A ROLE FOR CAMKII AND ERK1/2 PATHWAYS IN ALCOHOL SELF-ADMINISTRATION AND RELAPSE-LIKE BEHAVIOR

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ABSTRACT

Michael Charles Salling: A Role for CaMKII and ERK1/2 Pathways in Alcohol Self-Administration and Relapse-like Behavior
(Under the direction of Dr. Clyde Hodge)

Alcoholism is a debilitating neuropsychiatric disorder that adversely affects many people worldwide. Understanding the neurobiological mechanisms that cause alcohol addiction is paramount to its treatment. New evidence suggests that addictive behaviors emerge as a result of plastic changes in the neural circuitry that mediates drug reinforcement and reward-learning. The goal of this dissertation was to identify changes in neuroplasticity-related proteins following alcohol consumption in areas of the brain that mediate alcohol reward. Initially, we analyzed the amygdala proteome following chronic alcohol consumption and found 26 proteins that showed differential protein expression. Several of these proteins are involved in synaptic plasticity including CaMKIIα, a protein kinase that modulates receptor activity and is required for the induction of long-term synaptic plasticity. We further characterized CaMKII expression in the amygdala, and found that it is specifically increased in the central and lateral amygdala following twenty-eight days of alcohol-drinking at the start of the dark cycle when there were no detectable blood alcohol levels. Consistent with CaMKII's role in AMPAR trafficking, we found a concomitant increase in AMPA/NMDA ratio in the central amygdala. We extended these findings by measuring CaMKII expression following operant self-administration and found

that phosphorylated CaMKII was increased in the amygdala of alcohol-drinking mice. To determine if increased CaMKII activation played a role in alcohol's reinforcing properties, we infused CaMKII inhibitors into the amygdala prior to self-administration sessions. We found that CaMKII inhibition attenuates alcohol but not sucrose operant self-administration and concluded that CaMKII activity in the amygdala functionally regulates the reinforcing properties of alcohol. Lastly, we developed a mouse protocol for relapse-like behavior and tested the role of ERK1/2, a protein kinase that plays a role in plasticity, in relapse-like behavior. We found that inhibition of ERK1/2 phosphorylation potentiates cue-induced reinstatement of alcohol-seeking and induces sucrose-seeking. Collectively, these experiments demonstrate that inhibiting the activity of protein kinases that are involved in synaptic plasticity can affect alcohol-related behaviors in a reinforcer-specific manner and suggest that modulating these pathways has the potential for pharmacotherapeutic intervention in alcoholics.

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LIST OF ABBREVIATIONS

ACSF – Artificial cerebrospinal fluid

AMPA - α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BA - Basal nucleus of the amygdala

BK – Big potassium

CaMKII – Calcium-calmodulin dependent protein kinase II

CeA - Central nucleus of the amygdala

CREB – cAMP response binding element

EEF2 - Eukaryotic elongation factor 2

ERK1/2 - Extracellular regulated protein kinase II

GABA - Gamma-aminobutyric acid

KIF5C- Kinesin heavy chain 5C

LA - Lateral nucleus of the amygdala

LTP – Long-term potentiation

m-AIP – myristolated autoinhibitory peptide

mGluR – Metabatropic glutamate receptor

NAc – Nucleus accumbens

NMDA - N-Methyl-D-aspartic acid

NSF - N-Ethylmaleimide sensitive factor

PFC – Prefrontal cortex

PSD – Postsynaptic density

VTA - Ventral Tegemental Area

CHAPTER 1: INTRODUCTION

Societal impact of alcoholism

Alcohol is routinely consumed by many people and its use pervades many aspects of society. In the United States, most people will try alcohol in their lifetime, often in a social setting where drinking is encouraged to facilitate social interaction. It is estimated that 80% of Americans will try alcohol and 60% of them will continue drink alcohol throughout the duration of their life [1]. Although moderate alcohol use is common, 18% of people will abuse alcohol at some point [2]. Alcohol abuse engaging in daily use or repeated heavy drinking episodes called binge drinking that can pose problems like missed work, strained relationships, or criminal offenses [3-5]. In some individuals, continued alcohol abuse can progress to alcohol dependence, described in the DSM IV by several factors including alcohol tolerance, somatic symptoms related to alcohol withdrawal, inability to limit or abstain from drinking, and increased time spent obtaining alcohol or recovering from its effects. Alcoholism shares features with other addictive disorders, namely, that alcohol is continually abused despite serious, negative consequences.

The cause of alcoholism not fully understood. However, many variables have been identified that contribute to alcohol dependence, including family history, age of onset, drinking patterns, and complex factors like environmental and psychiatric illnesses [6, 7]. Biomedical research has identified emerging changes in brain

function following alcohol abuse that are thought to contribute to its behavioral symptoms. In the clinic, an alcoholic's lack of control over their drinking is treated a symptom of a larger disease, and not as a personal shortcoming, with treatment directed at medical and psychological interventions [8].

The consequences of alcohol use on the American public are enormous along the lines of both cost and an individual's well-being. The overall economic impact of alcohol abuse in the United States has been estimated at $235 billion annually [5]. Health-related costs, loss of productivity, and law enforcement are cited as 3 major factors contributing to this debt. Strikingly, there are an estimated 85,000 deaths attributed to alcohol use annually, more than four times the deaths related to illicit drug use [9]. Furthermore, alcohol use impacts others and accounts for of a large proportion of reported incidences of harassment, property damage, domestic violence, and murders, among other problems which have their own lasting consequences [10].

A major problem with alcoholism is that there are limited treatment options. Currently there are only three FDA approved medications available for the treatment of alcohol use disorders: disulfiram, aimed at preventing drinking by causing aversive hangover-like symptoms following alcohol ingestion, naltrexone, shown to reduce relapse frequency and severity by antagonizing opioid receptors and likely decreasing dopamine release, and acamprosate, shown to prolong abstinence possibly through a reduction in excitatory glutamate neurotransmission [11, 12]. These medications are not prescribed very often due to their high rate of attrition, negative side effects and uncertainty regarding their mechanism of action [11, 13].

Therefore, it is a major goal of alcohol research to better understand the neurobiological underpinnings of alcoholism. Due to alcohol's complex actions on the brain, identification of its effects has proven challenging, however, decades of alcohol research coupled with new approaches in molecular biology and physiology are elucidating these neurobiological mechanisms and moving the field closer to comprehensive treatment of alcohol use disorders.

Alcohol's effect on the human brain

Human research has established several lines of evidence that demonstrates brain dysfunction in alcoholics. Postmortem studies have shown significant loss of white matter in human alcoholics, as well as grey matter in the frontal lobes [14]. Examining volumetric changes in the brains of living alcoholics using neuroimaging techniques like magnetic resonance imaging has confirmed significant loss in the frontal lobe [15] and led to the identification of several additional brain regions affected by chronic alcohol use including the corpus callosom, thalamus, hypothalamus, hippocampus, and cerebellum [16]. Additionally, brain imaging performed in concert with neuropsychological testing has revealed that alcoholics showing decreased brain volume have deficits in impulsivity, memory, and motor disturbances compared to healthy controls [17]. The cause of this loss is not fully understood, but evidence supports the hypothesis that neuroinflammation [18] and glutamate excitotoxicity [19, 20] are consequences of chronic alcohol abuse which can lead to axonal demyelination, synapse loss, and cell death [21].

In addition to the neurodegenerative effects of alcohol, chronic alcohol use and subsequent withdrawals can lead to a hyper-excitable central nervous system.

Associated symptoms appear upon withdrawal and include insomnia and anxiety. These symptoms are believed to be a result of alcohol-induced neurochemical adaptations that include inhibitory desensitization through the down regulation of alpha containing gamma-aminobutyric acid receptors ($GABA_A$) [22, 23] and excitatory sensitization through the up regulation of N-Methyl-D-aspartic acid (NMDA) sensitive glutamate receptors [24, 25]. An escalation in the severity of these symptoms is thought to occur through a phenomenon referred to as the kindling effect, where repeated alcohol withdrawals exacerbate withdrawal symptoms. For instance, in binge drinking an individual will cycle between heavy drinking episodes and detoxifications that increase seizure susceptibility and anxiety-like behavior through an accumulation of neurotransmitter adaptations [26-28].

Neurodegeneration and neuroexicitability are outcomes of chronic alcohol consumption and can be directly measured; however, why an individual continually abuses alcohol despite its negative consequences is unclear. A good starting point is to understand how individuals maintain behaviors related to obtaining natural rewards. Sensory stimuli in the environment are encoded and used to detect the presence of rewards like food. Successful stimulus-reward outcomes are encoded via associative memory neural mechanisms. Associative memory has been shown to be crucial to maintaining these fundamental behaviors when it is related to obtaining rewards like food, water, and sex it is called appetitive conditioning. Data obtained from functional magnetic resonance imaging studies have demonstrated that mesocorticolimbic structures are activated following a form of appetitive conditioning where cues are paired with food rewards [29]. In human alcoholics,

exposure to alcohol cues activates several mesocorticolimbic regions including the ventral tegmental area (VTA), prefrontal cortex (PFC), basal ganglia, orbitofrontal cortex, nucleus accumbens (NAc), and amygdala to a greater extent than in abstinent or social drinkers [30-33] and to a greater degree of activation than appetitive controls (juice versus alcohol taste) [30]. In other drugs of abuse like cocaine and nicotine [34, 35], similar findings have been reported and shared adaptations to this neurocircuitry may underlie common addictive behaviors including compulsive drug-use and relapse.

Alcohol and animal models

To further our understanding of alcohol's effects on the brain, rodent models have been employed to elucidate the cellular and molecular actions of alcohol on the brain. Using myriad experimental approaches, researchers have delineated many of alcohol's effects on neurotransmitter receptor expression and activity. These findings have revealed several neuroadaptations that occur in mesocorticolimbic structures like the VTA, NAC, amygdala, and PFC, brain regions involved in reward processing. The use of behavioral models has led to significant progress in determining the neural circuitry involved and permits the testing of hypotheses regarding the functional consequence of these adaptations on addictive behaviors.

Alcohol is a polar molecule that freely passes the blood-brain barrier to access many central nervous system targets. At behaviorally-relevant doses (0-70 mM), acute alcohol enhances GABA transmission by acting as a positive allosteric modulator of the $GABA_A$ receptor [36] and inhibits a specific subset of excitatory glutamate receptors like NMDA receptors (5-50 mM) [37]. These two actions

decrease neuronal depolarization and cause alcohol's depressant effects like intoxication and sedation. Other neurotransmitter receptors affected by alcohol include acetylcholine (10 mM, [38]), serotonin (>25 mM, [39]), glycine (>35 mM, [40]) and the potassium channels GIRK (>20 mM, [41]) and BK (>20 mM, [42]). Acute alcohol has also been shown to alter neurotransmitter release through several mechanisms [43] including increased dopamine release from the VTA to the NAc [44, 45]. This effect on dopamine release is shared by many other drugs of abuse, is believed to be indicative of reward value, and is known to contribute to the development of addiction through associative learning mechanisms [46]. The mechanism(s) by which alcohol causes dopamine release is the subject of enduring debate. Several candidate mechanisms include 1) disinhibition of circuitry affecting dopamine neurons in the VTA through its actions on GABA and NMDA receptors, 2) affecting presynaptic mechanisms on dopamine neurons, or 3) through the pharmacological actions of the alcohol metabolite acetaldehyde [47]. Overall, the complexity of alcohol's interactions make it a very challenging to study especially compared to other drugs of abuse like cocaine which have a more specific pharmacological effects. Alternatively, it may be more important to focus on how repeated exposures to alcohol affect neurotransmission as these adaptations are thought to underlie addictive behaviors.

Chronic exposure paradigms have led to the discovery of several adaptations in neurotransmitter systems caused by alcohol. In animal models, prolonged alcohol exposure can be accomplished by administering alcohol directly (e.g. systemic injection, intragastric delivery, or exposure to alcohol vapor) or by using a mouse or

rat line that will consume alcohol voluntarily. Studies using these methods have demonstrated that chronic alcohol exposure causes homeostatic adaptations related to alcohol's acute actions. Chronic alcohol exposure reorganizes $GABA_A$ receptor expression and increases the expression of specific glutamate receptors including NMDA, AMPA, and metabatropic glutamate receptors (mGluRs) [48-50]. The end result of these adaptations is a hypeglutamatergic state that has been described previously in humans upon alcohol withdrawal [19, 43] and that has been measured through electrophysiological techniques [43] and microdialysis in rodents [51].

Neurochemical adaptations in neurotransmitter systems are thought to underlie specific behavioral aspects of alcohol addiction. One group of behavioral assays used to quantify anxiety in rodents involves assessing the performance on validated tasks like the elevated plus maze. When performing these tasks, alcohol-dependent animals experiencing withdrawal will demonstrate increased anxiety-like behavior [52]. Treatment with GABA agonists such as benzodiazepines or NMDA inhibitors have been shown to oppose the anxiogenic like effects of alcohol withdrawal, suggesting that alcohol withdrawal symptoms are mediated by GABA- and NMDA-specific pathways [53, 54]. In addition, causing dependence in rats using alcohol vapor exposure potentiates the reinforcing effects of alcohol [55, 56]. Thus, alcohol exposure may cause maladaptive activation of neurotransmitter systems and improper encoding of neural information. These physiological adaptations appear to mediate the positive (reward) and negative (withdrawal) behaviors in alcohol addiction.

For many receptors, neurotransmitter binding leads to the activation of downstream signaling cascades including protein kinases which can affect gene transcription. Several protein kinases are altered by drugs of abuse. These alterations can have a dynamic and lasting impact on neurotransmission, and have are proposed as potential molecular mechanisms underlying addiction [57]. Several protein kinases that modulate receptor activity, gene transcription and synapse maturation are affected by drugs of abuse. Two of these protein kinases, calcium-calmodulin dependent kinase II (CaMKII) and extracellular regulated kinase 1 and 2 (ERK1/2), are phosphorylated following increased levels of intracellular calcium caused by depolarization and through g-protein mediated release from internal calcium stores .

Addiction is believed to be caused by maladaptive changes to neural circuits that regulate reward and motivation. Repeated drug exposures are believed to cause aberrant synaptic plasticity in these regions that leads to improper encoding of drug-related cues, and shifts an individual's behavior towards the obtaining and using drugs despite its negative consequences [58, 59]. Heavy drinking can produce a hyperactive state in which increased glutamate release and upregulation of glutamate receptors occur in several brain regions that regulate reward behavior [60]. Increased glutamate activity activates both the CaMKII and ERK1/2 signaling pathways [61] which have multiple effector molecular targets including transcription factors like cAMP response element-binding (CREB) and Elk-1 that are involved in synaptic plasticity [62]. These pathways have been shown to be required in some forms of long-term plasticity, where synaptic connections of neurons are

strengthened through increased surface expression of glutamate receptors. As a result of these properties, CaMKII and ERK1/2 have been investigated as molecular candidates that underlie drug-induced adaptations that contribute to addictive behaviors.

Calcium/calmodulin dependent protein kinase II (CaMKII)

CaMKII is a serine-threonine protein kinase activated by the presence of calcium-calmodulin. It constitutes 1-2% of brain total protein and is the most abundant protein of the post-synaptic density (PSD). Four genes have been identified (α, β, γ, and δ) that form a CaMKII 12 subunit heteromeric complex [63]. The α and β subunits of CaMKII are the most highly expressed in the brain [64]. Each subunit can be activated by the association of calcium-bound calmodulin to its catalytic domain, autophosphorylate its neighboring subunit, and remain active for up to several hours after calcium levels have decreased [65]. Following activation, CaMKII is able to localize to the PSD where it can modulate receptor activity by interacting with several receptors including NMDA, AMPA, dopamine, GABA and L-type voltage gated channels [66, 67]. In addition, phosphorylated CaMKII can translocate to the nucleus and phosphorylate multiple transcription factors including Neurogenic differentiation factor 1 (NeuroD), Nf-κB, and CREB, transcription factors that have been implicated in regulating mechanisms of neuroplasticity [68]. Other complex actions of CaMKII emerge from crosstalk among kinase pathways [69]. For instance, CaMKII can bind and directly activate map kinase kinase 1 (MEK1) which phosphorylates extracellular regulated kinase 1/2 (ERK1/2), another calcium-signaling protein kinase involved in gene transcription [70, 71].

The ability of CaMKII to switch on, remain active, and have dynamic actions on receptor activity and gene transcription has led to extensive characterization of its role in synaptic plasticity. Importantly, it is required for the induction of NMDA-dependent long-term-potentiation (LTP), a leading candidate mechanism for the physiological basis of memory formation [72]. Following pre- and postsynaptic activation and calcium influx, CaMKII is thought to initiate LTP by phosphorylating AMPAR GLUR1 subunits on serine 831 which has been shown to increase AMPAR conductance [73]. In addition, CaMKII regulates LTP by increasing AMPAR delivery to the synapse through its interactions with the PSD [74]. NMDA-dependent LTP induction is blocked by preventing CaMKII phosphorylation using CaMKII antagonists that block the binding of calcium/calmodulin and the generation of transgenic mice with a point mutation that blocks phosphorylation of the threonine 286 site on CaMKIIα [75]. Bathing postsynaptic cells with a constitutively activated form of CaMKII, CaMKII T286D can induce LTP. Further, mutant mice that express CaMKII T286D occludes LTP induction as the LTP response is already maximized [76]. Additional *in vivo* work demonstrated that rewiring of cortical networks of the visual system does not occur in CaMKIIα thr286 mutants [77]. Collectively, these results demonstrate that CaMKII is necessary and sufficient for LTP induction *in vitro* and *in vivo* and is required for experience-dependent plasticity.

CaMKII's involvement in LTP makes it a major molecular target of learning and memory research, a notion that is supported by behavioral data collected from genetic and pharmacological manipulation of CaMKII activity. Heterozygous mutant mice deficient in CaMKIIα protein expression have memory deficits and higher

thresholds for LTP induction [78-80]. The CaMKIIα thr286A mutant mouse which lacks CaMKIIα autophosphorylation and hippocampal LTP has severe spatial memory deficits on the Morris water maze, a hippocampal-dependent task. Overexpression of CaMKIIα in the forebrain, including the amygdala, leads to enhanced fear conditioning and increased anxiety in mice, two behaviors mediated by the amygdala [81]. Furthermore, pharmacological inhibition of CaMKIIα T286 phosphorylation in the lateral amygdala using site-specific infusions of a CaMKII inhibitor KN-62 prevents the acquisition, but not expression of fear conditioning in rats [82]. Due to the amygdala's role in encoding drug-related cues [83], amygdala CaMKII may play an important role in drug conditioning as well.

CaMKII's dual roles in plasticity and learning have made it an intriguing candidate for the actions of drugs of abuse. Pharmacological inhibition of CaMKII phosphorylation with KN-93 decreases amphetamine self-administration [84] and blocks cue-induced reinstatement of cocaine-seeking when injected into the NAc [85]. Surprisingly, alcohol's actions on CaMKII are not well understood despite the many known actions of alcohol on receptors that regulate the CaMKII pathway. Previous work with alcohol demonstrated that CaMKII is upregulated in rats with prenatal exposure to alcohol [86] and CaMKII sensitizes BK channels to alcohol [87] which may modulate alcohol tolerance. Overall, very few studies have investigated the effects of alcohol on CaMKII and no studies exist that examine the role of CaMKII in alcohol-specific behaviors.

Extracellular-Regulated Kinase 1 and 2 (ERK1/2)

The mitogen activated protein kinase (MAPK) system is a complex set of signal transduction pathways that are conserved among all eukaryotic organisms. These kinases form sequential cell signaling pathways that integrate the activity of diverse extracellular signals within the cell [88]. MAP kinases are activity-dependent modulators of rapid cellular responses and short- or long-term changes in gene transcription [89]. It is this ability to modulate cellular responses to extracellular signals that underscores the ability of MAP kinases to transduce neuronal activity into enduring changes in functional activity of the CNS such as those seen during chronic alcohol use or abstinence.

The ERK/MAPK pathway integrates activity of a variety of extracellular and intracellular factors to produce coordinate changes in gene transcription that lead to long-term changes in CNS structure and function, including cell growth, neuroplasticity and addiction [88, 90-93]. The two closely related isoforms of ERK (ERK1 and ERK2, or $ERK_{1/2}$) are phosphorylated within the activation loop of the kinase on both a threonine and a tyrosine residue by $MEK_{1/2}$. $ERK_{1/2}$ activity requires phosphorylation of both of these sites [94]. Activated $ERK_{1/2}$ phosphorylates cellular targets or translocates to the nucleus where it activates specific gene transcription factors [95]. By regulating gene transcription, the ERK/MAPK pathway transduces cellular events into long-term changes in neural and behavioral functions, such as those seen in learning, memory, and addiction.

Role of Amygdala in Self-administration and Relapse-like Behavior

The amygdala is major structure of the limbic system that mediates reward-related learning. The neurocircuitry of the amygdala lends itself to the integration of information related to the reward valence and procurement [83]. It receives primary afferent dopaminergic terminals from the VTA and reciprocal glutamatergic connections between the hippocampus, NAc, and PFC [96]. Acute exposures to drugs of abuse including alcohol cause an increase of dopamine release from the VTA into the NA and amygdala [97, 98]. Dopamine release from the VTA is believed to be indicative of reward value and can modulate amygdala activity [99]. Sensory information specific to environmental cues is processed by glutamatergic projections to amygdala neurons [100] and inhibition of amygdala activity is regulated by its glutamatergic afferents on amygdala interneurons from the PFC [101, 102]. Amygdala circuitry has been proposed to be critical for the formation and maintenance of addictive behaviors like operant self-administration and cue-induced reinstatement of drug seeking [103].

A critical role of the amygdala is learning which stimuli predict the availability of a reward. The amygdala is made up of several nuclei including the central (CeA), basal (BA) and lateral nucleus (LA), with the BA and LA often described together as basolateral nucleus (BLA), that have been shown to contribute to distinct behavioral functions using models of associative memory, including fear-conditioning and reward-learning. The BLA has been shown to process incoming sensory information regarding cues, including auditory, visual, gustatory, and olfactory, that predict the onset of an unconditioned stimulus, like rewarding stimuli [104-108]. Moreover,

neuronal firing in the BLA is altered prior to encounters with food rewards which varies according to reward size [109]. The CeA has been shown to be necessary for conditioned stimulus output, like freezing following fear conditioning [110]. These behavioral functions are relevant to drug addiction as they can signal the availability of a drug and mediate drug-specific behaviors. For instance, functional inactivation of the BLA by excitotoxic lesions [111], microinjection of tetrodotoxin [112] or lidocaine [113] have all been shown to inhibit relapse-like behavior in rats. Additionally, lesions and NMDA receptor blockade attenuate behavioral sensitization to cocaine or amphetamine [114, 115]. Therefore, amygdala subregions appear to be critical substrates for drug conditioning and drug reinforcement.

The amygdala is particularly relevant to alcohol addiction as it is highly sensitive to its pharmacological actions and plays an important role in mediating its behavioral effects. Acute alcohol exposure inhibits NMDA receptors and enhances GABA transmission in the amygdala [116]. It is known that the discriminative stimulus properties of alcohol are modulated by $GABA_A$ receptors in the amygdala [117] and multiple site injections of the GABA antagonist, SR 95531, have revealed that the most sensitive region for decreasing alcohol self-administration is the CeA [118]. This evidence suggests that the amygdala plays a major role mediating alcohol's rewarding properties. In experiments using the conditioned place procedure (CPP), ethanol conditioning and expression is disrupted by amygdala lesions and dopamine antagonism disrupts CPP expression [119, 120]. Long-term exposure to alcohol causes neuroadaptations to occur in the amygdala. Following chronic exposure to alcohol, the amygdala compensates to alcohol's acute effects

by increasing NMDA receptor sensitivity and releasing additional glutamate [60, 121]. Increased glutamate activity is likely to influence amygdala function including associative learning. Supporting this idea, chronic alcohol has been shown to cause deficits in fear conditioning, possibly by blocking the encoding of new information as LTP induction is occluded following this treatment [122]. Alternatively, acute alcohol has been shown to enhance consolidation of reactivated fear memories [123]. An interpretation of these results may be that alcohol increases synaptic efficiency during the consolidation time period, but reduces the capacity for further plasticity and learning [122]. Collectively, these studies demonstrate that the amygdala is critical to behaviors implicated in addiction including alcohol reinforcement and relapse-like behavior.

Behavioral Models

Although the human condition is not entirely reproducible in animals, behavioral features of addiction can be effectively modeled in rodents. The models used in this dissertation include the two-bottle choice procedure, the operant self-administration procedure, and the reinstatement procedure. The two-bottle procedure is useful in examining the long-term neurobiological consequences of alcohol consumption. It has inherent face validity and requires common neural circuitry between species [124]. One drawback of this procedure is that the reinforcing effects of alcohol cannot be measured in most paradigms. A complementary approach is the operant self-administration procedure where mice perform a response like a lever press to receive an alcohol reward with an inactive or water lever present. Increased responding on the alcohol lever is a definitive and

quantitative demonstration of alcohol's reinforcing properties in mice which can then be experimentally manipulated. An advantage to this procedure over the two-bottle procedure is its temporal acuity related to alcohol specific behaviors. It can used to assess the maintenance of self-administration and may be useful in determining adaptations following a discrete number of sessions. In reinstatement models, self-administering mice are given a period of extinction and then exposed to stimuli that are thought to promote relapse in humans including stress, cues or contexts associated with alcohol, or a priming dose of alcohol [124]. This paradigm can be use to examine the neurobiological consequences of stimulus-induced alcohol-seeking independent of alcohol's pharmacological actions.

Rationale

Aberrant synaptic plasticity has been proposed to play a major role in addiction. Protein kinase activity regulates plasticity and may be an important molecular mechanism in the development of addictive behaviors [58]. The experiments in this dissertation were designed to characterize and investigate the role of two protein kinases, CaMKII and ERK1/2, in alcohol-related behaviors. Initially, we focused on the amygdala due to its role in alcohol reinforcement and associative memory. Using and unbiased proteomics approach, CaMKIIα and a network of proteins involved in CaMKII synthesis were identified as having a higher level of expression in the amygdala of mice following chronic alcohol consumption at the start of the dark cycle when there were no detectable blood alcohol levels. CaMKII expression was further characterized and the electrophysiological properties of amygdala neurons were measured to determine if increased CaMKII

corresponded with increased glutamate activity in alcohol-drinking mice. To determine if increased CaMKII in the amygdala had a functional consequence, its role in the reinforcing effects of alcohol was tested by training mice to self-administer alcohol in operant chambers and then infusing CaMKII inhibitors into the amygdala prior to operant sessions. Finally, relapse is a major issue in treating alcoholics that is dependent on the learning of associations between environmental cues and alcohol availability. To address this issue, a mouse model of relapse-like behavior was developed and used to test the role of ERK1/2 in cue-induced reinstatement of alcohol-seeking.

CHAPTER II: CHRONIC ALCOHOL CONSUMPTION INCREASES CAMKII EXPRESSION IN THE AMYGDALA

INTRODUCTION

Human alcohol consumption is a pervasive and enduring practice integrated into many aspects of society. In developed countries, nearly 50% of adults drink alcohol in a manner that imparts little risk of abuse or dependence [125]. Approximately 7% of the population, however, transitions from moderate use to dependence [1], contributing to serious physical, psychiatric, and social problems [126-131]. Although the transition from moderate alcohol drinking to addiction is undeniably influenced by complex psychosocial factors [132] chronic alcohol use produces discernable physiological adaptations in neural systems that regulate adaptive behaviors of the organism, such as alterations in synaptic plasticity ([58, 133]. The adaptations that occur during the transition to alcohol dependence are thought to underlie the long-term persistent nature of addiction and their identification is crucial to treatment of the disorder [134].

Alcohol-induced synaptic modifications may have their most profound consequences on the neural circuitry of the amygdala, a central component of the brain's reward system uniquely positioned to process alcohol reward, alcohol-related cues, and influence alcohol-seeking behavior [83, 135]. The amygdala is an assembly of interconnected anatomically and physiologically defined nuclei that

include the CeA, LA, and BA [100, 136, 137]. Evidence from aversive and appetitive learning experiments has delineated unique contributions to associative memory for several of these subregions [100]. The LA integrates sensory information related to environmental cues and primary reinforcers transmitted via thalamic and cortical afferents [138] whereas the BLA has been implicated in cue-induced reinstatement of reward-seeking behavior through projections to the nucleus accumbens (NAc) [139]. The CeA is widely regarded as being critical for the expression of anxiety [83], but perhaps more importantly, it has been identified as a primary neural substrate of alcohol reinforcement [118].

To evaluate alcohol's effects on the amygdala, unbiased proteomic analysis provides an objective approach for identifying molecular networks that underlie long-term adaptations to alcohol exposure. Here, we evaluated changes in the amygdala proteome induced by chronic alcohol consumption using 2D- differential in gel electrophoresis (2D-DIGE) followed by identification of affected proteins by mass spectrometry (MALDI-TOF/TOF). We successfully identified 26 proteins that were significantly altered by long-term voluntary drinking and focused on the alpha subunit of calcium/calmodulin dependent kinase II (CaMKIIα), and several proteins involved in its local synthesis. CaMKII is a dynamic protein kinase that has been described as a molecular candidate for learning and memory due to its unique properties [65]. CaMKII is phosphorylated following increased intracellular levels of Ca^{2+} that can occur following neuronal depolarization and remain phosphorylated long after increased Ca^{2+} levels have subsided ([140, 141]. It can influence the activity of multiple neurotransmitter receptors including increasing glutamate transmission by

phosphorylating AMPA and NMDA receptor subunits and modulating the trafficking of AMPA receptors to the synapse [142]. Importantly, CaMKII is required for multiple forms of synaptic plasticity including the induction of LTP ([78, 143] and the structural remodeling of dendritic spines [144], effects that demonstrate its ability to cause enduring changes in synaptic signaling. Pharmacological and transgenic inhibition of CaMKII has established that it regulates several forms of learning and memory, including Pavlovian conditioning in the amygdala [145-147]. These properties of CaMKII make it an intriguing target for the induction of long-term synaptic changes that contribute to the persistent nature of addiction, particularly in subregions of the amygdala which mediate the consolidation of stimulus-reward learning as well as the reinforcing effects of alcohol [118].

Following the identification of CaMKIIα, we sought to further characterize its expression and functional properties following voluntary alcohol consumption. To determine if CaMKIIα is increased globally, we measured its expression in multiple brain regions. As amygdala subregions have unique roles in behavior, we looked at CaMKIIα expression in the CeA, LA, and BA and found that it demonstrated its largest effect in the CeA. CaMKII has many effects on synaptic plasticity; therefore, we measured the electrophysiological properties of CeA neurons following voluntary consumption of alcohol, and in agreement with known functions of CaMKII, we observed increased AMPA/NMDA ratio in CeA neurons following the two-bottle procedure.

METHODS

Subjects: Adult male C57BL/6J mice (7-8 weeks old, Jackson Laboratories) were maintained on a 12 hour reverse light dark cycle. Mice were single-housed in Plexiglas chambers with food and water available *ad libitum* except where noted. All animals were treated in accordance with the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and NIH guidelines for the Care and use of Laboratory Animals (National Research Council, 1996).

2-bottle choice procedure: Initially, mice were given either one bottle of alcohol (10% v/v) or water for 3 days. On the 4th day, all mice were given a second bottle of water. Every other day, bottles and mice were weighed and the position (left or right) of the bottles were switched to prevent side bias. Daily g/kg (alcohol), fluid intake and bottle preference were calculated for each mouse. On the 28th day, at the end of the light cycle, mice were injected with sodium pentobarbital (6 mg per mouse), trunk blood was collected for blood alcohol concentration and mice were perfused with ice cold phosphate buffered saline (0.1 M) before being processed for 2D-DIGE, immunoblotting, or immunohistochemistry.

Blood collection and assays: For tail blood collection, mice were briefly immobilized (<1 min) in a restraint tube (Braintree Scientific, Braintree, MA) and a scalpel was used to make a small nick at the end of their tail. Trunk blood was collected from the body cavity during perfusions prior to PBS flush. All blood was collected in heperanized tubes and immediately centrifuged to separate and collect plasma. BAC

of plasma collected (5 µl) was measured via Analox G-5 analyzer (Analox Instruments, Lunenburg, MA). Corticosterone concentration was quantified from plasma (10 µl) using a Radioimunnoassay Kit (MP Biomedicas, Solon, OH) and calculated as ng/ml.

2D-DIGE: To reduce variations in alcohol dose, mice demonstrating consistent levels of alcohol consumption (n = 12) throughout the drinking period and control mice (n = 12) that had similar fluid consumption were selected for the proteomics experiment. To obtain enough tissue for six gels (4 2D-DIGE gels, 1 phosphostain gel, and 1 gel for protein collection), mice were matched on fluid preference and alcohol consumption and then pooled into samples (3 mice per sample, 4 alcohol and 4 water samples). Following perfusions to eliminate blood contamination, mice brains were rapidly removed and frozen on dry ice. Using a cryostat, coronal sections (0.5 mm) that included the majority of the amygdala were collected (1.0 mm posterior to bregma, Franklin and Paxinos, 2008) and bilateral tissue punches (1 mm diameter) were directed at the amygdala region. Tissue punches were submerged in homogenization buffer that contained protease and phosphatase inhibitors I and II (Sigma Aldrich, St. Louis, MO). Remaining tissue was fixed by submerging in 4% paraformaldehyde overnight, mounted using super glue on slides and observed under a light microscope to confirm correct location of amygdala punches.

2D-DIGE was performed by Applied Biomics (Hayward, Ca) using the following method. Tissue punches were washed (10mM Tris-HCl, 5 mM magnesium acetate, pH= 8.0), resuspended in 2D cell lysis buffer (30mM Tris-HCl, pH= 8.8 with

7 M urea, 2M thiourea and 4% CHAPS) and sonicated at 4 °C. Tubes were spun at 14,000 rpm for 30 min to collect supernatant and protein concentration was determined using the Bio-Rad protein assay method. CyDye (1:5 diluted with DMF from 1 nmol/µl stock) was then added 30 µg of cell lysate along with 10 mM lysine. Cy2, Cy3, and Cy5 labeled samples were mixed and diluted with 2X 2D sample buffer (8 M urea, 4% CHAPS, 20 mg/ml DTT, 2% pharmalytes and trace amount of bromophenol blue). Next, 100 µl of destreak solution and rehydration buffer 7 M urea, 2 M thiourea, 4% CHAPS, 20 mg/ml DTT, 1% pharmalytes and trace amount of bromophenol blue) was added to 250 µl for the 13 cm IPG strip. Samples were mixed well and spun before loading into strip holder with 1 ml mineral oil on top of face down 13 cm strip. The IEF was run following the protocol provided (Amersham Biosciences, Buckinghamshire, UK) under dark at 20° C. Next, IPG strips were incubated in fresh equilibration buffer (50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, trace amount of bromophenol blue and 10 mg/ml DTT) for 10 minutes with slow shaking, rinsed in SDS gel running buffer, transferred into gradient SDS-Gel (9-12% SDS), and ran at 15° C.

Immediately following SDS-PAGE, image scans were obtained with Typhoon Trio (Amersham Biosciences, Buckinghamshire, UK) using the provided protocols. Images were analyzed using QuantL software (GE-Healthcare, Buckinghamshire, UK) and in-gel analysis and cross-gel analysis was performed using DeCyder software 6.5 (GE-Healthcare, Buckinghamshire, UK) and changes were expressed as ratio. All protein spots that met the following *a priori* criterion (average change greater than 25%, change appeared in all 4 gels, significance determined by t-test, p

< 0.05) were picked up by Ettan Spot Picker (GE-Healthcare, Buckinghamshire, UK). Selected spots were digested in-gel with trypsin, peptides were extracted, desalted and subjected to MALD-TOF/TOF (Applied Biosystems, Foster City, CA) analysis to identify each protein.

Immunoblotting: Additional mice (n = 12) underwent the 2-bottle choice procedure where tissue was collected in the same manner as the 2D-DIGE experiment with the exceptions that individual amygdala samples were collected and homogenized (Branson Sonifier 5000) in buffer that contained SDS, protease and phosphatase inhibitors I and II (Sigma Aldrich, St. Louis, MO). Protein concentration was determined using a BCA assay (Invitrogen, Carlsbad, CA). Western blots were performed by loading 8 ug of protein on 8-12% bis tris minigels (Invitrogen, Carlsbad, CA) and transferring protein to PVDF membranes using iblot semi dry blotting system (Invitrogen, Carlsbad, CA). Blots were initially blocked with 1% BSA in buffer solution and probed with the following primary antibodies overnight in blocking buffer at 4 °C: monoclonal mouse anti-CaMKIIα (Millipore, 1:10,000), polyclonal rabbit anti-pCaMKII$_{thr286}$ (Abcam, 1:1500), monoclonal rabbit anti pGLUR1$_{ser831}$ (1:2000), polyclonal rabbit anti-NSF (Cell Signaling, 1:2000), and monoclonal mouse anti-GAPDH (1:10,000)). Blots were washed several times and incubated in horseradish peroxidase-conjugated secondary antibodies (rabbit anti-mouse (1:10,000) or goat anti-rabbit (1:10,000) in blocking buffer) for one hour and visualized using a chemiluminescent ECL kit (Thermoscientific, Waltham, MA). Optical density of each band at corresponding molecular weight was measured

using Scion imaging software and values were calculated as percent GAPDH (loading control). All values were calculated as percent control (water) from each individual blot.

Immunohistochemistry: Coronal sections (40 microns) were collected using a vibratome (Leica) and stored in cryoprotectant at -20°C. Sections were washed with phosphate buffer solution (0.1 M PBS), followed by inhibition of endogenous peroxidase activity with 1% H_2O_2, antigen retrieval using citra buffer at 70°C, blocked with 5% goat serum in PBS with 0.1% Triton-x (Sigma,) and then incubated with primary antibodies (phospho-GluR1ser831 1:1500 (Abcam) or CaMKIIα (Millipore) 1:10,000) overnight in blocking buffer. Antibody bound protein was visualized using ABC vectastain kit (Vector labs, Burlingame, CA) or mouse on mouse kit (Vector labs, Burlingame, CA) with DAB as chromagen. Sections were mounted on slides, cover slipped with cytoseal and images were taken using a camera mounted on a light microscope. Quantification of positive cells and pixels was performed using Bioquant software. Pixel density and cell count measurements was calculated from a circumscribed field (e.g., brain region) and divided by the area of the region and expressed as pixels/mm^2 and cells/mm^2. All analyses were carried out by experimenters blind to each condition.

Brain Slice Preparation: Following the 2-bottle choice procedure, male C57BL/6J mice were decapitated under anesthesia (Isoflurane) 0-6 hours following the final exposure to alcohol. The brains were quickly removed and placed in ice-cold

sucrose-artificial cerebrospinal fluid (ACSF): (in mM) 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$ saturated with 95% O_2/5% CO_2. Slices of 300 µm in thickness were prepared using a Tissue Slicer (Leica). Slices were then stored in a heated (approximately 28°C), oxygenated (95% O_2–5% CO_2) holding chamber containing 'normal' ACSF [ACSF: (in mM) 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10.0 glucose, and 26.0 NaHCO3] or transferred to a submerged recording chamber where they were perfused with heated (28–30°, unless otherwise noted), oxygenated ACSF at a rate of about 2 ml/min. Slices were allowed to equilibrate in normal ACSF for 1 h before experiments began. 25 µM picrotoxin was included in the extracellular solution in all experiments to block GABAergic transmission.

Whole-Cell Voltage Clamp Recordings (Performed by Tom Kash's Lab): Slices were placed in a submerged chamber (Warner Instruments, Hamden, CT) and neurons of the CeA were directly visualized with infrared video microscopy (Olympus, Tokyo, Japan). In general, recordings were focused on the medial aspect of the CeA, where the greatest change in CaMKIIα immunohistochemistry occurred. Recording electrodes (3–6 MΩ) were pulled on a Flaming-Brown Micropipette Puller (Sutter Instruments, Novato, CA) using thin-walled borosilicate glass capillaries. Following successful break in, spontaneous excitatory post synaptic currents (sEPSCs) were recorded at -70mV. Immediately following this, we evaluated the paired pulse ratio of evoked EPSC in the same neuron using a 50 ms interstimulus interval. EPSCs were evoked by local fiber stimulation with bipolar ni–chrome electrodes. Stimulating

electrodes were placed 100–500 µm lateral from the recorded neuron, and electrical stimuli (5–40 V with a 100–150 µs duration) was applied at 0.2 Hz unless otherwise noted. Paired-pulse ratios are defined as $EPSC_2/EPSC_1$. AMPA/NMDA ratios were calculated as the ratio of the magnitude of the EPSC at +40 mV at 50 ms following stimulation (NMDA) to the peak of the EPSC at –70 mV (AMPA) similar to the approach used in [148]. Recording electrodes were filled with (in mM) Cs^+-gluconate (135), NaCl (5), HEPES (10), EGTA (0.6), ATP (4), GTP (0.4), pH 7.2, 290–295 mOsmol. Signals were acquired through a Multiclamp 700B amplifier (Axon Instruments), digitized and analyzed through pClamp 10.2 software (Axon Instruments). Input resistance, holding current, and series resistance were all monitored continuously throughout the duration of experiments. Experiments in which changes in series resistance were greater than 20% were not included in the data analysis.

RESULTS

Chronic drinking increases CaMKII pathway in the amygdala

In our initial experiment, we sought to identify changes in the amygdala proteome following chronic alcohol consumption using the standard 2-bottle choice procedure. Male C57BL/6J mice (n = 40) consumed 10% alcohol (v/v) or water for 28 days. We selected a subset of alcohol-drinking mice (n = 24) that drank alcohol consistently throughout the experiment, demonstrated a preference for the alcohol solution compared to water (alcohol preference = 75%), and voluntarily consumed large amounts of daily alcohol (averaged 11.55 g/kg per day) **(Figure 1a)**. Tail bloods were taken at 2 time points to measure BACs: at the beginning of the dark cycle

BACs were not different then water drinking mice and 6 hours into the dark mice obtained moderate blood alcohol levels (BACs = 39.1 mg/dl) **(Figure 1b).** On the 28th day, at the start of the light cycle when there were no detectable BACs **(Figure 1b)**, differences in corticosterone levels or differences in withdrawal and anxiety measures (unpublished data), amygdala tissue from water and alcohol drinkers was collected and compared using 2D-DIGE.

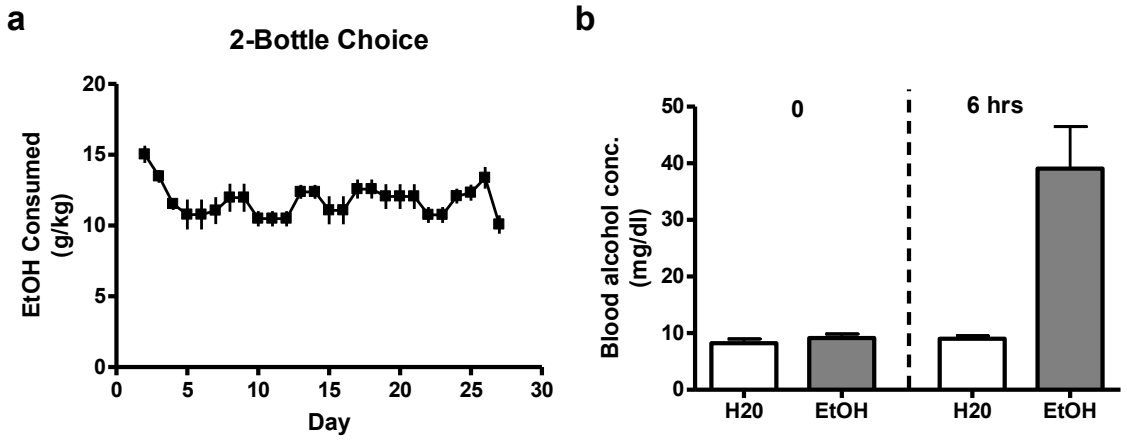
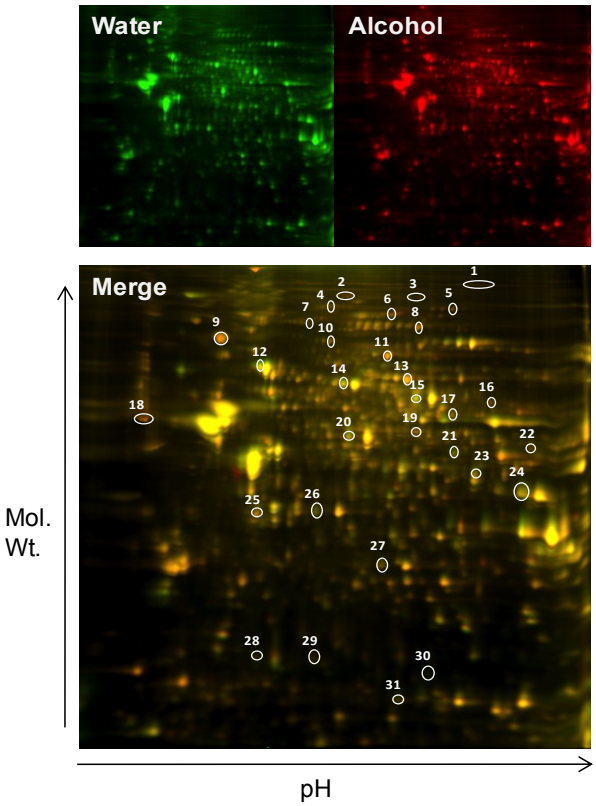


Figure 1: The 2-bottle choice procedure. C57BL/6J mice selected for the proteomics experiment drank 10% alcohol (v/v) for 28 days (a) showing consistent levels of alcohol consumption (mean =11.55 g/kg per day). (b) Mice demonstrated a pattern of drinking where and no detectable levels at the start of the dark cycle and they showed moderate blood alcohol levels (39.1±7.08 mg/dl) 6 hours into dark cycle.

A total of 28 proteins met *a priori* criterion for identification and 27 of them were successfully identified using mass spectrometry (MALDI TOF/TOF) (**Figure 2b**). Due to our initial hypothesis that alcohol consumption alters plasticity-related proteins, our focus shifted to the significantly increased expression (+35%) in CaMKIIα ($F(1,7) = , p = 0.0048$) (**Figure 3**), a regulator of synaptic strength as well as other identified proteins that regulate its local synthesis including kinesin heavy chain 5C (KIF5C) (+35%, p = 0.004, t-test), [149], a retrograde transporter of CaMKIIα mRNA, and eukaryotic elongation factor 2 (EEF2) (+31%, p = 0.0009, t-test) which locally

translates CaMKIIα mRNA [150] (**Figure 4**). Additionally, N-ethylmaleimide sensitive factor (NSF), a postsynaptic stabilizer of AMPA receptors [151] was increased (+45%, p = 0.0051, t-test) and, collectively, this network of proteins suggests an enhancement of glutamatergic signaling in the amygdala following chronic alcohol consumption.

a



b

	Gene ID	Spot #	p Value	Change
Alcohol Metabolism				
Aldehyde dehydrogenase	Aldh1	7	0.0052	1.26
Malate dehydrogenase, cytosolic	Mdh1	26	0.0007	-1.31
Glycolysis				
Hexokinase, type 1	Hk1	6	0.0052	1.72
ATP Citrate Lyase	Acly	5	0.002	1.42
Adenylate kinase	Ak1	28	0.00083	-1.28
Phosphoglycerate mutase 1	Pgm	27	0.021	-1.32
Aldolase 1, A isoform	Aldoa	24	0.0017	-1.35
Cytidine monophosphate (UMP-CMP) kinase 1	Cmpk1	29	0.016	-1.35
Fumarate hydratase	Fh1	23	0.021	-1.41
Enolase 1	Eno1a	20	0.012	-1.45
Mitochondrial				
Mitofilin	Immt	11	0.01	1.45
Leucine-rich ppr motif containing protein	Lrppc	2	0.044	1.43
Chaperone Proteins				
Chaperonin subunit 7	Cct7	16	0.003	1.34
Heat shock protein 90 beta	Hsp90	9	0.00088	1.29
Heat shock protein 70	Hsp70	12	0.004	-1.42
Development/Plasticity				
N-ethylmaleimide sensitive fusion protein	Nsf	10	0.0051	1.45
Syntaxin binding protein 1A	Stxbp1	13	0.0022	1.44
Kinesin heavy chain 5C	Kif5c	4	0.004	1.35
Calcium/calmodulin dependent kinase 2 alpha	Camk2a	19	0.0038	1.35
brain abundant, membrane attached signal protein 1	Basp1	18	0.0084	1.33
Eukaryotic elongation factor 2	EeF2	8	0.0009	1.31
Adenylate cyclase-associated protein 1	Cap1	17	0.0096	-1.27
Dihydropyrimidinase-like 2	Crmp2	14	0.024	-1.29
G-protein beta 2 subunit	Gnb2	25	0.0007	-1.31
Down syndrome cell adhesion like protein	Dscam	15	0.014	-1.33
Truncated platelet-activated factor 1	Lis1	21	0.034	-1.39
Cannabanoid receptor interacting protein 1A	Crip1a	30	0.0012	-2.03

Figure 2: Proteomics identification of altered protein expression following alcohol consumption. Amygdala tissue from each group was labeled, combined and ran on 4 gels. (a) Representative gel showing protein staining in water (Cy2, green), alcohol (Cy3, red), and merged image with protein spots exhibiting differential protein abundance between groups numerically labeled (white circles). Molecular weight decreases from top to bottom and isolelectric point increases from left to right. (b) A list of proteins categorized by function shows corresponding spot on gel, significance level (p-value, t-test) and fold change.

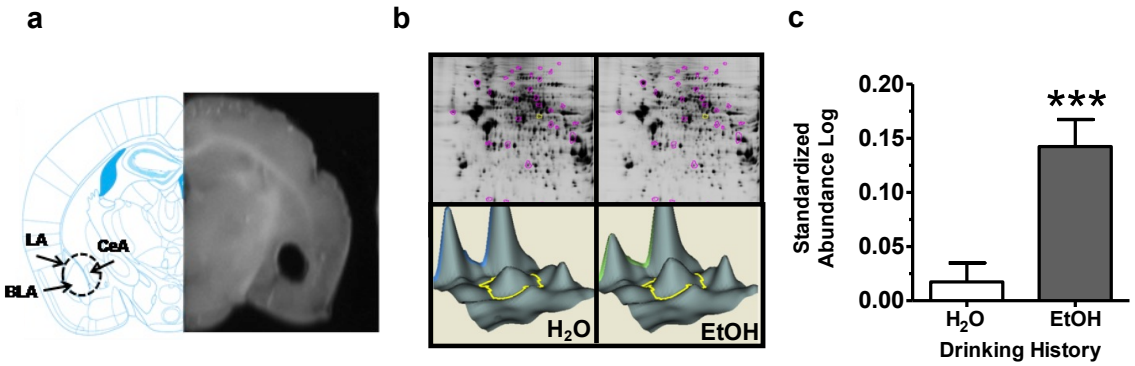


Figure 3: Proteomics analysis revealed increased CaMKIIα in the amygdala of alcohol-drinking mice. (a) Image taken of an amygdala punch (right) used in proteomics experiment with amygdala subreginos identified (left). (b) Top panel shows representative gels of water (left) and alcohol (right) conditions depicted in black and white 2D gels of amygdala tissue with CaMKIIα spot circled in yellow. Other proteins meeting criterion for selection were circled in pink. Lower panels illustrate 3D rendition of CaMKIIα flourescence, indicative of protein abundance, between groups and demonstrating increased levels in alcohol-drinking group. (c) Graph of CaMKIIα protein abundance revealed by 2D DIGE analysis, *** $p < 0.005$, t-test.

Alcohol increases CaMKIIα, not CaMKIIβ in the amygdala

To validate findings from the proteomics study, additional two-bottle mice (n = 28) were ran and tissue was collected using similar methods for western blot analyses to confirm protein changes. To increase our chances at replicating our results, we selected mice (n = 14) that matched the drinking patterns, preference and volumes as the proteomics experiment (12.85±0.9 g/kg daily, 69.5±3.0% preference). Immunoblots confirmed that CaMKII α (+60%, $p = 0.0154$, t-test), and not CaMKIIβ, had increased expression in the amygdala of alcohol drinking mice (**Figure 4a,b**). Levels of p-CaMKII$_{thr286}$ and p-GLUR1$_{ser831}$ were elevated (+33% and +52%, respectively) in alcohol drinkers, but not significantly different between groups (**Table 1**). Additionally, NSF was probed and shown to have increased expression in the amygdala of alcohol-drinking mice (+41%, $p < 0.049$, Student's t-test) (**Figure 4c)**. To determine if increased CaMKII expression is specific to the amygdala, the nucleus accumbens (NAc), striatum (STR), and motor cortex (MCTX) were probed

for CaMKIIa expression. The NAc demonstrated increased CaMKIIα ($p = 0.026$, t-test), but not CaMKIIβ expression, and alternatively, the PFC, STR, and MCTX did not exhibit changes in either CaMKII protein levels **(Table 1).** Our data demonstrate that alcohol-drinking increased CaMKIIα expression in brain regions that form the greater amygdala (a region known to regulate reward and associative memory processing) compared to brain regions involved in motor activity. As a result, these effects are likely to have functional consequences related to alcohol consumption.

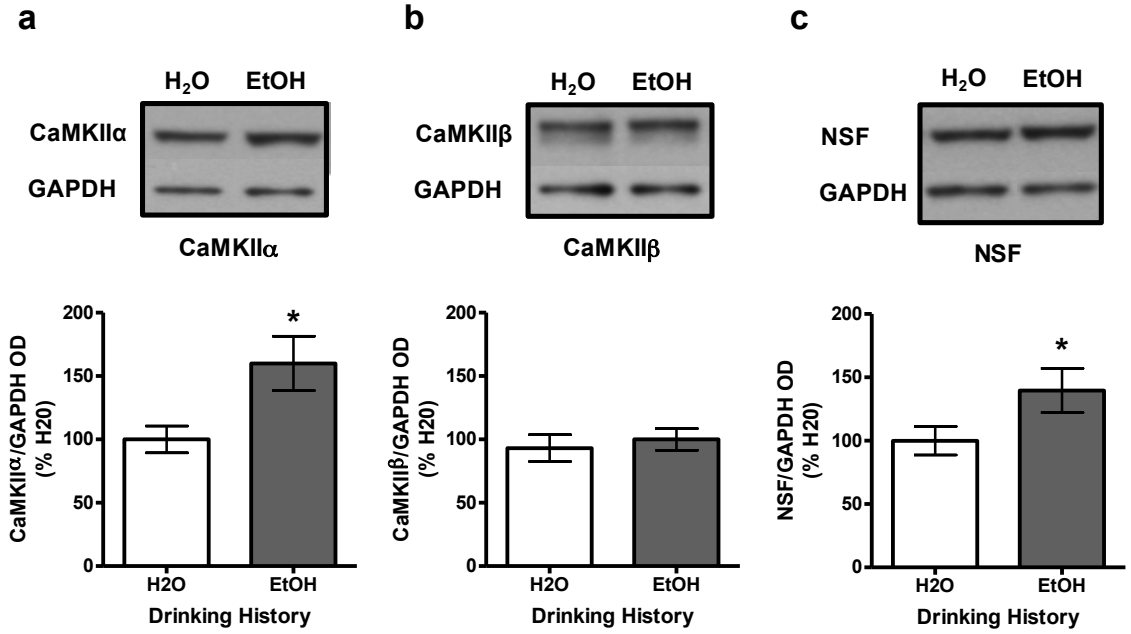


Figure 4: Western blot analyses was used to confirm altered protein expression seen in proteomics experiment. Comparison of amygdala tissue from water and alcohol drinking mice demonstrated increased CaMKIIα and NSF expression (a,c) but not CaMKIIβ (b) expression in alcohol-drinking mice as evidenced by representative blots with optical density (OD) measurements below that were calculated as loading control (GAPDH) ratio and as the percentage of their levels in water-drinking mice. *, $P < 0.05$, Student's t-test.

Table 1: Western blot results

		H2O	EtOH
PFC	CaMKIIα	100 ±4.3	101.6 ±10.2
	CaMKIIβ	100 ±5.6	94 ±4.7
NAC	CaMKIIα	100 ±4.3	**136.6 ±10.8***
	CaMKIIβ	100 ±10.2	142 .8 ±33.0
STR	CaMKIIα	100 ±3.0	108.3 ±9.5
	CaMKIIβ	100 ±3.8	109.2 ±12.0
AMY	CaMKIIα	100 ±10.5	**160.0 ±21.4***
	CaMKIIβ	100 ±13.2	96.2 ±14.0
	pCaMKII	100 ±15.4	132.9 ±37.8
	pGLUR1	100 ±20.3	152.0 ±35.1
	NSF	100 ±11.3	139.6 ±17.4
MCTX	CaMKIIα	100 ±11.9	120.1 ±20.3
	CaMKIIβ	100 ±11.4	114.4 ±11.4

*, $p < 0.05$, Student's t-test

Increased CaMKIIα and p-GluR1 in specific subregions of the amygdala

The amygdala is a heterogeneous structure comprised of several subregions with diverse projections and neuronal subtypes. Accordingly, they are known to mediate different aspects of reward-seeking behavior. Using immunohistochemistry, our first goal was to determine if increased CaMKIIα expression in the amygdala showed subregional specificity. Second, despite a lack of p-GLUR1 $_{Ser831}$ effect found in western blot analysis, we observed increased expression, but not significant in alcohol-drinking mice. Because tissue punches lack regional specificity and likely include some neighboring brain region tissue, we examined p-GLUR1 $_{Ser831}$ expression using immunohistochemistry to reveal subregional effects. Tissue was collected from an additional group of 2-bottle mice that exhibited similar drinking patterns alcohol preference and fluid consumption as prior experiments.

Immunoperoxidase staining and quantification revealed that CaMKIIα IR (CaMKIIα positive cell counts per mm^2) was significantly increased in the CeA ($p = 0.011$, t-test) and the LA ($p = 0.045$, t-test) (**Figure 5**), but not the BA substructures of alcohol-drinking mice. In addition, p-GluR1$_{Ser831}$ IR (positive pixels per mm^2) was increased in the CeA of alcohol drinking mice, but not the LA, or BA (**Figure 6**).

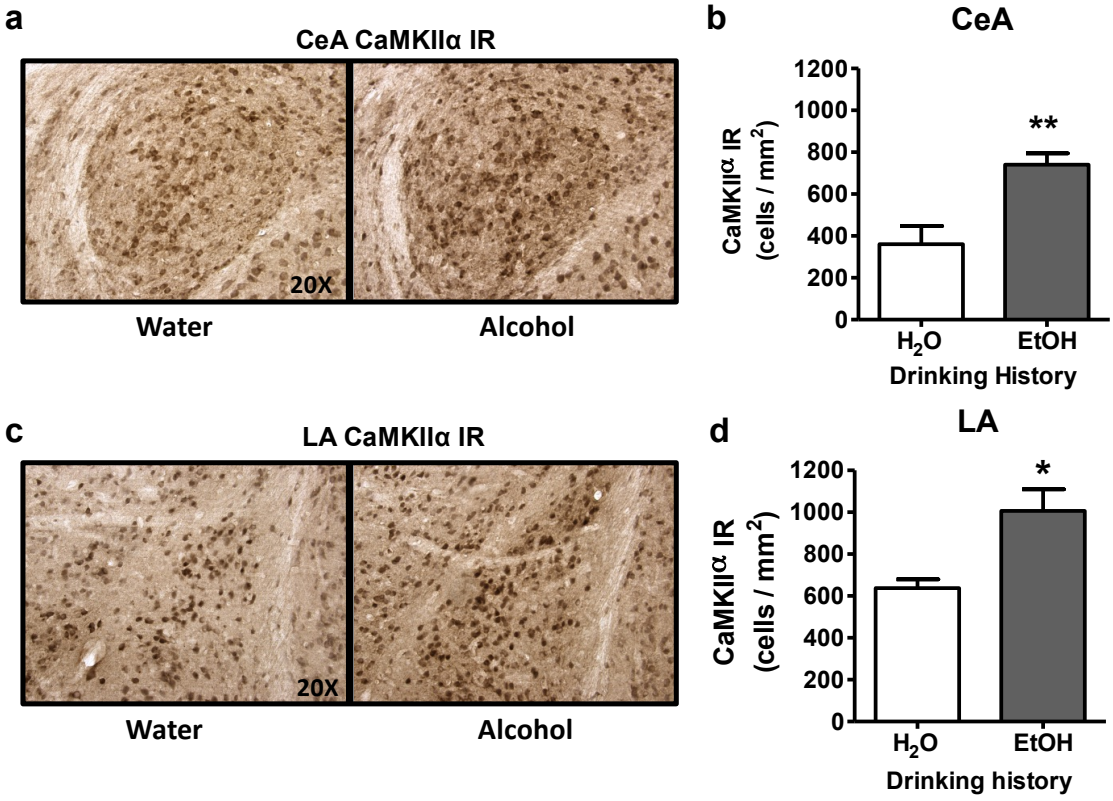


Figure 5: CaMKIIα immunohistochemistry reveals increased expression in specific amygdala subregions. (a, c) Representative micrographs (20X) demonstrating that chronic alcohol consumption increased expression of CaMKIIα in the CeA and LA. (b,d) Immunoreactivity (IR) was measured as positive cells per mm² and quantified in subregional fields. Significant increases were seen in the CeA (b) and LA (d) of alcohol-drinking mice. *, $p< 0.05$, **, $p < 0.01$; Student's t-test.

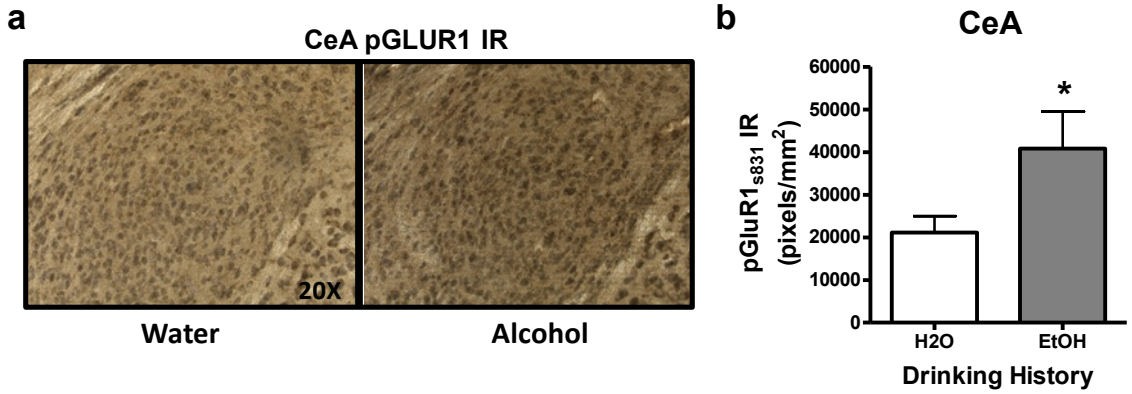


Figure 6: p-GLUR1$_{Ser831}$ immunohistochemistry reveals increased expression in the central amygdala (CeA). (a) Representative micrographs (20X) depicting expression pattern of p-GLUR1$_{Ser831}$ in the CeA water and alcohol-drinking mice. (b) Immunoreactivity (IR) was measured as positive pixels per mm² and quantified in subregional fields. Significant increases were seen in the CeA of alcohol-drinking mice. *, p< 0.05; Student's t-test.

Alcohol drinking led to increased glutamatergic transmission in CeA

Growing evidence indicates that chronic alcohol exposure alters synaptic activity in the sub-regions of the amygdala [43, 60, 121, 152]. Evidence is lacking, however, regarding the influence of chronic voluntary alcohol self-administration. Given, the observed alcohol-induced increases in CaMKIIα and the known role of these proteins in excitatory synaptic activity [153], we predicted that a history alcohol drinking would increase AMPAR-mediated synaptic activity in the CeA. Additional alcohol and water drinking mice under the same two-bottle drinking protocol (n=6/group) were used. [154, 155]. Alcohol-drinking mice showed a significantly higher AMPA/NMDA ratio compared to water drinking controls ($p < 0.005$, Student's t-test) (**Figure 7a,b**). When taken together with the observed increase in p-GluR1$_{Ser831}$, this result suggests that chronic voluntary alcohol drinking increases synaptic strength/AMPAR function in the CeA. Since exposure to chronic ethanol vapor increases ethanol-induced glutamate levels in CeA [60], we measured

response to paired pulses as an index of the probability of transmitter release [156]. Paired-pulse ratio was not different between alcohol (1.293±0.19) and water controls (1.45±0.13) (**Figure7c**), a result that suggests alcohol drinking did not alter probability of transmitter release. We also examined stimulated EPSCs (sEPSC) to further characterize potential synaptic sites of action. Alcohol drinking mice showed an increase in sEPSC frequency ($p < 0.01$, Student's t-test) (**Figure 7d,e**) but no change in amplitude (alcohol: 21.4±1.2, water: 21.0±1.3) (**Figure 7f**). An increase in the frequency of sEPSCs in the absence of change in paired-pulse ratio has been interpreted to reflect a potential increase in the number of functional AMPARs at previously silent post-synaptic synapses [155, 157]. Combined with results shown above, these results suggest that voluntary drinking leads to post-translational adaptations in AMPAR receptor activation (e.g., phosphorylation at $GluR1_{Ser831}$, the CaMKII phosphorylation site) that are associated with increased AMPAR-mediated synaptic activity in the CeA.

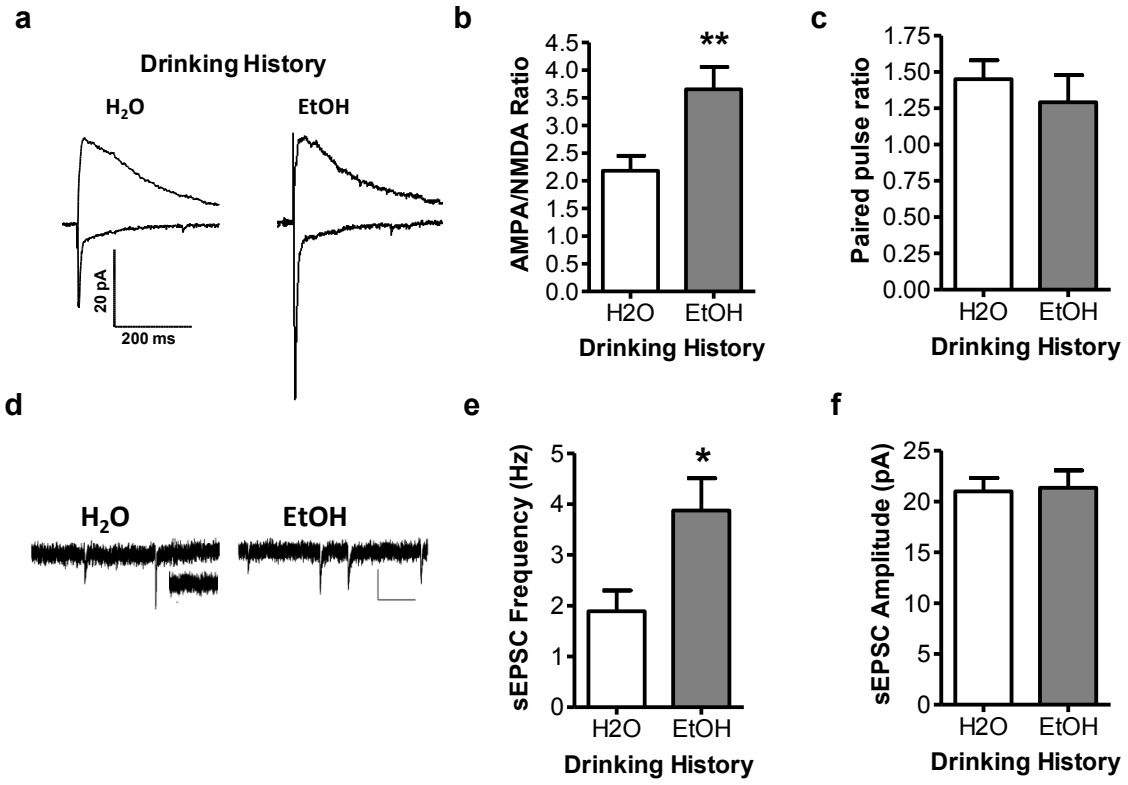


Figure 7: Electrophysiological properties of central amygdala neurons. (a) Representative tracings of AMPA and NMDA currents taken from whole cell patched central amygdala in water and alcohol drinking mice. (b) Alcohol drinking mice show increased AMPA/NMDA ration compared to water drinking mice, but without an changes in PPR (c). (d) Representative tracings of sEPSCs from each group demonstrating increase in sEPSC frequency, but not amplitude and quantified in (e) and (f) (*, p<.05; **, p < 0.01; Student's t-test).

DISCUSSION

An understanding of the molecular mechanisms by which alcohol use causes long-term changes to reward-related neurocircuitry may lead to novel pharmacotherapeutic interventions for addictive behaviors. Here, we demonstrate using multiple techniques that expression of the protein kinase CaMKIIα is increased in the amygdala, particularly the CeA, following chronic alcohol self-administration. As CaMKII is known to have a requisite role in many forms of synaptic plasticity [69], we measured electrophysiological properties of CeA neurons and found they

exhibited an increased AMPA/NMDA ratio in alcohol-drinking mice in agreement with evidence that CaMKII promotes the delivery of AMPARs to the synapse [64].

In our initial screen, we identified 26 proteins exhibiting differential protein abundance with an unbiased proteomics approach in the amygdala of alcohol- compared to water-drinking mice. An intriguing subset of these proteins included CaMKIIα and related proteins implicated in CaMKIIα translation and AMPA receptor trafficking. This subset comprised KiF5C, a transport molecule that shuttles cargos that include CaMKIIα and GluR2 subunits to the PSD [158], EEF2, an elongation factor that, when phosphorylated, participates in direct CaMKIIα mRNA translation [150] and NSF, an ATPase that binds to GluR2 and stabilizes surface expression of AMPA receptors in the synapse [159]. Collectively, these proteins represent potential cellular machinery for increasing CaMKII expression and the insertion and stabilization of AMPA receptors in the synapse. Their concomitant increase in amygdala expression following voluntary alcohol consumption points to an enhancement of glutamate transmission. In addition to this subset, previous proteomics studies on brains of human alcoholics revealed several homologous proteins that we identified including Aldh1, Crmp2, Gnbp, Immt, Eno1a, and Hsp70 [160], suggesting that our findings may translate to the human condition. Our largest change was a 2-fold decrease in the expression of cannabanoid interacting protein 1A (Crip1a), a relatively understudied protein which has been suggested as a novel target for substance abuse disorders [161] through its actions on CB1 activity, a receptor known to affect alcohol consumption [162].

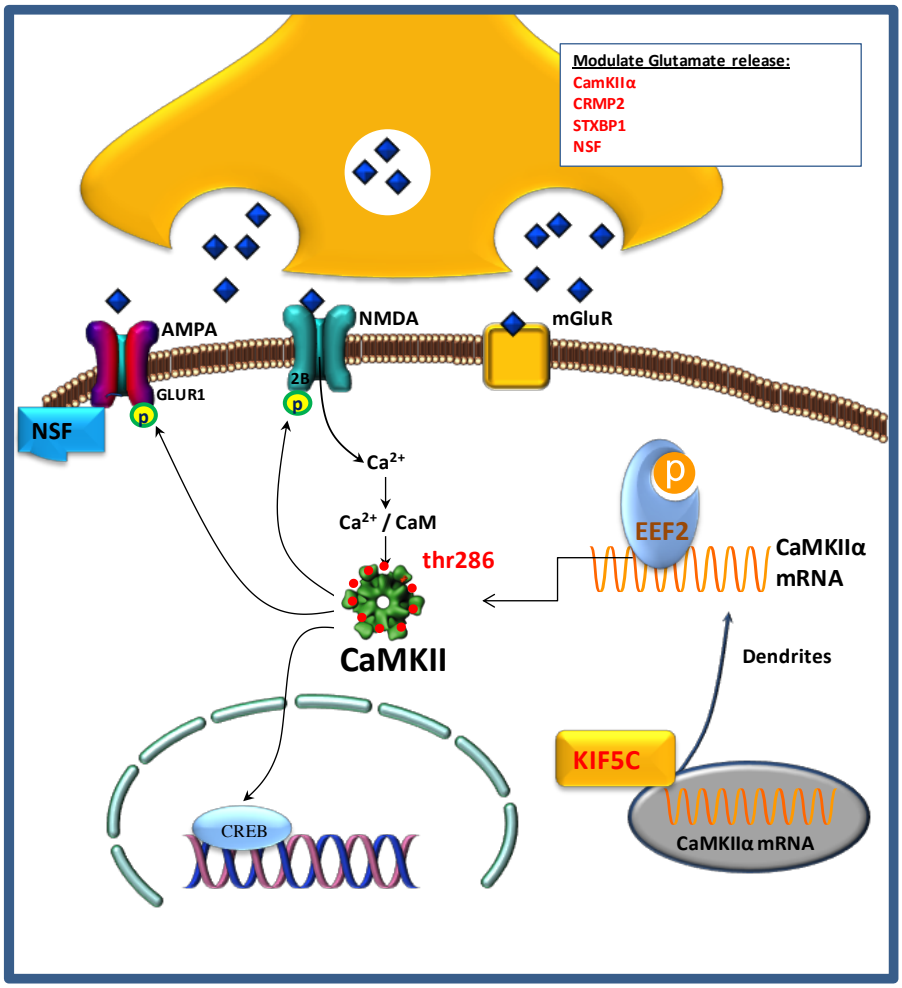


Figure 8: Schematic depicting glutamatergic synapse and relation to proteins identified by 2D-DIGE as being upregulated in the amygdala. CaMKII is activated following increased levels of calcium (Ca^{2+}) and the binding of the calcium/calmodulin (Ca^{2+}/CaM) complex. Once phosphorylated at its thr286 site, CaMKII has multiple actions, including phosphorylation of several substrates involved in plasticity including NR2B NMDA subunit, GLUR1 AMPA subunit, and CREB, following translocation to the nucleus. In addition to CaMKIIα, proteins involved in its synthesis were also upregulated by alcohol including KIF5C, which transports CaMKII mRNA and AMPAR subunits towards dendrites, and EEF2, which directly participates in local CaMKIIα translation. NSF plays a role in stabilizing AMPARs in the synapse. In addition to postsynaptic actions, several identified proteins affect presynaptic glutamate activity (CaMKIIα, CRMP2, STXBP1, NSF).

We focused our efforts on CaMKII because it is an ideal molecular candidate for initiating and sustaining maladaptive plasticity following drug exposure. The CaMKII pathway is affected by many drugs of abuse and has been proposed as a downstream converging point for multiple neurotransmitter systems [85]. In addition, it regulates surface expression of inhibitory and excitatory neurotransmitter receptors

[163, 164] as well as phosphorylating transcription factors associated with long-term neuroplasticity and synapse maturation including CREB and NeuroD [68, 165]. Previous work has identified increased CaMKII expression in the nucleus accumbens following cocaine and morphine exposure [166, 167]. However, much less is known about how drug exposure affects CaMKII expression in the amygdala, and only a few studies overall have tested how alcohol affects CaMKII expression [86]. Therefore, we sought to further characterize the effect of chronic alcohol consumption on CaMKII expression in multiple brain regions using immunochemical methods. We found that total expression of CaMKIIα, but not CaMKIIβ, was increased in the amygdala and NAc of alcohol-drinking mice, but not the striatum or motor cortex. Increased CaMKIIα compared to CaMKIIβ expression found in these regions has been reported following increased synaptic activity and learning [168]. To determine CaMKII activity, we probed for $pCaMKII_{Thr286}$ and $pGLUR1_{Ser831}$ and although we saw increased protein levels in alcohol-drinking mice, these changes were not significant using western blot analysis. Additionally, we confirmed that the ATPase NSF was increased in the amygdala of alcohol-drinking mice as found in the proteomics experiment. In addition to AMPAR stabilization, NSF is involved in GABAR endocytosis and presynaptic vesicular release [169, 170] which may contribute to the increased electrophysiological activity observed in the amygdala. Finally, our method for collecting amygdala tissue was not specific to amygdala subregions, so we used immunohistochemistry and found that CaMKIIα is increased in the CeA and LA, with the largest change occurring in the CeA. In addition, we determined that $pGluR1_{Ser831}$ is increased in the CeA of alcohol-drinking mice.

Adaptations of glutamate transmission are a consequence of exposure to many drugs of abuse including alcohol. Behaviorally relevant doses of alcohol in acute preparations inhibit glutamate receptors with NMDARs being primarily affected in the brain regions that have been studied [37]. Alternatively, chronic exposure to alcohol has been widely shown to result in hyperglutamatergic adaptations throughout the brain. Compensatory increases in NMDA subunit expression and subsequent sensitization to NMDA agonists have been reported in many limbic structures including the amygdala [43]. Following prolonged exposure to alcohol vapor, the CeA shows increased expression of NMDA subunits as well as increased glutamate release in response to acute alcohol [43, 60, 171, 172]. Our finding that chronic alcohol consumption increases synaptic response in the CeA as indicated by increased sEPSC frequency and AMPA/NMDA is consistent with findings that chronic alcohol exposure enhances glutamate in the CeA. However, our specific findings that AMPA currents are increased relative to NMDA currents and that there were no detectable changes in PPR differ from previous reports in the CeA [60, 171] and BLA [121, 173]. This may be explained by the increased BACs seen in these studies or may be due other methodological issues. A key difference in exposure methods is that these studies use forced alcohol exposure and which has inherent motivational differences compared to voluntary alcohol consumption. Additionally, the unique circumstance that acute alcohol inhibits AMPAR EPSCs in the CeA as opposed to what has been reported in the BLA may lend itself to adaptive increases in AMPAR function [173]. Similar findings to our own have been reported in the VTA following voluntary alcohol consumption in rats, where increased AMPA/NMDA ratio

and spontaneous and miniature EPSC frequency were found in the absence of alterations in EPSC amplitude or PPR [174]. To address these discrepancies, direct comparisons of voluntary and involuntary alcohol exposure would need to be accomplished possibly through intra-catheter self-administration of alcohol and yoked controls, however this would be difficult to accomplish. Lastly, it is important to note that we did not establish a clear link between increased CaMKII activity and increased AMPA currents in the CeA, however, previous research has shown that CaMKII inhibition decreases NMDA dependent synaptic insertion of AMPA receptors [175].

In summary, voluntary drinking upregulated CaMKII and a network of proteins involved in its synthesis as well as the functional properties of amygdala neurons. These initial adaptations are likely to contribute to increased excitatory transmission through plastic changes in glutamatergic synapses that causes the hyperglutamatergic state seen in alcohol dependence. Disrupted glutamate homeostasis has been viewed as an adaptation that underlies the loss of control over drug-seeking seen in addicts [176]. New therapies are being developed to reverse synaptic glutamate including targeting pre- and postsynaptic mGLuRs, which can decrease neuronal glutamate release and receptor activity, as well as targeting cysteine-glutamate exchange proteins on glia like GLT1 which regulate extracellular glutamate levels [176]. Both of these approaches have been successful in reducing drug-seeking in preclinical models [177-180]. A second strategy is to regulate glutamate receptor activity through intracellular mechanisms, like the modulation of CaMKII, which has had its own success in reducing drug-seeking in

animal models [85]. A potential drawback of targeting CaMKII is that it regulates a wide array of cellular functions and could likely lead to undesirable side effects. While additional research is needed to better understand CaMKII's role in addiction, the results presented here indicate that it may regulate alcohol-related behaviors.

CHAPTER III: CAMKII ACTIVITY FUNCTIONALLY REGULATES ALCOHOL REINFORCEMENT

INTRODUCTION

The development of alcohol addiction is a complex multiphasic process that is characterized initially by repeated intoxication episodes that give way to compulsive drug intake and later stages of addiction that include dependence/withdrawal syndromes [181]. Contemporary theories of addiction suggest that alcohol produces maladaptive changes in molecular cell signaling pathways that lead to enduring changes in the function of specific neural circuits, such as the mesocorticolimbic system [182-184]. Accordingly, these drug-induced adaptations are thought to regulate behavioral pathologies that occur in alcoholism [57, 185, 186]. In spite of these widely held views, the extent to which initial alcohol use during the intoxication stage produces functionally significant changes in molecular signaling systems, and whether these neural targets of alcohol regulate motivation to consume the alcohol, remain to be fully characterized.

Emerging evidence has established that CaMKII activity is altered by drugs of abuse and that it plays a role in addictive behaviors [57]. CaMKII is a family of Ca^{2+}-activated Ser/Thr protein kinases that mediates many intracellular responses in the brain including regulation of membrane current, neurotransmitter synthesis and release, cytoskeletal organization, gene expression, and synaptic plasticity

[64, 187, 188]. CaMKII is activated when neuronal depolarization leads to Ca^{2+} entry into the cell through multiple sources including ionotropic glutamate receptors, L-type voltage-gated calcium channels, and via release from internal stores following G protein receptor activation. Following activation, CaMKII can translocate to the membrane and/or postsynaptic density where it regulates receptor (i.e., NMDA, AMPA) activity [142]. Thus, the CaMKII pathway is a candidate molecular mechanism for the drug-induced neurodaptations thought to contribute to addictive behaviors.

A number of targets of CaMKII, including NMDA and AMPA receptors, PSD proteins, CREB, BDNF, and the MAPKs are known to regulate alcohol-related behaviors including self-administration and relapse (e.g., [186, 189-193]). Thus, CaMKII may represent a molecular point of convergence in the dynamic regulation of maladaptive behaviors associated with alcoholism, but it is unknown if CaMKII functionally regulates these critical behaviors. In view of this concept, research suggests that the development of addiction involves dysregulated glutamate transmission in neural circuits that regulate normal adaptive functions of the organism [176, 194-196]. These ideas are supported by numerous studies showing dependence-induced changes in glutamate-mediated biochemical, physiological, and behavioral functions (e.g., [59, 60, 121, 197-200]). However, it remains a significant goal for research to identify molecular and cellular adaptations in glutamate systems induced by initial alcohol use that mechanistically regulate self-administration behavior.

In chapter II, we found that CaMKII expression is increased in the CeA following alcohol consumption. In agreement with CaMKII's actions on glutamate receptor trafficking and synaptic plasticity [201], we found that alcohol drinking increased the AMPA/NMDA ratio in CeA neurons. The amygdala is a heterogeneous set of nuclei consisting of several anatomically and functionally distinct structures. The lateral structures (LA and BLA) appear cortex-like and send major glutamatergic projections to the CeA, which consists primarily of striatum-like GABAergic projection neurons [202]. Processing of primary reinforcement is directed via the CeA and lateral structures regulate associative learning [203, 204]. Accordingly, current evidence indicates that the CeA is an integral part of the neural circuitry that underlies the intoxication/binge stages of addiction, which involve a major influence by positive reinforcement mechanisms [181]. For example, amygdala activity correlates with craving in alcoholics [33] and a variety of transmitter systems in the CeA have been shown to regulate alcohol reinforcement in rodent models [118, 205-208]. Interestingly, electrolytic lesions of the CeA reduced limited-access alcohol drinking (two-bottle procedure) in C57BL/6J mice but had no effect in mice that were made dependent on alcohol via vapor inhalation [209], providing strong support for involvement of the CeA in the pre-dependent stage of alcohol addiction. It is unknown if CaMKII activity in the amygdala regulates alcohol-seeking behavior.

To address this question, we first examined adaptations in CaMKII activation (phosphorylation) in the CeA following operant alcohol vs. sucrose self-administration. We found that operant self-administration of sweetened alcohol

elevated levels of p-CaMKII$_{thr286}$ and its receptor target p-GLUR1$_{ser831}$ in the CeA and LA. Enhanced glutamate activity in the amygdala has been shown to occur following alcohol exposure [43, 121] and play a role in alcohol-related behaviors including conditioned-place preference [171] and increased anxiety-related behavior during withdrawal from chronic alcohol exposure [121]. Due to CaMKII's effects on glutamate activity, we hypothesized that increased CaMKII activity plays a role in alcohol self-administration and influences operant self-administration of alcohol differently than sucrose. Therefore, we performed mechanistic studies to evaluate regulation of operant alcohol self-administration behavior by CaMKII in the amygdala. We found that inhibition of CaMKII phosphorylation in the amygdala attenuates alcohol- but not sucrose-self-administration in the absence of spontaneous locomotor deficits. This finding led us to conclude that CaMKII functionally and selectively regulates alcohol reinforcement. A better understanding of how changes in CaMKII signaling regulates behavioral pathologies in alcohol addiction has the potential to lead to new pharmacotherapeutic strategies for the treatment of alcohol addiction.

METHODS

Animals: Male C57BL/6J mice (Jackson Labs, Bar Harbor, ME) were group housed in a colony maintained at 27ºC on a reverse 12 hour light-dark cycle. Experiments were conducted during the dark cycle. Mice were 8-10 weeks at the onset of experiments. Food and water are available ad lib in the home cage unless otherwise noted.

Apparatus: Operant self-administration sessions were conducted in two-lever operant chambers (Med Associates, Georgia, VT). Chambers were interfaced to Windows-based PCs for control of experimental sessions and data recording. Responses on the “active” lever were reinforced by delivery of a liquid solution (0.014 ml) into an adjacent drinking trough. Reinforcement delivery was paired with 4-sec visual (light above the lever) and auditory (pump sound) stimuli. Responses on the “inactive” lever were recorded but produce no programmed consequence. Head entries into the trough were recorded when an infrared photo beam is broken.

Operant self-administration: To facilitate acquisition of the lever-pressing behavior, mice were deprived of fluids for ~20 hours prior to initial training in the operant chamber. The first 3 training sessions lasted 16 hours and began with 5% sucrose (w/v) as the reinforcing solution. During the 1st session, one lever press on the active lever results in a reinforcer delivery (FR1). For the 2nd session, the response requirement is increased from FR1 to FR2 to FR4 following completion of 15 reinforcements at each response requirement and for the 3rd session the response requirement is FR4. The response requirement for all subsequent sessions is FR4 and each daily session is 1 hour. Mice were then trained to self-administer alcohol using a sucrose fading procedure [189] during which the concentration of alcohol was gradually increased from 0% to 9% (v/v) and sucrose is decreased from 5% to 2% (w/v). A minimum of 2 testing sessions were conducted at each concentration. Mice drank a final sweetened alcohol solution of 9% ethanol (v/v) and 2% sucrose

(w/v) for the number of sessions outlined in each experiment. Sucrose mice were trained similarly, but did not have alcohol faded into their solutions.

Immunohistochemistry: Immediately following the self-administration session mice were euthanized with sodium pentobarbital, perfused with first ice cold PBS and then fixed with 4% paraformaldehyde. Coronal sections (40 microns) were collected using a vibratome (Leica) and stored in cryoprotectant (30% glycerol, 30% ethylene glycol in 0.1 PBS) at -20°C. Sections were washed with phosphate buffer solution (PBS), followed by inhibition of endogenous peroxidase activity with 1% H_2O_2, antigen retrival using citra buffer at 70°C, blocked with 5% goat serum in PBS with 0.1% Triton-x (Sigma, St. Louis, MO) and then incubated with primary antibodies (phospho-CaMKII$_{thr286}$ 1:1500 (Abcam), phosphor-GLUR1$_{Ser831}$ or CaMKIIα (Millipore) 1:10,000) overnight in blocking buffer. Positively labeled cells were visualized using DAKO kit (Carpinteria, CA) or mouse on mouse kit (Vector labs, Burlingame, CA) each using DAB as chromagen.

Immunoreactivity was visualized using an Olympus CX41 light microscope (Olympus America, Center Valley, PA). Images were acquired using a digital camera (Regita model, QImaging, Burnaby, BC) interfaced to a computer (Dell, Round Rock, TX). Image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, TN) was used to quantify immunoreactivity. The microscope, camera, and software were background corrected and normalized to preset light levels to ensure fidelity of data acquisition. Pixel density and cell count measurements were calculated from a circumscribed field (e.g., brain region) and

divided by the area of the region and expressed as pixels/mm^2 and cells/mm^2. Data were acquired and analyzed by a researcher blind to group condition from a minimum of 4 sections/brain region/animal and averaged to obtain a single value per subject.

Surgery: At least 24 hours following their 30th operant session, mice were anesthesized with a cocktail of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.), placed in a stereotaxic instrument (Kopf Instruments, Tujunga, CA) and a midline incision was made over the skull using aseptic techniques. Two bilateral holes were drilled in the skull and bilateral injector guide cannulae (26 gauge; Plastics One, Roanoke, VA) were implanted and secured to the skull with dental cement directed. Guide cannulae were aimed 2 mm above their amygdala (A-P: -1.1 mm, M-L: ±3.0 mm, and D-V: -2.5 mm D-L (Paxinos and Franklin, 2008) to prevent damage to the amygdala and allow space for drug diffusion. The wound was treated with bacitracin ointment, and closed with 3-0 silk. Obturators (33 gauge) that extend 0.5 mm beyond the tip of the guide were inserted into the guide cannulae following surgery and were moved daily to prevent cannulae blockage and scarring. Mice were given 4 days to recover from surgery before returning to operant sessions where they had at least 10 sessions before beginning microinjection experiments.

Microinjection Procedure: After lever pressing returned to pre-surgical baseline levels, sham injections were conducted to habituate mice to the microinjection procedure. Here, mice were hand restrained and shortened injectors were inserted into their guide cannulae for 5 minutes prior to sessions and injection pumps were

operated, but no fluid was infused. Mice were given repeated sham injections prior to operant sessions until they demonstrated total response values in 2 consecutive sessions that was equal to their baseline performance. Next, mice were given one microinjection of ACSF prior to an operant session before beginning drug injections that were ordered pseudorandomly and included an additional ACSF microinjection that was used as the control value for each dose response. Injectors were 2.0 mm longer than the cannulae and were directed at the amygdala. ACSF, CaMKII or AMPA inhibitors (see Drugs) were infused (0.5 µl of solution per side injected over 4 minutes) in the amygdala immediately before operant sessions. Mice received no more than 8 injections during the experiment. Following completion of the experiment, mice were fixed using procedures described above and placements were verified visually using a large magnifying glass to identify placements while brains were being sectioned. Experimenters identifying placements were blind to the results.

Locomotor Activity: To determine if drug-induced changes in operant behavior were due to nonspecific motor effects of the drugs, locomotor activity was assessed following operant self-administration testing. Mice were initially habituated to chambers for two hours following a sham injection. Seven and 14 days later, mice were counterbalanced and infused drug (KN-93 (10 ug/per side), m-AIP (1 ug/per side), or ACSF prior to 1 hour locomotor sessions.

Drugs: Cell-permeable CaMKII inhibitors used in these studies were: 1) *KN-93*, 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methyl benzylamine; EMD Biosciences; Gibbstown, NJ), a widely-used inhibitor of CaMKII that selectively binds to the CaM binding site of CaMKII and thereby prevents Ca2+/CaM from activating CaMKII [210]; 2) *m-AIP* (myristoylated autocamtide-2 inhibitory peptide; N-Myr-Lys-Lys-Ala-Leu-Arg-Arg-Gln-Glu-Ala-Val-Asp-Ala-Leu-OH ; Biomol Research Labs, USA), a highly selective and potent cell-permeable inhibitor of CaMKII that selectively inhibits the phosphorylation and activation of CaMKII [211] . This compound binds directly to the calmodulin binding site of the enzyme; 3) *NBQX* (2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[***f***]quinoxaline-7-sulfonamide) a potent inhibitor of AMPA and kainate receptors. Dose ranges for these studies were based on the literature [84, 212-214] and preliminary data. All drugs were dissolved in ACSF prior to test sessions.

Data Analyses: Data from all behavioral and IHC studies were analyzed statistically by t-test or repeated measures analysis of variance (RM ANOVA) where appropriate. Significant main effects of interactions were followed by post-hoc multiple comparisons (Dunnet's) using SigmaStat (SPSS, Chicago, IL, USA).

RESULTS

Operant self-administration increases CaMKII activity

Following chronic alcohol consumption, synaptic strengthening in amygdala neurons may enhance alcohol's reinforcing properties and the salience of alcohol-related cues. In chapter II, we showed that voluntary alcohol consumption increases CaMKII expression and glutamate transmission in the CeA. Here we sought to extend these findings by measuring the active (or phosphorylated) form of CaMKII (p-CamKII$_{thr286}$) and its primary substrate on AMPA receptors GluR1$_{ser831}$ (p-GluR1$_{ser831}$) immediately following operant self-administration in mice (n = 16). Protein expression was compared between groups of mice that self-administered a sweetened alcohol solution (9% alcohol/2% sucrose) and mice that self-administered sucrose alone (2% sucrose), upon completion of their 30th operant session. Importantly, there were no differences between alcohol and sucrose reinforcement groups in total reinforced responding, which includes active and inactive lever responses, percent active lever presses, or reinforcers delivered during operant sessions. These findings control for the potential confound of overall activity (**Figure 9**). On the 30th session, alcohol mice consumed an average of 1.14±0.1 g/kg during the hour long session and had an average BAC of 53.1±11.8.

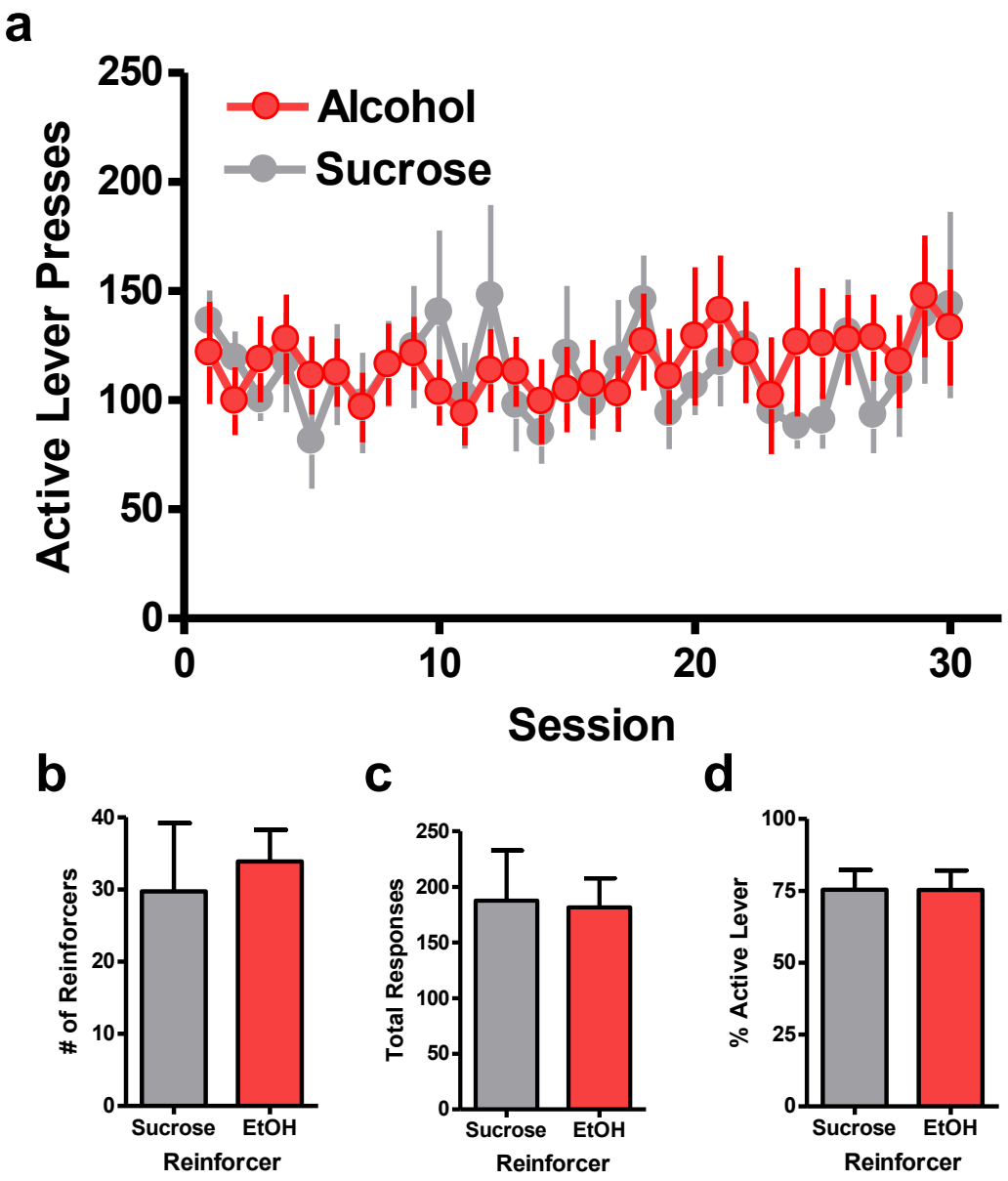


Figure 9: Mice self-administered alcohol or sucrose in operant chambers for 30 sessions following training. (a) Mice in each group pressed the active lever similarily throughout the 30 sessions. In the last operant session, mice from each group received a similar number of reinforcers delivered (b), did not differ on total lever responses (c) or in the percentage of active levers pressed (d) demonstrating similar activity and performance on their last session.

To compare CaMKII activity between alcohol and sucrose mice, we probed for p-CaMKII $_{thr286}$ and p-GluR1 $_{ser831}$ in amygdala sections using immunoperoxidase methods. Results showed that p-CaMKII$_{thr286}$ IR (positive cells per mm^2) was

significantly increased in the CeA ($p < 0.05$, Student's t-test) and LA ($p < 0.05$, Student's t-test), (**Figure 10a-b**) but not BA (data not shown). p-GluR1$_{ser831}$ immunoreactivity (IR) was significantly increased in the CeA ($p < 0.05$, Student's t-test) and LA ($p < 0.05$, Student's t-test), (**Figure 11a-b**) but not BA (data not shown) of alcohol-self administering mice.

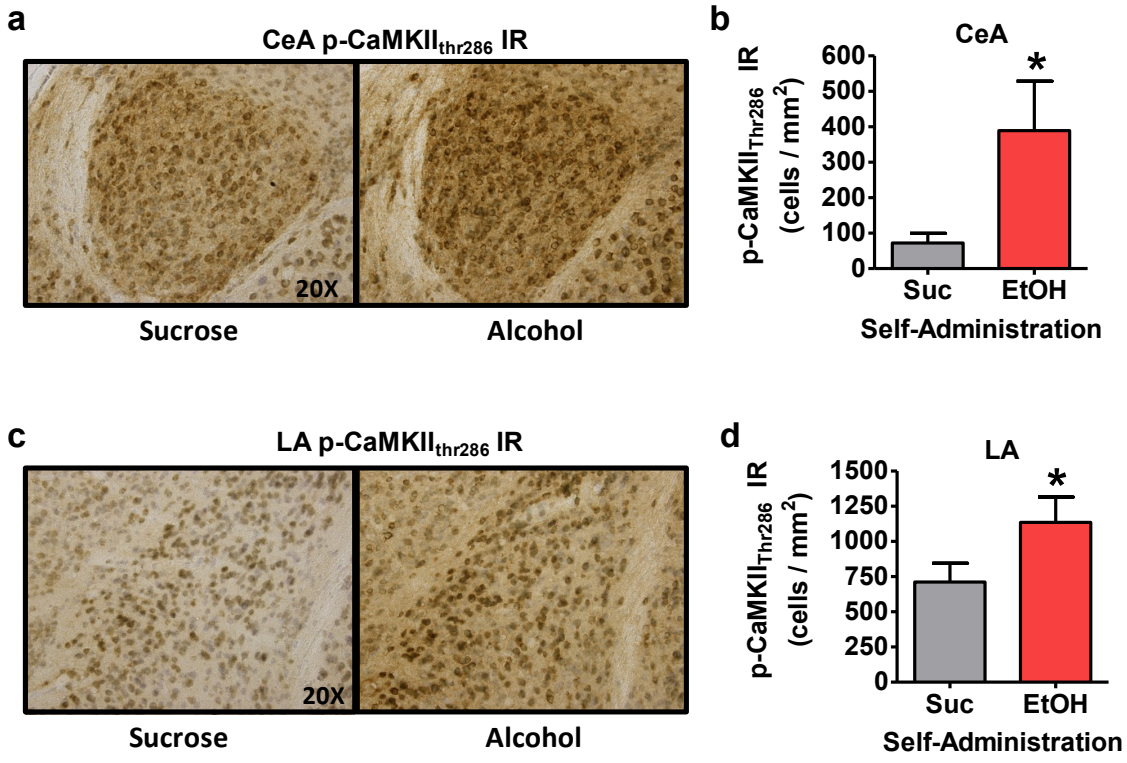


Figure 10: p-CaMKII$_{thr286}$ immunohistochemistry reveals increased expression in specific amygdala subregions. (a, c) Representative micrographs (20X) demonstrating that chronic alcohol consumption increased expression of p-CaMKII$_{thr286}$ in the central amygdala (CeA) and lateral amygdala (LA). (b,d) Immunoreactivity (IR) was measured as positive cells per mm² and quantified in subregional fields. Significant increases were seen in the CeA (b) and LA (d) of alcohol compared to sucrose-drinking mice. *, $p < 0.05$; Student's t-test.

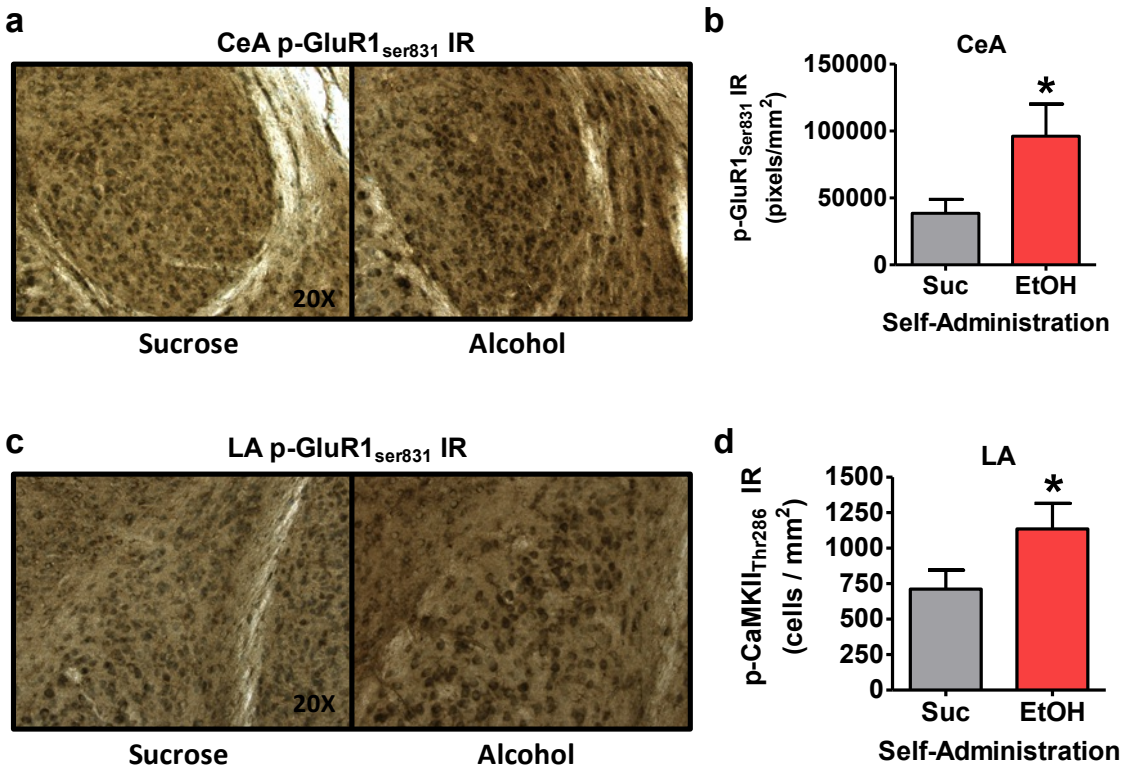


Figure 11: p-GLUR1$_{ser831}$ immunohistochemistry reveals increased expression in specific amygdala subregions. (a, c) Representative micrographs (20X) demonstrating that chronic alcohol consumption increased expression of p-GLUR1$_{ser831}$ in the CeA and LA. (b,d) Immunoreactivity (IR) was measured as positive pixels per mm² and quantified in subregional fields. Significant increases were seen in the CeA (b) and LA (d) of alcohol compared to sucrose-drinking mice. *, p< 0.05; Student's t-test.

CaMKII inhibition in amygdala decreases alcohol reinforcement

The finding that alcohol self-administration increased phosphorylation of CaMKII and its kinase substrate GluR1$_{ser831}$ in the CeA and LA lead us to hypothesize that increased CaMKII activity regulates alcohol reinforcement. To determine the functional role of CaMKII activity, we inhibited calcium-dependent CaMKII phosphorylation using microinjections of the inhibitors KN-93 and a myristoylated version of the autoinhibitory domain of CaMKII (m-AIP) into the amygdala. Of the 13 mice that received surgery, 8 recovered successfully from surgery, completed the experiment and had correct placements (**Figure 15**). We found that the compound KN-93 (10 ug/per side) decreased alcohol reinforced

responding (-35%, p<0.05, paired t-test) without producing significant deficits in locomotor spontaneous activity (p > 0.05, paired t-test) (**Figure 14a**). Due to KN-93's known off-target effects, we tested another CaMKII inhibitor, m-AIP (1.0 ug/per side) and found that alcohol self-administration was decreased (-35%, p < 0.05, paired t-test) to near identical levels as KN-93 (**Figure 12a-b**). Interestingly, there were no overall effects of the CaMKII inhibitors on alcohol reinforced responses from mice with placements outside of the amygdala (data not shown), but alcohol reinforced responding decreased during the last 15 minutes of the session, possibly a consequence of delayed diffusion of the peptide into the amygdala from the distal sites.

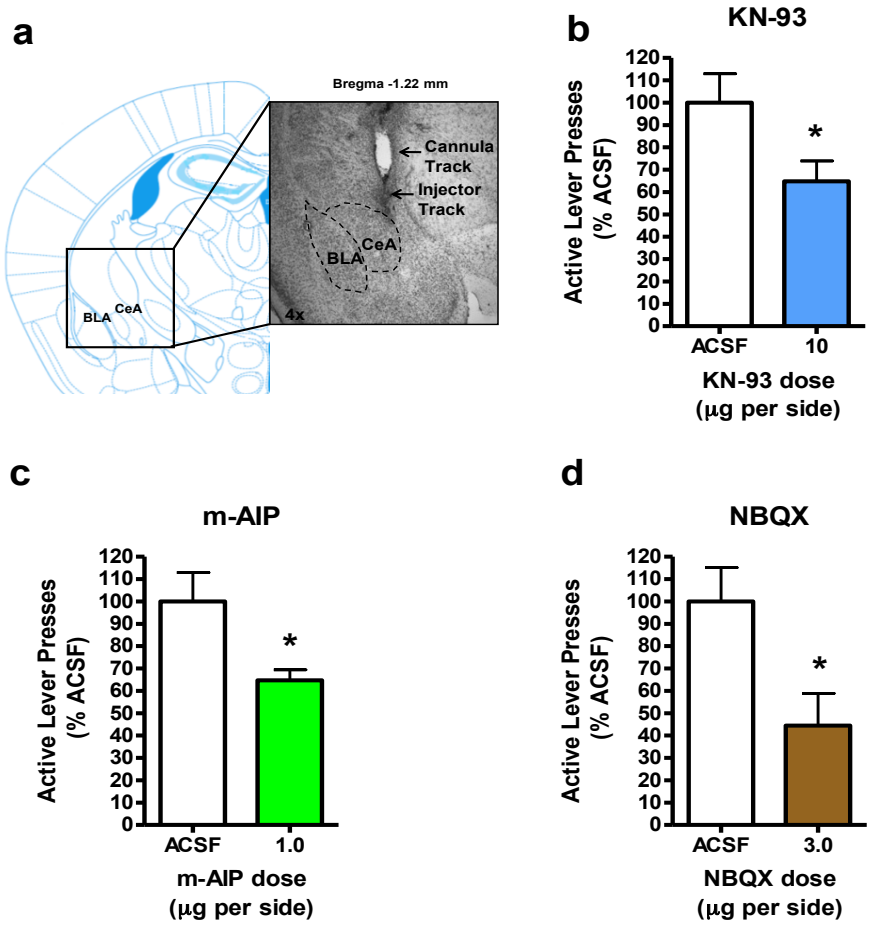


Figure 12: Microinjections of KN-93, m-AIP, and NBQX decrease alcohol self-administration. (a) Representative micrograph of cannula placed in amygdala at Bregma -1.22 mm. Image demonstrates injector placement into the amygdala via guide cannula track and amygdala subregions targeted. Microinjections of the CaMKII inhibitors, KN-93 (10 ug/per side) (b) and m-AIP (1.0 ug/per side) (c), as well as the AMPA/kainate receptor antagonist, NBQX (3.0 ug/per side) all significantly decreased responding for alcohol relative to ACSF microinjction. Data are comprised of mice with correct amygdala placements. *, p< 0.05; paired t-test.

In the amygdala, CaMKII may regulate alcohol self-administration behavior through its role in modulating AMPAR activity. In previous experiments, systemic AMPAR/kainate inhibition was shown to reduce alcohol self-administration, but with the caveat that it reduces locomotor activity [215]. By infusing NBQX, a potent AMPA/kainate antagonist, into the amygdala of 6 of the same mice (n = 6, two mice were removed due to clogged cannulae) that previously showed reductions following CaMKII inhibition, we sought to determine if these receptors regulate alcohol self-administration. Infusion of NBQX (3.0 ug/per side) into the amygdala significantly reduced ($p < 0.05$, paired t-test) responding for alcohol by 56% (**Figure 12d**), a finding indicating a role for AMPARs in the reinforcing effects of alcohol.

These results suggest that the amygdala is a specific neuronal substrate where CaMKII activity regulates alcohol reinforcement. However, these data do not discern whether this effect is specific for alcohol. To determine reinforcer specificity, effects of CaMKII inhibition in the amygdala using m-AIP (0.3, 1.0, 10.0 µg/per side) were compared independently in groups of alcohol and sucrose self-administering mice. For placements, out of 13 alcohol mice, 8 recovered from surgery and had correct placements and in the sucrose group, 8 had surgery and 6 had correct placements (**Figure 15**). For behavioral measures, groups were analyzed separately using a repeated measures ANOVA. For alcohol group, we found a main effect for drug ($F(3,21) = 10.3$, $p < 0.001$) on active lever presses and post-hoc analyses

revealed that two doses (1.0 and 10.0 ug/per side) significantly decreased alcohol-reinforced responding ($p < 0.05$, Dunnet's). Importantly, inactive lever presses were not changed ($F(3,21) = 0.90$, $p = 0.457$). Data are graphed as percentage ACSF responses (**Figure 13a**). Other behavioral measures were not changed. For sucrose self-administering mice, there was no drug effect found on active ($F(3,15)=0.574$, $p = 0.641$) or (**Figure 13b**) inactive lever presses ($F(3,15)=0.559$, $p = 0.65$) in sucrose self-administering mice as well as other behavioral variables. Additionally, we tested potential nonspecific effects of m-AIP on motor activity and found the 1.0 dose of m-AIP had no effect on spontaneous locomotor activity in either alcohol (n = 8) or sucrose (n = 5) mice condition ($p > 0.05$, paired t-test). Data for alcohol and sucrose groups are graphed together (**Figure 14b**).

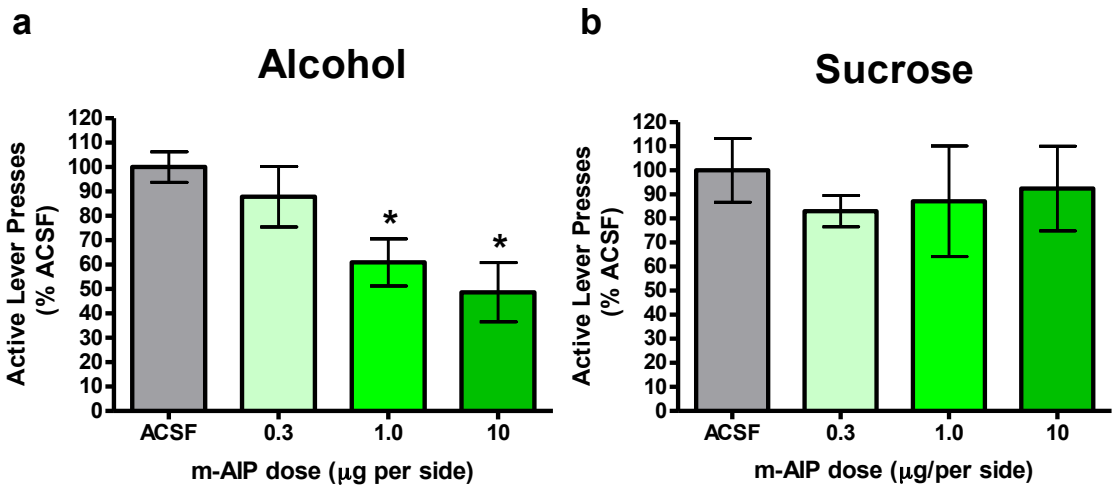


Figure 13: m-AIP dose dependently decreases alcohol but not sucrose self-administration. (a) Microinjections of three doses of m-AIP (0.3, 1.0, or 10 ug/per side) were tested in (a) alcohol and (b) sucrose self-administering mice. Two concentrations of m-AIP (1.0, or 10 ug/per side) significantly decreased responding for alcohol relative to the ACSF microinjection. Data are from mice with correct amygdala placements. *, $p< 0.05$; RM-ANOVA, followed by Dunnets post-hoc analysis.

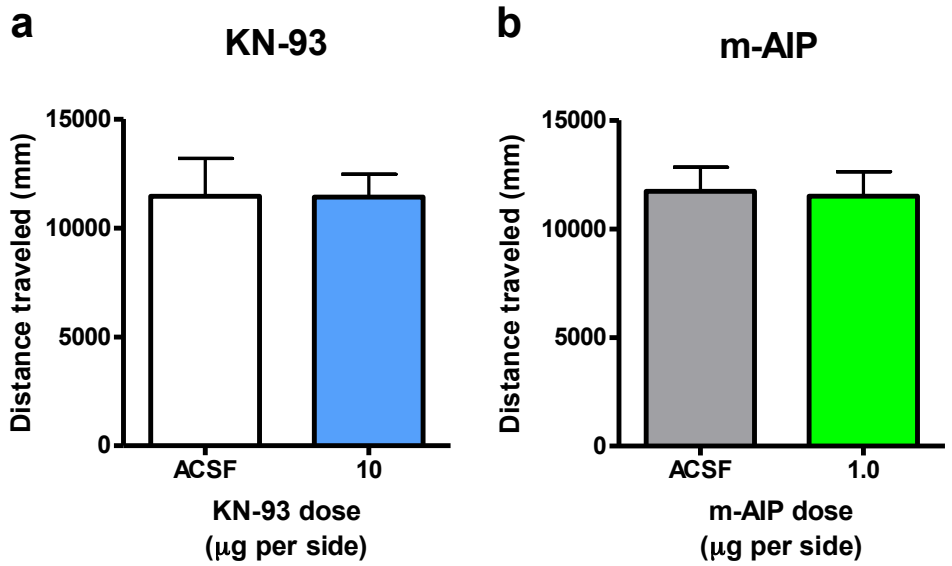


Figure 14: Microinjections of CaMKII inhibitors do not affect spontaneous locomotor activity. Microinjection of KN-93 (10 ug/per side) (a) or m-AIP (1.0 ug/per side) (b) did not significantly alter locomotor activity on the open-field test where total distance traveled (mm) over 1 hour was measured.

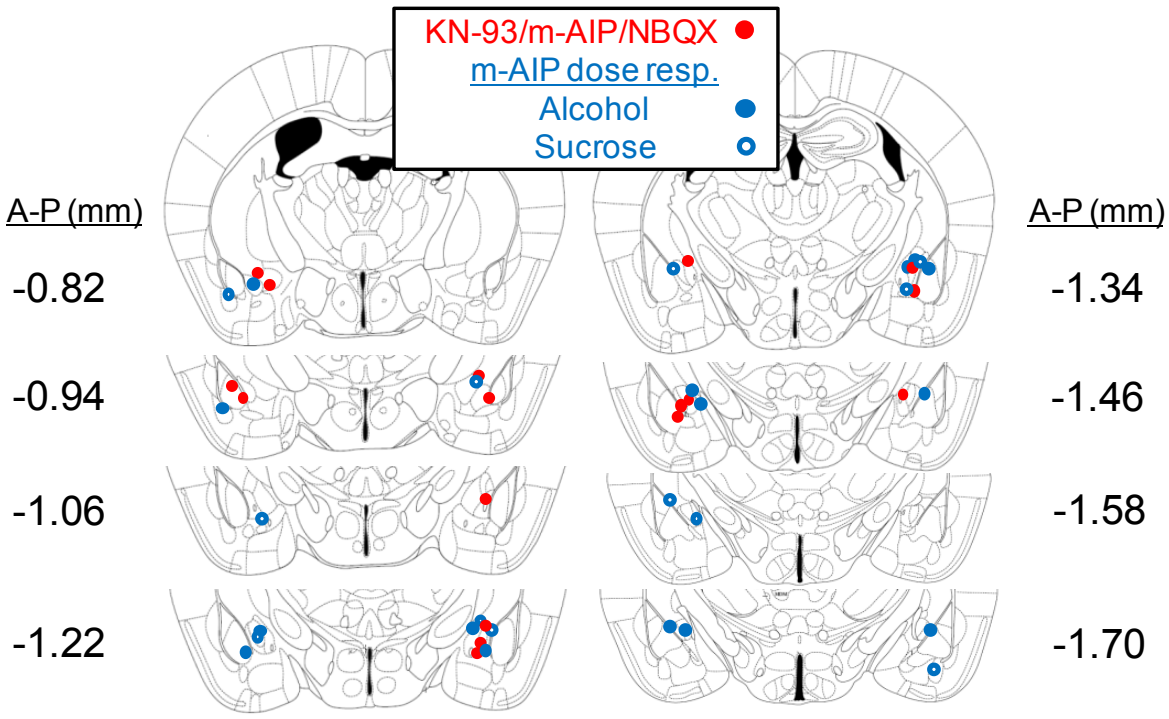


Figure 15: Injector placements in the amygdala. Schematic demonstrating anatomical specificity of cannulae placements in amygdala subregions and distance (mm) posterior to Bregma. Placements are identified with circles for both KN-93/m-AIP/NBQX (red) and m-AIP dose response curve (Blue, alcohol:closed, sucrose:open) experiments. Missed placements are not included.

DISCUSSION

The purpose of this study was to extend prior findings showing the effects of alcohol drinking on CaMKII expression to include evaluation of activation (e.g., phosphorylation) and function. Initially, we sought to determine if operant alcohol self-administration, which evaluates the reinforcing effects of the drug, is associated with altered CaMKII activation in the amygdala. In this behavioral procedure, mice are trained to press a lever with auditory and visual cues signaling the delivery of a contingent reinforcer. In mice matched to their operant activity (i.e. lever presses), sweetened alcohol self-administration increased p-CaMKII$_{thr286}$ immunoreactivity in the CeA and LA compared to sucrose self-administration alone a finding that supports the notion that alcohol enhances synaptic activity in amygdala subregions that mediate learning and motivational behaviors. Moreover, the unique structure and localization of CaMKII allows it to remain phosphorylated long after Ca^{2+} levels have decreased permits continuous modulation of receptor function including the phosphorylation of the AMPAR subunit GLUR1, a proposed mechanism for the strengthening of synapses and consolidating associative memory [216]. Therefore, we measured protein levels of phosphorylated GLUR1 at the CaMKII phosphorylation site, serine 831 (p-GLUR1$_{ser831}$), and found elevated levels in alcohol self-administering mice, which suggests increased CaMKII activity in the CeA and LA. In addition, p-GLUR1$_{ser831}$ shows increased conductance of calcium and has been associated with the presence of LTP [217]. Enhancement of CaMKII and AMPA activity in these regions may refine and stabilize synaptic connections that encode primary and secondary reinforcers. Evidence from cocaine self-

administration experiments has shown that CaMKII and p-GLUR1$_{ser831}$ expression in the NAc, a region critical to psychostimulant reward, is increased following cocaine self-administration and that it functionally and selectively regulates motivation for cocaine self-administration [85]. Thus, due to the amygdala's primary role in the reinforcing properties of alcohol, we proposed that increased CaMKII activity in the amygdala plays a key role in alcohol self-administration.

Our previous experiments established a link between alcohol self-administration and its effects on CaMKII expression and activity in the amygdala. Emerging research supports a functional role for CaMKII in addictive behaviors [57]. To date, studies directly investigating the role of CaMKII in alcohol-related behaviors have not been conducted. We hypothesized that observed increases in p-CaMKII$_{thr286}$ in the CeA and LA are required for the maintenance of alcohol self-administration. To determine the role of CaMKII in the reinforcing effects of alcohol, we infused inhibitors of CaMKII activity, KN-93 and m-AIP into the amygdala prior to alcohol self-administration sessions. KN-93 is a compound that prevents phosphorylation of CaMKII by blocking the Ca^{2+}/calmodulin binding site on CaMKII's catalytic domain with reported off-target actions on L-type channels [218]. A more selective inhibitor, m-AIP is a synthetic cell permeable peptide designed to mimic the autoinhibitory region of CaMKII. Each inhibitor significantly reduced responding for alcohol by approximately 30%. These results suggest that CaMKII activity in the amygdala is required for the full expression of the reinforcing effects of alcohol.

Alternative explanations should be considered. First, there is an extensive literature supporting a role for CaMKII in learning and memory. Thus, an alternative

hypothesis is that CaMKII inhibition may have produced deficits in task memory. However, there were no significant changes in response accuracy or sucrose self-administration, which argues against this interpretation. Second, CaMKII regulates glutamate transmission and may cause deficits in locomotor activity, yet spontaneous locomotor activity was not affected in a dose that decreased responding. To assess reinforcer specificity, a dose response curve of m-AIP was carried out on mice self-administering sweetened alcohol and sucrose alone and two doses of m-AIP selectively decreased responding for the alcohol solution, but not sucrose. This study provides additional conformation that CaMKII specifically and functionally regulates the reinforcing effects of alcohol.

Our results complement a growing collection of studies implicating CaMKII in the regulation of drug-specific behaviors. Exposure to several drugs of abuse including cocaine, amphetamine, and morphine increase CaMKII expression. In addition, drug-specific behaviors regulated by CaMKII have been identified. KN-93 injected into the VTA [219] or NAc [220] decreased sensitization to cocaine. Using a model for motivation to cocaine reinforcement, it was shown that CaMKIIα expression was positively correlated with motivation for cocaine and lentiviral knockdown of CaMKIIα in the NAc attenuated motivation for cocaine reinforcement [221]. Cocaine reinstatement increases $pCaMKII_{thr286}$ and $GLUR1_{ser831}$ in the NAc [85] which was blocked by either CaMKII inhibition or blockade of GluR1 surface expression [85]. Conditioned place preference (CPP) is a model of drug reinforcement and CPP training with amphetamine increases CaMKII activity in the hippocampus [222, 223] and infusion of KN-93 into the hippocampus decreased

amphetamine CPP [222, 223]. CaMKII has also been shown to regulate addictive behaviors caused by morphine. Naloxone-induced morphine withdrawal symptoms are decreased by the CaMKII antagonist KN-62 infused into the hippocampus. Additionally, the development of morphine CPP is blocked by infusion of KN-62 into either the hippocampus or amygdala [224]. Importantly, the maintenance and reactivation of morphine CPP is prevented by amygdala injections of KN-62 [224], a result consistent with our finding that CaMKII inhibitors decreased alcohol self-administration.

The features of the CaMKII molecule make it a promising candidate for the aberrant plasticity that is thought to underlie addiction. CaMKII is activated by neuronal depolarization via Ca^{2+} entry and release from intracellular stores, can remain phosphorylated for long periods of time after the Ca^{2+} signal diminishes, and can reorganize synapses towards increased glutamate activity and increased Ca^{2+} entry. Thus, altered CaMKII activity is likely to be affected by and to contribute to increased brain activity in through positive feedback mechanisms. Increased CaMKII activity caused by alcohol could very well contribute to the enhancement of glutamate signaling that is a characteristic of alcoholism. Our finding that alcohol-self-administration increases CaMKII activity in the amygdala differently than self-administration of a natural reward provides evidence that alcohol differentially affecting brain regions involved in reward processing through its effects on CaMKII activation. Accordingly, inhibiting this activity leads to specific reductions in the reinforcing effects of alcohol and may be useful as a strategy for treating alcoholism. These findings support the hypothesis that CaMKII activity may functionally regulate

enduring pathological behaviors associated with alcoholism, such as chronic self-administration.

As an individual develops alcohol dependence, increased alcohol intake is influenced by the positive reinforcing effects of alcohol. In preclinical models, the amygdala is critical to the associative memory processes required to maintain alcohol self-administration. Following alcohol consumption, plastic changes in amygdala neurons may enhance alcohol's reinforcing properties and the salience of alcohol-related cues, which may in turn narrow the behavioral repertoire towards alcohol procurement and away from natural rewards. This study shows for the first time that that CaMKII is a potential molecular mechanism of drug-induced behavioral pathologies that occur during the initial stages of addiction. Understanding the molecular mechanisms behind alcohol's effects on neuroplasticity, and delineating whether those mechanisms contribute to alcohol reinforcement may give way to therapeutic interventions that could prevent the transition to alcohol abuse to alcohol dependence.

CHAPTER IV: SYSTEMIC INHIBITION OF ERK1/2 PHOSPHORYLATION POTENTIATES ALCOHOL-SEEKING AND INITIATES SUCROSE-SEEKING IN C57BL/6J MICE

INTRODUCTION

Relapse to alcohol-seeking after abstinence is a hallmark of alcoholism and a major clinical problem. It has been estimated that approximately 80% of abstinent alcoholics relapse [225], a phenomenon adding permanence to the already substantial economic and social burden of alcoholism. Emerging evidence indicates that chronic alcohol exposure produces maladaptive changes in molecular cell signaling pathways that lead to long-term changes in brain function [116, 182-184]. Accordingly, these drug-induced adaptations are thought to regulate enduring behavioral pathologies that occur in alcoholism [57, 185, 186], such as relapse. A better understanding of the molecular and cellular mechanisms that regulate the behavioral pathologies in alcoholism has the potential to lead to new pharmacotherapeutic strategies.

Research has suggested that associative learning and memory processes (i.e., forms of neuroplasticity) may play a principal role in relapse as evidenced by the fact that exposure to environmental cues associated with alcohol drinking promote craving and relapse in abstinent alcoholics [226, 227]. For these reasons, recent theories of addiction emphasize the importance of determining if alcohol and

other drugs of abuse usurp basic mechanisms of neuroplasticity to produce cue-induced relapse [134, 228]. Moreover, cell signaling systems that subserve synaptic plasticity are primary candidate mechanisms because they transduce pleiotropic drug effects, such as changes in membrane receptor activity, into enduring modifications in neural function [46]. Thus, a key challenge for the field is to determine if cell signaling systems that regulate normal associative learning and memory also regulate maladaptive cue-induced alcohol-seeking behavior.

Animal models of relapse can be divided into two subcategories: those that fully express relapse and involve alcohol-drinking and models that are directed at the initiation of relapse, often described as craving [229]. The basic criterion for relapse models is straightforward: rodents consume alcohol for an allotted time period, are given a period of abstinence, and then the rodents consume alcohol or perform actions that previously rewarded them with alcohol at a higher rate than during the abstinence phase. One commonly used procedure of relapse expression that produces an escalation of alcohol drinking is called the alcohol deprivation effect (ADE). In ADE procedures, rodents are given a period of access to alcohol, then a period of abstinence followed by a re-exposure to alcohol self-administration. During this re-exposure, a number of animal models (mice, rats, monkeys, and humans) will show an escalation of alcohol intake compared to previous levels [229]. This increase is the phenomenon known as the ADE. Alternatively, in reinstatement experiments, rodents are trained to self-administer alcohol by performing an action like lever pressing with a contextual or explicit cue that signals the availability of alcohol. They then undergo extinction sessions in which the operant behavior does

not have any consequences, and subsequently, the rodents decrease responding. Finally, in a reinstatement session, the conditioned stimulus is presented. This conditioned stimulus, typically a cue, an associated context, or a priming exposure, is presented. In reinstatement experiments, reinstatement behavior is operationally defined as increased responding compared to extinction responding in the absence of alcohol delivery. Importantly, an advantage of reinstatement tests over tests like ADE is that they allow the researcher to evaluate conditioned reinforcement separately from alcohol's direct behavioral and pharmacological effects. Therefore, the neurobiological activity proceeding relapse that underlies associated drug-cue memories and drug-seeking can be studied independently.

The mitogen-activated protein kinase (MAPK) cell signaling pathway is receiving growing attention as a potential molecular mechanism of alcohol-related behavioral pathologies [230]. In particular, the extracellular-regulated kinase (ERK) MAPK pathway is of interest because it integrates activity of a variety of extracellular and intracellular factors to produce coordinated changes in gene transcription that lead to long-term changes in CNS structure and function. These include cell growth, neuroplasticity and addiction [88, 90-93]. The two closely related isoforms of ERK (ERK1 and ERK2, or ERK1/2) are phosphorylated within the activation loop of the kinase on both a threonine and a tyrosine residue by MEK [231]. ERK1/2 activity requires phosphorylation of both of these sites [94]. Activated ERK phosphorylates cellular targets or translocates to the nucleus where it activates specific gene transcription factors [95]. By regulating gene transcription, the ERK/MAPK pathway

can transduce cellular events into long-term changes in neural and behavioral functions, such as those seen in learning, memory, and addiction.

The MAP kinases have been associated with many of the negative health effects of ethanol including liver disease, pancreatitis, cancer risk, neurotoxicity, and cardiovascular disease [230]. Recent *in vivo* evidence indicates that ethanol may alter activity of the ERK/MAPK signaling cascade in brain regions that are of behavioral significance. Acute injection of alcohol (1.5 – 3.5 g/kg) produces a dose- and time-dependent decrease in p-ERK1/2 in mouse cortex [232]. The ability of acute ethanol to reduce p-ERK1/2 levels in brain is not dependent on age and found from postnatal day 7 through adulthood [232, 233]. Interestingly, blockade of p-ERK1/2 with a MEK/ERK1/2 inhibitor prevented ethanol-induced increases in c-Fos expression in the Edinger-Westphal nucleus [234], a finding that suggests ethanol-mediated changes in gene expression are regulated by the ERK/MAPK system. Taken together, these studies demonstrate that alcohol produces rapid changes in the active form of ERK1/2 in specific brain regions.

Recent evidence also shows that the ERK/MAPK system is altered by chronic alcohol and relapse. Exposure to chronic ethanol vapor (BAL of approximately 200 mg/dl) suppressed p-ERK1/2 immunoreactivity in the amygdala, cortex, cerebellum, and dorsal striatum. Conversely, withdrawal from ethanol vapor resulted in a time-dependent increase in p-ERK1/2 levels that peaked at 13-h post withdrawal and remained elevated at 24-h, which was the last time point measured [235]. These data are consistent with findings from our lab showing that cue-induced reinstatement of alcohol-seeking behavior is associated with increased pERK1/2

immunoreactivity in the basolateral amygdala and nucleus accumbens shell [178]. When taken together with our finding that the ERK1/2 inhibition produces dose-dependent biphasic effects of alcohol self-administration [189], these data suggest that ERK/MAPK activity may underlie behavioral pathologies in alcohol addiction, including chronic drug use and relapse.

The purpose of this experiment is to determine the mechanistic role of ERK1/2 in cue-induced reinstatement using a specific pharmacologic inhibitor of the upstream kinase MEK1/2, α-[Amino[(4-aminophenyl)thio]methyle ne]-2-(trifluoromethyl)benzeneacetonitrile (SL327). To do this, we developed a model of reinstatement for C57BL/6J mice using an adapted version of the cue-induced reinstatement procedure previously described [178] that demonstrated significant responding on an alcohol lever compared to extinction session responding. To determine the role of ERK1/2 in alcohol reinstatement, mice were pretreated with SL327 (30 mg/kg), a dose that has previously not been associated with locomotor effects [189], or vehicle prior to reinstatement sessions. Mice given vehicle reliably demonstrated response-contingent cue-induced reinstatement of alcohol-seeking behavior. In mice pretreated with SL327, cue-induced reinstatement of alcohol-seeking was potentiated compared to vehicle and, interestingly, cue-induced reinstatement of sucrose-seeking was also observed in a separate group of sucrose drinking mice. Our finding that C57BL/6J mice demonstrate cue-induced reinstatement of alcohol but not sucrose seeking behavior and that systemic ERK1/2 inhibition potentiates cue-induced responding for both alcohol and sucrose, lead us to conclude that $ERK_{1/2}$ functionally modulates relapse-like behavior.

METHODS

Animals: Male C57BL/6J mice (n = 80), 8 weeks old, were housed in groups of four in standard Plexiglas cages (17.8 W x 29.2 L x 12.7 H cm). Cages were lined with corn cob bedding, contained a PVC tube for environmental enrichment, and had a wire stainless steel top. Food and water were available *ad libitum* unless otherwise noted. The mouse vivarium was maintained on a reverse 12 h light-dark cycle (lights off at 8:00 a.m.). All animals were treated in accordance with the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill and NIH guidelines for the care and use of laboratory animals (National Research Council, 1996).

Self-administration apparatus: Alcohol and sucrose self-administration, extinction and reinstatement sessions were conducted in Plexiglas operant chambers designed for mice (Med Associates, Georgia, VT) measuring 15.9 x 14 x 12.7 cm with stainless steel grid floors. Each chamber was housed in a sound-attenuating cubicle equipped with a house fan that provided ventilation and helped mask external noise. The left and right wall of each operant chamber was equipped with one ultra-sensitive stainless steel response lever and a liquid delivery system with a stimulus light located above each lever. Solutions were delivered via a syringe mounted to a programmable pump (PHM-100, Med Associates), which delivered 0.014 ml per activation into a stainless steel cup located to the right of the associated response lever. The inactive lever was located on the opposite wall relative to the active lever. Pressing this lever did not produce any programmed

consequence, but responses on it were recorded. The operant conditioning chambers were interfaced (Med Associates) to an IBM compatible PC, which was programmed to record all lever presses, port entries, and liquid deliveries.

Self-administration, extinction, and reinstatement procedure: Following one week of acclimation to housing conditions, mice were deprived of fluids for 20 hours prior to initial training in the operant chamber. The first three days of training were 16 hr sessions with a 5% sucrose reward on FR1, FR1-FR4 progressive ratio, and FR4 schedules respectively. All subsequent sessions followed a FR4 schedule and lasted 1 hr. Mice were then trained to self-administer either sucrose (2% w/v) or a sweetened alcohol solution (9% alcohol (v/v)/2% sucrose (w/v)) by using a sucrose fading procedure where sucrose was decreased from 5% to 2% in both groups and alcohol concentration was gradually increased from 0% to 9% for the alcohol group. The solutions used (9% alcohol/2% sucrose and 2% sucrose) were chosen because in previous experiments, mice self-administering 9% alcohol would frequently leave large amounts of residual fluid in cup. With sweetened alcohol, residual fluid is rarely seen, and because 2% sucrose leads to similar levels of active lever presses as the sweetened alcohol solution it can serve as a natural reinforcer control with the percentage of sucrose remaining constant. Mice were maintained on these solutions for 35 sessions and after all sessions, fluid cups were monitored for residual fluid to ensure fluid consumption.

The extinction sessions were similar to self-administration sessions except for the absence of programmed consequences (no light, sound, or liquid deliveries) following active lever presses. Matching to reinstatement and drug groups was

carried out by averaging active and inactive lever presses over the last 5 training sessions and the last 5 extinction sessions and ensuring that these values did not have significant differences. For each measure, mice with outlier values were determined by Grubb's test (Graphpad software). Mice were given habituation injections of vehicle following the 29th self-administration session and 2 hours prior to the 34th self-administration session, and extinction sessions 7 and 13.

Two hours prior to either a reinstatement test or a 15th day of extinction, mice were injected intraperitoneally with either vehicle (15% DMSO) or 30 mg/kg SL327. For the reinstatement test, 28 µl of the solution (sucrose or sweetened alcohol) was placed in the cup prior to the session and the light cue and the pump sound were reactivated and followed an FR4 schedule. Importantly, the reinforcing solution was not delivered during reinstatement sessions.

Drugs: Alcohol solutions were prepared by diluting 95% Ethyl Alcohol (Pharma, CT (v/v)) with tap water to the desired concentration. Sucrose solutions were made by dissolving granulated sugar into tap water (w/v). SL327 (Tocris Bioscience, Ellisville, MO) was dissolved in 100% DMSO and diluted with DI H_2O (final concentration 15% DMSO) and injected intraperitoneally (i.p.) in a volume of 10 ml/kg body weight with a 27 gauge needle.The SL327 dose used (30 mg/kg) was chosen because it previously was found to have no significant effects on spontaneous locomotor activity in C57BL/6J mice [189].

Data analysis: For each self-administration, extinction and reinstatement session, multiple behavioral parameters were collected. Specifically, # of reinforcers delivered, active lever presses, inactive lever presses, percent active lever presses, and port entries were recorded for all sessions. Matching to treatment groups was needed to ensure that extinction and reinstatement groups responded similarly. For experiment 1, data used to match groups (average active and inactive lever presses over last 5 days of alcohol self-administration and extinction sessions) and used to compare reinstatement test sessions (test session dependent variables/average last 5 days extinction for those variables) were analyzed using an independent t-test. Extinction data for experiment 1 were analyzed using a repeated measures ANOVA. In experiment 2 and 3, data used to match groups were similar to experiment 1 except it was analyzed using a two-way analysis of variance (ANOVA) for comparison of each subgroup. Extinction data were analyzed using two way repeated measures ANOVA for lever (active vs. inactive) and session. For matching data in all experiments, Grubb's test was used to test for active or inactive lever presses, and outliers were removed. Test session data were analyzed using two-way ANOVA followed by post hoc comparisons (Tukey test). Significance was assigned for all p values < 0.05 for all analyses.

RESULTS

Protocol promoted relapse-like behavior in mice

Initially, our goal was to create a mouse model of reinstatement using operant self-administration procedures. Mice (n=16) were successfully trained using the

sucrose fading procedure to self-administer alcohol for 45 days. One mouse was removed from the experiment due to high levels of inactive lever presses during extinction identified using an outlier criterion. Throughout the alcohol self-administration sessions, mice pressed the active lever significantly more on the inactive lever and this demonstrates that the alcohol solution has reinforcing properties. Over the last 5 days of alcohol self-administration, mice averaged 136.5±6.0 active lever presses, 60.7±8.7 inactive lever presses, 31.5±1.3 alcohol deliveries, 74.2±2.2 % active lever presses and consumed an average of 1.17±0.05 g/kg of alcohol during the 1 hr session.

During the last 5 days of extinction, mice averaged 44.1±2.7 active lever presses, 68% reduction in responding compared to the last 5 days of alcohol self-administration sessions. In addition, mice averaged 39.3±4.6 inactive lever presses and responded on the active lever 61.2±3.2% of their total responses. Following alcohol-self administration sessions, mice began 14 daily extinction sessions in which levers were extended, but there were no programmed consequences. Data from extinction sessions were analyzed using a repeated measures two-way ANOVA. There was a main effect for lever ($F(1,182) = 9.837$, $p < 0.01$) and extinction session ($F(13,182) = 20.717$, $p < 0.001$) as lever responses decreased over time which is due to the decreased responding on the active lever (**Figure 16a**) indicated by the lever x extinction session interaction ($F(13,182) = 8.821$, $p < 0.001$). Post hoc analyses (Tukey test) revealed that responding on active and inactive were significantly different on extinction session 1-6.

After matching mice to conditions so that their last 5 days of self-administration and extinction in terms of active and inactive lever presses were similar, mice were placed in either reinstatement (n=7) or extinction groups (n=8). Because mice were matched to their last 5 days of extinction, reinstatement test session lever presses were calculated as a percentage of the average lever presses from the last 5 extinction sessions. Mice in the reinstatement group had a significantly higher number of active lever presses expressed as percentage of last five days of responding ($p < 0.01$, Student's t-test), but not inactive lever presses (**Figure 16b**). This finding demonstrated cue-induced reinstatement of alcohol-seeking behavior. Other behavioral measures, including number of headpokes and percent responding on the active lever, did not differ significantly between groups of mice.

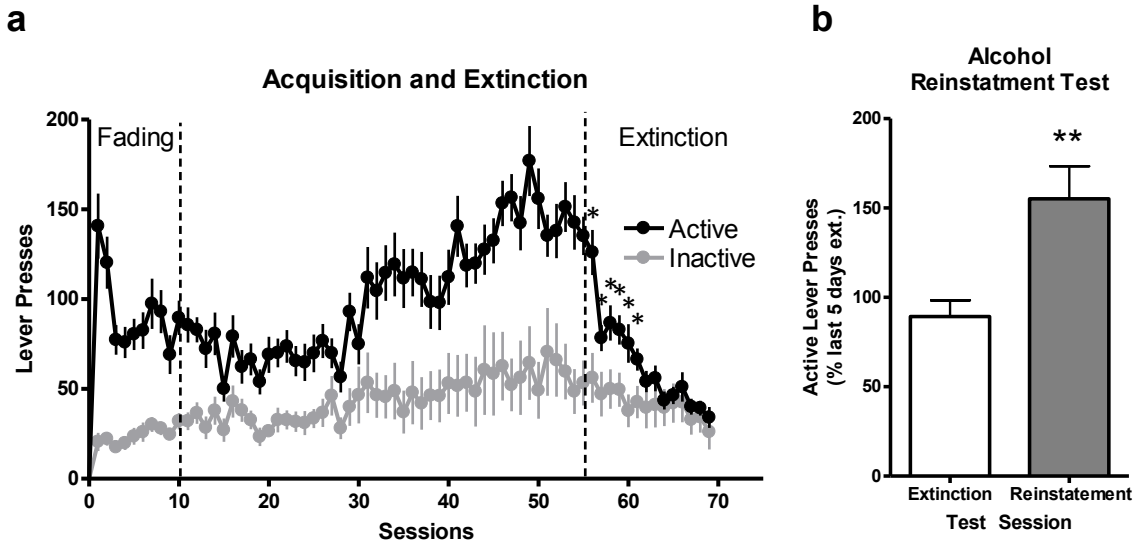


Figure 16: Cue-induced reinstatement of alcohol-seeking behavior in the mouse. (a) Graph showing active and inactive lever presses during reinstatement procedure where mice are initially trained using sucrose fading (first 10 days), then drink a sweetened alcohol solution for 45 days, enter an extinction phase. During extinction, active and inactive lever presses were significantly different until day 7 ((*, $p < 0.05$;Two-way RM ANOVA, followed by Tukey's post hoc, (b) Mice were divided into a 15th day of extinction or reinstatement session. Mice in the reinstatement test responded on the active lever significantly more than in the extinction session. (*, $p< 0.05$; **, $p < 0.01$; Student's t-test).

SL327 potentiated relapse-like behavior in alcohol self-administering mice

In experiment 1, mice demonstrated high active lever presses by day 35. Therefore in experiments 2 & 3, the number of self-administration sessions was reduced from 45 to 35. In experiment 2, a separate group of mice (n=41) acquired lever pressing behavior following sucrose fading and self-administered alcohol solution for 35 days (**Figure 17a**). One mouse was excluded from the experiment due to outlier inactive lever presses during the extinction sessions. Over the last 5 self-administration sessions, the remaining mice (n=40) averaged 134.5±8.9 active lever presses and 43.6±5.2 inactive lever presses, and a 78% preference of the active lever. During this time, mice received on average 30.6±2.0 deliveries of alcohol solution, averaged 63.6±5.5 port entries, and did not leave residual fluid at the end of the sessions. After correcting for differences in bodyweight, it was determined that the mice averaged 1.0±0.2 g/kg of alcohol over these last 5 days.

Following alcohol-self administration sessions, mice began 14 daily extinction sessions in which levers were extended, but there were no programmed consequences. There was a main effect for the extinction session as lever responses decreased over time ($F(13,39) = 17.104$, $p < 0.001$), which is due to the decreased responding on the active lever indicated by the lever x extinction session interaction ($F(13,39) = 21.148$, $p < 0.001$). Post hoc analyses (Tukey test) revealed that active and inactive lever presses ceased to be significantly different on extinction session 6 -14. In addition, there were no significant differences of inactive lever presses between groups throughout the extinction sessions, a finding

demonstrating that responding on this lever was quite stable, while the active lever differed significantly for multiple extinction sessions.

Following the 14th extinction session, mice were matched to four separate groups using average active and inactive lever presses over the last 5 extinction sessions. Matching data from each alcohol group are summarized (**Table 2**) and importantly there were no significant differences in the averages of active lever responses, inactive lever responses, and percent active lever responses over the last 5 days of self-administration and the last 5 days of extinction.

Table 2: Alcohol matching to groups

Alcohol Matching	**Active Lever**		**Inactive Lever**		**% Active**	
Subgroup	Last 5 SA	Last 5 Ext	Last 5 SA	Last 5 Ext	Last 5 SA	Last 5 Ext
Vehicle Extinction	142.1±18.4	45.5±10.1	49.0±12.0	47.9±13.2	75.5±3.8	55.3±5.8
Vehicle Reinstatement	121.6±18.0	38.8±4.0	46.1±9.0	44.4±9.2	74.2±3.5	51.5±4.7
SL327 Extinction	135.9±20.9	50.1±13.0	35.3±9.4	39.5±12.9	77.8±4.4	60.3±3.4
SL327 Reinstatement	140.3±12.3	36.3±5.7	41.1±13.5	39.5±15.5	79.0±5.6	56.2±6.7

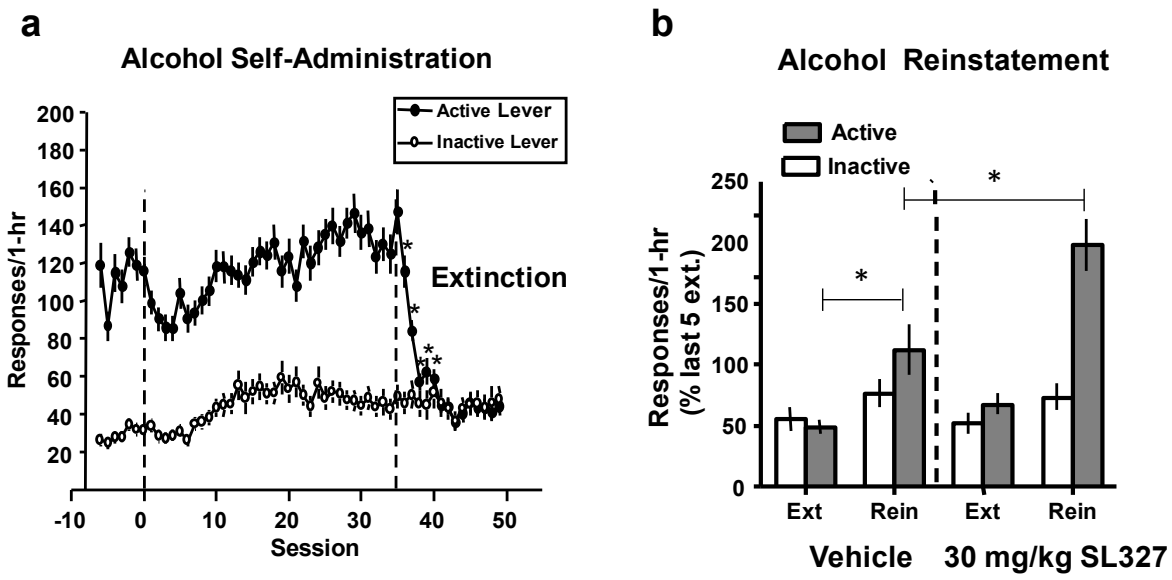


Figure 17. ERK1/2 inhibition potentiates reinstatement of alcohol-seeking behavior. (a) Graph depicting lever presses during acquisition and extinction phases of reinstatement procedure. Mice pressed significantly more on the active lever for the first 5 extinction sessions. *, p< 0.05; RM-ANOVA (b) Lever responses shown as percent last 5 days of extinction. Two-way ANOVA revealed a significant main effect for both drug and reinstatemnt test as well as an interaction. Post-hoc analyses revealed that vehicle-treated mice successfully reinstated alcohol-seeking behavior and that mice that were pretreated with SL327 displayed a potentiation of active lever responding.

As mice were matched on the average of their last 5 extinction sessions, reinstatement results are calculated as a percentage of this average. Active and inactive lever presses are shown (**Figure 17a**). Following a two-way ANOVA on active lever responses as a percentage of the last 5 days of extinction, it was determined that there was a main effect for drug ($F(1,36) = 12.074$, $p = 0.001$) as SL327 active lever responding was increased over vehicle. In addition, there was a main effect of test session ($F(1,36) = 41.34$, $p < 0.001$) as reinstatement mice responded significantly more on the active lever. There was also a significant interaction ($F(1,36) = 5.042$, $p = 0.031$). Post-hoc analysis using (Tukey's) revealed that within vehicle, reinstatement mice had increased active lever responses, which

demonstrated a proof of principle of the reinstatement procedure. Drug within extinction sessions did not differ significantly. However, drug within reinstatement session was significantly different. Specifically, SL327 significantly enhanced reinstatement of alcohol-seeking behavior by 86%. (**Figure 17b**) A two-way ANOVA on inactive lever responses as a percentage change from the last 5 days of extinction revealed that there were no significant differences between groups.

SL327 induced relapse-like behavior in sucrose self-administering mice

For the sucrose group, 1 mouse was excluded from the experiment due to outlier values of inactive lever presses over the last 5 days of extinction. Mice (n=36) acquired lever pressing behavior following sucrose fading and self-administered sucrose for 35 days as shown in figure 2A. Over the last 5 self-administration sessions, mice averaged 121.4±13.5 active lever presses and 51.0±7.5 inactive lever presses demonstrating a 69% preference for the active lever. Mice averaged 27.1±3.0 deliveries of sucrose and 58.0±6.2 port entries.

For extinction sessions, sucrose self-administering mice showed decreased responding for the active lever a seen in Figure 2A which was confirmed by statistical analysis. A RM two way ANOVA revealed that there was a main effect of extinction session on lever responding ($F(13,35) = 3.646$, $p < 0.001$) as responding decreased over the extinction sessions. In addition, there was a significant interaction of lever x extinction session $F(13,35)= 7.501$, $p < 0.001$) and post hoc analysis (Tukey test) revealed that active lever pressing was significantly higher than inactive lever presses until extinction session 4.

Table 3: Sucrose matching to groups

Sucrose Matching	Active Lever		Inactive Lever		% Active	
Subgroup	Last 5 SA	Last 5 Ext	Last 5 SA	Last 5 Ext	Last 5 SA	Last 5 Ext
Vehicle Extinction	127.5±24.9	46.8±7.8	67.0±19.3	71.4±27.3	65.9±5.1	48.5±6.6
Vehicle Reinstatement	119.1±29.0	39.4±11.2	60.0±19.1	49.4±21.2	65.8±6.7	49.4±7.5
SL327 Extinction	95.8±21.2	33.6±7.3	37.8±6.8	27.4±7.7	69.8±2.6	56.1±4.3
SL327 Reinstatement	136.2±30.3	47.6±11.2	37.0±6.3	29.7±8.6	74.5±3.5	62.9±5.8

Matching data from each group are summarized in table 2 and analyses using one-way ANOVA demonstrated that there were no significant differences among roups. Similar to the alcohol group, reinstatement results are calculated as a percentage of lever presses over last the last 5 days of extinction. Active and inactive lever presses are shown (**Figure 18a**). A two-way ANOVA on active lever presses, revealed a main effect for test ($F(1,32)= 10.672$, $p = 0.003$) indicating increased responding in the reinstatement group. There was no main effect for drug, ($p = 0.064$), and no statistically significant interaction ($p = 0.059$). In the alcohol reinstatement experiment, relapse-like behavior was potentiated by SL327 and this experiment was designed to determine if the effect was specific to alcohol. Due to our *a priori* hypothesis that SL327 modulates reinstatement, a planned comparison (Student's t-test) was applied to drug within reinstatement condition which revealed a significant ($p = 0.0156$) increase in active lever responding in the SL327 group, suggesting that ERK/12 inhibition potentiates cue-induced reinstatement seeking of sucrose as well as alcohol. A two-way ANOVA on inactive lever response (% last 5 days extinction) was performed and revealed that there was a main effect for drug ($F(1,32)=4.855$). Planned comparisons (Tukey test) show that this effect is due to

increased inactive lever responding in the SL327 reinstatement group compared to extinction (p = 0.027) and within reinstatement groups (p = 0.009) (**Figure 18b**).

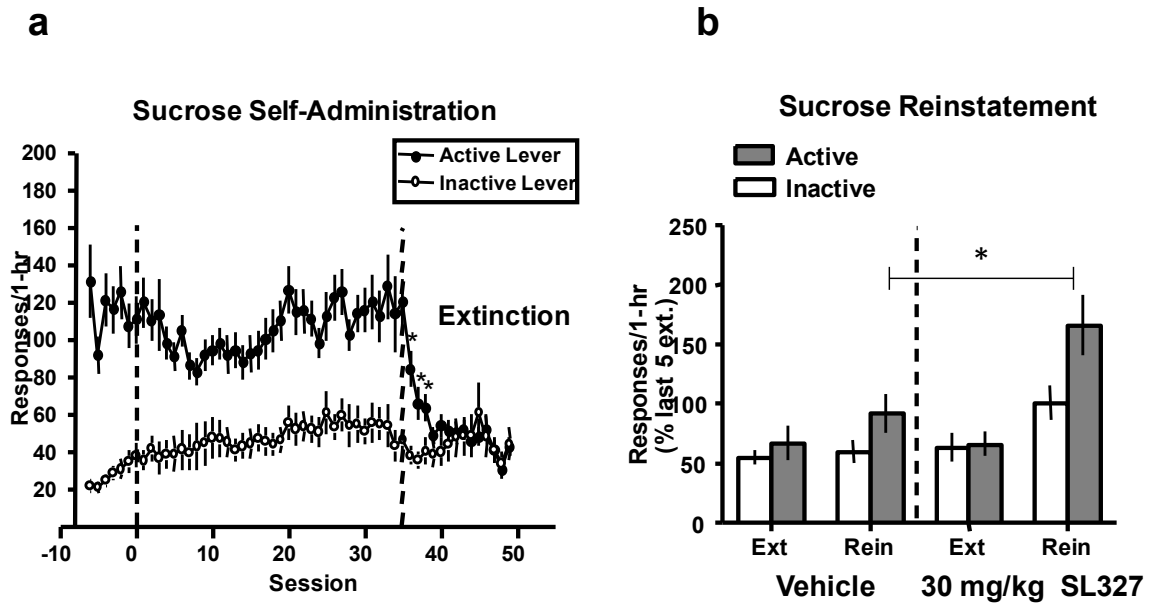


Figure 18: ERK1/2 inhibition potentiates reinstatement of sucrose-seeking behavior. (a) Graph depicting lever presses during acquisition and extinction phases of reinstatement procedure for sucrose self-administering mice. Mice pressed significantly more on the active lever for the first 3 extinction sessions. *, $p < 0.05$; RM-ANOVA (b) Lever responses shown as percent last 5 days of extinction. Two-way ANOVA revrelaed a significant main effect for reinstatement. A planned comparison revealed that mice that were pretreated with SL327 displayed a potentiation of cue-induced active lever responding.

DISCUSSION

This study had two primary goals. First, we sought to develop a mouse behavioral model of response-contingent cue-induced reinstatement of alcohol seeking behavior. C57BL/6J mice were first trained to self-administer alcohol in operant chambers. Then, following an extinction phase, mice were tested for cue-induced reinstatement. Results showed that alcohol-reinforced responses by C57BL/6J mice extinguished to levels of responding that occurred on an inactive lever, indicating full extinction of performance. Presentation of alcohol-associated cues resulted in a significant increase in responding on the lever that formerly

produced an alcohol solution, demonstrating relapse-like behavior. Second, given the prominent role of ERK1/2 MAPK in neural and behavioral plasticity [91], we hypothesized that modulating this critical cell signaling pathway regulates cue-induced reinstatement of alcohol-seeking. Our results showed that ERK1/2 inhibition by SL327 potentiated reinstatement of alcohol-seeking behavior. Interestingly, ERK1/2 inhibition promoted reinstatement in sucrose controls that did not otherwise demonstrate reinstatement. Overall, these findings demonstrate a reliable method for evaluating cue-induced reinstatement of alcohol-seeking behavior in mice and suggest that ERK/MAPK activity may regulate aspects of relapse-like behavior.

Our finding that ERK1/2 inhibition modulates alcohol-seeking behavior adds to a growing list of experiments linking drugs of abuse to ERK1/2 activity. Previous research has implicated ERK1/2 in cue-induced reinstatement of drug-seeking behavior. Similar to other psychostimulants, cocaine has been shown to increase levels of pERK1/2 in the mesocorticolimbic circuitry [71, 236] and has been proposed as a neuroadaptive mechanism involved in addictive behavior. To determine the role of ERK1/2 in cocaine relapse-like behavior, a reinstatement model in rats was developed where reinstatement-responding was time-dependent [237]. In this procedure, following 10 days of cocaine self-administration, rats will reliably reinstate after 30 days, but not 1 day, of withdrawal. At the 30 day cue-induced reinstatement test, rats were found to have increased levels of phosphorylated pERK1/2 in the CeA. At this time point, infusion of the ERK1/2 inhibitor, U0126, into the CeA blocked the increased reinstatement responding, demonstrating that ERK1/2 activity functionally regulates drug-seeking behavior

[237]. ERK1/2 expression has also been shown to be affected by alcohol reinstatement. Our lab has shown that reinstatement of alcohol-seeking behavior increased levels of pERK1/2 in the BLA and NAc shell compared to extinction behavior in rats [178]. Systemic mGluR5 inhibition by MPEP attenuated reinstatement behavior as well as decreased pERK1/2 expression in the BLA and NAc. Alternatively, we found in mice that direct inhibition of pERK1/2 with SL327 enhanced cue-induced reinstatement of alcohol- and sucrose-seeking behavior. Several experimental differences may explain these discrepancies. First, mGluR5 is expressed in specific brain regions compared to the global expression pattern of ERK1/2 and its modulation has multiple downstream effects including influencing NMDA receptor activity [238]. On the contrary, the effect of directly modulating ERK1/2 systemically using the MEK1/2 inhibitor, SL327 on alcohol self-administration is more consistent with our findings. Using the same SL327 dose (30 mg/kg) and pretreatment time, ERK1/2 potentiated alcohol self-administration in mice without affecting motor activity or motivation using a progressive-ratio paradigm [189]. They conclude that this effect is most likely due to a reduction in alcohol's reinforcing properties, and subsequent escalation of responding to obtain alcohol's reinforcing effects similar to what is observed in D1 inhibition and cocaine self-administration [239]. Second, systemic inhibition of ERK1/2 could lead to a disinhibition of the neural circuitry that regulates reinstatement behavior by causing increased activity in the amygdala, NAc, or VTA. Similarly, ERK1/2 inhibition in the VTA has been shown to block GDNF-mediated decreases in alcohol self-administration and thus suggest that decreased alcohol consumption is related to

increased activity of pERK in this region [240]. Systemic ERK1/2 inhibition may lead to increased dopamine release from the VTA into the NAc, a phenomenon observed in cocaine-seeking behavior [241], or could affect other neural systems like decreasing PFC function which is known to regulate drug extinction and would be predicted to increase NAc and amygdala activity [122, 242]. Follow-up experiments should determine the brain regional expression pattern of pERK1/2 after reinstatement tests in mice. The use of site-specific microinjections into discrete brain regions like the NAc and amygdala that show increased levels of pERK1/2 expression or that are known to regulate reinstatement behavior would obviate many of the potential problems related to systemic inhibition.

Due to the high incidence of relapse in alcoholics, a variety of rodent behavioral models have been developed for the evaluation of behavior that corresponds conceptually with human relapse. To date, only a few mouse models of reinstatement have been described, despite the need to test this behavior in mice. The lack of these models has prevented reinstatement testing in genetic models and limits many researchers interested in reinstatement to working with rats. Additionally, positive findings regarding mechanisms behind other alcohol-related behaviors in mice cannot be extended to relapse-like behavior. To our knowledge, only two other mouse protocols of reinstatement have been described [243, 244]. One protocol has been successful in causing high levels of alcohol reinstatement using an FR1 schedule and has been used to demonstrate that GLUR3 mutant mice do not reinstate the same levels as their littermates [243]. In the second protocol, mice were trained to self-administer on a FR3 schedule and contextual and multimodal

cues were used to promote reinstatement [244]. In our model, we also used a complex multimodal cue (cue light, pump sound, and single delivery of the alcohol or sucrose solution as an olfactory/gustatory cue, which seems to be necessary in mouse models of reinstatement, but not rats [178]. Some key difference between the protocols is training length, the use of discriminative contextual cues, and lower levels of alcohol consumption (0.55 vs 1.17 g/kg alcohol). An advantage of both of these alternative methods is that mice do not require the addition of sucrose to consume alcohol which in our procedure presents a potential confound as our sucrose control mice did not demonstrate significant reinstatement behavior. Nevertheless, the development of reinstatement models in mice has many advantages and should open up a number of opportunities, including the assessment of the many genetic models of mice and the use of optogenetics to determine the precise neural circuitry involved in reinstatement.

ERK1/2 serves as a point of convergence for a large array of neuronal events and integrates extracellular signals that affect gene transcription that leads to synapse modification. The ability of ERK1/2 to cause long-term synaptic changes has made it a molecular candidate for the neuroadaptations believed to underlie addictive behaviors. Chronic alcohol exposure affects the activity of ERK1/2 in brain regions that regulate the enduring behavioral pathologies that define alcoholism, such as relapse. In a mouse model of cue-induced reinstatement of alcohol-seeking, ERK1/2 inhibition led to the potentiation of relapse-like behavior, an effect that generalized to a natural reinforcer. The results from these experiments further

implicate the MAPK pathway as a regulator of addictive behavior, yet the neural circuitry involved remains unclear, and should be the focus of future experiments.

CHAPTER V: LIMITATIONS AND FUTURE DIRECTIONS

Over the last decade, research aimed at identifying the molecular mechanisms behind addiction have increasingly focused on proteins that play a role in synaptic plasticity. The studies presented here examined the role of two plasticity-related protein kinase pathways, CaMKII and ERK1/2, in multiple behavioral models of addiction.

First, the alpha subunit of CaMKII was identified using 2D-DIGE proteomics as being significantly increased in mice that voluntarily drank alcohol for 28 days in the two-bottle choice procedure. Further analyses of amygdala subregions revealed that the largest increase in CaMKIIα expression occurred in the CeA along with concomitant increased levels of the CaMKII phosphorylation site $pGluR1_{Ser831}$. Altered CaMKII activity in the CeA suggested physiological differences in alcohol dinking mice and whole cell patch electrophysiology performed in amygdala slices revealed an increase in the AMPA/NMDA ratio of alcohol-drinking mice in the CeA.

Through a wide array of experimental methodologies, CaMKII has been established as a regulator of synaptic plasticity, and this action likely occurs through its effects on AMPAR trafficking [64]. In the present experiment, increased AMPAR activity was consistent with increased expression of CaMKIIα and pGLUR1, however, the direct relationship between the two was not addressed. The following strategy could prove useful in establishing this relationship. First, co-expression of

CaMKII and AMPAR in central amygdala neurons needs to be demonstrated in alcohol-drinking mice. Second, biochemical methods can determine membrane insertion of phosphorylated and total AMPAR subunits. Third, patched neurons should be visualized via fluorescent dye tracer assays and fixed for immunohistochemistry of CaMKIIα and $pCaMKII_{Thr286}$. Lastly, a mechanistic link would be established by the disruption of increased AMPAR activity by CaMKII inhibition. This could be accomplished by microinjecting a CaMKII inhibitor into the amygdala prior to recordings or infusing the inhibitor into the bath solution, however, direct infusion of an inhibitor directly into the patched neuron would more clearly establish a postsynaptic CaMKII mechanism for unsilencing synapses through increased AMPAR function. Alternatively, other proteins identified that play a role in CaMKII mRNA transport, (KIF5C), and translation, (EEF2), as well as AMPAR transport, (KIF5C), and stabilization in the synapse, (NSF), may be targeted to disrupt this mechanism. Additionally, increased CaMKII and AMPAR activity suggests that chronic alcohol consumption has established LTP in CeA neurons. Occlusion of LTP induction would be predicted in alcohol-consuming mice if LTP is already established and could be interpreted as a mechanism for the persistent nature of alcohol behaviors regulated by the CeA. As CaMKII and AMPA are involved in synapse maturation, a demonstration of increased dendritic spine density would further support this mechanism and have a high impact regarding the idea that chronic alcohol use causes synaptic rigidity in the emotional part of the brain. Finally, we found that CaMKIIα was also increased in the NAc, a brain region known to process reward and to have major inputs from the amygdala. Additional

experiments should characterize these effects in a similar fashion to the present study due to its important role in regulating addictive behaviors.

Due to its role in plasticity, research has been primarily focused on CaMKII's role in learning and memory, and in the case of addiction, aberrant learning and memory. Alternatively, the strengthening of synapses through imbalances in CaMKII activity could have a number of maladaptive consequences including generating anxiety and seizures, symptoms that present in alcohol withdrawal. The two-bottle procedure used here did not produce overt signs of withdrawal (i.e open field, tail spin, or increased corticosterone levels) at the start of the light cycle, however 8 hours later these symptoms emerge (unpublished data). Follow up experiments should evaluate CaMKII expression and the electrophysiological properties of CeA neurons at this time point or using a model that causes dependence such as alcohol vapor exposure or forced alcohol ingestion. Mice that overexpress CaMKIIα in the forebrain display increased anxiety-like behavior [81] and given recent work demonstrating the CeA's role in anxiety [245], we predict that increased CaMKIIα would contribute to withdrawal-related anxiety.

Second, findings from the two-bottle procedure suggested that alcohol exposure caused increased glutamate activity in the CeA, a subregion known to play a central role in the reinforcing effects of alcohol, which may be a consequence of increased CaMKII activity that were found. To determine the role of CaMKII in alcohol reinforcement, these findings were extended to the operant self-administration paradigm. Comparison of alcohol and sucrose self-administering mice revealed that pCaMKII and pGLUR1 had increased expression levels in the CeA and

LA of alcohol self-administering mice. Preventing CaMKII activity, accomplished by infusing inhibitors in the amygdala, decreased alcohol but not sucrose self-administration in the absence of locomotor deficits and lead to the conclusion alcohol reinforcement is functionally and selectively regulated by CaMKII.

Elevated CaMKII and GluR1 phosphorylation seen in alcohol compared to sucrose self-administering mice supports the induction of long-term plasticity by alcohol. Electrophysiology experiments would be useful in determining synaptic activity including measuring AMPA/NMDA ratio and LTP in this eparadigm in the CeA and LA and may be related to alcohol reinforcement and cue processing respectively. For instance, enhanced activity in the LA may be related to increasing the salience of alcohol-related cues, a finding consistent with learning and memory hypotheses on addiction [58].

Here we found that large reductions of alcohol self-administration occurred through AMPA/kainate inhibition with NBQX. Because CaMKII was the focus of this experiment, AMPA modulation was not fully characterized. Future experiments can address this by determining if the reduction is reinforcer specific. Additionally, other AMPA subunit selective antagonists like the AMPA non-GluR2 subunit inhibitor NASPM would provide more compelling evidence of AMPA regulation. Additional evidence for AMPA regulation would be through specific agonists. Other considerations would be to block the phosphorylation site of GluR1 with synthetic inhibitory peptides directed at this site.

Third, mouse models of alcohol relapse are uncommon, despite extensive research characterizing the effect of alcohol on mouse brain and behavior and the

relevance of relapse to alcoholism. To address this deficiency, a mouse model of cue-induced reinstatement was developed. In this behavioral paradigm, mice were trained to self-administer alcohol in operant chambers where audio and visual cues signaled the delivery of the alcohol reinforcer. Once high levels of drinking were established, response contingent alcohol delivery and cues were removed. Mice demonstrated a reliable extinction of active lever presses demonstrated by similar response levels on the previously active and inactive levers and following the reinstatement test, mice given cues had significantly more active lever responses than the extinction group, an effect demonstrating cue-induced reinstatement of alcohol-seeking behavior. In a follow-up experiment, the role of ERK1/2 was evaluated by pretreating mice with SL327, an ERK1/2 inhibitor, prior to alcohol reinstatement tests. Reinstatement was observed in vehicle-treated mice reinstated and a potentiation of reinstatement was seen mice treated with SL327. To determine reinforcer specificity, a second group of mice was trained to self-administer sucrose and tested on reinstatement using the same procedure. Vehicle-treated mice did not reinstate sucrose-seeking behavior, however, SL327 pretreatment led to robust levels of reinstatement in sucrose mice. We concluded that reinstatement behavior can be modulated by ERK1/2 activity.

The procedure used was successful in promoting cue-induced reinstatement of alcohol-seeking behavior. In the second experiment, this result did not appear true for sucrose-seeking behavior when looking at vehicle-pretreated reinstatement tests between alcohol and sucrose groups. Adjusting the behavioral parameters, like lowering the response requirement or using a discriminate context for extinction

sessions, could promote this behavior as it possible with SL327 injections. Although a limitation of this procedure, this phenomenon does appear consistent with the idea that alcohol causes long-term changes to appetitive conditioning resulting in an enduring vulnerability to relapse. Similar findings have occurred in cocaine reinstatement where environmental stimuli paired with a single injection of cocaine elicited drug-seeking behavior even up to one year later while the highly palatable natural reinforcer sweetened condensed milk could not produce reinstatement behavior immediately after extinction [246]. These findings demonstrate that drug-related cues exert stronger and more persistent control over behavior than those related to natural reinforcers. The neurobiological mechanism for this phenomenon is likely mediated by aberrant synaptic plasticity that leads to maladaptive learning.

Collectively, the preclinical studies reported here support a growing literature on protein kinase regulation of addictive behaviors. Several protein kinases have been shown to modulate alcohol-specific behaviors including FYN, PKC, PKA, ERK1/2 [57] and from our data, CaMKII. CaMKII and ERK1/2 are especially intriguing because they are activated by increased intracellular calcium levels, are known regulators of LTP, and can enter the nucleus and affect transcription factors involved in plasticity [247]. Thus, they are candidate mechanisms for immediate effects such as receptor modulation and long-term functional changes that contribute to addictive behaviors. A large scale meta-analysis of literature pertaining to drug effects on protein translation and gene transcription determined that CaMKII and MAPK pathways are the 1st and 3rd most common pathways affected by drugs of abuse and that CaMKII represented a positive feedback loop that could be induced,

persist long-term, and be resistant to noise [248]. Furthermore, crosstalk between the two pathways occurs, activation of ERK1/2 by nicotine [249] and amphetamine [250] is blocked by CaMKII inhibition. Collectively, these findings along with our own, demonstrate that CaMKII and ERK1/2 are candidate mechanisms for the development and expression of addiction and indicate a need for further study.

Lastly, drug therapies that target protein kinases are difficult to achieve, especially in the case of CaMKII and ERK1/2. These kinases have a number of roles throughout the body including cell proliferation and survival [247] and their inhibition would likely cause a number of side effects. Alternative treatment strategies include identifying and inhibiting the specific interaction between protein kinases and their brain-specific substrates. For instance, from our studies the CaMKII substrate $pGLUR1_{Ser831}$ was increased in the amygdala of alcohol-drinking mice and inhibiting the specific interaction between CaMKII and p-GluR1 would potentially target the alcohol elevated CaMKII and pGluR1 activity. This target may have potential as clinical trials have shown that more general AMPAR antagonists appear to be well-tolerated and their use in treating addiction is being considered [251]. Another treatment strategy is aimed at breaking cue-drugs associations like enhancing the extinction of drug-related cues. For instance, repeatedly exposing an addict to drug cues without giving them the drug may disrupt the ability for the cue to promote craving and relapse. Targeting the consolidation of drug-cue memories by activating a drug memory and then interfering with memory consolidation is another strategy. As both CaMKII and ERK1/2 play roles in associative memory and memory consolidation, modulating their activity directly or through upstream targets during

these interventions would likely accelerate extinction or arrest consolidation mechanisms, and promote long-term abstinence.

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