slower decay kinetics of glutamate-induced AMPA receptor current (Isaacson and Nicoll, 1991). Interestingly ethanol has been shown to inhibit AMPA receptor function by stabilizing desensitization of AMPA receptors (Moykkynen et al., 2003). These data suggest that intra-amygdala aniracetam may be counteracting the actions of ethanol to stabilize desensitization and inhibit AMPA receptors. Indeed, other AMPA receptor positive modulators counteract the intoxicating effects of ethanol as

measured by attenuation of ethanol-induced motor impairment in various tasks (Jones et al., 2008). Thus desensitization inhibition may serve to reduce overall ethanol sensitivity; an interesting possibility for future studies to examine. Related to this point, it would be interesting to determine if there are endogenous substrates that function as AMPA receptor desensitization inhibitors and are modulated by ethanol.

One of the key hallmarks of alcohol use disorders is the development of ethanol dependence. Dependence is often characterized by compulsive craving, increased tolerance, and physical withdrawal symptoms including: tremors, insomnia, hyperactivity, and anxiety (American Psychiatric Association, 2013). Our data indicate that enhanced AMPA receptor activity can promote increased self-administration of moderate doses of ethanol in non-dependent animals. However; it is not clear as to how potentiation of AMPA receptor activity would alter ethanol self-administration or relapse-like behavior in a dependence model. Others have recently shown that glutamate receptor subtypes differentially modulate reinforcement and ethanol-seeking in dependent versus non-dependent rats (Sidhpura et al., 2010). Thus, it is possible that potentiation of AMPA receptor activity may function differently in a dependent rodent model; an interesting possibility for future experiments to examine. Furthermore, dependence and withdrawal have been associated with increased anxiety-like phenotypes; an effect attributed to maladaptive neuroadaptations within brain regions such as the amygdala (Roberto et al., 2010; Sparta et al., 2007; Wills et al., 2010). Studies indicate that AMPA receptor positive modulators may mediate anxiety-like behavior as indicated by alterations in response to fear-inducing stimuli in a fear-conditioning procedure (Yamada et al., 2009). Given that we observed behavioral

changes in this region after positive modulation of AMPA receptors, it may be possible that chronic ethanol induced-anxiety could be attenuated by aniracetam pretreatment.

Effects were observed on the maintenance of ethanol self-administration and cue-induced reinstatement of ethanol-seeking behavior. However, the role of enhanced AMPA receptor activity in modulating ethanol-seeking during extinction was not fully examined. Others have recently shown that nootropic compounds may have some efficacy at promoting extinction learning, which could be important as a therapeutic tool to reduce sensitivity to drug-related cues (Kufahl et al., 2012). Indeed, aniracetam is a nootropic drug with actions to promote learning and memory processes (Lebrun et al., 2000; Vaglenova et al., 2008). As a control experiment, we tested whether aniracetam could potentiate ethanol-seeking during an extinction session, which resulted in no significant effect on ethanol lever responding. However, it is not clear if extinction learning can be facilitated by enhancing activity at AMPA receptors in the presence of ethanol-associated cues. Future experimentation by chronically administering aniracetam during cue-exposure would address this possibility.

In conclusion, we have determined a functional role for enhanced activity of AMPA receptors to facilitate ethanol reinforcement and relapse-like behavior in an alcohol-preferring rodent model of alcoholism. These studies add to a small, yet growing, body of literature demonstrating the significance of AMPA receptor-mediated neuroadaptations and signaling in modulating various aspects of ethanol drinking. These data also highlight the role of potentiated glutamate signaling in promoting increased drinking and vulnerability to relapse and suggest that therapeutics targeting glutamatergic systems or AMPA receptors could be of significant clinical relevance.

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