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Potentiation of amygdala AMPA receptor activity selectively promotes escalated alcohol self-administration in a CaMKII-dependent manner

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Abstract

Growing evidence indicates that drugs of abuse gain control over the individual by usurping glutamate-linked mechanisms of neuroplasticity in reward-related brain regions. Accordingly, we have shown that glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) activity in the amygdala is required for the positive reinforcing effects of alcohol, which underlie the initial stages of addiction. It is unknown, however, if enhanced AMPAR activity in the amygdala facilitates alcohol self-administration, which is a kernel premise of glutamate hypotheses of addiction. Here we show that low-dose alcohol (0.6 g/kg/30-min) self-administration increases phosphorylation (activation) of AMPAR subtype GluA1 S831 (pGluA1 S831) in the central amygdala (CeA), basolateral amygdala, and nucleus accumbens core (AcbC) of selectively bred alcohol-preferring P-rats as compared to behavior-matched (non-drug) sucrose controls. The functional role of enhanced AMPAR activity was assessed via site-specific infusion of the AMPAR positive modulator, aniracetam, in the CeA and AcbC prior to alcohol self-administration. Intra-CeA aniracetam increased alcohol- but not sucrose-reinforced responding, and was ineffective following intra-AcbC infusion. Since GluA1 S831 is a Ca2+/calmodulin-dependent protein kinase II (CaMKII) substrate, we sought to determine if AMPAR regulation of enhanced alcohol self-administration is dependent on CaMKII activity. Intra-CeA infusion of the cell-permeable CaMKII peptide inhibitor m-AIP dose-dependently reduced alcohol self-administration. A sub-threshold dose of m-AIP also blocked the aniracetam-induced escalation of alcohol self-administration, demonstrating that AMPAR-mediated potentiation of alcohol

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DISCLOSURE/CONFLICTS OF INTEREST

The authors declare no conflict of interest.

reinforcement requires CaMKII activity in the amygdala. Enhanced activity of plasticity-linked AMPAR-CaMKII signaling in the amygdala may promote escalated alcohol use via increased positive reinforcement during the initial stages of addiction.

Keywords

alcohol drinking; amygdala; AMPA receptor; CaMKII; glutamate; GluA1

INTRODUCTION

The fundamental behavioral process of positive reinforcement reflects the tendency of all animals, human and non-human, to repeat responses that produce a desired outcome. Accordingly, positive reinforcement mechanisms underlie the repetitive nature of alcohol seeking-behavior during the initial binge/intoxication stages of addiction (Koob and Volkow, 2010; Wise and Koob, 2014). At the neurochemical level, evidence suggests that alcohol and other drugs of abuse gain control over behavior by usurping glutamatergic mechanisms of synaptic plasticity in reward circuits (Kalivas, 2009; Kauer and Malenka, 2007; McCool et al., 2010; Winder et al., 2002). Thus, identifying glutamatergic mechanisms of the positive reinforcing effects of alcohol is crucial for understanding the etiology of addiction.

Glutamate is the primary excitatory neurotransmitter in the mammalian brain and plays an essential role in regulating many of the biochemical, physiological, and behavioral effects of alcohol (Backstrom et al., 2004; Besheer et al., 2006; Gass and Olive, 2008; Hodge and Aiken, 1996; Hodge et al., 2006; McCool et al., 2010; Siggins et al., 2003; Weight et al., 1991; Woodward et al., 2006). Growing evidence indicates that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors (AMPARs) are important modulators of alcohol self-administration and relapse-like behavior (Agoglia et al., 2015; Backstrom and Hyytia, 2004; Cannady et al., 2013; Salling et al., 2014; Sanchis-Segura et al., 2006; Wang et al., 2012). AMPARs are ligand-gated ion channel receptors comprised of four subunits (GluA1-4) and mediate rapid excitatory glutamate neurotransmission and regulate synaptic strength (Bredt and Nicoll, 2003). AMPARs are involved in a variety of biological functions and dysregulation of their activity has been implicated in several neurological, psychiatric, and addictive disorders (Zhang and Abdullah, 2013), including alcoholism (Kryger and Wilce, 2010).

Repeated exposure to alcohol leads to AMPAR-mediated neuroadaptations that enhance synaptic strength in brain regions known to regulate reinforcement and reward processes (Stuber et al., 2008; Wang et al., 2012), which may promote alcohol consumption through alterations in reinforcement processes. Consistent with this concept, alcohol exposure increases extracellular glutamate levels in reward-related brain regions (Gass et al., 2011a; Griffin et al., 2015; Roberto et al., 2004). AMPAR GluA1 expression is increased in the striatum after systemically administered alcohol (Wang et al., 2012) and in nucleus accumbens by chronic intermittent consumption (Neasta et al., 2010). Surface GluA1 is also upregulated in the amygdala after chronic intermittent alcohol vapor exposure (Christian et al., 2012), and we have shown that both voluntary alcohol drinking (home-cage) and low-

dose operant alcohol self-administration increase GluA1 S831 phosphorylation in the amygdala (Salling et al., 2014). Thus, AMPAR activity and expression are targets of alcohol in specific brain regions that mediate positive reinforcement.

Glutamate activity in reward-related brain regions has a well-defined role in modulating alcohol consumption, reinforcement, and seeking behaviors (Besheer et al., 2010; Rassnick et al., 1992; Schroeder et al., 2008). Systemic enhancement of glutamate activity at AMPARs increases alcohol self-administration and seeking-behavior, which shows that heightened activity at these receptors has the potential to augment alcohol reinforcement processes (Cannady et al., 2013). Further, AMPAR activity in the amygdala is required for the positive reinforcing effects of alcohol (Salling et al., 2014). However, it is not known if enhancement of AMPAR signaling in specific reward-related brain regions potentiates the positive reinforcing effects of alcohol, which would be consistent with a role for these systems in the etiology of addiction.

Recent evidence also indicates that drug use, withdrawal, and craving are associated with post-translational modification of AMPARs in reward circuits (Wolf, 2010), including phosphorylation by Ca2+/calmodulin-dependent protein kinase II (CaMKII) (Anderson et al., 2008). CaMKII is a family of Ca^{2+}-activated Ser/Thr protein kinases that mediates many intracellular responses in the brain including glutamate release, AMPA receptor function, and synaptic plasticity (Colbran and Brown, 2004; Hudmon and Schulman, 2002; Lisman et al., 2002). A crucial function of CaMKII is to phosphorylate AMPARs at GluA1 S831 (pGluA1 S831), which leads to potentiation of AMPAR-mediated activity (Barria et al., 1997b; Mammen et al., 1997). Importantly, elevated glutamate levels after repeated alcohol use (Roberto et al., 2004) might activate CaMKII, which then phosphorylates AMPARs at GluA1 S831, promotes membrane insertion, and enhances their function (Colbran and Brown, 2004; Hudmon and Schulman, 2002; Lisman et al., 2002). We have shown that operant alcohol self-administration increases expression and phosphorylation of the alpha isoform of CaMKII (CaMKIIα) in mouse amygdala (Salling et al., 2014) and that CaMKII inhibition in the frontal cortex increases the reinforcing effects of alcohol (Faccidomo et al., 2015). However, it remains to be determined if AMPAR-mediated regulation of the positive reinforcing effects of alcohol requires CaMKII signaling.

The goal of the present preclinical studies was to examine the functional role of enhanced glutamate activity at AMPARs, specifically within limbic reward-related brain regions, in the positive reinforcing effects of alcohol. Effects of operant alcohol self-administration were first assessed on AMPAR GluA1 S831 phosphorylation and total GluA1 immunoreactivity in the nucleus accumbens and amygdala. Guided by these results, we evaluated potential mechanistic regulation of escalated alcohol self-administration via site specific infusion of the AMPAR positive modulator aniracetam. Finally, we sought to determine if aniracetam-induced increases in alcohol self-administration were dependent on CaMKII activity. These studies were conducted using selectively-bred alcohol-preferring (P-) rats, which are a genetic model of high alcohol preference (Li et al., 1979). The P-rat line has been shown to fulfill requirements of an animal model of alcoholism (Lester and Freed, 1973), including development of tolerance and dependence through voluntary drinking, and maintaining preference for ethanol over other palatable solutions (Kampov-

Polevoy et al., 2000; Lankford et al., 1991); thus, providing translational relevance of the current findings to individuals with genetic risk for high alcohol preference.

MATERIALS AND METHODS

Animals

Adult male inbred alcohol-preferring (P-) rats were pair-housed in Plexiglas cages and handled daily prior to any training procedures, and subsequently single-housed following surgeries. All rats had ad libitum home-cage access to food and water. The colony room was maintained on a 12 hour light/dark cycle (lights on at 7 am) and all experiments were conducted during the light cycle. Body weights for rats in each experiment are shown in Table S1 (Supplementary Materials). All procedures were conducted in accordance with the National Institute of Health guidelines, and approved by the University of North Carolina Institutional Animal Care and Use Committee.

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

To examine the effects of operant alcohol self-administration on expression and post-translational modifications of the GluA1 subunit, P-rats were trained to self-administer either alcohol (15% v/v; n=9) or sucrose (0.4% w/v; n=9) as we describe in (Besheer et al., 2010; Cannady et al., 2013); see also Supplementary Materials and Methods). Brain tissue was collected immediately after the 28th self-administration session. Using immunohistochemistry (IHC), coronal brain sections were processed for phosphorylated GluA1 S831 (pGluA1 S831) or total GluA1. Immunoreactivity (IR) in subregions of the amygdala (lateral - LA, basolateral - BLA, and central nuclei - CeA), subregions of the nucleus accumbens (core - AcbC and shell - AcbSh; see Supplementary Methods), the prelimbic region (prefrontal cortex), and the dorsal medial striatum were evaluated as previously described (Besheer et al., 2012; Cannady et al., 2011; Spanos et al., 2012) (Supplementary Methods).

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

To assess the effects of the AMPA positive modulator aniracetam, alcohol self-administering P-rats received site-specific infusion of aniracetam aimed at the CeA (0, 1, 3, μg/ 0.5 μl/side; n = 10) or AcbC (0, 1, 3, 6 μg/0.5 μl/side; n = 11) immediately prior to operant alcohol (15%, v/v) self-administration sessions as previously described (Schroeder et al., 2003); also see Surgery and Microinjection Methods in Supplementary Methods. Drug testing began on the 71st day of self-administration using a within-subjects design in which each aniracetam dose was administered in a randomized order. Following testing, rats in the amygdala group underwent a locomotor assessment to determine whether aniracetam affected general locomotor behavior (see Supplementary Methods). To assess reinforcer specificity, sucrose-trained P-rats (n = 9) received intra-CeA aniracetam (0, 1, 3, 6 μg/0.5 μl/side,) immediately before operant sucrose (0.4%, w/v) self-administration sessions beginning on the 39th day of self-administration.

Experiment 3: Examination of CaMKII involvement in aniracetam-induced potentiation of alcohol self-administration

To test if inhibition of CaMKII phosphorylation reduced alcohol self-administration, P-rats (n = 7) received intra-CeA Myristolated Autocamtide-2-related inhibitory peptide (m-AIP; 0, 5, 10, 20 μg/0.5μl/side) immediately before operant alcohol (15%, v/v) self-administration sessions. A second group of alcohol self-administration trained P-rats (n = 9) received intra-amygdala microinjections of vehicle, aniracetam (1 μg), m-AIP (10 μg), and a cocktail solution consisting of aniracetam (1 μg) + m-AIP (10 μg) to assess whether aniracetam-induced alterations in alcohol self-administration were dependent on CaMKII activity. All drug doses were administered in a randomized order using a within subjects design beginning on the 35th day of self-administration.

Drugs

Alcohol (95% (w/v); Pharmco-AAPER, Shelbyville, KY) was diluted in distilled water to 15% (v/v). The AMPAR positive modulator aniracetam (Tocris, Ellisville, Missouri), and the CaMKII peptide inhibitor myristolated autocamtide-2 -related inhibitory peptide (m-AIP; Enzo Life Sciences), were dissolved in 50% DMSO in artificial cerebral spinal fluid (aCSF). Aniracetam is an allosteric positive modulator of AMPARs that slows deactivation and desensitization of the receptor (Vyklicky et al., 1991) and has no effect on NMDA-mediated synaptic potentials (Boxall and Garthwaite, 1995). Evidence also indicates that aniracetam metabolites enhance AMPAR activity through a CaMKII-dependent mechanism (Nishizaki and Matsumura, 2002). m-AIP is a highly potent cell-permeable inhibitor that selectively inhibits the phosphorylation and activation of CaMKII and has no effect on cyclic AMP-dependent protein kinase, protein kinase C, calmodulin-dependent protein kinase IV (Ishida et al., 1995). The aniracetam and m-AIP dose ranges and were chosen based on demonstrated efficacy in altering behavior in previous studies (Masuoka et al., 2008; Rao et al., 2001; Salling et al., 2014).

Data Analysis

Alcohol- (or sucrose-) responses were analyzed by one-way repeated measures analysis of variance (RM ANOVA). Cumulative responses, and cumulative distance traveled (cm) were analyzed by between groups or RM ANOVA where appropriate. Intake (g/kg) was analyzed by one-way RM ANOVA and calculated based on rat body weight and number of reinforcers delivered. Tukey post-hoc comparisons were performed to identify differences between treatments/treatment groups. T-tests were performed to identify differences in pGluA1 S831 and total GluA1 immunoreactivity between alcohol- and sucrose self-administering rats within brain regions. Statistical significance was determined at $p \leq 0.05$.

RESULTS

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

To examine chronic alcohol-induced neuroadaptations of AMPAR subunits, immunohistochemistry (IHC) was performed to assess phosphorylation and expression of

the GluA1 subunit in alcohol- and sucrose self-administration trained P-rats. Mean ± S.E.M. sucrose and alcohol responses across the 28 days were 152.20 ± 28.35 and 110.90 ± 11.31 (corresponding to alcohol intake of 0.60 ± 0.06 g/kg), respectively (**Fig 1A and 1B**). This level of alcohol self-administration is consistent with prior studies in P-rats following 30-60 min operant sessions (Besheer et al., 2010; Cannady et al., 2013; Schroeder et al., 2005; Schroeder et al., 2008). Two-way RM-ANOVA indicated that there was no difference between baseline sucrose- and alcohol-reinforced responses analyzed over the 28 day period [$F(1, 16) = 1.831$, $p = 0.1948$]. These data indicate that operant performance was similar between alcohol and sucrose self-administering P-rats, allowing for direct comparisons of GluA1 immunoreactivity between the two self-administration groups.

Amygdala pGluA1 S831 IR was significantly increased in rats with a history of alcohol self-administration in the basolateral nucleus (BLA; $t(16) = 3.671$, $p=0.002$; **Fig 1C**) and the central nucleus (CeA; $t(16) = 2.173$, $p=0.045$; **Fig 1D**). In contrast, pGluA1 S831 IR in the lateral nucleus of the amygdala (LA) did not differ between groups (**Fig 1E**). Significant increases in pGluA1 S831 were observed in the nucleus accumbens core, but not the shell (Supplementary Fig 1 and Results). pGluA1 S831 IR in the dorsal medial striatum and prefrontal cortex was also examined, and no statistical differences were observed between groups (**Table 1**). Additionally, total GluA1 expression was measured to determine if alterations in phosphorylated GluA1 were the result of changes in GluA1 expression and no significant differences in any of the examined regions were observed (**Table 2**). Given a slight trend for an increase in total GluA1 protein following alcohol (**Table 2**), the ratio of pGluA1 to total GluA1 was compared within subject for brain regions showing a change in pGluA1. Results indicated that the changes in pGluA1 S831 IR were preserved when assessed relative to total protein: Mean±SEM pGluA1 / total GluA1 IR: BLA (sucrose=0.96±0.14 vs. alcohol=1.4±0.09; $t(13)=2.6$,$p=0.01$); CeA (sucrose=0.98±0.10 vs. alcohol=1.29±0.14; $t(13)=1.8$,$p=0.04$); and AcbC (sucrose=1.06±0.12 vs. alcohol=1.66±0.12; $t(13)=3.4$,$p=0.002$). Together, these data suggest that operant alcohol self-administration produces increases GluA1 S831 phosphorylation (e.g., activation) in the amygdala and nucleus accumbens to a greater extent that the nondrug palatable reinforcer, sucrose.

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

Baseline self-administration parameters are detailed in **Table 3**. To assess whether potentiation of AMPAR activity within limbic brain regions would alter alcohol self-administration, aniracetam (AMPAR positive modulator) was microinjected into the CeA or ACbC of P-rats trained to self-administer alcohol (15% v/v). One-way RM ANOVA indicated that intra-amygdala infusion of aniracetam significantly increased alcohol-reinforced lever responding [$F(2,9) = 4.2$, $p<0.03$; **Fig 2A**], with a significant increase observed following pretreatment with the lowest aniracetam dose (1 μg) relative to vehicle ($p=0.03$). The increase in responding resulted in an increase in alcohol intake following the aniracetam (1 μg) dose (**Table 4**). Analysis of cumulative alcohol-reinforced responses across the session (**Fig 2B**) showed a significant main effect of intra-amygdala aniracetam dose [$F(2,18) = 3.78$, $p=0.04$] and time [$F(5,45) = 26.88$, $p<0.001$] as alcohol-reinforced

responding increased over the duration of the session. No significant aniracetam dose by time interaction was observed. Water-lever responses (vehicle, mean±SEM = 2.0±0.39) were unchanged by aniracetam. Accurate cannulae placements were verified for all rats in this experiment (**Fig 2C**). Importantly, intra-CeA aniracetam (1 μg) had no effect on locomotor activity in an open field (**Supplementary Fig 2**), suggesting that the increase in alcohol responses was not due to a nonspecific increase in locomotor behavior.

Examination of alcohol-reinforced responding following intra-AcbC aniracetam (1-6 μg) by one-way RM ANOVA showed a significant effect of accumbens aniracetam dose [$F(3,30)=3.4$, $p=0.03$]; however, post-hoc analyses indicated no significant differences from vehicle. Water-lever responses (vehicle, mean±SEM = 7.3±2.5) were unchanged by aniracetam. As such, this data pattern suggests that in contrast to the functional role of amygdala AMPARs in modulating alcohol self-administration, intra-accumbens aniracetam does not significantly alter alcohol self-administration (**Supplementary Fig 3A-C**).

To determine if the observed increase in alcohol self-administration following intra-amygdala aniracetam pretreatment (**Fig 2A**) was specific to alcohol, aniracetam was assessed in sucrose (0.4%) self-administering P-rats. Intra-amygdala aniracetam had no significant effect on sucrose-reinforced responses across all doses tested (**Fig 2D**). Cumulative sucrose-reinforced responses over time showed a main effect of time [$F(5,40) = 13.53$, $p<0.001$], but no significant main effect of intra-amygdala aniracetam dose or interaction (**Fig 2E**). Water-lever responses (vehicle, mean±SEM = 5.3±1.9) were unchanged by aniracetam. Accurate cannulae placements were verified for all rats included in this experiment (**Fig 2F**). Together, the results from this experiment show that pharmacological potentiation of intra-amygdala AMPAR activity results in an escalation in alcohol self-administration that is specific to alcohol reinforcement and is not related to a nonspecific increase in motor activity.

Experiment 3: Examination of CaMKII involvement in aniracetam-induced potentiation of alcohol self-administration

Intra-amygdala infusion of the CaMKII inhibitor m-AIP significantly reduced alcohol-reinforced lever responses [$F(3,18) = 3.92$, $p=0.03$], at the highest dose m-AIP dose (20 μg; $p= 0.03$; **Fig 3A**). Examination of cumulative alcohol responses across the session indicated a significant main effect time [$F(5,30) = 21.73$, $p<0.001$], and a significant interaction of m-AIP dose and time [$F(15,90) = 2.04$, $p=0.02$]. Specifically, alcohol-reinforced responding was significantly reduced by m-AIP (20 μg) at 25 and 30 minutes (**Fig 3B**). The reduction in alcohol-reinforced responding resulted in a significant decrease in alcohol intake (**Table 4**). Water-lever responses (vehicle, mean±SEM = 17.0±7.4) were unchanged by m-AIP. Accurate cannulae placements were verified for all rats included in this experiment (**Fig 3C)**.

To determine if aniracetam (1 μg)-induced potentiation of alcohol self-administration (**Fig 2A**) depends on CaMKII activity, a cocktail of aniracetam (1 μg) and the highest m-AIP dose that did not alter alcohol self-administration (10 μg; **Fig 3A**) was administered in the amygdala. Significant differences in alcohol-reinforced responding were observed after

intra-amygdala aniracetam+AIP cocktail infusion [F(3,24) = 12.52, p<0.001]. Intra-amygdala aniracetam (1 μg) alone significantly increased alcohol-reinforced responses (p=0.02; **Fig 3D**) and intake (**Table 4**) and m-AIP (10 μg) alone had no effect on alcohol self-administration, confirming the results of Experiment 2 (**Fig 2A**) and the initial assessment in this experiment 3 (**Fig 3A**), respectively. Interestingly, intra-amygdala infusion of the aniracetam+AIP cocktail blocked the aniracetam-induced increase in alcohol-reinforced lever responses (p<0.001; **Fig 3D**) with a concomitant decrease in alcohol intake (**Table 4**). Examination of the pattern of alcohol-reinforced responses across the sessions by a three-way ANOVA, indicated a significant main effect of time [F(5,40) = 53.22, p<0.001], an aniracetam by m-AIP interaction [F(1,8)=53.95, p<0.001], an m-AIP dose by time interaction [F(5,40)=6.66, p<0.001], and a aniracetam by m-AIP by time interaction [F(5,40) = 10.1, p<0.001]. Post-hoc analysis showed escalated alcohol-reinforced responding following intra-amygdala aniracetam (1 μg) beginning at 15 minutes and persisting for the duration of the session (**Fig 3E**). Moreover, this aniracetam-induced escalation in alcohol responses was significantly reduced by m-AIP (aniracetam+AIP cocktail) beginning early and lasting throughout the session (**Fig 3E**). Responses on the water lever (vehicle, mean ±SEM = 13.4±4.1) were unchanged by drug treatment. Accurate cannulae placements were verified for all rats included in this experiment (**Fig 3F**). Importantly, co-administration of aniracetam and m-AIP did not alter locomotor activity in an open field (**Supplementary Fig 4**). Together these data indicate that aniracetam-induced increases in alcohol self-administration are dependent on amygdala CaMKII activity.

DISCUSSION

Recent evidence indicates that acute systemic enhancement of glutamate activity at AMPARs enhances the positive reinforcing effects of alcohol (Cannady et al., 2013), which is consistent with the hypothesis that heightened glutamate activity may underlie escalated alcohol use during the initial stages of addiction (Kalivas, 2009; Wise and Koob, 2014). In this study, we first sought to determine if the positive reinforcing effects of alcohol are associated with increased AMPAR phosphorylation at GluA1 S831, which is indicative of facilitated receptor activity. Operant alcohol self-administration increased pGluA1 S831 IR in subregions of the amygdala (BLA and CeA) and nucleus accumbens (AcbC) of genetically selected alcohol-preferring P-rats. These results led us to pharmacologically target AMPARs in the CeA and AcbC to assess functional involvement of enhanced AMPAR activity in potentiating alcohol reinforcement. Pharmacological enhancement of AMPAR activity, via local infusion of aniracetam in the CeA, but not AcbC, escalated alcohol self-administration. Moreover, reinforcer specificity was confirmed as intra-CeA aniracetam did not alter self-administration of the non-drug reward, sucrose. Finally, enhanced AMPAR-mediated facilitation of alcohol reinforcement was dependent on CaMKII as site-specific pretreatment with the CaMKII inhibitor m-AIP in the CeA blocked aniracetam-induced increases in alcohol self-administration. These data suggest that alcohol-induced upregulation of CaMKII-dependent AMPAR activity in the CeA may underlie the escalated positive reinforcing effects of alcohol that are required for the development of addiction (Wise and Koob, 2014).

AMPAR activity is enhanced by several molecular processes, including phosphorylation at GluA1 S831 (Diaz, 2010; Partin et al., 1996; Pei et al., 2009). Our data show that operant alcohol self-administration significantly increased GluA1 S831 phosphorylation in the BLA, CeA and AcbC relative to sucrose self-administering controls. This observation is highly significant given that GluA1 S831 phosphorylation is required for long-term potentiation (LTP; a molecular correlate of learning and memory) and facilitates trafficking of AMPARs to synaptic sites within the plasma membrane (Barria et al., 1997b; Esteban et al., 2003); suggesting that chronic alcohol self-administration may potentiate synaptic strength within these two well-characterized brain regions that regulate drug-taking and seeking behaviors. Moreover, given the prominent role of AMPAR GluA1 activity in learning, memory, and motivational processes (Crombag et al., 2008; Maren, 2005), it was important to compare potential alcohol-induced changes in GluA1 S831 phosphorylation to a behaviorally-matched control group that experienced the same complex operant conditioning and ongoing motor activity. Thus, these data show that alcohol increases AMPAR GluA1 phosphorylation (activation) to a relatively greater extent than the non-drug highly palatable reinforcer sucrose, but do not address potential sucrose-only or learning-only based effects.

Changes in phosphorylated protein may reflect altered receptor expression. This seems unlikely, however, since no significant changes were observed in total GluA1 immunoreactivity after operant alcohol self-administration (**Table 2**). These results contrast with studies showing upregulation of total GluA1 in the nucleus accumbens after home-cage alcohol drinking (Ary et al., 2012; Neasta et al., 2010). This difference may reflect effects of higher doses of alcohol on total GluA1 protein expression following home-cage consumption as compared to the present operant methods that resulted in lower doses. It is also possible that total GluA1 expression was upregulated in the sucrose-trained animals in our study since GluA1 subunit expression/trafficking is altered by sucrose intake (Peng et al., 2011), which could mask alcohol-induced changes in total GluA1 expression when compared to sucrose. Regardless, alcohol self-administering P-rats exhibited a greater degree of GluA1 S831 phosphorylation than parallel sucrose controls, which provides novel evidence of low-dose operant alcohol-mediated alterations in AMPAR activation in the amygdala and nucleus accumbens in a genetic model of high alcohol preference.

Given the prominent role of upregulated glutamate activity in the etiology of alcohol addiction (Gass et al., 2011b; Koob et al., 1998; Kryger and Wilce, 2010; McCool et al., 2010; Stuber et al., 2008), we hypothesized that enhanced AMPR activity in these reward-related brain regions might increase operant alcohol self-administration. Site-specific infusion of the AMPAR positive modulator aniracetam (1 μg) in the CeA increased operant alcohol self-administration indicating that heightened glutamate signaling at AMPARs functionally enhances the reinforcing effects of alcohol. Furthermore, these data suggest that enhanced AMPAR activity in the CeA might have contributed to our previous finding that systemic administration of aniracetam facilitates alcohol self-administration (Cannady et al., 2013). Interestingly, intra-CeA aniracetam produced an increase in alcohol-reinforced responding at the low dose but failed to alter performance at a single higher dose. This data pattern is consistent with our previous findings following systemically administered aniracetam (Cannady et al., 2013), and other studies showing inverted U-shaped dose-

response function by aniracetam (Pizzi et al., 1993), which may indicate off-target effects of high doses. Overall, these are the first results to show that enhanced AMPAR activity in the CeA potentiates the positive reinforcing effects of alcohol.

Pharmacological studies have shown that AMPARs modulate alcohol reinforcement and seeking behavior (Backstrom and Hyytia, 2004; Sanchis-Segura et al., 2006; Stephens and Brown, 1999; Wang et al., 2012) and we recently showed that AMPAR activity in the amygdala is required for alcohol positive reinforcement in mice (Salling et al., 2014). To our knowledge, however, this is the first study to show potentiation of alcohol reinforcement via site-specific activation of the amygdala. Consistent with this result, recent evidence shows that potentiating activity of a specific amygdala-accumbens circuit via optogenetic stimulation drives motivated behavior as evidenced by increases in nose-poke-contingent self-stimulation (Stuber et al., 2011). Given this evidence, it is plausible that potentiation of amygdala AMPAR activity could alter non-drug reinforcement. However, sucrose self-administration was not altered by intra-CeA aniracetam at doses as high as 6 μg/side, which supports prior evidence that enhanced glutamate activity at AMPARs does not alter non-drug reinforcement (Cannady et al., 2013). Similarly, the effective dose of aniracetam (1 μg/side) did not alter locomotor activity, indicating the absence of nonspecific motor activation. Overall, these data suggest that activation of AMPARs in the CeA potentiates the positive reinforcing effects of alcohol, which results in escalated intake. It is important to note, however, that centrally administered drugs can diffuse to adjacent nuclei within or between brain region (Perez de la Mora et al., 2006), which may have engaged the lateral amygdala or other proximal structures.

AMPARs are widely expressed in the mammalian brain (Petralia and Wenthold, 1992) and may mediate alcohol reinforcement in striatal regions (Wang et al., 2012); however, aniracetam infusion in the AcbC had no statistically significant effect on total alcohol-reinforced responses, suggesting a relatively greater mechanistic role of the amygdala. This lack of effect in the nucleus accumbens is somewhat surprising given the well-established role for glutamate activity in this region to modulate alcohol reinforcement. Indeed, intra-accumbens blockade of NMDA and mGluR5 receptors has been shown to attenuate alcohol self-administration (Besheer et al., 2010; Gass and Olive, 2009; Rassnick et al., 1992). It is not clear if varied AMPAR expression between the amygdala and nucleus accumbens contributed to the differential effect of aniracetam on alcohol self-administration. However, this is unlikely as an intra-AcbC aniracetam dose six times higher than that of the effective dose in the amygdala was ineffective at changing behavior. That said, aniracetam infusion in the AcbC produced a dose-dependent trend for changes in alcohol-reinforced responding as evidenced by small increases and decreases both in total responding and response rate throughout the session (see Supplementary Results). Based on these trends in behavior, and the observed alcohol-induced increase in pGluA1 S831 IR in the AcbC, an alternative explanation is related to microinjection site. Several lines of evidence suggest discrete roles for each accumbens subregion (core vs shell) in mediating the rewarding properties of alcohol (Chaudhri et al., 2010). Thus, future experimentation examining potentiated AMPAR signaling in the shell may show different effects on alcohol self-administration.

A highly significant finding of this study is that AMPAR-mediated escalation of alcohol self-administration in the CeA was dependent on CaMKII. We have shown that operant alcohol self-administration increases phosphorylation of GluA1 S831 and CaMKIIα T286, and that inhibition of AMPAR or CaMKII activity in the amygdala decreases self-administration (Salling et al., 2014). It was unknown, however, if AMPAR-mediated potentiation of alcohol self-administration requires CaMKII signaling. To address this question, we first evaluated effects of amygdala infusion of the cell permeable CaMKII peptide inhibitor m-AIP on operant alcohol self-administration. In agreement with prior results in mice (Salling et al., 2014), m-AIP dose-dependently decreased alcohol self-administration in P-rats. Then, we pretreated rats with a behaviorally inactive dose of m-AIP (10 μg/side) in combination with the effective dose of aniracetam (1 μg/side) in the CeA. m-AIP blocked the aniracetam-induced increase in operant alcohol self-administration and reduced responding and intake. Although it is unclear why alcohol self-administration following the drug combination was reduced below control levels, these data provide evidence that AMPAR-mediated facilitation of alcohol reinforcement by aniracetam functions through a CaMKII-dependent mechanism within the amygdala.

Importantly, when taken together with prior data (Salling et al., 2014), these results show bidirectional effects on alcohol self-administration by AMPAR activation, or inhibition, in the amygdala, which strongly supports mechanistic regulation. We propose that alcohol self-administration increases glutamate levels in the amygdala after chronic alcohol exposure (Roberto et al., 2004) leading to phosphorylation of CaMKIIα (Salling et al., 2014) and subsequent activation of AMPARs by CaMKIIα at S831 (Barria et al., 1997a). Since CaMKII-dependent AMPAR activity underlies synaptic plasticity (Barria et al., 1997b), this suggests that moderate alcohol use may mimic plasticity-inducing events in the amygdala (Christian et al., 2012). Moreover, since CaMKII-dependent AMPAR activity is required for behavioral plasticity, such as learning and memory (Crombag et al., 2008), these plasticity-like adaptations may promote a gain in alcohol reinforcement learning and function, which supports the development of addiction (Wise and Koob, 2014). Overall, these data suggest that targeted reductions in AMPAR or CaMKII activity may be of therapeutic value for treatment of alcohol use disorders without side effects on non-drug reward processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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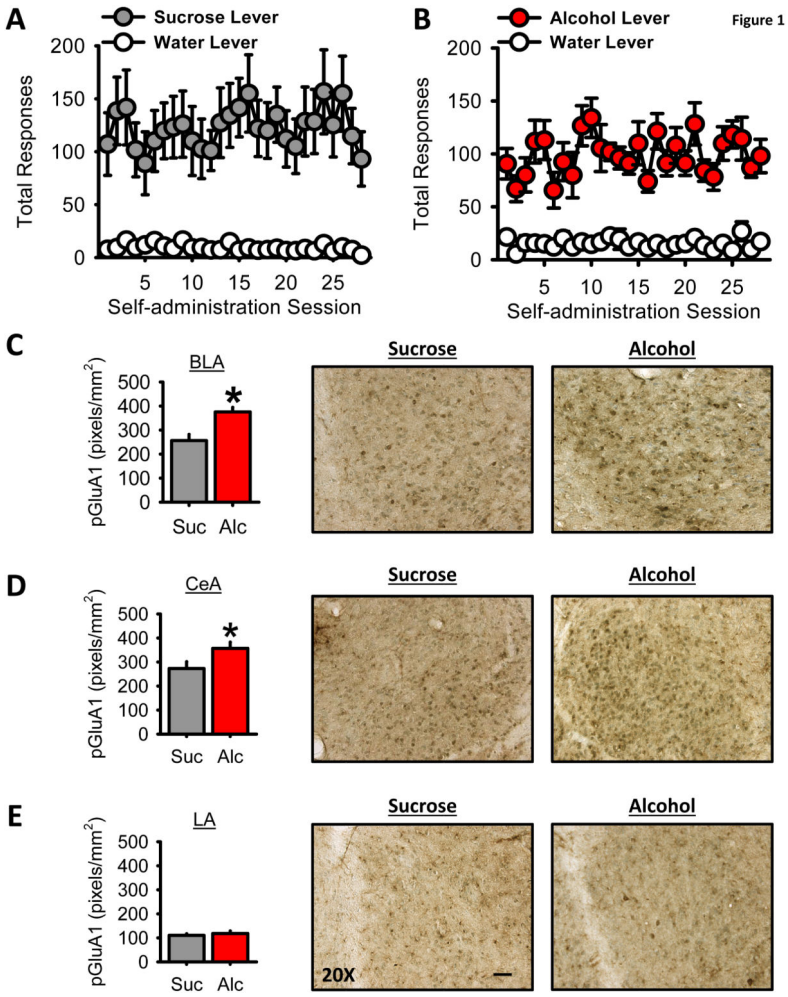

Figure 1. Alcohol self-administration upregulates phosphorylation of AMPAR GluA1 S831 subunit in subregions of the amygdala

Total lever presses plotted as a function of daily 30-min self-administration sessions for **(A)** sucrose- or **(B)** alcohol exposed P-rats prior to tissue collection for immunohistochemistry (IHC). Mean (± SEM) immunoreactivity of the pGluA1 S831 positive area (pixels/1000/mm^2) in the **(C)** basolateral amygdala (BLA), **(D)** central amygdala (CeA), and **(D)** lateral amygdala (LA) following sucrose **(left)** or alcohol **(right)** self-administration. Representative 20X photomicrographs of the BLA, CeA, and LA are of pGluA1 S831 IR after 28 days of sucrose or alcohol self-administration. Scale bar represents 50 microns. Graphed values are expressed as means ± SEM; * $p < 0.05$ – alcohol (ALC) vs sucrose (SUC), t-test.

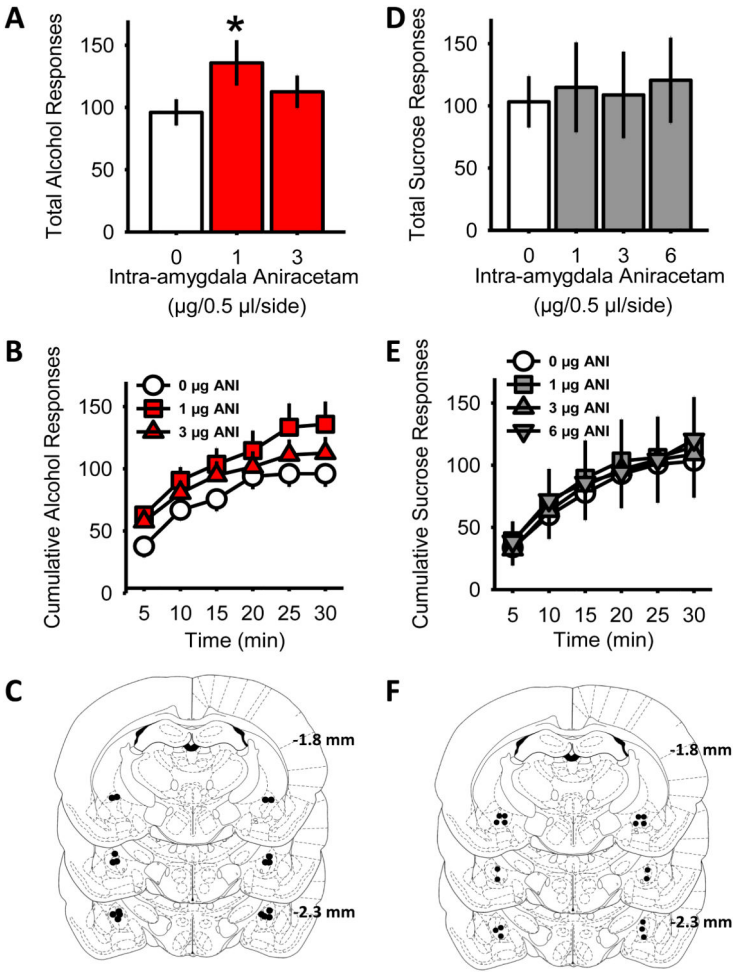

Figure 2. Positive modulation of amygdala AMPA receptors potentiates alcohol self-administration in a reinforcer-specific manner

Total alcohol **(A)** and sucrose **(D)** reinforced lever presses plotted as a function of intra-CeA aniracetam dose in selectively bred alcohol preferring P-rats (n = 10). Cumulative alcohol **(B)** and sucrose **(E)** reinforced responses plotted as a function of time (min) during 30-min session. Slope of the cumulative plot represents response rate. **(C and F)** Schematic showing injection location in the CeA for alcohol- and sucrose-trained P-rats. Graphed values are expressed as means ± SEM; *$p<0.05$ relative to vehicle treatment.

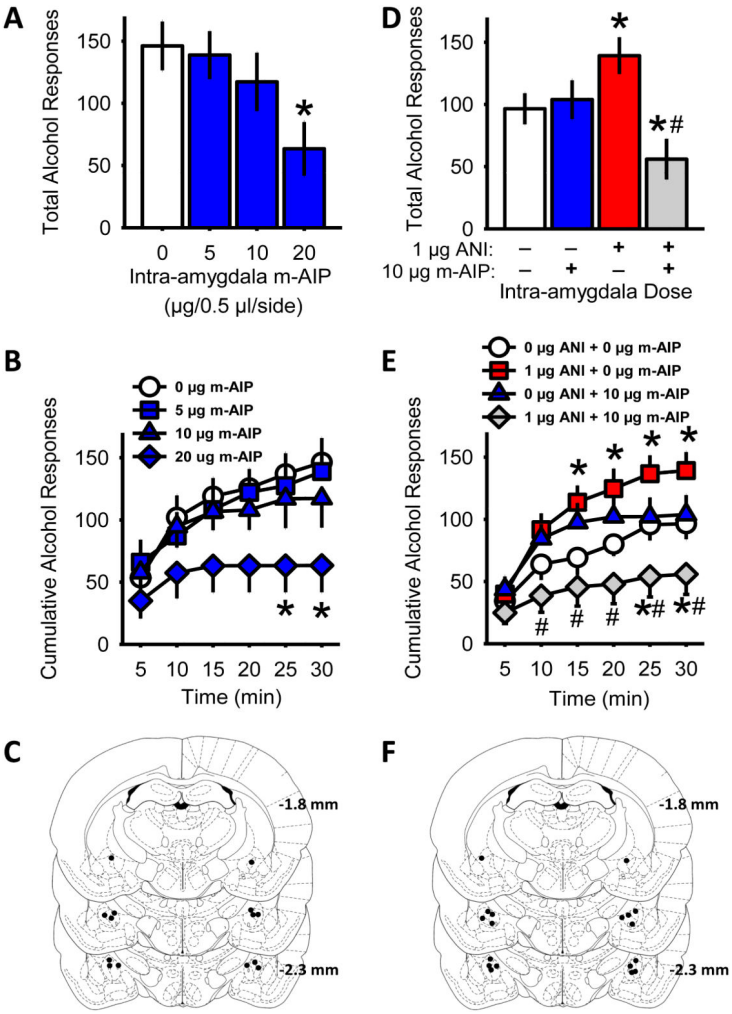

Figure 3. Intra-amygdala inhibition of CaMKII activity blocks aniracetam-induced increase in alcohol self-administration

(A) Total alcohol reinforced responses plotted as a function of the CaMKII peptide inhibitor m-AIP dose in P-rats (n = 7) showing a dose-dependent reduction in alcohol reinforcement. **(B)** Cumulative alcohol-reinforced responses were significantly decreased by intra-amygdala m-AIP pretreatment (20 µg). **(D)** Intra-CeA co-administration of the CaMKII inhibitor m-AIP (10 µg) blocked the aniracetam (1 µg) induced increase in alcohol reinforced responses in P-rats (n = 9). **(E)** Aniracetam-induced changes in response rate (cumulative alcohol-reinforced responses) was significantly increased by aniracetam but blocked by co-infusion of m-AIP (10 µg). **(C and F)** Illustrations showing accurate injector cannulae placements in the CeA. Graphed values are expressed as means ± SEM; *p<0.05 relative to vehicle treatment and #p<0.05 relative to 1 µg aniracetam (ANI).

Table 1

pGluA1 S831 immunoreactivity (pixels/1000/mm^2) after alcohol and sucrose self-administration. Data represent mean ± SEM (n=9/group).

Brain region	Alcohol Self-administration	Sucrose Self-administration
Dorsal Striatum		
pGluA1 S831 IR	578.06 ± 38.09	512.88 ± 41.40
Prefrontal Cortex		
pGluA1 S831 IR	90.76 ± 11.68	95.38 ± 10.78

Table 2

Total GluA1 immunoreactivity (pixels/1000/mm^2) after alcohol and sucrose self-administration. Data represent mean ± SEM (n=9/group).

Brain region	Alcohol Self-administration	Sucrose Self-administration
BLA		
Total GluA1 IR	278.59 ± 15.48	226.08 ± 26.98
CeA		
Total GluA1 IR	279.89 ± 19.99	231.79 ± 22.23
LA		
Total GluA1 IR	101.79 ± 10.20	78.13 ± 16.18
AcbC		
Total GluA1 IR	537.87 ± 34.17	496.89 ± 31.82
AcbSh		
Total GluA1 IR	556.85 ± 42.52	555.22 ± 35.62

Table 3

Behavioral baseline from 5 groups of P-rats showing number of responses emitted on the alcohol and water associated lever prior to infusion of the AMPAR positive modulator aniracetam (ANI) or the CaMKII inhibitor myristolated AIP (m-AIP) infusion. Alcohol intake was confirmed at the end of each session and converted to g/kg. Data represent mean ± SEM (n=7 – 11/group).

	Responses		Alcohol Intake (g/kg)
Test Group	**Alcohol Lever**	**Water Lever**	
Alcohol Reinforcement			
Amygdala ANI	117.75 ± 7.19	9.20 ± 1.95	0.69 ± 0.05
Nucleus Accumbens ANI	105.82 ± 6.49	8.27 ± 1.84	0.61 ± 0.05
Amygdala AIP	129.14 ± 15.58	21.79 ± 4.02	0.78 ± 0.09
Amygdala ANI + m-AIP	115.50 ± 10.69	9.78 ± 2.63	0.68 ± 0.05
Sucrose Reinforcement			
Amygdala ANI	133.27 ± 25.25	15.89 ± 5.22	--

Table 4

Alcohol intake (g/kg) after AMPAR positive modulator aniracetam or the CaMKII inhibitor myristolated AIP (m-AIP) infusion. Alcohol intake was confirmed at the end of each session and converted to g/kg. Data represent mean ± SEM (n=7 – 11/group).

	Aniracetam Dose (μg)			
Brain Region	**0**	**1**	**3**	**6**
Amygdala	0.54 ± 0.07	0.77 ± 0.12 *	0.65 ± 0.10	N/A
N. Accumbens	0.58 ± 0.09	0.54 ± 0.12	0.68 ± 0.11	0.39 ± 0.11
	m-AIP Dose (μg)			
Brain Region	**0**	**5**	**10**	**20**
Amygdala	0.81 ± 0.12	0.79 ± 0.12	0.79 ± 0.12	0.36 ± 0.13 *
	Aniracetam + m-AIP Dose (μg)			
Brain Region	**0 + 0**	**1 + 0**	**1 + 10**	**0 + 10**
Amygdala	0.52 ± 0.07	0.75 ± 0.09 *	0.31 ± 0.10 *#	0.56 ± 0.09

* $p < 0.05$ relative to 0 or 0 + 0;

# $p < 0.05$ relative to 1 μg aniracetam + 0 m-AIP