Alcohol-seeking behavior elicited by a discrete Pavlovian alcohol cue is invigorated by an alcohol context and requires AMPA glutamate receptors in the basolateral amygdala

Joanna M. Sciascia

A Thesis in the
Department of Psychology

Presented in Partial Fulfillment of the Requirements
For the Degree of Master of Arts (Psychology) at
Concordia University
Montreal, Quebec, Canada

August, 2015

CONCORDIA UNIVERSITY

School of Graduate Studies

This is to certify that the thesis prepared

By: Joanna Marie Sciascia

Entitled: Alcohol-seeking behavior elicited by a discrete Pavlovian alcohol cue is invigorated by an alcohol context and requires AMPA glutamate receptors in the basolateral amygdala

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Signed by the final examining committee:

Dr. Wayne Brake Chair

Dr. Uri Shalev Examiner

Dr. James Pfaus Examiner

Dr. Nadia Chaudhri Supervisor

Approved by: _____

Chair of the Department or Graduate Program Director

_____ _____

Date: Dean of Faculty

Abstract

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Joanna Marie Sciascia

Environmental cues associated with alcohol consumption can trigger craving and facilitate relapse in abstinent alcoholics. We hypothesized that alcohol-seeking behavior evoked by a discrete cue associated with alcohol, would be influenced by context and require glutamate transmission in the basolateral amygdala. Male, Long-Evans rats that had previously consumed ethanol (EtOH; 15%; v/v) received Pavlovian conditioning sessions in which a 10-sec auditory stimulus (CS; 15 trials per session) was paired with EtOH (0.2 ml/CS). Entries into a fluid port where EtOH was delivered were measured. Pavlovian conditioning occurred in a specific context (alcohol context) and was alternated with sessions in a different context (non-alcohol context) where neither the CS nor EtOH was presented. At test, the CS was presented without EtOH in either the alcohol context or the non-alcohol context. In a separate study, rats received a bilateral microinfusion (0.3 µl/hemisphere) of 0, 0.3, or 1.0 µg of the AMPA glutamate receptor antagonist NBQX [2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione)] in the BLA. The effect of NBQX administration in the CPu on alcohol-seeking behavior elicited by an alcohol-predictive CS was also tested in a non-alcohol context. Alcohol-seeking elicited by the CS was invigorated in the alcohol context relative to the non-alcohol context. NBQX in the BLA attenuated CS responding at test in both contexts, but had no effect when infused into the CPu. These data highlight an important role of context in modulating the vigor of Pavlovian-conditioned alcohol-seeking, and suggest that AMPA receptors within the BLA are required for the expression of this behavior.

Keywords: Alcohol, Pavlovian conditioning, basolateral amygdala, NBQX, Context, Cues, Ampa receptors

Acknowledgments

I wish to express my sincerest gratitude to Dr. Nadia Chaudhri. Thank you for your support, constant guidance, and your perseverance in training me to be a researcher. Most of all, thank you for believing in me. I would also like to thank Patricia Janak for her invaluable feedback on the experimental design used in this thesis, and Rebecca Reese for collecting part of this data. Our research would not be possible without support from Heshmat Rajabi, Steven Cabilio who assisted with MED-PC programming and data extraction, and Aileen Murray who guided me through the proper handling and care of laboratory animals. I would also like to acknowledge Jose Mendoza, Lindsay Sparks, Zarish Abbas and Sarah Nolan Poupart for their endless support and encouragement, as well as all the dedicated C-lab members for their feedback. Furthermore, I would like to extend my gratitude to Dr. Uri Shalev, and Dr. Jim Pfaus for accepting to be part of my defense committee.

I would like to extend my deepest gratitude to my parents and sisters. Mom and Dad, you are the light of my life. Thank you for giving me everything you have, and working so hard to support our family. To my big sisters and their beautiful children, words can not describe the joy you bring to my life. I'm dedicating this thesis to all of you.

Contribution of Authors

Nadia Chaudhri and Patricia Janak designed the experiments and obtained funding. Rebecca Reese conducted experiment1. Joanna Sciascia analyzed data from experiment 1, collected, and analyzed data for experiment 2 and wrote the manuscript under the guidance of Nadia Chaudhri.

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Thesis overview

Environmental stimuli that are reliably paired with the pharmacological effects of alcohol can, through associative learning, come to serve as cues that predict alcohol. Alcohol-predictive cues can evoke craving in abstinent alcoholics, which in turn can lead to alcohol-seeking behaviors that increase the risk of relapse (Cooney, Litt, Morse, Bauer, & Gaupp, 1997; Nees, Diener, Smolka, & Flor, 2012; Schneider et al., 2001). Thus, it is of value to investigate the behavioral and neurobiological mechanisms underlying relapse elicited by cues that predict alcohol.

Cues that predict alcohol can be separated into at least 2 categories, based on their temporal relation to the pharmacological effects of alcohol. 'Discrete alcohol cues' are stimuli that are closely linked to alcohol consumption, and are reliably experienced immediately before the pharmacological effects of alcohol take effect. 'Contextual alcohol cues' are multimodal stimuli that routinely occur in the background during alcohol consumption (Cassaday, Horsley, & Norman, 2005; Janak & Chaudhri, 2010; Nees et al., 2012; Remedios, Woods, Tardif, Janak, & Chaudhri, 2014; Sciascia, Mendoza, & Chaudhri, 2014; Sparks, Sciascia, Ayorech, & Chaudhri, 2014). Although discrete and contextual cues can both independently trigger alcohol-seeking behaviors (Chaudhri, Sahuque, & Janak, 2008, 2009; Millan & McNally, 2011; Remedios et al., 2014; Sciascia et al., 2014; Zironi, Burattini, Aicardi, & Janak, 2006), less is known about how contextual alcohol cues can influence alcohol-seeking behaviors elicited by discrete alcohol cues. A recent study in our laboratory found that alcohol-associated contexts can invigorate alcohol-seeking behavior elicited by a discrete Pavlovian cue that predicts alcohol in rats (Remedios et al., 2014). The current research sought to replicate this finding using a new behavioral procedure, and to investigate the neurobiological processes that mediate alcohol-seeking behavior elicited by discrete Pavlovian alcohol cues that are experienced in different environmental contexts.

The glutamatergic system has been implicated in alcohol dependence in humans (Holmes, Spanagel, & Krystal, 2013) and operant conditioned alcohol-seeking behavior in animal models (Bäckström & Hyytiä, 2004; Cannady, Fisher, Durant, Besheer, & Hodge, 2013;

Gass, Sinclair, Cleva, Widholm, & Olive, 2011; Rodd et al., 2006). For instance, preclinical research shows that systemic injections of AMPA (α-Amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid) and NMDA (N-methyl-d-aspartate) glutamate receptor antagonists decrease cue-induced reinstatement of alcohol-seeking behavior (Bachteler, Economidou, Danysz, Ciccocioppo, & Spanagel, 2005; Bäckström & Hyytiä, 2004). In addition, enhancing glutamate activity increases cue-induced reinstatement of alcohol self-administration in rats (Cannady et al., 2013). It is evident that glutamate transmission plays a critical role in behavior maintained by alcohol cues, but the role of glutamate in alcohol-seeking behavior elicited by discrete Pavlovian cues has not been investigated.

The basolateral amygdala (BLA) is critically involved in the formation of Pavlovian associations between environmental stimuli and rewards (Meil & See, 1997). The BLA receives and integrates input from cortical and subcortical regions about auditory and olfactory conditioned cues (Grace & Rosenkranz, 2002). Functional inactivation of the BLA attenuates context-induced renewal of alcohol-seeking, suggesting that the BLA is required for the renewal of alcohol-seeking behavior triggered by an alcohol-associated context (Chaudhri, Woods, Sahuque, Gill, & Janak, 2013). In addition, glutamate activity in the BLA increases during reinstatement of alcohol-seeking but not food-seeking behavior, indicating that glutamate in the BLA is an important mediator of alcohol-seeking behavior (Gass et al., 2011). Therefore, the BLA appears to be a critical brain region that is involved in the formation of memories that involve CS-US associations, and a prime candidate in the study of alcohol-seeking behavior mediated by discrete and contextual alcohol-conditioned cues.

The present thesis research sought to first examine whether alcohol-seeking behavior elicited by a discrete alcohol-associated cue would be enhanced in an alcohol context relative to a context where alcohol had never been consumed. In order to test this hypothesis, we used an experimental design that involved alternating Pavlovian conditioning sessions in one context, referred to as the 'alcohol context,' with exposure to second context where alcohol was never experienced, called the 'non-alcohol context'. During Pavlovian conditioning sessions, a discrete cue is paired with the delivery of alcohol, while no cues or alcohol are delivered during sessions in a non-alcohol context. At test, the discrete cue is presented in the alcohol context and the non-alcohol context using a within-subjects design, and alcohol-seeking behavior is indexed by the number of port entries made into the fluid port. We also determined if AMPA glutamate

receptors within the BLA are required for alcohol-seeking behavior elicited by a discrete Pavlovian cue in both contexts. In a control study, we tested the role of AMPA glutamate receptors in the caudate putamen (CPu), just dorsal to the BLA, on alcohol-seeking behavior elicited by a discrete cue in a non-alcohol context.

Background

Relapse to alcohol use and abuse can be precipitated by cues that predict alcohol

Alcohol is one of the most commonly used addictive substances in the world, (Ferreira & Willoughby, 2008; Vonghia et al., 2008) and a leading cause of death and disability. In 2011, 16.7 million people aged 12 and above were classified as alcohol dependent in the United States of America (*Results from the 2011 National Survey on Drug Use and Health: Summary of National Findings*, 2012). It is estimated that of those seeking treatment from alcohol dependence, 85% will relapse within the first year of recovery (Sinha, 2011).

Given that relapse is such a substantial problem, considerable effort has been made to understand the factors that promote relapse. Exposure to stimuli that have been previously linked to alcohol consumption can evoke subjective and physiological indications of craving (Field & Duka, 2002; Nees et al., 2012; Schneider et al., 2001), which may in turn facilitate relapse (Cooney et al., 1997; Evren, Cetin, Durkaya, & Dalbudak, 2010; Field & Cox, 2008)**.** A link between environmental stimuli and craving has been demonstrated in studies that have used classical conditioning techniques in order to create associations between arbitrary cues and alcohol. For example, Field & Duka (2002) gave social drinkers two drinks that distinctively differed in taste, smell and color of the glass in which the drink was presented. One drink always contained 0.2 g/kg of 90% alcohol and was designated as the conditioned stimulus (CS+), and the other drink never contained alcohol and was designated as the CS-. During conditioning sessions, subjects were instructed to smell and consume five 50 ml glasses of the CS+, and later were asked to drink five 50 ml glasses of the CS- in a counterbalanced order. Following successive conditioning sessions, subjects were presented with the CS+ and CS- (which did not contain alcohol), and were asked to choose which beverage they would prefer to consume after they had smelled and tasted each drink. The results showed that on the last conditioning session, the CS+ evoked higher skin conductance relative to the CS-, and gazing behavior towards the

CS+ was significantly higher than the CS- during the subsequent test day. In addition, subjective ratings of craving were elevated in response to the CS+, while ratings of craving to the CS- decreased across conditioning sessions. This experiment demonstrates that an initially neutral cue, such as taste, can come to acquire incentive properties through repeated pairings with alcohol (Ludwig & Wikler, 1974). Similar findings have been replicated in studies using alcohol dependent subjects (Pomerleau, Fertig, Baker, & Cooney, 1983). For instance, sniffing a preferred alcoholic beverage increases swallowing, salivation and heart rate in abstinent alcoholics relative to non-alcoholic controls (Pomerleau et al., 1983). In addition, when alcohol-dependent individuals were asked to think about personal alcohol cue-related situations, subjects displayed increased craving and negative affect, along with a decrease in positive affect relative to thinking about neutral situations (Fox, Bergquist, Hong, & Sinha, 2007).

Alcohol-associated cues not only elicit subjective and physiological changes in alcohol dependent individuals, but also induce changes in brain activity (Fryer et al., 2013; Myrick et al., 2004). For instance, in a study conducted by Myrick and colleagues (2004), alcohol dependent and social drinkers were placed into a magnetic resonance imaging scanner (MRI) and given a sip of alcohol before viewing images of alcoholic beverages, non-alcoholic beverages, and neutral images. Results showed that several brain regions including the nucleus accumbens, ventral tegmental area and insula were activated during alcohol images and not for images depicting non-alcoholic beverages. Furthermore, social drinkers did not display activation in these areas when alcohol images were displayed. Importantly, within the alcohol group, craving elicited by alcohol-related images was correlated with activation in the left nucleus accumbens, anterior cingulate and orbitofrontal cortex. These results indicate that brain activation in response to alcohol-related images differs between alcoholic and non-alcoholic individuals, and that self reported craving in alcohol dependent individuals is correlated with activation of discrete brain regions. Taken together, these data support the hypothesis that environmental stimuli associated with prior intake of alcohol can be an important determinant of continued alcohol abuse. Therefore, a deeper understanding of the behavioral and neural mechanisms that govern alcohol-seeking behaviors evoked by cues that predict alcohol is needed.

The context in which a discrete alcohol cue is experienced can modulate the vigor of conditioned responses elicited by the discrete cue

A wealth of research from human and animal studies has shown that multiple different types of environmental stimuli can come to signal alcohol via classical conditioning (Beirness & Vogel-Sprott, 1984; Chaudhri et al., 2008; Drobes, Saladin, & Tiffany, 2001; Field & Duka, 2002; Fryer et al., 2013; Glautier, Drummond, & Remington, 1994; Janak & Chaudhri, 2010; Ludwig & Wikler, 1974; Myrick et al., 2004; Nees et al., 2012; Shapiro & Nathan, 1986). These environmental stimuli can be divided into two broad categories based on their proximal relation with drug intake. *Discrete cues* are conditioned stimuli that are most directly linked to drug or alcohol consumption. For example, the sight, smell, and taste of alcohol are sensory properties that repeatedly precede alcohol intoxication. In contrast, *contextual cues* are not temporally linked with drug administration, and are instead described as a constellation of stimuli that are routinely present in the background during drug intake (Remedios et al., 2014). For example, entering a local bar may entice an individual to have a drink, if he or she has frequently consumed alcohol in a similar context. A goal of the current experiments was to investigate the independent and combined effect of discrete and contextual cues on alcohol-seeking behavior.

Mounting evidence indicates that both discrete and contextual cues are important during the learning and expression of conditioned drug-seeking behavior (Chaudhri et al., 2008; Conklin, Robin, Perkins, Salkeld, & McClernon, 2008; Nees et al., 2012; Sciascia et al., 2014; Zironi et al., 2006). The capacity of contexts associated with alcohol to trigger relapse-like behavior in animals was initially investigated using an operant conditioning procedure in which rats were trained to make an operant response in a distinct context (called 'context A') in order to obtain an appetitive stimulus. This behavior was then extinguished by withholding the drug in a different environmental context (context B). Upon placement into context A, the operant response was reinstated in the absence of alcohol delivery, suggesting that the specific memory of the context in which rats received alcohol was sufficient to reinstate alcohol-seeking behavior (Chaudhri et al., 2008). This ABA renewal model has been widely used to study the effect of context on relapse for a number of abused drugs, including heroin (Bossert et al., 2011; Bossert, Liu, Lu, & Shaham, 2004), cocaine (Crombag, Grimm, & Shaham, 2002; Fuchs, Eaddy, Su, & Bell, 2007), and nicotine (Diergaarde, de Vries, Raasø, Schoffelmeer, & De Vries, 2008).

The influence of context on alcohol-seeking behavior elicited by a discrete, Pavlovian alcohol cue has also been investigated using the ABA renewal procedure (Chaudhri et al., 2008, 2009; Sciascia et al., 2014). In this procedure, rats were trained to discriminate between two 10-

sec auditory stimuli in context A. One auditory stimulus (CS+) was consistently paired with ethanol, and the other stimulus (CS-) was presented without ethanol. Rats received 16 presentations of each CS during daily Pavlovian discrimination training sessions. During these sessions, entries into a fluid port where ethanol was delivered for oral consumption were measured during the CS+ and CS-. Following training, conditioned port-entries were extinguished in context B, where the CS+ and CS- were presented as during training, but ethanol was not delivered. Twenty-four hours after the last extinction session, renewal was assessed by placing the rats back into context A and exposing them to the CS+ and CS- without ethanol. Using this procedure, Chaudhri and colleagues (2008, 2009) showed that during training, rats learned to reliably discriminate between the CS+ and CS-, as indicated by more responses during the CS+ rather than the CS-. Responding to both auditory cues diminished across extinction sessions in context B. At test, when rats were placed back into the training context (context A), responding increased during the CS+ compared to extinction, whereas CS- responding remained low. These findings are indicative of context-induced renewal of alcohol-seeking behavior, and support the hypothesis that contexts can acquire the capacity to predict alcohol availability through Pavlovian conditioning.

In the everyday experience of drug abusers, it is unlikely that individuals will be exposed to discrete or contextual cues associated with alcohol in isolation. Instead, these cues often co-occur, and together can enhance subjective and physiological indications of craving, more so than when experienced independently (Nees et al., 2012)**.** This hypothesis was recently tested by Remedios et al (2013) using an animal model of Pavlovian conditioned alcohol-seeking in which rats were trained in a distinctive context to discriminated between a CS+ that was paired with alcohol and a CS- that was presented without alcohol. Following training, rats were repeatedly exposed to a different context, referred to as the non-alcohol context, where the CS+, CS- and alcohol was not presented. In order to assess the impact of context on behavior elicited by an alcohol predictive cue, half of the rats were tested in the alcohol context and half were tested in the non-alcohol context. In both contexts the discrete cues (CS+ and CS-) were presented, but no alcohol was delivered. In a second, similar experiment, rats were also tested in a third, novel context in which the cues were presented without alcohol. The results showed that port entries during the CS+ were significantly elevated in the alcohol context relative to the non-alcohol

context and novel context, suggesting that the combined experience of discrete and contextual cues associated with alcohol may be the strongest trigger for craving, and potentially relapse.

In the present research, we sought to test the hypothesis that responding elicited by a discrete alcohol predictive cue will be enhanced in an alcohol context relative to a non-alcohol context, while addressing some important limitations of the previous study. Specifically, the procedure used by Remedios and colleagues (2014) involved training rats to discriminate between a CS+ and CS- in an alcohol context over 14 consecutive sessions, followed by 8 consecutive sessions of exposure to a non-alcohol context prior to test. This procedure did not allow for equal exposure to both contexts, which may have contributed to the elevated levels of responding at test in the alcohol context. To address this limitation, Pavlovian training sessions in the present study were alternated daily with sessions of exposure to a non-alcohol context, thereby ensuring that all rats received an equal amount of exposure to either context prior to testing procedures. Another limitation of the prior design was that performance at test may have been influenced by the fact that animals had not received alcohol for a prolonged period of time prior to test. To avoid this potential limitation, our revised procedure allowed for a maximum time of 3 days without alcohol prior to test.

Glutamate neurotransmission contributes to alcohol-seeking behavior elicited by cues that predict alcohol

There is a growing interest in novel pharmacotherapy for alcohol addiction, and considerable focus has been placed on the glutamatergic system. Glutamate is an excitatory neurotransmitter that is abundant in the brain, and mediates approximately 70% of synaptic transmission in the central nervous system (Iverson, Iverson, Bloom, & Roth, 2009). Glutamate receptors can be either ionotropic, which mediate fast excitatory transmission, or metabotropic, which mediate slower, modulatory transmission in the brain (Iverson et al., 2009). Ionotropic receptors consist of N-methyl-D-asparate receptors (NMDAr), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAr) and kainite receptors. The glutamate system is of particular interest due to its involvement in various aspects of alcohol addiction, including glutamatergic neuroadaptations that arise following chronic and acute exposure to alcohol. For instance, chronic exposure to ethanol inhibits NMDAr functioning (Lovinger, White, & Weight, 1989), and as an adaptive consequence, an up-regulation of NMDAr functioning occurs which

causes increased excitatory neurotransmission. These effects have been shown to contribute to ethanol tolerance and withdrawal in humans (Tsai et al., 1998) and in animals (Bienkowski et al., 2001; Dahchour, De Witte, & Witte, 2003; Valverius, Crabbe, Hoffman, & Tabakoff, 1990). In addition, post-mortem studies of alcohol dependent patients reveal a correlation between NMDA receptor upregulation and human alcohol dependence (Freund & Anderson, 1999).

Glutamate plays a critical role in synaptic plasticity, and is fundamental in learning and memory processes (Riedel, Platt, & Micheau, 2003). Experiments in this thesis tested the hypothesis that glutamate neurotransmission at AMPAr is needed for the expression of alcohol-seeking behavior elicited by discrete cues that predict alcohol. This hypothesis is based on a rich literature identifying a role for glutamate in Pavlovian learning, memory formation and behavior maintained by cues that predict alcohol. For instance, studies using various types of glutamate receptor antagonists have revealed a critical role of glutamate in context and cue-induced relapse-like behavior (Bachteler et al., 2005; Bäckström & Hyytiä, 2004; Bossert et al., 2004; Van den Oever et al., 2008). In one study, Backstrom and Hyytia (2004) examined the effects of blocking ionotropic glutamate receptors on cue-induced ethanol-seeking behavior using a reinstatement model of oral self-administration. Rats were conditioned to associate an anise odour with the delivery of a 10% ethanol solution. In addition, delivery of ethanol was accompanied by a 3s light stimulus. Next, rats received extinction sessions in which lever presses had no programmed responses, and olfactory stimuli signalling delivery were withheld. In order to investigate the effects of ionotropic glutamate receptors on reinstatement of ethanol-seeking behavior, rats were given systemic injections of saline, a non competitive NMDA receptor antagonist (MK-801), the competitive NMDA receptor antagonist (CGP39551), an NMDA/glycine site antagonist (L-701,324), a competitive AMPA/kainate receptor antagonist (CNQX), or an opioid receptor antagonist, naltrexone. During reinstatement tests, the rats were placed into operant conditioning chambers containing the ethanol-predictive odor, where each lever press resulted in the presentation of the light/tone stimulus. During the first two active lever presses, a small priming dose of ethanol was delivered for oral consumption, and subsequent lever presses had no programmed consequences. Systemic injections of the NMDA/glycine antagonist, the AMPA/kainate glutamate receptor antagonist, and the opioid receptor antagonist, attenuated reinstatement of responding relative to saline-infused animals. These data suggest that blocking glutamate transmission can reduce cue-induced reinstatement of alcohol-seeking

behavior. One limitation of this study, however, is that the AMPAr antagonist CNQX also has affinity to the glycine site located on NMDArs (Lester et al, 1989), making it difficult to isolate a specific role of AMPAr functioning in cue-induced reinstatement of alcohol-seeking behavior. To circumvent this possible confound, the present studies used NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), a compound that selectively targets AMPA receptors, and does not have affinity for the glycine site located on NMDAr (Goldstein & Litwin, 1993).

Like NMDA receptors, chronic exposure to ethanol upregulates AMPA receptor subunit proteins in animal models (Neasta, Ben Hamida, Yowell, Carnicella, & Ron, 2010) and in post mortem brain preparations on human alcoholics (Breese, Freedman, & Leonard, 1995). In behavioral models of alcohol self-administration, systemic infusions of an AMPA receptor antagonist block reinstatement of alcohol-seeking in rats (Bäckström & Hyytiä, 2004) and mice (Sanchis-Segura et al., 2006). Further support for the involvement of AMPAr functioning in alcohol-seeking behavior is provided by a recent study conducted by Cannady and colleagues (2012), which investigated the effect of enhancing AMPA receptor activity on alcohol self-administration and cue-induced reinstatement using a positive allosteric modulator, aniracetam (ANI). Rats were first trained to press a lever in order to receive a 15% ethanol solution, which was accompanied with a light cue. Following stable self-administration, rats were pretreated with 0, 1, 5, 10, and 30 mg/kg (i.p) of ANI using a within-subjects design. The results revealed that following infusions of 1 and 5 mg/kg of ANI, responses made on the active lever were potentiated relative to pretreatment with saline. Interestingly, ANI did not alter operant sucrose responding under identical training procedures, which suggests that enhanced glutamate activity at AMPA receptors induced by ANI pretreatment selectively potentiated alcohol-seeking behavior. To test the effects of ANI on cue-induced reinstatement of alcohol-seeking behavior, rats were given extinction sessions following stable self administration, in which previously reinforced responses were extinguished by withholding the light cue and alcohol. At test, rats were infused with saline or 5 mg/kg of ANI prior to the reinstatement test session, and lever responses resulted in the light cue without alcohol. During the reinstatement test, rats pretreated with saline and ANI displayed cue-induced alcohol-seeking behavior as shown by increased responding on the active lever compared with the last day of extinction. Interestingly, responses made on the active lever were potentiated following pretreatment with ANI relative to rats infused with saline. Collectively, these results suggest that AMPA receptor signaling can

invigorate alcohol self-administration and facilitate cue-induced relapse of alcohol-seeking behavior.

The BLA is important for conditioned responses elicited by cues that predict either aversive or appetitive unconditioned stimuli.

The amygdala is critically involved in the acquisition (Fanselow & Kim, 1994), consolidation (Nader, Majidishad, Amorapanth, & LeDoux, 2001; Paré, 2003), and expression of Pavlovian associations between a CS and US (Wilensky, Schafe, Kristensen, & LeDoux, 2006). One subsystem of the amygdala is the basolateral complex (BLA), which is comprised of the lateral (LA), basolateral (BL), and basomedial nuclei. The BLA is anatomically well situated to integrate information from a variety of sensory modalities. For instance, it receives sensory information from the auditory cortex (Goosens & Maren, 2001; Quirk, Repa, & LeDoux, 1995) and the olfactory cortex (Nishijo, Uwano, Tamura, & Ono, 1998). In addition, projections from the hippocampus to the BLA underlie conditioning to contextual cues (Maren, 2001). These neuroanatomical data suggest that the BLA may be a locus of convergence for information about conditioned stimuli and unconditioned stimuli. Indeed, animals with damage to the BLA do not respond to an auditory or contextual cue that signals a fearful event, such as a footshock (Goosens & Maren, 2001; Nader et al., 2001). Furthermore, functional inactivation of the BLA prior to fear conditioning disrupts cue-induced fear learning (Wilensky et al., 2006) and place avoidance (Vafaei, Jezek, Bures, Fenton, & Rashidy-Pour, 2007).

The BLA is also essential for conditioned drug-seeking behaviors. For instance, excitotoxic lesions or reversible inactivation to the BLA impairs the acquisition of cocaine self administration on a second order schedule of reinforcement (Whitelaw, Markou, Robbins, & Everitt, 1996), and the reinstatement of extinguished cocaine-seeking behavior following exposure to cocaine-associated conditioned stimuli (Fuchs et al., 2005; Meil & See, 1997). The BLA has also been implicated in conditioned drug-seeking behaviors using other reinforcers including heroin (Fuchs & See, 2002), cocaine (Fuchs et al., 2005) and more recently, alcohol (Chaudhri et al., 2013; Millan & McNally, 2011). For example, context-induced renewal of Pavlovian-conditioned alcohol-seeking behavior is attenuated by bilateral or unilateral inactivation of the BLA (Chaudhri et al., 2013). Such findings suggest that the BLA is a key

brain region responsible for processing and integrating behaviors triggered by conditioned stimuli.

Consistent with the findings outlined above, studies have also implicated the BLA in cue-induced reinstatement of alcohol-seeking behavior. Based on previous literature indicating that glutamatergic transmission is required for learning and the formation of CS-US associations (Bachteler et al., 2005; Bäckström & Hyytiä, 2004; Bossert et al., 2004; Van den Oever et al., 2008)**,** Gass and colleagues (2010) quantified changes in extracellular glutamate in the BLA during cue-induced reinstatement of alcohol-seeking behavior or food-seeking behavior. Elevated glutamate activity was observed within the BLA during cue-induced reinstatement of alcohol-seeking behavior, relative to food-seeking behavior, suggesting that glutamate in the BLA may play a facilitatory role in alcohol-seeking induced by alcohol-predictive cues.

Specific aims of the current research

Mounting clinical and preclinical data support the hypothesis that discrete and contextual stimuli associated with alcohol can elicit craving, and precipitate relapse. Furthermore, the context in which a discrete cue is experienced can influence the vigor of alcohol-seeking behavior elicited by that cue. Given that glutamate receptors mediate synaptic plasticity involved in learning and memory, the conditioning of these cues to alcohol may be mediated by glutamatergic transmission in specific brain regions. The BLA is a prime candidate for investigation, as it is a locus of convergence of afferents from both subcortical and cortical sensory regions. To our knowledge, no studies have examined the effects of selective AMPA receptor antagonism in the BLA on alcohol-seeking behavior elicited by a discrete alcohol cue.

Experiments in this thesis tested the hypothesis that environmental context can influence conditioned responding elicited by discrete, Pavlovian alcohol cues. Briefly, rats that had previously consumed ethanol in the home cage, received Pavlovian conditioning training sessions in which an auditory CS was paired with alcohol for oral consumption. These training sessions occurred in a distinctive context, referred to as the alcohol context, and were alternated with sessions of exposure to a second, different context referred to as the non-alcohol context, where the CS and ethanol were never presented. At test, the CS was presented without ethanol in either context in the absence of alcohol. We predicted that responding to the discrete alcohol-predictive cue would be invigorated in an alcohol context relative to the non-alcohol context.

We also investigated the contribution of AMPA glutamate receptors in the BLA to Pavlovian-conditioned alcohol-seeking behavior. Using the method described above, at test rats received a bilateral microinfusion into the BLA of 0, 0.3, or 1.0 µg/0.3µl of the AMPA receptor antagonist NBQX. We predicted that alcohol-seeking elicited by a discrete CS would be diminished in rats infused with NBQX, and that this effect may occur in both contexts. In this experiment, the effect of NBQX infusions in the BLA was also investigated during a Pavlovian conditioning session where the discrete cue was paired with alcohol.

Finally, to ensure brain region specificity, the effect of AMPA receptor antagonism was also tested in the CPu, a region dorsal to the BLA that also expresses AMPA receptors. The CPu contains AMPA receptor on postsynaptic neurons (Tarazi, Campbell, Yeghiayan, & Baldessarini, 1998) and has been previously used in anatomical control studies for the BLA (Fuchs et al., 2005).

General Methods

Subjects

Male, Long-Evans rats (220-275g on arrival; n=75) were obtained from Harlan Laboratories (Indianapolis, USA). They were individually housed in polycarbonate shoebox cages in a temperature- (21°C) and humidity-controlled colony room on a 12-hour light/dark cycle (lights on at 0700-hr; procedures conducted during the light phase). Unrestricted access to rat chow (Ralston Purina, Canada) and water was provided throughout. Rats had 7-10 days to acclimate to the colony room, during which time they were weighed and handled on Monday, Wednesday and Friday. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Ernest Gallo Clinic and Research Center and the Animal Research Ethics Committee (AREC) at Concordia University, and are in agreement with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996).

Apparatus

Behavioral procedures were conducted in conditioning chambers (ENV-009A; 32.8 cm x 32.8 cm x 32.8 cm; Med Associates Inc., St-Albans, VT) that were housed in custom-made, ventilated, sound attenuating melamine cubicles (53.6 cm x 68.2 cm x 62.8 cm). The sidewalls of each chamber were made of stainless steel, and the rear walls, ceilings and front walls were made of clear Plexiglas. The floors were made of metal bars that extended from the rear wall to the front wall. A fluid receptacle (ENV-200R3AM) was located 2 cm from the floor, near the center of the right wall. Fluid was delivered into the receptacle via a 20-ml syringe that was mounted onto a pump (Med Associates Inc., PHM-100, 3.33 RPM) located outside the sound-attenuating cubicle. Entries into the fluid port were measured by interruptions of a photo beam located across its entrance. A white light (Med Associates Inc., 75W, 100 mA, ENV-215M) was located near the ceiling on the left side of the chamber. The same wall featured a white noise generator (Med Associates Inc., ENV-225SM, 80-85 dB) and clicker stimulus (Med Associates Inc., ENV-135M, 75-80 dB). A personal computer (PC) computer running Med PC IV software controlled fluid delivery and auditory stimulus presentations, and also recorded port entries.

Drugs

Ethanol (EtOH, 15%, *v/v)* was prepared by diluting 95% EtOH in tap water. AMPA glutamate receptors in the BLA and CPu were blocked using NBQX [2,3-Dioxo-6-nitro-1,2,3,4 - tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium salt)] (Abcam Inc, Cambridge, USA;118876-58-7). NBQX (10 mg) was dissolved in 0.6 ml of sterile 0.9% sodium chloride to obtain a concentraion of 5 µg/0.3 µl. Concentrations of 1.0 µg/0.3 µl and 0.3 µg/0.3 µl were made through serial dilution. Aliquots of each concentration were stored in a - 20° C freezer until needed. NBQX concentration was chosen based on preliminary research showing a robust decrease in port entries elicited by a discrete alcohol cue following bilateral infusion of 5 µg/0.3 µl or 1 µg/0.3 µl of NBQX into the BLA. The latter concentration was obtained from published data on fear conditioning studies that have used infusions of 3 µg of NBQX in the BLA (Walker & Davis, 1997; Walker, Paschall, & Davis, 2005).

General Procedures

Ethanol consumption in the home-cage

Ten days after arrival, rats were acclimated to the taste and pharmacological effects of EtOH in their home-cage using a 24-hr, intermittent-access schedule that induces high levels of EtOH consumption in rats (Simms et al., 2008; Sparks et al., 2014; Wise, 1973). Rats had access to water via a 400 ml plastic bottle for 7 days/week. However, on Monday, Wednesday and Friday a 100 ml graduated cylinder containing 15% EtOH was placed onto the lid of the home-cage for 24 hr. Before each session, EtOH cylinders, water bottles and rats were weighed, and 24 hr later EtOH cylinders and water bottles were re-weighed to record consumption. To mitigate the effects of side preference on intake, the placement of EtOH and water containers on the home-cage was alternated in each session. Spillage was accounted for by subtracting EtOH and water lost from bottles that were placed on empty cages from EtOH consumption during the corresponding session.

Surgery

Upon completing home-cage ethanol exposure, rats in experiments 2 and 3 were implanted with bilateral, 26-gauge guide cannulae (Plastics One, Roanoke, VA) targeting the BLA (AP: -2.8; ML: ± 5.1; DV -5.4; final DV at injector tip is -8.4) or CPu (AP: -2.8; ML: ±

5.1; DV -4; final DV at injector tip is -6), respectively. Rats were anesthetized with isoflurane, their heads shaved and placed into a stereotaxic apparatus. An incision was made to expose the skull, and DV coordinates at bregma and lambda were used to verify a flat skull position. Guide cannulae were anchored to the skull using dental cement and metal screws and occluded with metal obdurators of the same length. One hour into surgery and upon completion of surgery, 1 ml of 0.9% saline was administered (s.c) to maintain hydration. Following surgery, rats received a single injection of buprenophine (0.1 ml/kg, s.c.), and sweetened, softened rat chow was provided to encourage feeding. Weight gain was monitored during a subsequent 7-20 day recovery period.

Habituation to behavioral testing chambers

After recovery, rats were transported on a cart from the colony room to the behavioral testing room and handled individually for 1 min. The next day they were placed into a designated behavioral testing chamber for 20 min, during which time the house light was illuminated and entries into the fluid port were recorded. Each chamber was set up as context 1 on the first day of habituation, and context two on the subsequent day. Contexts were created by the addition of distinctive visual, olfactory and tactile stimuli to the chamber. Context 1 consisted of black walls, created by placing black cardboard paper over the Plexiglas wall of the conditioning chamber, with a smooth Plexiglas floor insert placed on the floor of the conditioning chamber. Brown paper towels were placed on a wastepan underneath the conditioning chamber floor, along with a strip of white non-absorbent paper on top of the brown paper, onto which 3 sprays of lemon odor was applied. Context 2 featured clear Plexiglas walls, and a wire mesh insert placed onto the conditioning chamber floor. White absorbent paper was placed on a waste pan underneath the conditioning chamber floor, along with a strip of white non-absorbent paper on top of the white absorbent paper, onto which 3 sprays of almond odor was applied. Odors were prepared by adding tap water to lemon oil (SAFC Supply Solutions, St Louis, MO) or benzaldehyde (almond odor; OMEGA Chemical Company Inc., Levis, QC, Canada) for final concentrations of 10% (v/v) and were applied to waste pans beneath the chamber floors.

Pavlovian Conditioning

Pavlovian conditioning sessions (Mon-Fri; 75 min) were conducted to train rats to associate a discrete auditory cue with EtOH. Once each rat was weighed and placed into its

assigned testing chamber the Med-Associate program was initiated, and 2 min later the house light in each chamber was illuminated to indicate the start of the session. During each session, rats received 15 presentations of an auditory conditioned stimulus (CS, 10-sec continuous white noise) that was delivered on a variable-time 260-second schedule. At 4 sec after CS onset 0.2 ml of EtOH was delivered into a fluid receptacle for oral consumption. A total of 3 ml of EtOH was delivered per session, and at the end of each session, ports were checked to ensure that it had been consumed. Pavlovian conditioning sessions were conducted in a specific environmental context, which consisted of context 1 for half the rats and context 2 for the remainder. Assignment to each context type was based on ethanol intake averaged over the last 3 sessions of ethanol exposure in the home-cage.

Pavlovian conditioning sessions were alternated with sessions in which rats were placed into the same chamber that was equipped with a different set of contextual cues. This second context was referred to as the non-alcohol context. The purpose of these sessions was to train rats to discriminate between a context where they receive alcohol and a context where they never received alcohol. During each 75 min session in the non-alcohol context, the house light was illuminated after a 2 min delay, but no auditory stimulus or EtOH were ever presented. During these sessions the pump remained activated from the onset of the session to the end, however, syringes containing alcohol were not mounted on the pumps.

Half of the rats received Pavlovian conditioning on session 1 and the remaining half received exposure to the non-alcohol context. Sessions in each context were alternated until rats had received an equal number of Pavlovian conditioning sessions, and sessions of exposure to the non-alcohol context. Training procedures were conducted Monday through Friday.

Test

Twenty four hours after the last training session, responding to the CS in the absence of EtOH was assessed in an alcohol context for half the rats, and a non-alcohol context for the remaining rats. At test, the CS was presented as in Pavlovian conditioning training, however, EtOH was withheld. Approximately 25 min prior to the test session rats received localized infusions of saline, 0.3, or 1 μg/0.3μl of NBQX in the BLA (Exp. 2) or the CPu (Exp. 3).

Intracranial microinfusions

Bilateral microinfusions were conducted in the behavioral testing room using 33 gauge injectors (Plastics One, Roanoke, VA) that extended 3 mm (experiment 2) or 2 mm (experiment 3) below the cannulae. Each injector was connected to a Hamilton syringe (10 µl; Fisher Scientific, 1701 RNR- #14-815-279) using polyethylene size 50 (PE-50) tubing (VWR International Co.). Hamilton syringes were placed on a microinfusion pump (Harvard Apparatus, PHD 2000) that infused at a rate of 0.3 µl/min. A total volume of 0.3 µl was infused, after which injectors were kept in place for 2 min to maximize diffusion. Rats were gently restrained during microinfusions in order to avoid detachment from the injectors. The travel of 2 small air bubbles within each line was used to indicate successful infusions. Rats were immediately placed into the testing chambers after the infusion, and session onset occurred 5-25 minutes later.

Experiment 1: Effect of context on alcohol-seeking elicited by a discrete cue that predicts alcohol.

Experiment 1 tested the hypothesis that environmental context can influence conditioned alcohol-seeking behavior elicited by a discrete, alcohol-predictive cue. One week after arrival, rats (n=21) received 12 sessions of EtOH exposure in the home-cage as described above. Subsequently, rats underwent 10 sessions of Pavlovian conditioning alternated with 10 sessions of exposure to a non-alcohol context. At 24 hrs after the last training session, rats were placed into either the alcohol context (n=8) or the non-alcohol context (n=8) where the CS was presented as before, but EtOH was withheld. A within-subjects design was used during test, such that the following day, each rat was tested in the alternate condition.

Experiment 2a: The effect of blocking BLA AMPA receptors on alcohol-seeking elicited by a discrete cue that predicts alcohol.

Experiment 2 investigated the role of glutamate transmission at AMPA receptors in the BLA on alcohol-seeking behavior elicited by a discrete, alcohol-predictive cue. After 11 days of acclimation to the lab, rats (n=42) received 15 sessions of EtOH exposure in the home-cage, followed by surgery to implant guide cannulae targeting the BLA. Following recovery from surgical procedures rats were habituated to context 1, and context 2 the subsequent day. After habituation to context, behavioral training began in which rats received a total of 11 Pavlovian conditioning sessions alternated with 11 sessions of exposure to non-alcohol context. In order to

habituate the rats to microinfusion procedures, a sham microinfusion was conducted with a cut injector on session 7 followed by a saline sham infusion on session 9 using a full length injector. The effect of AMPAr antagonism in the BLA was tested on session 12, in which rats were given bilateral microinfusions of either saline (n=12), 0.3 (n=7) or 1.0 (n=7) µg/0.3 µl of NBQX in the BLA using a between-subjects design. Rats were separated into each group based on average CS responding during the last 3 sessions of Pavlovian conditioning in the alcohol context. All subjects were tested in both the alcohol context and the non-alcohol context. Following test 1, rats received 2 additional Pavlovian training sessions and 2 additional sessions in a non-alcohol context. Test 2 followed identical procedures as in test 1, however, rats that received their first test in an alcohol context were now tested in the non-alcohol context, and vice versa.

Experiment 2b: The effect of blocking BLA AMPA receptors on responding to a discrete alcohol cue that is paired with EtOH.

Rats from Experiment 2a were used to investigate the effect of NBQX on responding to an alcohol-predictive cue in an alcohol context where alcohol was paired with the CS. Two days following the final testing session in experiment 2a, rats received 4 consecutive Pavlovian conditioning sessions in their respective Pavlovian conditioning contexts. On session 5, rats were either treated with saline, (n=8), 0.3 (n=10) or 1.0 (n=7) µg/0.3 µl of NBQX into the BLA using a between-subjects design. Drug group assignment was based on normalized CS responding (CS port entries minus PreCS port entries) and total port entries averaged over the last 4 sessions. Each dose included some rats from each of the prior doses in experiment 2a.

Experiment 3: The effect of NBQX in the CPu on responding to a discrete alcohol cue in a non-alcohol context.

In a separate group of rats (n=12), the effect of AMPAr antagonism on responding to a discrete cue that predicts alcohol in a non-alcohol context was investigated in the CPu. The purpose of this experiment was to ensure that the effect observed following administration of NBQX were specific to the BLA, and were not due to an upward diffusion of NBQX to anterior parts of the brain. Identical testing procedures were conducted as in experiment 2 in order to maximize direct behavioral comparisons, except that all rats were tested in the non-alcohol context, using saline or 0.3 µg/0.3 µl of NBQX as within-subject variables.

Histological verification of cannulae placements

Rats in experiment 2 and 3 were anesthetized with isoflurane and given a microinfusion of fast green into the BLA and CPu (0.1 µl/min, 0.5 µl). Injectors were kept in the cannulae for 5 min in order to optimize diffusion. Rats were then decapitated, brains were removed from the skull and post-fixed in formalin for 24 hrs. Brains were subsequently placed in 25% sucrose for one week, and then sliced on a cryostat (60 microns, coronal), which was maintained at -19^{o}C. Sections were mounted onto glass slides, stained with cresyl violet, and analyzed using light microscopy. Subjects in experiment 2 and 3 were included in the final analyses if the most ventral point of the injector tips touched the boundary, or were inside the boundary of the BLA and CPu as defined in the rat brain atlas of Paxinos and Watson (1997).

Statistical analyses

Five rats who attained < 3.8 g/kg EtOH intake were dropped prior to behavioral training in experiment 1 (final n=16). A total of 16 rats were dropped from experiment 2 (for data on dropped rats, see supplementary figure 1). Six of the lowest drinkers were dropped following ethanol exposure in the home-cage, and one rat was dropped during behavioral training sessions because of failure to acquire the CS-US association. In addition, 9 rats were dropped because of inaccurate placements (final n=26). Two rats from experiment 2a did not participate in experiment 2b due to aggressive behavior (final n=25). Finally, one rat was dropped from experiment 3 prior to test because he did not acquire the CS-US association during Pavlovian conditioning training (final n=11).

During ethanol consumption in the home cage, ethanol consumption (g/kg; grams of ethanol consumed per kilogram of body weight) and preference for ethanol (%; calculated as a ratio of ethanol intake in gm divided by the sum of water and ethanol intake in gm) were used as dependent variables. Each measure was analyzed using repeated-measures analyses of variance (RM-ANOVA) with *Session* as a within-subjects variable.

During Pavlovian conditioning sessions, entries into the fluid port were recorded during the following intervals: each 10 second CS trial (CS); 10 sec intervals preceding each CS trial (PreCS); 10 sec intervals after each CS trial (PostCS). In addition, port entries that occurred during inter-trial intervals (ITI) that did not include PreCS, CS, and PostCS (ITI; total port entries minus PreCS, CS, and PostCS) were recorded. Pavlovian conditioning sessions were

analyzed using RM-ANOVA with *Session* (EXP 1: 1-10; EXP 2: 1-11; EXP 3: 1-11) and *Interval* (CS and PreCS) as within-subjects factors.

Total port entries obtained during training in the alcohol and non-alcohol context were compared across behavioral training. Data were analyzed using repeated-measures analyses of variance (RM-ANOVA) with *Context* (alcohol and non-alcohol) and *Session* as within-subjects variables.

Test data for experiment 1 were analyzed using RM-ANOVA with *Context* (alcohol, non-alcohol context) and *Interval* (PreCS, CS) as within-subjects variables. Paired samples *t*-tests were used to investigate significant main effects and interactions. Frequency of port entries, and time spent in the port during each CS trial at test was analyzed using RM-ANOVA with *CS trial* and *Context* as within-subject variables.

Data obtained at test for experiment 2a was analyzed using RM-ANOVA. Port entries made during PreCS and CS intervals were analyzed together with *Interval* (PreCS, CS) as within-subjects factors and *Dose* (0, 0.3, 1 μg/0.3 μl NBQX) as between-subjects factors. PostCS and ITI were analyzed separately using RM-ANOVA with *Context* (alcohol, non-alcohol) as a within-subjects factors, and *Dose* as a between-subjects factor. Frequency of port entries per CS trial at test were analyzed using RM-ANOVA with *Trial* and *Context* as within-subject variables and *Dose* as a between-subjects variable. Data obtained from experiment 2b was analyzed using RM-ANOVA with PreCS and CS port entries as within-subjects factors and *Dose* (0, 0.3, 1 μg/0.3 μl NBQX) as between-subjects factors. Frequency of port entries per CS trial at test in the alcohol context were analyzed using RM-ANOVA, with *CS trial* as a within-subjects factor, and *Dose* as a between-subjects factor.

Test data collected from experiment 3 was analyzed using RM-ANOVA, with *Interval* (PreCS, CS) and *Drug* as within-subjects factors. Frequency of port entry during each CS trial was analyzed using RM-ANOVA with *Trial* and *Drug* as within-subjects variables. Finally, PostCS and ITI were analyzed separately using a paired-samples *t*-test, with *Drug* as the within-subjects variable.

Violations of homogeneity of variance were determined by Mauchly's test of sphericity and were corrected using the Huynh-Feldt correction. Analyses were conducted using SPSS (Version 18) with a significance level of $\alpha = 0.05$.

Results

As depicted in supplementary figure 2, ethanol intake and preference for ethanol over water increased across sessions of ethanol consumption in the home-cage for all 3 experiments.

Experiment 1: Effect of context on alcohol-seeking elicited by a discrete cue that predicts alcohol

Figure 1a illustrates intervals of interest, and temporal relation between the CS and US across sessions of Pavlovian conditioning. Rats learned to associate an auditory cue (CS) with EtOH across Pavlovian conditioning sessions in the alcohol context (Fig. 1b). A comparison of port entries made during the PreCS and CS intervals indicated that port entries during the CS increased as a function of session, whereas port entries during the PreCS did not [Session, $F(9,135) = 17.649$, $p = .000$; Interval, $F(1,15) = 59.275$, $p = .000$; Session × Interval, $F(9,135) = 25.091$, $p = .000$], Paired-samples *t*-tests revealed that CS responding was significantly higher than PreCS responding ($p \leq 0.5$ for all comparisons) on all sessions, except for session 1, where PreCS responses were higher [$t(15) = 2.628$, $p = .019$].

The total number of port entries (Fig. 1c) made in the alcohol context was higher relative to the non-alcohol context [Context, $F(1,15) = 77.752$, $p = .000$]. Furthermore, while total port entries remained elevated during Pavlovian training sessions, they steadily decreased across sessions in the non-alcohol context [Session, $F(9, 135) = 3.460$ $p = .001$; Context × Session $F(9,135) = 3.912$, $p = .000$]. Paired samples *t*-tests confirmed that total port entries on the first session in the non-alcohol context were significantly higher than the last session [$t(15) = 3.670$, $p = .002$], and that total port entries on the first session relative to the last in the alcohol context remained the same [$t(15) = .541$, $p = .597$]. Similar findings were observed during Pavlovian conditioning sessions in experiment 2 and 3 (supplementary Fig. 3).

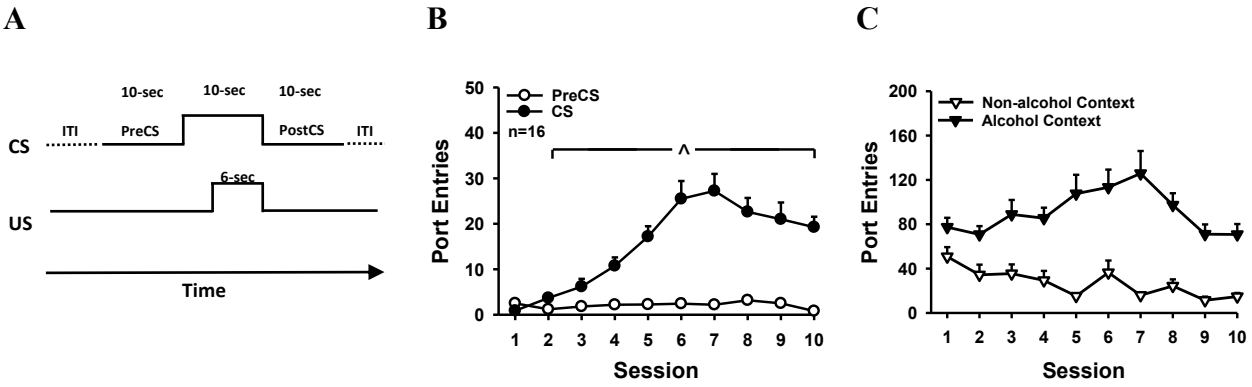
Presentations of the CS without alcohol elicited alcohol-seeking behavior in the non-alcohol context, and this effect was invigorated in the alcohol context (Fig. 1d-f). Rats made more port entries in the alcohol context than the non-alcohol context [Context, $F(1,15) = 39.671$ $p = .000$] and responding was higher during the CS than the PreCS interval [Interval, $F(1,15) = 10.857$, $p = .005$]. Furthermore, higher port entries were observed during CS trials in the alcohol context [Context × Interval, $F(1,15) = 12.391$, $p = .003$]. Follow-up *t*-test for paired-samples verified that there was no difference in port entries during the PreCS as a function of context

[$t(15) = .000$ $p = 1.00$], but that port entries during the CS were higher in the alcohol context than the non-alcohol context [$t(15) = 3.409$, $p = .004$]. Presentations of the CS without EtOH elicited more port entries relative to the PreCS baseline in both the non-alcohol context [$t(15) = 3.925$, $p = .001$] and the alcohol context [$t(15) = -6.223$ $p = .000$].

The number of port entries made during each CS trial at test in either context is depicted in Figure 1e. More port entries were made in the alcohol context than the non- alcohol context [Context, $F(1,15) = 10.517$, $p = .005$] and there was a reduction in port entries as a function of Trial [Trial, $F(14, 210) = 7.684$, $p = .000$]. No significant interaction [Trial × Context, $F(14, 210) = .606$, $p = .662$] suggested that the number of port entries made during CS trials did not change as a function of which context condition rats received at test. Paired samples *t*-tests comparing port entries during each trial indicated that port entries were lower in the non-alcohol context than the alcohol context on sessions 4, 6, 7, 8, 12, 14 & 15 (all p's < 0.05).

Similar results were obtained for the duration of time that rats spent in the fluid port during each CS trial at test (Fig. 1f). More time was spent in the port in the alcohol context compared to the non-alcohol context [Context, $F(1,15) = 17.185$, $p = .001$], and there was a reduction in time spent in the port as the test session progressed [Trial, $F(14, 210) = 12.691$, $p = .000$]. There was no interaction observed between Trial and Context, suggesting that time spent in the port during CS trials did not change as a function of which context condition rats received at test [Trial × Context, $F(14, 210) = .854$, $p = .543$]. Time spent in the port in the alcohol context was significantly higher relative to the non-alcohol context on sessions 2, 4, 5, 6, 7, 8, 12, 13, 14 and 15 (all p's < 0.05). There was no effect of context on port entries during the PostCS or ITI intervals (see Supplementary Fig. 4).

Acquisition of Pavlovian alcohol-seeking behavior



Alcohol-seeking behavior elicited by a discrete Pavlovian cue at test

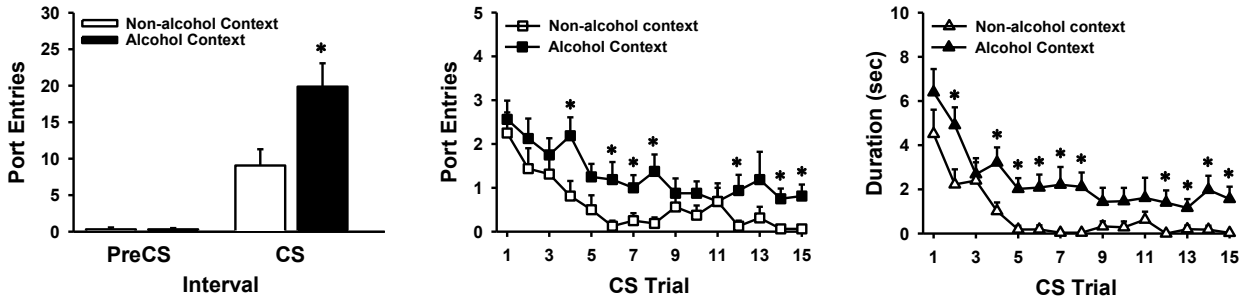


Figure 1. Alcohol-seeking behavior elicited by a discrete cue is invigorated in an alcohol context relative to a context where alcohol had never been consumed Port entries made during the CS were enhanced in an alcohol context relative to a non-alcohol context. **(A)** Depiction of data collected during each time interval, and temporal relation between the CS and delivery of alcohol. **(B)** Mean (± SEM) number of port entries made during CS presentations (filled circles), and 10 seconds prior to CS onset (PreCS; open symbols) during Pavlovian conditioning training in an alcohol context. **(C)** Mean (± SEM) total number of port entries made during Pavlovian conditioning training in an alcohol context (filled triangles) and exposure to a second context where the CS and EtOH delivery was never presented (open triangles). **(D)** Mean (± SEM) port entries made during PreCS and CS intervals at test in the alcohol context (filled bar) and the non-alcohol context (open bar). **(E)** Mean ± (SEM) number of port entries made during each CS trial at test in an alcohol context (filled squares) and non-alcohol context (open squares). **(F)** Mean (±

SEM) time spent (sec) in the port during each CS trial at test in an alcohol (filled triangles) and non-alcohol (open triangles) context. * $p < .05$, alcohol > non-alcohol context.

Experiment 2a: The effect of blocking BLA AMPA receptors on alcohol-seeking elicited by a discrete cue that predicts alcohol

In order to investigate the hypothesis that alcohol-associated contexts can invigorate responding to a discrete CS, we conducted a 2×2 ANOVA analyzing PreCS and CS responding for saline-treated animals in the alcohol and non-alcohol context (Fig. 2a). Similar to findings obtained in experiment 1, port entries during the CS were invigorated in an alcohol context relative to a non-alcohol context [Context, $F(1,11) = 5.121$, $p = .045$; Interval, $F(1,11) = 61.969$, $p = .000$; Context x Interval, $F(1,11) = 6.960$, $p = .023$].

Blocking AMPA glutamate receptors in the BLA attenuated alcohol-seeking behavior elicited by an alcohol-predictive CS, and this effect was observed in both an alcohol context and a non-alcohol context (Fig. 2a). Overall, port entries were higher in the alcohol context compared to the non-alcohol context at test [Context, $F(1,23) = 8.652$, $p = .007$], with higher port entries made during the CS than PreCS interval [Interval, $F(1,23) = 67.043$, $p = .000$]. Importantly, while responses made in the alcohol context were higher relative to the non-alcohol context, this effect was only observed during CS presentations [Context × Interval, $F(1,23) = 10.691$, $p = .003$]. NBQX in the BLA reduced port entries made during the CS in both contexts [Dose, $F(2,23) = 14.610$, $p = .000$; Interval × Dose, $F(2,23) = 14.844$, $p = .000$]. No interactions between context, interval and dose were found [Context × Dose, $F(2,23) = .569$, $p = .574$; Context × Interval × Dose, $F(2,23) = .690$, $p = .512$], suggesting that the effect of NBQX did not differ between test conditions during either interval.

To further investigate the effect of NBQX on alcohol-seeking behavior, responses made during the CS were investigated in isolation. Port entries made during the CS were higher in an alcohol context relative to a non-alcohol context [Context, $F(1,23) = 9.664$, $p = .005$] and this effect was attenuated by infusion of NBQX, regardless of context [Dose $F(2,23) = 14.767$, $p = .000$; Context × Dose, $F(2,23) = .629$, $p = .542$]. The main effect of dose was further investigated using Bonferroni post hoc tests comparing CS responses at each dose collapsed across context. NBQX attenuated responding to the CS following pretreatment of 0.3 and 1 µg/0.3 µl doses [saline vs. 0.3µg, $p = .005$; saline vs. 1.0 µg, $p = .000$; 0.3 µg vs. 1.0 µg, $p = 0.328$]. Identical analyses were conducted to investigate the effect of NBQX on PreCS port entries. The results showed that responses made during the PreCS remained stable across context conditions, and did

not vary as a function of treatment with NBQX [Context, $F(1,23) = 1.248$, $p = .275$; Context × Dose, $F(2,23) = .226$, $p = .799$; Dose $F(2,23) = .541$, $p = .589$].

There was no impact of test context on port entries made during PostCS intervals [Fig. 2b; Context, $F(1,23) = 1.010$, $p = .325$]. However, NBQX reduced PostCS responding regardless of which context condition rats received at test [Dose, $F(2,23) = 7.462$, $p = .003$; Context × Dose, $F(2,23) = .891$, $p = .424$]. Bonferroni post hoc analyses revealed a significant difference in port entries made between saline and 1.0 µg [$p = .003$] of NBQX when context was collapsed across dose, while no significant effects were found between saline and 0.3 µg [$p = .957$] or 0.3 µg and 1.0 µg/0.3 µl doses [$p = .059$]. Furthermore, port entries made during ITI were not affected by NBQX (Fig. 2c; Context, $F(1,23) = 1.894$, $p = .182$; Context × Dose, $F(2,23) = 1.341$, $p = .281$, $p = .275$; Dose, $F(2,23) = .464$, $p = .635$].

Figure 2d-f depict port entries made during each CS trial in the alcohol and non-alcohol context at test for each dose of NBQX. Port entries made during CS trials decreased across test, with significantly reduced responding for rats infused with NBQX relative to saline [Trial, $F(14,322) = 10.315$, $p = .000$; Dose, $F(2,23) = 14.788$, $p = .000$; Trial × Dose, $F(28,322) = 2.719$, $p = .000$]. While responding in the alcohol context was overall higher than test in a non-alcohol context [Context, $F(1,23) = 9.810$, $p = .005$], this difference in behavior did not occur as a function of Trial or Dose of NBQX [Context × Dose, $F(2,23) = .643$, $p = .535$; Trial × Context, $F(14,322) = .495$, $p = .894$; Trial × Context × Dose $F(28,322) = 2.175$, $p = .720$). To further investigate the significant Trial × Dose interaction, RM-ANOVA was used to investigate port entries made during the first trial at test in both contexts across drug groups. No significant main effect of context, or interactions with drug was found [Context, $F(1,23) = .006$, $p = .940$; Context x Drug $F(2,23) = .244$, $p = .786$], however, a main effect of dose was found, suggesting that overall, NBQX reduced port entries made during the first CS trial regardless of context [Dose, Drug, $F(2,23) = 6.468$, $p = .006$]. To further investigate the effect of NBQX on port entries made during the first CS trial, bonferroni post hoc tests were conducted, which revealed a significant reduction in port entries made for rats infused with 1.0 µg/0.3µl of NBQX relative to rats infused with saline ($p = .005$). No differences in port entries were found between saline and 0.3 µg/0.3µl treated animals ($p = .558$) or between 0.3 µg and 1.0 µg treated animals ($p = .177$).

Similarly, more time was spent in the ports during each CS trial in the alcohol context relative to the non-alcohol context, and this effect was significantly reduced with for rats treated with NBQX (Supplementary Fig. 5).

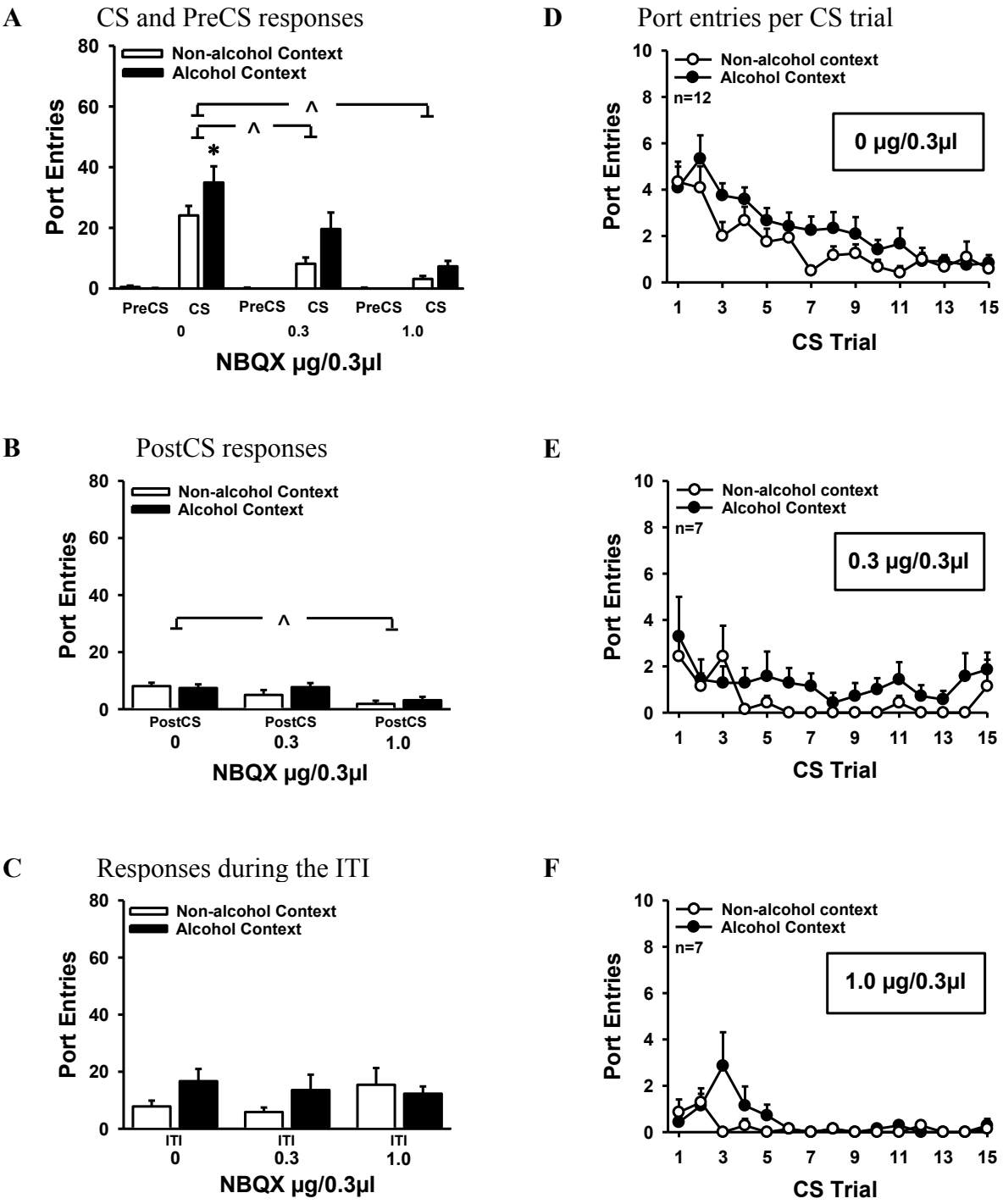


Figure 2. Blocking glutamate at AMPAr attenuates Pavlovian cue-induced alcohol-seeking in an alcohol (filled symbols) and non-alcohol context (open symbols). **(A)** Mean (± SEM) port entries made during CS and PreCS intervals. **(B)** Mean (± SEM) port entries made during PostCS intervals. **(C)** Mean (± SEM) port entries made during the variable ITI, calculated by subtracting

port entries made during the PreCS, CS, and PostCS intervals from total port entries. * $p < .05$, alcohol > non-alcohol context. ^ $p < .05$, saline > NBQX on data collapsed across context. **(D-F)** Mean (± SEM) number of port entries during each CS trial at test in both contexts.

Experiment 2b: The effect of blocking BLA AMPA receptors on responding to a discrete alcohol cue that is paired with alcohol.

Presentation of the CS followed by delivery of alcohol at test in an alcohol context elicited a significant increase in port entries relative port entries made during the PreCS [Fig. 3a; Interval, $F(1,22) = 118.520$, $p = .000$]. Administration of NBQX in the BLA had no effect on CS port entries when the CS was paired with alcohol [Interval × Dose, $F(2,22) = .775$, $p = .473$; Dose, $F(2,22) = 1.133$, $p = .340$]. Furthermore, the pattern of responding during CS trials at test remained stable across test, and was not affected by NBQX [Fig. 3b; Trial, $F(14,308) = .906$, $p = .530$; Dose, $F(2,22) = .954$, $p = .400$; Trial x Dose, $F(28,308) = 1.302$, $p = .145$].

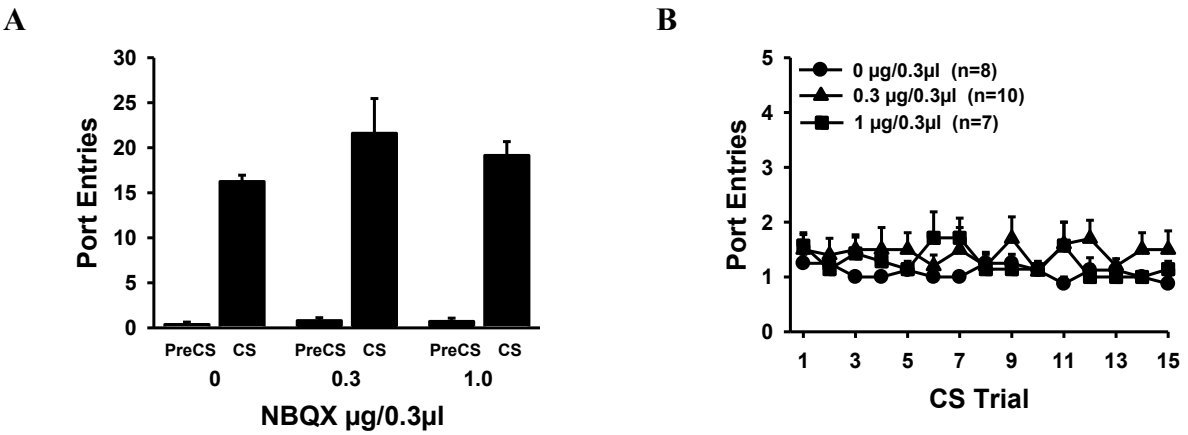


Figure 3. Blocking glutamate at AMPAr had no effect on alcohol-seeking behavior when the CS was paired with alcohol. **(A)** Mean (± SEM) port entries made during PreCS and CS intervals at test in an alcohol context. **(B)** Port entries made during each CS trial at test for rats infused with 0, 0.3, or 1.0 µg/0.3µl of NBQX in the BLA.

Experiment 3: The effect of NBQX in the CPu on responding to a discrete alcohol cue in a non-alcohol context.

Infusion of NBQX in the CPu had no effect on alcohol-seeking behavior elicited by the CS in the absence of alcohol at test in a non-alcohol context (Fig. 4). Port entries made during the CS were significantly higher than PreCS responding following infusions of saline and NBQX [Fig. 4a; Interval, $F(1,10) = 78.536$, $p = .000$; Dose, $F(1,10) = .358$, $p = .563$; Interval × Dose, $F(1,10) = .435$ $p = .524$]. In addition, there was no impact of NBQX on frequency of port entries during each CS trial at test [Fig. 4b: Trial, $F(14,70) = 6.332$, $p = .000$; Drug, $F(1,5) = .137$, $p = .726$; Trial × Drug, $F(14,70) = .620$, $p = .840$]. Analysis of port-entries made during PostCS intervals and ITI indicated that there was no impact of NBQX on port entries during either interval [Fig. 4c: Post CS, $t(10) = 1.677$, $p = .124$; ITI, $t(15) = 1.449$, $p = .178$].

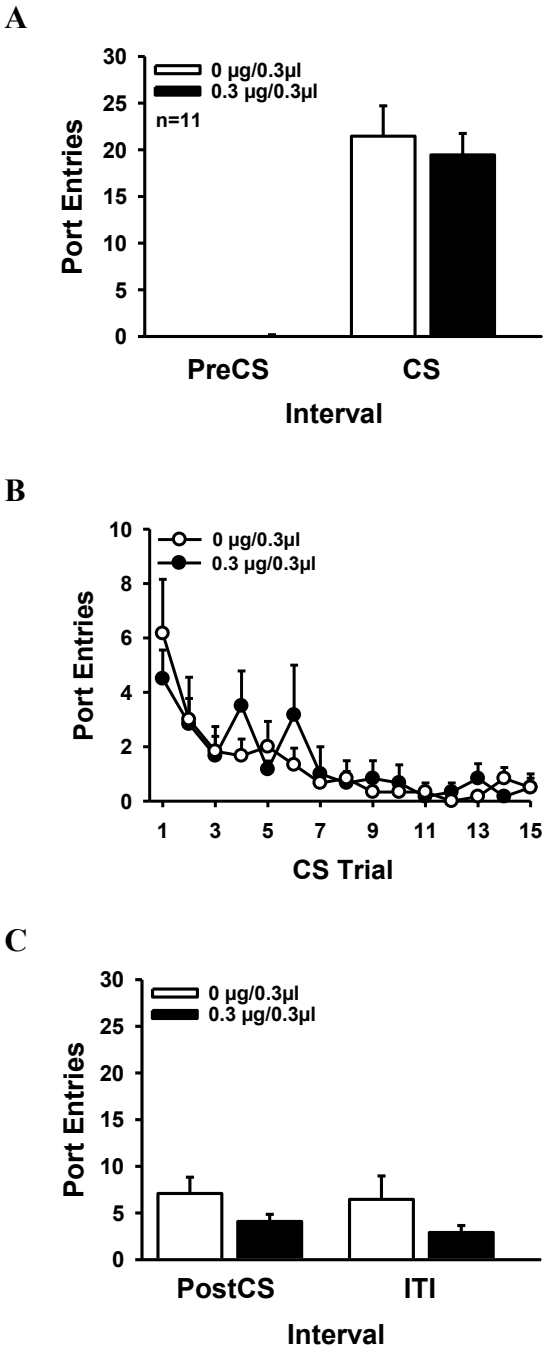


Figure 4. Infusion of NBQX in the CPu had no effect on alcohol-seeking behavior at test in a non-alcohol context. **(A)** Data represent Mean (± SEM) PreCS and CS port entries. **(B)** Mean (± SEM) port entry per CS trial. **(C)** Mean (± SEM) PostCS and variable ITI.

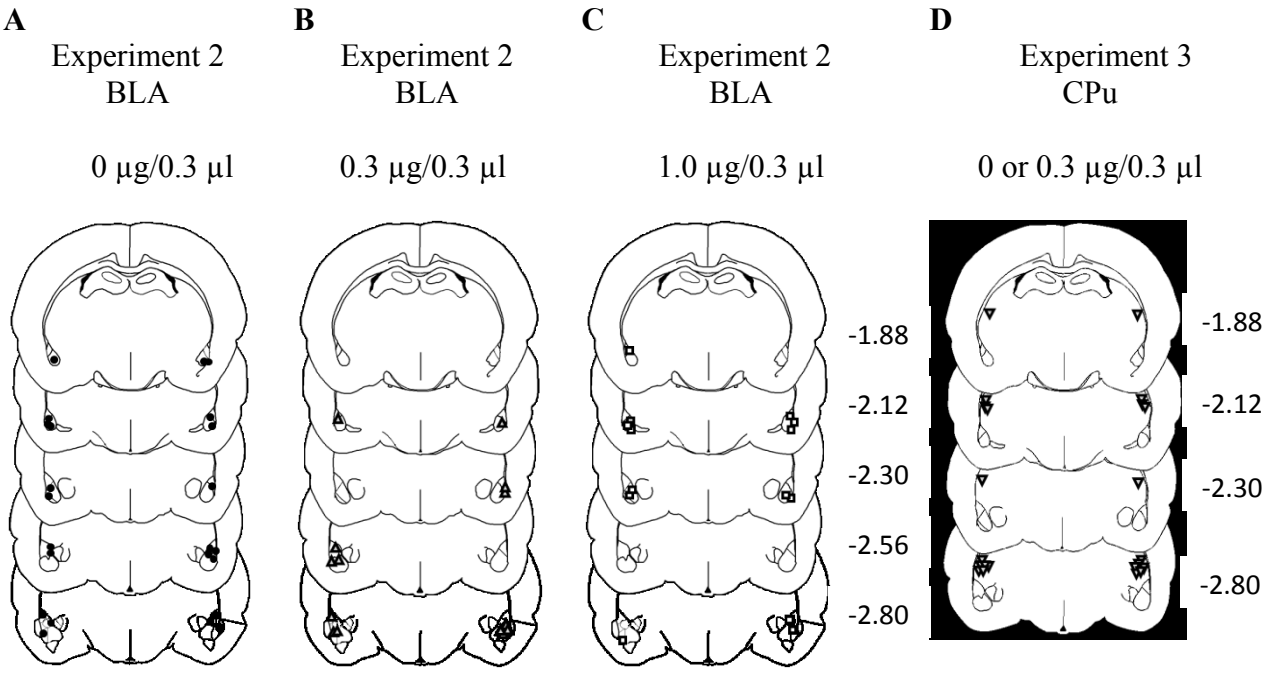


Figure 5. Location of injector tips on or within the boundaries of the BLA (A-C) and CPu (D).

General Discussion

In the present research, presentations of a discrete alcohol-predictive CS without alcohol elicited alcohol-seeking behavior in a non-alcohol context, and this effect was invigorated in an alcohol-associated context. This pattern of results was replicated in saline-treated rats in a second study that investigated the role of AMPA glutamate receptors in the BLA in alcohol-seeking elicited by the discrete CS in both contexts. Intra-BLA infusions of NBQX attenuated alcohol-seeking behavior elicited by the CS in both an alcohol context and a non-alcohol context. Control experiments revealed that port entries elicited by the CS were not influenced by NBQX in a test session in which the CS was paired with alcohol, and that NBQX administered into the CPu had no impact on CS responding in the absence of alcohol in a non-alcohol context. These findings highlight that the context where a discrete alcohol-predictive CS is encountered can serve as an important determinant in the level of conditioned responding elicited by the CS, and provide novel evidence that AMPA glutamate receptors in the BLA are needed for alcohol-seeking behavior elicited by discrete Pavlovian alcohol cues.

Acquisition of CS-alcohol association, and behavioral discrimination between an alcohol context and a non-alcohol context

Paired presentations of the CS with alcohol in the present studies resulted in the acquisition of Pavlovian alcohol-seeking behavior. In all three experiments, port entries made during PreCS intervals remained low, while port entries made during the CS escalated across sessions, indicating that the CS triggered alcohol-seeking behavior. This explanation is corroborated by unpublished data from our laboratory, in which paired presentations of a CS and alcohol resulted in the acquisition of alcohol-seeking behavior, whereas unpaired presentations of a CS and alcohol did not (Supplementary Fig. 6). Additional support comes from studies that incorporated a second cue during Pavlovian conditioning sessions that was never paired with alcohol (CS-). Across Pavlovian discrimination training, port entries made during the CS+ increases, while port entries made during the CS- remain low, suggesting that rats learned to discriminate between a cue that predicted alcohol and one that did not when both were presented in the same session (Chaudhri et al, 2008a, 2008b, 2009, 2010, Sciascia et al, 2013; Sparks et al, 2013). Such findings support the conclusion that the CS acquired the capacity to predict alcohol

through repeated pairings with alcohol delivery, and that the observed increase in behavior was not due to sensitization or pseudoconditioning. Our findings also support substantial human laboratory studies indicating that neutral environmental cues can come to acquire incentive motivational properties through repeated pairings with alcohol, and can lead to craving in humans (Field & Duka, 2002; Ludwig & Wikler, 1974).

Another important question to be considered before interpreting the test data is whether or not rats learned to discriminate between the alcohol context and the non-alcohol context during training. An examination of total port entries made during training sessions suggests that they did. Total port entries increased across Pavlovian conditioning sessions in the alcohol context, but decreased across sessions in the non-alcohol context. The latter finding suggests that rats learned to stop checking the port for alcohol in the non-alcohol context. These results are consistent with our prior data showing that total port entries in a non-alcohol context diminish across training sessions (Sparks et al, 2013; Remedios et al, 2014), and parallel human studies that show higher stimulation and positive expectancies in a bar setting relative to a laboratory setting (Wall, McKee, & Hinson, 2000; Wall, McKee, Hinson, & Goldstein, 2001)

The effect of context on alcohol-seeking behavior elicited by a discrete alcohol-predictive cue

Presentation of the CS without alcohol in a non-alcohol context evoked alcohol-seeking behavior, as indexed by elevated port entries during the CS relative to port entries during the PreCS. These findings are in accordance with previous studies demonstrating that a non-extinguished discrete CS can elicit alcohol-seeking behavior in a context where alcohol was never previously delivered (Chaudhri et al, 2010; Remedios et al, 2013; Sparks et al, 2013). Furthermore, these findings parallel clinical studies in which craving for alcohol is measured in environments that are not typically reminiscent of alcohol consumption. For instance, Myrick and colleagues (2004) measured craving for alcohol while subjects were in an fMRI scanner. Although subjects had presumably never consumed alcohol in an fMRI scanner, subjective craving in response to alcohol associated pictures was observed, suggesting that alcohol cues are reliably triggered even in environments where alcohol was never consumed.

Importantly, average port entries made during CS trials, and time spent in the port during the CS were elevated in an alcohol-associated context relative to the non-alcohol context, a finding that was replicated in experiment 2a. This finding suggests that experiencing an alcohol-

predictive discrete cue in an environment where alcohol has previously been consumed may serve as a more potent trigger for relapse than exposure to either the discrete or contextual cue alone. This interpretation is supported by clinical studies that show an increase in subjective ratings of craving in response to picture presentations of alcoholic beverages in a bar setting, relative to pictures of alcohol beverages alone (Nees et al., 2012). These data also provide important considerations for clinical models that assess craving in environments that are not realistic. For instance, the assessment of craving triggered by pictures of alcoholic beverages may be underestimated in lab settings, as opposed to naturalistic settings. Therefore, future studies should expand on cue-based research methods by incorporating more ecologically valid environments. Indeed, research has now begun to assess craving in response to discrete and contextual cues through virtual simulations, in which drug-predictive cues can be experienced in environments where the drug have previously been consumed (Bordnick et al., 2008; Bordnick, Graap, Copp, Brooks, & Ferrer, 2005; Paris et al., 2011).

These findings support prior research conducted using a similar Pavlovian conditioning procedure. Remedios and colleagues (2013) found that alcohol-seeking behavior elicited by a discrete cue is enhanced in an alcohol context relative to a non-alcohol context. However, these results may have been influenced by the fact that rats had more exposure to the alcohol context relative to the non-alcohol context prior to test. In addition, alcohol-seeking behavior may have been enhanced due to a prolonged absence of alcohol prior to test. Our novel procedure addressed these limitations by alternating Pavlovian conditioning sessions with exposure to a non-alcohol context, thereby allowing an equal amount of exposure to both contexts prior to test. Furthermore, our current procedure allowed a maximum time 3 days without alcohol prior to test in order to minimize any effect of alcohol deprivation on behavior.

Previous research supports the hypothesis that context associated with the presence or absence of alcohol can stimulate or suppress alcohol-seeking behavior. For instance, Tsiang and Janak (2008) investigated the impact of context on cue-induced reinstatement of alcohol seeking using inbred C57BL/6 mice. Self-administration training took place in a distinctive context, where each active lever press resulted in the presentation of a compound light/tone cue, and the delivery of alcohol for oral consumption. Following stable self-administration, extinction sessions were conducted in a different context where lever presses no longer resulted in the presentation of the compound cue or alcohol. The ability of context to motivate alcohol-seeking

behavior was assessed by testing rats in the self-administration context, where lever presses either resulted in the presentation of the compound cue or did not. In addition, reinstatement was assessed in the extinction context, where active lever presses resulted in the presentation of the compound cue. At test, active lever presses were enhanced in the self-administration context when the compound cue was presented relative to when it was not, suggesting that the combination of a discrete cue in a context where alcohol has previously been consumed can enhance alcohol-seeking behavior relative to exposure to an alcohol context alone. Interestingly, active lever presses in the extinction context were significantly lower relative to active lever presses in the self administration context, which suggest that environmental context in which discrete cues are experienced can either enhance or suppress alcohol-seeking behavior, and are thus in accordance with our current findings.

AMPA receptors in the BLA are needed for the expression of alcohol-seeking behavior elicited by a discrete Pavlovian alcohol cue, independent of the context in which the cue is experienced.

Our data indicate that alcohol-seeking behavior elicited by a discrete alcohol-predictive cue requires AMPA glutamate receptors in the BLA. Localized infusions of 0.3 and 1.0 μg/0.3μl of NBQX in the BLA attenuated port entries made during the CS, an effect that was observed in both contexts. In addition, the frequency of port entries and time spent in the port at test during each CS trial decreased, with steeper reductions observed with rats infused with NBQX. By contrast, NBQX had no effect on port entries made during the PreCS interval, or during the variable ITI that occurred between CS trials, suggesting that AMPA receptors in the BLA are specifically required for alcohol-seeking elicited by a discrete CS. However, the highest dose of NBQX reduced port entries during the PostCS interval, indicating that NBQX continued to reduce port entries following presentations of the CS. This finding is not surprising considering that the PostCS interval occurs immediately after the CS, and is thus considered an interval that may still be associated with the presence of alcohol. Alternatively, the observed reduction in CS and PostCS port entries following infusions of NBQX may have been attributable to a deficit in locomotion. This interpretation is unlikely because NBQX did not reduce average port entries or frequency of port entries during CS trials at test when the CS was paired with alcohol. In addition, there was no effect of NBQX on port entries made during the PostCS or variable ITI.

One interpretation for the reduction of alcohol-seeking behavior following AMPAr blockade in the BLA is that the memory of the CS-US association may have been disrupted. Our data indicate that port entries during the first CS trial were reduced following treatment with NBQX. This finding is intriguing because it suggests that AMPA receptors in the BLA are potentially required to access the initial memory of the CS-alcohol association. Accordingly, rats with BLA lesions are capable of acquiring CS-US associations, but are severely impaired during tasks where they are required to access information regarding the CS (Blundell, Hall, & Killcross, 2001). In support of this interpretation, NBQX administration in the BLA did not affect CS port entries when the CS was paired with alcohol, but reduced CS port entries when alcohol was not delivered following CS presentations. Similarly, unilateral inactivation of the BLA reduces responding triggered by a Pavlovian cue, but not when it is paired with alcohol (Chaudhri et al., 2013). Therefore, it is possible that AMPArs in the BLA are necessary for the ability for the CS to access the memory of the US.

Alternatively, it is possible that glutamate blockade in the BLA disrupted the capacity to attribute incentive salience to the CS, which would account for the reduction in CS port entries observed at test. Incentive salience is a term used to describe a motivational component of reward that makes stimuli paired with a reinforcer especially attractive and highly desired (Robinson & Berridge, 2001).This interpretation is plausible considering that the BLA is required for Pavlovian second-order conditioning (Hatfield, Han, Conley, Gallagher, & Holland, 1996; Setlow, Holland, & Gallagher, 2002) and acquisition of a new response with conditioned reinforcement (Burns, Robbins, & Everitt, 1993). In addition, discrete infusions of NMDA receptor antagonists in the NAC core disrupts Pavlovian conditioned sign-tracking performance in rats (Ciano, Cardinal, Cowell, Little, & Everitt, 2001). Taken together, glutamate activity in the BLA may be required for the ability of the CS to maintain its conditioned reinforcing properties, through which to motivate conditioned approach behaviors.

In a separate control study, we investigated the possibility that the reduction of port entries made during the CS following NBQX administration in the BLA could be attributable to an upward diffusion of NBQX into the dorsally-situated CPu. The CPu receives heavy glutamatergic inputs from the frontal cerebral cortex (Fonnum, Storm-Mathisen, & Divac, 1981) and has been shown to be involved in associative learning (Haruno & Kawato, 2006). Our current data show that NBQX administration in the CPu did not reduce port entries made during

the CS relative to saline-treated animals at test in a non-alcohol context. These findings negate the possibility that our results are due to an upward diffusion of NBQX into the CPu, and provide additional support for the role of AMPAr in the BLA in alcohol-seeking behavior elicited by an alcohol-predictive cue. It is also possible that NBQX diffused outwards, thereby affecting structures ventral or medial to the BLA. One such structure is the central nucleus of the amygdala, which has been implicated in Pavlovian conditioning and memory consolidation (Wilensky et al., 2006) as well as alcohol consumption in rodents (McBride, 2002). Future studies should therefore investigate the contribution of AMPAr activity in the central nucleus of the amygdala on Pavlovian conditioned alcohol-seeking behavior.

Our current finding are in accordance with research showing that glutamate activity in the BLA increases during cue-induced reinstatement of alcohol-seeking relative to food-seeking behavior (Gass et al., 2011). However, metabotropic glutamate receptors in the BLA also contribute to alcohol-seeking behavior in operant conditioning tasks. For example, metabotropic glutamate receptor antagonists reduce reinstatement of alcohol-seeking behavior either systemically (Bäckström & Hyytiä, 2004; Schroeder, Overstreet, & Hodge, 2005), or in the BLA (Sinclair, Cleva, Hood, Olive, & Gass, 2012). The contribution of these receptors to alcohol-seeking behavior elicited by Pavlovian alcohol cues still needs to be assessed.

Our current findings suggest that glutamate activity in the BLA is required for conditioned behavior guided by discrete Pavlovian cues, however, the question still remains as to where glutamate projections to the BLA originate from. The Prelimbic (PL) medial prefrontal cortex sends and receives glutamatergic input from the BLA (McDonald, 1991; Mcdonald, 1998), and has been implicated in reactivity to alcohol cues in humans (Fryer et al., 2013; George et al., 2001; Grüsser et al., 2004) and conditioned responding to discrete Pavlovian cues in rats (Sierra-Mercado, Padilla-Coreano, & Quirk, 2011; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). Interestingly, optogenetic inhibition of BLA-to-PL pathway reduces cue-induced reinstatement of cocaine seeking, suggesting that this pathway may be especially involved in the modulation of discrete conditioned cues on drug-seeking behavior (Stefanik & Kalivas, 2013). By contrast, contextual cues seem to be modulated by the ventral hippocampus (VH), a brain region that heavily projects to the BLA (Ishikawa & Nakamura, 2006), and is involved in the processing and integration of spatial and contextual information (for review see Jarrard, 1993). Accordingly, reversible inactivation of the ventral subiculum/CA1 of the

hippocampus attenuates context-induced reinstatement of heroin-seeking (Bossert & Stern, 2012) and inactivation of the dorsal hippocampus reduces context but not discrete cue-induced reinstatement of cocaine- seeking (Fuchs et al., 2005). Future studies should investigate the hypothesis that excitatory input relaying information about discrete alcohol cues to the BLA may originate from the PL, while the VH-to-BLA projection may contribute to the invigoration of alcohol-seeking behavior elicited by discrete cues that predict alcohol.

In summary, our data demonstrate that alcohol-seeking behavior elicited by an alcohol-predictive cue is invigorated in an alcohol-associated context, suggesting that the summation of discrete and contextual cues may be a stronger trigger for craving and relapse in abstinent alcoholics relative to either cue alone. This finding is particularly important for clinical research that assess craving in response to alcohol cues, as craving may be underestimated in laboratory settings relative to realistic drinking environments. In addition, infusion of NBQX in the BLA reduced port entries made during CS presentations regardless of environmental context. These findings provide novel evidence for a pivotal role of AMPA glutamate receptors in the BLA in the expression of Pavlovian-conditioned alcohol-seeking behavior maintained by a discrete Pavlovian CS, and offer new insight into the neurobiological processes that mediate conditioned responding elicited by alcohol-predictive stimuli.

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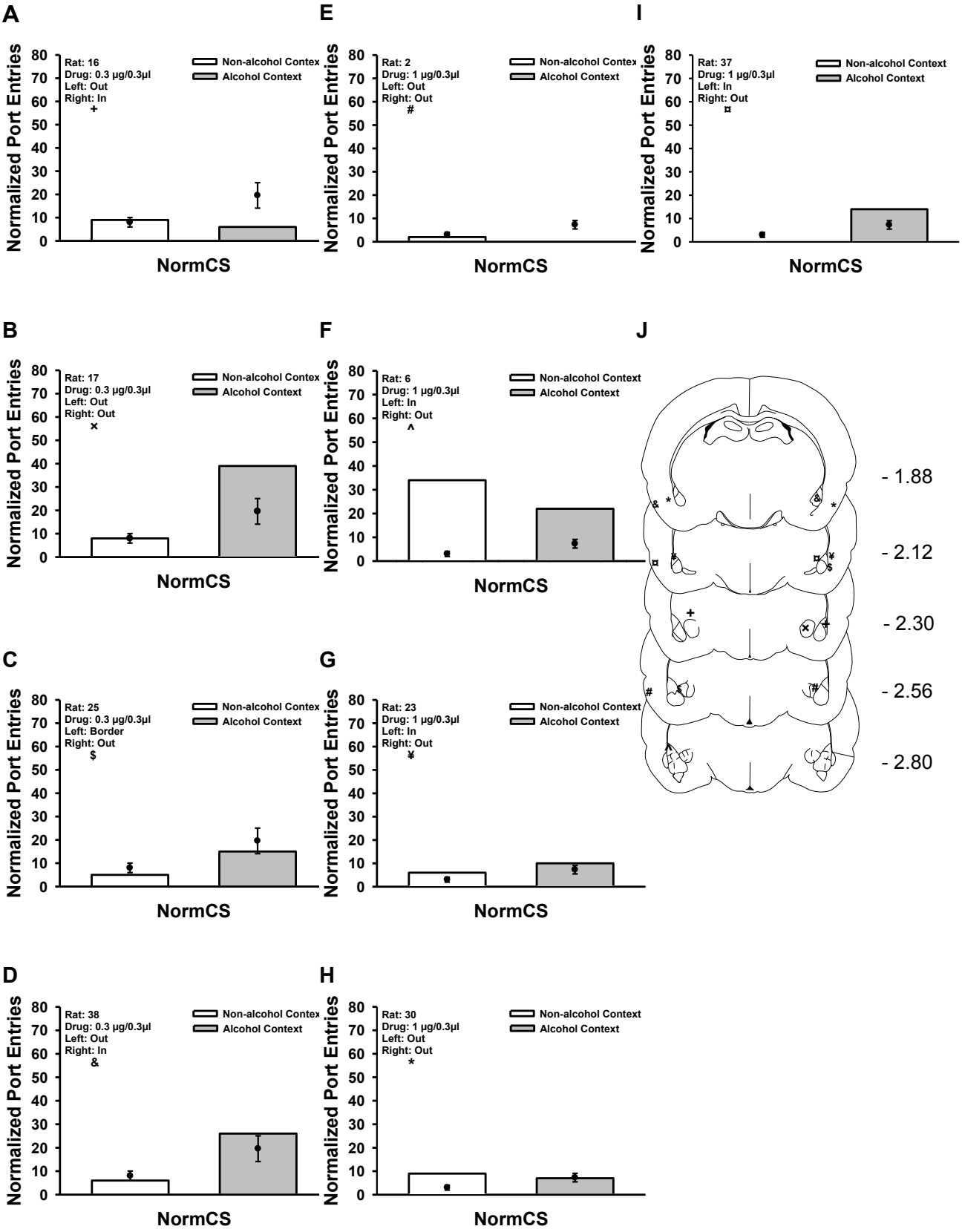
Supplementary Material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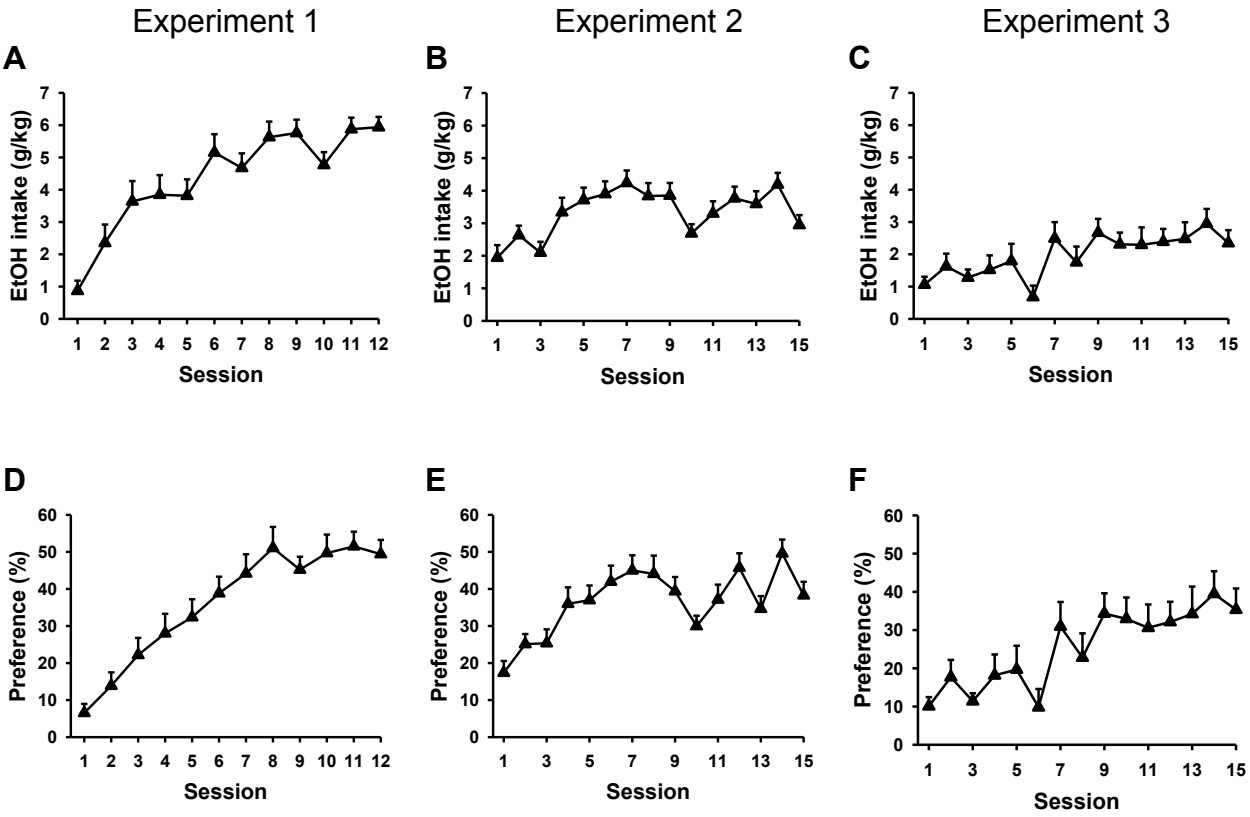
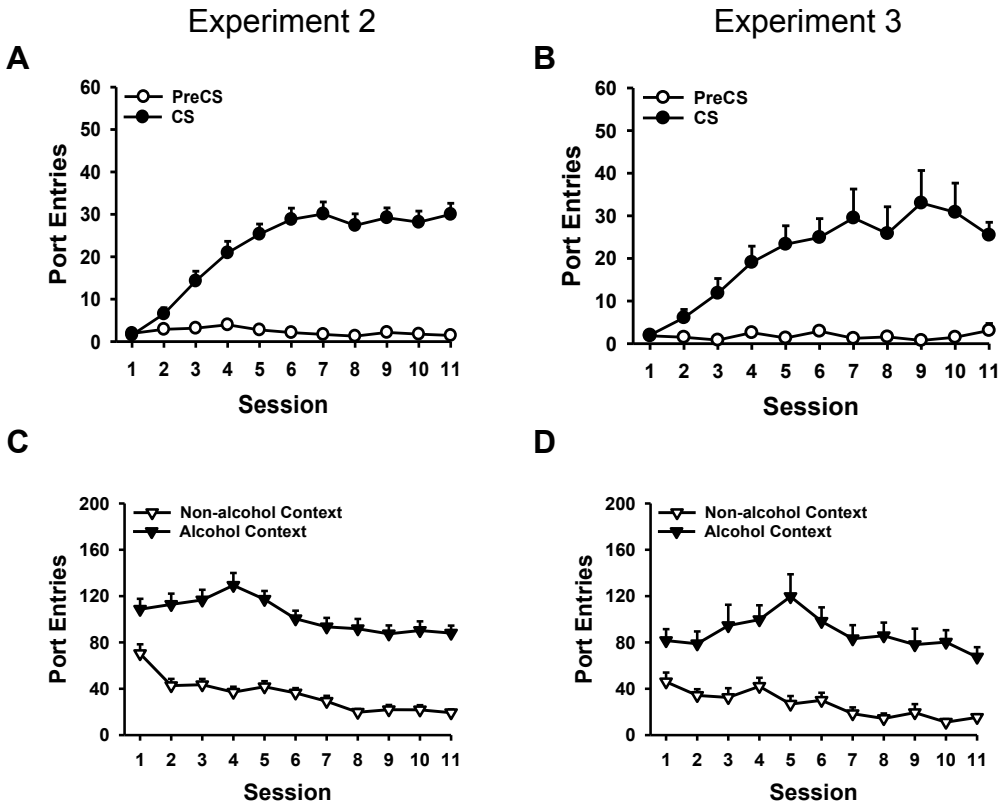
Supplementary Figure 1

Blocking glutamate at AMPAr outside the BLA had no effect on alcohol-seeking behavior elicited by the CS when it was presented without ethanol in the non-alcohol context or the alcohol context. (**A-I**) Normalized port entries at test in the non-alcohol context (open bars) and the alcohol context (gray bars) for 9 rats from Experiment 2a/2b that were dropped because either one or both injector tips were located outside the basolateral amygdala. Normalized port entries were calculated by subtracting port entries made during the PreCS from port entries made during the CS interval. Dose assignment for each rat is indicated within each graph. Mean (± SEM) test data for each dose and context condition obtained from rats with correct cannula placements are indicated within each graph (filled circles). Each individual rat was assigned a unique symbol that illustrates cannula placements. (**J**) Placement of injector tips from each corresponding dropped rat.



Supplementary Figure 2

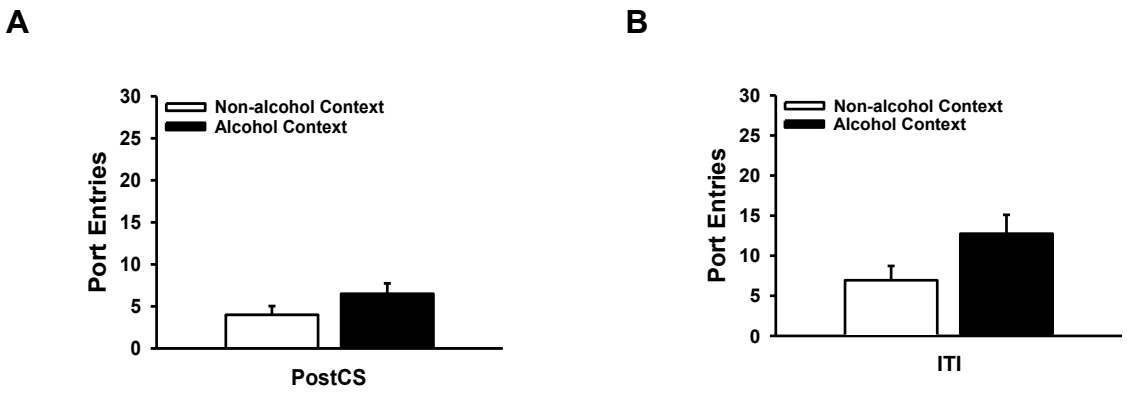
Ethanol consumption and preference for ethanol over water increased across sessions of ethanol consumption in the home-cage. Data from Experiments 1 (n=16), 2 (n=26) and 3 (n=11) are depicted. (**A-C**) Mean (± SEM) ethanol intake increased across sessions [Experiment 1; Session, $F(11,165) = 25.96$, $p < .001$; session 12 > session 1, $t(15) = 13.74$, $p < .001$: Experiment 2; Session, $F(14,350) = 8.80$, $p < .001$; session 15 = session 1, $t(25) = 1.964$, $p = .061$: Experiment 3: Session, $F(14,140) = 4.70$, $p < .001$, session 15 > session 1, $t(10) = 2.58$, $p = .027$]. (**D-F**) Mean (± SEM) ethanol preference over water increased across sessions [Experiment 1; Session, $F(11,165) = 35.42$, $p < .001$; session 12 > session 1, $t(15) = 14.16$, $p < .001$: Experiment 2; Session, $F(14,350) = 10.20$, $p < .001$; session 15 > session 1, $t(25) = 4.10$, $p < .001$: Experiment 3: Session, $F(14,140) = 6.93$, $p < .001$, session 15 > session 1, $t(10) = 3.81$, $p = .003$].



Supplementary Figure 3

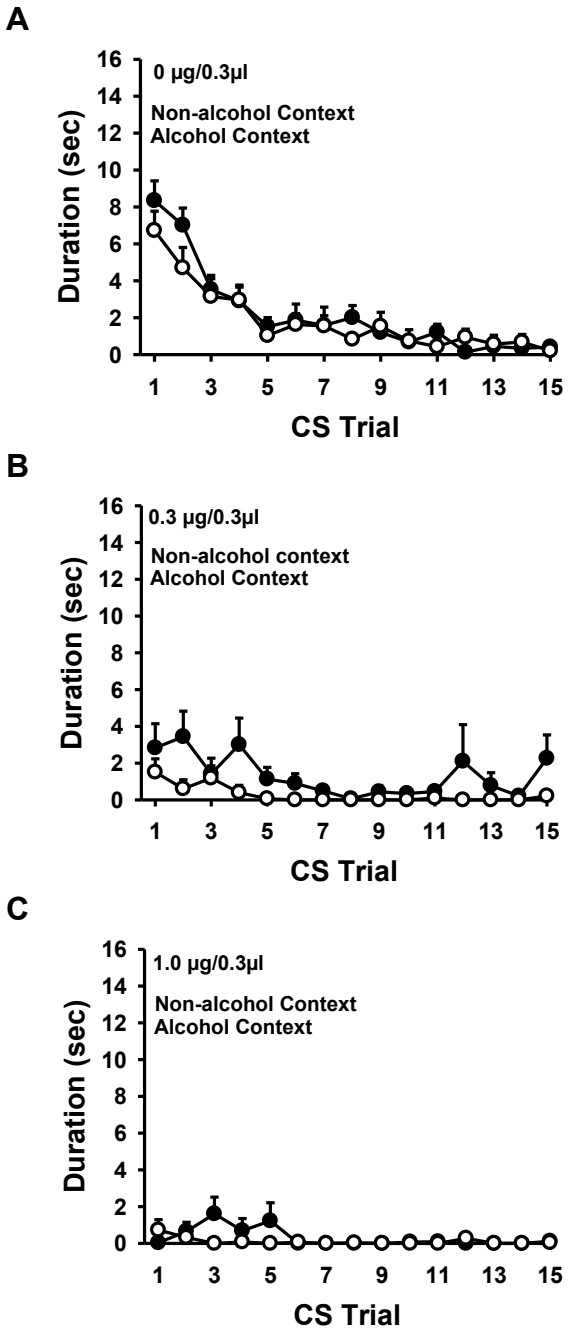
Rats learned to associate a discrete cue with the delivery of alcohol during Pavlovian conditioning sessions. Data from Experiments 2 (n=26) and 3 (n=11) are depicted. (**A-B**) Mean (± SEM) number of port entries during the CS (filled circles) and PreCS intervals (open circles). Port entries during the CS increased across sessions while PreCS responses remained low. [Experiment 2; Session, $F(10,250) = 21.78$, $p < .001$, Interval, $F(1,25) = 234.70$, $p < .001$; Session × Interval, $F(10,250) = 26.80$, $p < .001$: Experiment 3; Session, $F(10,100) = 6.68$, $p = .002$, Interval, $F(1,10) = 46.98$, $p < .001$; Session × Interval, $F(10,100) = 7.27$, $p = .001$]. ^ $p < .05$, CS versus PreCS. (**C-D**) Mean (± SEM) total number of port entries during each Pavlovian conditioning session in an alcohol context (filled triangles) and in alternating sessions in a non-alcohol context where neither the CS nor alcohol were presented (open triangles). More port entries were made during Pavlovian conditioning sessions in an alcohol context [Experiment 2; Context, $F(1,25) = 261.93$, $p < .001$; Experiment 3, Context, $F(10,100) = 92.24$, $p < .001$]. Total port entries were higher across sessions during Pavlovian conditioning and decreased across session in the non-alcohol context in experiment 2 [Session, $F(10,250) = 8.83$ $p < .001$; Context

× Session $F(10,250) = 3.04$, $p = .001$]. Total port entries decreased across sessions in experiment 3 [Session, $F(10,100) = 2.44$, $p = .012$, Session × Context, $F(10,100) = 1.64$, $p = .106$].



Supplementary Figure 4

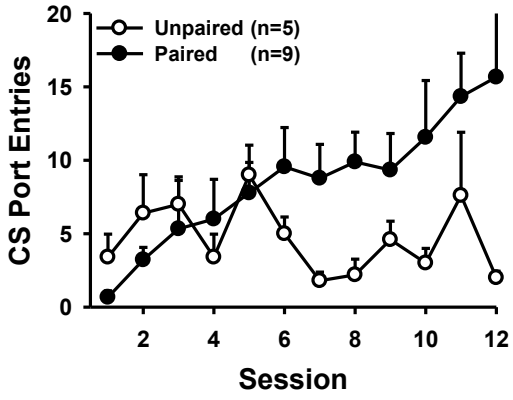
There was no effect of context on port entries made during the PostCS [$t(15) = 1.826$, p= .088] or ITI [$t(15) = 1.787$, p= .094] intervals at test for rats in experiment 1. Filled bars represent port entries obtained in an alcohol context, and open bars represent port entries made in a non-alcohol context.



Supplementary Figure 5

Blocking glutamate at AMPAr in the BLA reduced time spent in the fluid port during CS trials when the CS was presented without ethanol in the alcohol context (filled circles) or the non-alcohol context (open circles). Graphs depict mean (± SEM) time spent (sec) in the port in the alcohol context (filled circles) and non-alcohol context (open circles) for rats infused with (**A**) 0 (n=12) (**B**) 0.3 (n=7) and (**C**) 1.0 µg/0.3µl (n=7) of NBQX in the BLA. Overall, rats spent more

time in the fluid port during CS trials in the alcohol context relative to the non-alcohol context [Context, $F(1,23) = 7.16$, $p = .013$]. The amount of time spent in the port decreased across CS trials in both contexts [Trial, $F(14,322) = 12.86$, $p < .001$]. Infusion of NBQX in the BLA reduced time spent in the fluid port relative to rats infused with saline, particularly at the start of the test session [Dose, $F(2,23) = 26.40$, $p < .001$; Trial × Dose, $F(28,322) = 6.08$, $p < .001$]. This effect occurred in both test contexts [Context × Dose, $F(2,23) = 1.51$, $p = .242$; Trial × Context, $F(14,322) = 1.01$, $p = .443$; Trial × Context × Dose, $F(28,322) = 0.81$, $p = .738$].



Supplementary Figure 6

Port entries made during the CS for rats that received paired presentations (filled circles) of the CS and US increased across training sessions, while CS port entries made for rats in the unpaired group (open circles) decreased [Session, $F(11,132) = 1.780$, $p = .134$; Session × Group, $F(11,132) = 2.319$, $p = .012$].