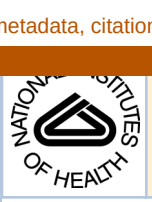

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Enhanced AMPA Receptor Activity Increases Operant Alcohol Self-administration and Cue-Induced Reinstatement

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Abstract

Long-term alcohol exposure produces neuroadaptations that contribute to the progression of alcohol abuse disorders. Chronic alcohol consumption results in strengthened excitatory neurotransmission and increased AMPA receptor signaling in animal models. However, the mechanistic role of enhanced AMPA receptor activity in alcohol reinforcement and alcohol-seeking behavior remains unclear. This study examined the role of enhanced AMPA receptor function using the selective positive allosteric modulator, aniracetam, in modulating operant alcohol self-administration and cue-induced reinstatement. Male alcohol-preferring (P-) rats, trained to self-administer alcohol (15%, v/v) versus water were pretreated with aniracetam to assess effects on maintenance of alcohol self-administration. To determine reinforcer specificity, P-rats were trained to self-administer sucrose (0.8%, w/v) versus water, and effects of aniracetam were tested. The role of aniracetam in modulating relapse of alcohol-seeking was assessed using a response-contingent cue-induced reinstatement procedure in P-rats trained to self-administer 15% alcohol. Aniracetam pretreatment significantly increased alcohol-reinforced responses relative to vehicle treatment. This increase was not attributed to aniracetam-induced hyperactivity as aniracetam pretreatment did not alter locomotor activity. AMPA receptor involvement was confirmed because DNQX (AMPA receptor antagonist) blocked the aniracetam-induced increase in alcohol self-administration. Aniracetam did not alter sucrose-reinforced responses in sucrose-trained P-rats, suggesting that enhanced AMPA receptor activity is selective in modulating the reinforcing function of alcohol. Finally, aniracetam pretreatment potentiated cue-induced reinstatement of alcohol-seeking behavior versus vehicle treated-P-rats. These data suggest that enhanced glutamate activity at AMPA receptors may be key in facilitating alcohol consumption

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Authors contribution:

RC, JB, and CWH were responsible for the study concept and design. RC, BD, and KRF contributed to the acquisition of animal data. RC, JB, and CWH assisted with data analysis and interpretation of findings. RC drafted the manuscript. RC, JB, and CWH provided critical revision of the manuscript for important intellectual content. All authors critically reviewed content and approved final version for publication.

and seeking behavior which could ultimately contribute to the development of alcohol abuse disorders.

Keywords

Alcohol; AMPA; Glutamate; Reinforcement; Relapse; Self-administration

Introduction

Glutamate signaling has been widely implicated in modulating addiction to alcohol and other drugs of abuse (Gass and Olive, 2008; Kalivas, 2009). The role of N-Methyl-D-aspartic acid (NMDA) receptors and metabotropic glutamate receptors in alcohol reinforcement processes and relapse have been studied extensively (Backstrom and Hyytia, 2004; Besheer et al., 2010; Bienkowski et al., 1999; Schroeder et al., 2008); however, the functional role of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPARs) in these behaviors has received less attention. AMPARs are a subclass of ionotropic glutamate receptors that are ubiquitously expressed throughout the brain (Petralia and Wenthold, 1992). These receptors mediate fast excitatory glutamate neurotransmission and play a key modulatory role in major neural functions and processes, including synaptic plasticity (Kessels and Malinow, 2009). This is significant given that chronic exposure to drugs of abuse is associated with molecular maladaptations that influence synaptic plasticity events within brain reward circuitry (Kalivas, 2009; Nestler and Aghajanian, 1997). Interestingly, AMPARs have been implicated in modulating the addictive properties of a variety of drugs of abuse (Bowers et al., 2010; Choi et al., 2011; Van den Oever et al., 2008). Thus, a better understanding of how activity at AMPARs might influence drug-taking and seeking behaviors may provide novel insights into the etiology of the multiple phases of drug addiction.

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

Thus, the goal of the present study was to determine the effect of acute potentiation of glutamate activity at AMPARs on operant alcohol self-administration and cue-induced reinstatement of alcohol-seeking behavior using the positive allosteric modulator,

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

Materials and Methods

Animals

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

Apparatus

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

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

Operant Self-administration

Training—One day prior to training, rats were fluid-restricted for approximately 24 hrs. Immediately afterwards, rats were placed in the operant conditioning chambers for an initial 16-hr lever-press training session in which presentation of a 0.1 ml solution of concurrently

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

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

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

To test whether potentiation of AMPAR signaling might influence alcohol self-administration, P-rats (n = 9) were pretreated with aniracetam (0, 1, 5, 10, 30 mg/kg, i.p.) 30 minutes prior to operant alcohol (15% v/v) self-administration sessions (30-min). Aniracetam doses within this range have been shown to be effective at altering behavior in previous studies (Knapp et al., 2002; Lebrun et al., 2000). Testing was conducted using a within-subjects design in which each aniracetam dose was administered in a randomized order. As such, blood-alcohol content (BAC) measurements were not conducted after each drug test session as blood sampling has the potential to disrupt ongoing self-administration behavior. There were at least 2 self-administration sessions between injections and test sessions occurred no more than two times per week. Baseline self-administration remained relatively stable throughout testing. Additionally, each liquid receptacle in the operant chambers was monitored for residual fluid after all test sessions and no sign of residual fluid was detected.

Test for aniracetam-induced alterations in locomotor behavior

Two weeks following aniracetam testing, the P-rats were tested to examine if potentiated aniracetam-induced alcohol self-administration was the result of changes in locomotor activity. Animals received aniracetam pretreatment (0 and 5 mg/kg, i.p.) 30 minutes prior to placement in locomotor chambers where distance traveled was measured and recorded via photo beam breaks. The aniracetam dose of 5 mg/kg was tested because it elicited the greatest effect on alcohol lever responses and intake during self-administration test sessions. Each rat experienced two 30-minute locomotor sessions in which aniracetam dose order (0 and 5 mg/kg) was randomized so that dose order would not influence behavioral outcomes. Locomotor sessions were interspersed with self-administration sessions with at least 3 days between tests. On the locomotor test days, self-administration sessions were withheld.

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

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

Test for aniracetam-induced alterations in alcohol clearance

To determine whether aniracetam pretreatment altered alcohol clearance, another cohort of P-rats (n=6 per treatment group) with a history of alcohol self-administration (at least of 28 baseline sessions). Groups were matched based on the average alcohol intake (g/kg) for the previous 7 days prior to this assessment (vehicle: 0.65 ± 0.08; aniracetam: 0.64 ± 0.07). Rats were pretreated with aniracetam (0; or 5 mg/kg; i.p.) 30 minutes prior to orally administered alcohol (1 g/kg, IG) and tail blood was collected 20, 30, 60, 120 and 240 min later. Plasma supernatant (5 μl) was analyzed for alcohol content using an Analox Alcohol Analyser (Model AM1, Analox Instruments USA Inc., Lunenburg, MA).

Test for reinforcer specificity via sucrose-self-administration

To test if enhanced AMPAR activity was selective at modulating alcohol reinforcement, behavior-matched sucrose-trained P-rats (n = 11) were pretreated with aniracetam (0, 1, 5, 10, 30 mg/kg, i.p.) 30 minutes prior to operant sucrose (0.8%, w/v) self-administration sessions (30-min). Aniracetam testing was conducted as described for the alcohol self-administration tests sessions.

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

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

To test the effects of aniracetam on extinction responding, the same animals were reintroduced to alcohol 15% (v/v) for 10 baseline self-administration sessions. Rats then underwent 12 daily extinction sessions (30 min; see above) and on the 13th extinction

session, animals were pretreated with vehicle (n=7) or aniracetam (n=7). One rat from the vehicle group did not resume reliable baseline responding after the reinstatement session and was not tested during extinction. Because aniracetam did not alter sucrose self-administration (see below), the role of aniracetam in sucrose reinstatement was not tested in this study.

Drugs

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

Data Analysis

Alcohol- (or sucrose-) and water-reinforced responses, cumulative responses, and cumulative distance traveled (cm) were analyzed by two-way repeated measures analysis of variance (RM ANOVA). Alcohol intake (g/kg) was estimated based on rat body weight and number of reinforcers delivered and was analyzed by one- or two-way RM ANOVA (where appropriate). Responding was “extinguished” when alcohol-lever responses were not statistically different than water-lever responses for at least two consecutive days (two-way ANOVA). Lever responses in the reinstatement session were analyzed by two-way RM ANOVA (with test condition as a repeating factor). BAC time course data were analyzed by two-way RM ANOVA. As described in (Gorin-Meyer et al., 2007), linear regression analysis was performed for each animal and derived parameters from the linear portion of the clearance curve (60 – 240 min) were analyzed by t-test for treatment group comparisons. Derived regression analysis parameters include: estimated BAC at 0 min (y-intercept), estimated clearance rate (mg/dl/min; slope of the regression line), and estimated clearance time (min; x-intercept of regression line). The estimated volume of distribution accounting for body weight (dl/g) was calculated by dividing the amount of administered alcohol (mg) by the product of body weight (g) and the estimated BAC(mg/dl) at 0 min (y-intercept)). The area under the clearance curve was also determined for each treatment group. Tukey post-hoc comparisons were performed to identify differences between treatments/treatment groups. Significance was determined at $p \leq 0.05$. Baseline data (mean ± S.E.M.) for the day preceding initial testing of aniracetam is reported in Table 1.

Results

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

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

showed a significant increase in alcohol intake ($F(4,32)=5.64$, $p=0.001$) after aniracetam pretreatment, with over a 60% increase in consumption (5 mg/kg aniracetam vs. vehicle treatment, $P<0.05$). The discrepancy between the significant increase in lever responses and no significant difference in intake after 1 g/kg aniracetam could be due to the presence of nonreinforced responses (i.e., responding during reinforcer delivery is recorded but does not count toward subsequent reinforcer delivery). Furthermore, statistical analysis of the rate of alcohol lever responding over time (cumulative lever responses) by two-way RM ANOVA showed significant main effects of time ($F(5,40)=47.20$, $p<0.001$), and dose ($F(4,32)=4.72$, $p=0.004$), and a significant interaction of time x dose ($F(20,160)=4.04$, $p<0.001$). Post-hoc analysis indicated that the aniracetam-induced increase in alcohol-reinforced responses emerged early (by 10 min) and persisted throughout the duration of the 30-minute test session (5 mg/kg aniracetam; Figure 1B). Together these data suggest that enhanced glutamate activity at AMPA receptors enhances the reinforcing function of alcohol (e.g., the behavioral process) as evidenced by increased alcohol-reinforced lever responses.

Test for aniracetam-induced alterations in locomotor behavior

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

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

Rats received double injections of DNQX and aniracetam to verify the role of AMPA receptors in potentiating alcohol-reinforced responses during operant self-administration sessions. Analysis by two-way RM ANOVA showed a main effect of DNQX dose ($F(1,8)=15.36$, $p=0.004$), but no main effect of aniracetam dose ($F(1,8)=0.15$, $p=0.71$). There was a significant interaction of aniracetam dose x DNQX dose ($F(1,8)=,18.28$, $p=0.003$). Post-hoc analysis indicated that DNQX (3 mg/kg) was ineffective at altering alcohol self-administration when administered in combination with vehicle (Aniracetam 0 mg/kg; Figure 2 left panel). Importantly, pretreatment with DNQX (3 mg/kg) significantly blocked aniracetam-induced increased alcohol self-administration (aniracetam 5 mg/kg; Figure 2). Alcohol intake followed the same statistical trends as lever responding. Intake values (g/kg) are as follows: 0.64 ± 0.03 (0 mg/kg DNQX and 0 mg/kg ANI), 0.61 ± 0.07 (3 mg/kg DNQX and 0 mg/kg ANI), 0.89 ± 0.06 (0 mg/kg DNQX and 5 mg/kg ANI), 0.46 ± 0.05 (3 mg/kg DNQX and 5 mg/kg ANI). A main effect of DNQX dose ($F(1,8)=24.48$. $p= 0.001$), and a significant interaction of DNQX and aniracetam dose ($F(1,8)=17.38$. $p= 0.003$) on alcohol intake was observed as analyzed by two-way RM ANOVA. Water lever responding was not altered by DNQX and aniracetam pretreatment. Two-way ANOVA showed no main effects of DNQX dose ($F(1,8)=1.82$, $p=0.21$), aniracetam dose($F(1,8)=1.74$, $p=0.22$) and no significant interaction of DNQX x aniracetam ($F(1,8)=0.03$, $p=0.86$). These data confirm the role of AMPA receptors in mediating the aniracetam-induced increases in alcohol-reinforced responding.

Test for aniracetam-induced alterations in alcohol clearance

To test if increased alcohol self-administration was the result of treatment-induced alterations in alcohol clearance, P-rats were challenged with a 1 g/kg (IG) dose of alcohol following aniracetam (0 vs. 5 mg/kg; i.p) pretreatment (n=6 per treatment group).

Pretreatment with aniracetam did not alter BAC over a 4-hr period relative to vehicle-treated P-rats (Figure 3). Analysis by two-way RM ANOVA showed a main effect of time ($F(4,40)=76.65$, $p<0.001$), but no effect of dose ($F(1,40)=1.139$, $p=0.31$) or interaction of time x dose ($F(4,40)=1.648$, $p=0.18$). Moreover, comparisons of estimated clearance rates (slope of the linear portion of the clearance curve; 60 – 240 min), estimated clearance times (x-intercept), estimated BAC at 0 min (y-intercept), volume of distribution accounting for body weight (dl/g), and area under the curve (AUC) showed no significant differences after aniracetam pretreatment as analyzed by t-tests (Table 2). Collectively, these data indicate that increased alcohol self-administration following aniracetam pretreatment was not related to a change in alcohol clearance.

Test for reinforcer specificity via sucrose self-administration

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

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

To examine the role of enhanced glutamate activity at AMPA receptors in modulating alcohol-seeking behavior in P-rats a response contingent cue induced-reinstatement procedure was used. Removal of response contingencies extinguished alcohol-associated lever responding over a period of 13 consecutive days (Figure 5A). Statistical analysis by two-way ANOVA showed a main effect of lever ($F(1,364)=324.14$, $p<0.001$), extinction session ($F(12,364)=29.39$, $p<0.001$), and a significant interaction of lever x extinction session ($F(12,364)=25.87$, $p<0.001$). Post-hoc comparisons showed significant differences in lever responses on the alcohol and water levers, with the exception of the last three extinction sessions ($p>0.05$), which confirms extinction. During reinstatement, both treatment groups (vehicle and aniracetam) displayed response-contingent cue-induced reinstatement of alcohol-seeking behavior, as evidenced by increased responding on the alcohol-associated lever compared to their last extinction session (Figure 5B). Two-way RM ANOVA (with test condition as a repeating factor) showed significant main effects of aniracetam dose ($F(1,13)=10.74$, $p=0.006$) and test condition ($F(1,13)=89.74$, $p<0.001$) and a significant interaction of aniracetam dose x test condition ($F(1,13)=11.16$, $p=0.005$). Interestingly, responding on the alcohol-associated lever was potentiated in P-rats after pretreatment with aniracetam (5 mg/kg vs. vehicle group) during reinstatement ($p\leq0.05$, Tukey post-hoc). Responses on the water lever during reinstatement were not altered by aniracetam pretreatment (Figure 5C) as no main effect of dose ($F(1,13)=0.12$, $p=0.74$), test condition ($F(1,13)=1.90$, $p=0.19$), or interaction ($F(1,13)=0.02$, $p=0.88$) was evident. Moreover, an examination of operant responses during extinction did not reveal any differences between the aniracetam-treated and vehicle-treated groups. The mean values (± SEM) for lever responding during extinction after pretreatment with aniracetam were as follows: 4.286 ± 0.92 (vehicle; alcohol lever), 1.286 ± 0.75 (vehicle; water lever), 8.286 ± 3.15 (5 mg/kg; alcohol lever), 1.71 ± 0.36 (5 mg/kg; water lever). The variability in alcohol

lever responses following aniracetam was caused by a high number of responses by a single rat as reflected by the large S.E.M.. Analysis by two-way ANOVA (with test condition as a repeating factor) showed no main effects of dose ($F(1,12)=0.41$, $p=0.53$), test condition ($F(1,12)=0.70$, $p=0.42$) and no interaction of dose X test condition ($F(1,12)=2.16$, $p=0.17$). These data suggest that potentiated glutamate activity at AMPARs exacerbates cue-induced alcohol-seeking behavior.

Discussion

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

The reinforcing effect of drugs of abuse like alcohol is a major controlling process by which subsequent drug-taking episodes are facilitated (Stolerman, 1992; White, 1996). Here, a novel neuronal mechanism is provided that could contribute, in part, to increased alcohol drinking. That is, enhanced glutamate activity at AMPA receptors alters alcohol reinforcement function in a manner that facilitates self-administration. These data are highly relevant given that excessive alcohol consumption has been shown to increase the likelihood for the development of alcoholism and/or alcohol dependence in humans (Saha et al., 2006). Interestingly, previous work by Stephens and Brown (1999) showed that administration of NBQX (AMPA receptor antagonist) not only reduced operant alcohol self-administration on a progressive ratio schedule of reinforcement, but sucrose and saccharin self-administration as well as locomotor activity; suggesting that AMPARs were either not selective in modulating alcohol reinforcement or results could not be determined due to non-specific motor-impairing effects of NBQX (Stephens and Brown, 1999). In contrast, the present work demonstrates a selective role for enhanced AMPAR activity in modulating alcohol reinforcement since AMPAR activation resulted in increased alcohol self-administration on a fixed ratio schedule of reinforcement. Further, reinforcer specificity was confirmed because aniracetam pretreatment was void of effects on sucrose self-administration.

An intriguing feature of the alcohol self-administration experiments is the inverted U-shaped dose response curve following aniracetam pretreatment. Lower aniracetam doses were effective at significantly increasing operant self-administration, whereas higher doses did not alter alcohol-reinforced responses. Given the role of aniracetam as a positive modulator of glutamate activity at AMPA receptors (Tang et al., 1991), the inverted-U dose-response curve may reflect changes in extracellular glutamate levels after chronic alcohol exposure. Specifically, low doses of aniracetam may have potentiated alcohol-induced elevated glutamate activity at AMPARs and promoted alcohol self-administration, while higher aniracetam doses may have exceeded the necessary threshold to alter alcohol reinforcement

function. Indeed, other studies have shown biphasic effects of aniracetam in response to glutamate challenge (Pizzi et al., 1993). Moreover, evidence shows that chronic alcohol exposure increases extracellular glutamate levels as measured by in vivo microdialysis (Lallemand et al., 2011; Melendez et al., 2005; Roberto et al., 2004) in brain regions that have been shown to regulate alcohol reinforcement. Future investigations measuring glutamate levels/signaling after a history of alcohol self-administration or after aniracetam pretreatment might assist in elucidating the underlying mechanism of this biphasic dose-response.

It could be argued that positive modulation of AMPA receptors induced changes in locomotor behavior which could have augmented operant responses. However, aniracetam pretreatment did not alter water or sucrose lever responding, and did not alter locomotor behavior in an open field, suggesting that increased alcohol self-administration was not due to a treatment-induced hyperactive state. It is also possible that habituation to the testing environment on the first test may have masked aniracetam-induced changes in motor behavior on the second test. However, this explanation is less likely given that locomotor behavior on the first and second tests for each dose was similar. Further, the lack of an aniracetam effect on general motor activity is consistent with others showing that aniracetam does not alter locomotor behavior in rats (Ventra et al., 1994). Future examinations of interactions of consumed alcohol and aniracetam may provide additional insights into potential alterations in locomotor behavior.

Operant self-administration procedures have a critical learning and memory element (White, 1996) that could possibly have been influenced by aniracetam. Indeed, aniracetam is a nootropic compound that has cognitive-enhancing effects in rodents (Lebrun et al., 2000; Ventra et al., 1994). However, increased operant responding after aniracetam pretreatment did not generalize across reinforcers (i.e. no effect on sucrose self-administration), making an explanation of general enhancement of cognitive function less plausible. An alternative explanation is that aniracetam produced an alcohol-like effect that primed further alcohol self-administration. This is highly unlikely due to evidence suggesting that the actions of AMPAR positive modulators counteract those of alcohol. Specifically, alcohol has been shown to stabilize rapid sensitization of AMPARS after activation by glutamate (Moykkynen et al., 2003; Moykkynen et al., 2009), whereas AMPAR positive modulators act as desensitization inhibitors (Tang et al., 1991); allowing for increased AMPAR current decay time and essentially opposing the actions of alcohol (Jones et al., 2008). Interestingly, the possibility of the opposition of the effects of alcohol by aniracetam may explain the increased alcohol self-administration. That is, aniracetam may blunt the subjective or pharmacological effects of consumed alcohol which may lead to greater alcohol self-administration (Besheer et al., 2012; Hodge et al., 2001). Future investigation assessing the role of AMPAR positive modulation on the discriminative stimulus effects of alcohol could provide insight into whether AMPAR positive modulators alter how alcohol is perceived. Regardless of the underlying mechanism, our findings demonstrate a novel role for enhanced activity at AMPARs in potentiating on-going alcohol self-administration.

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

reinstatement was not tested. Given that a small amount of alcohol (0.1 ml) was added to the drinking well prior to the reinstatement session, it is plausible that aniracetam increased sensitivity to these orosensory cues which in turn contributed to the potentiation of alcohol-seeking behavior. Examining the contributions of AMPAR activity in modulating response to orosensory cues and non-drug seeking behavior might provide insights for a specific role of these receptors in modulating cue-induced relapse to alcohol-seeking. Several studies have shown that reducing glutamate signaling at AMPARs attenuates relapse to alcohol-seeking behavior after exposure to alcohol cues (Backstrom and Hyytia, 2004; Sanchis-Segura et al., 2006). We extend those findings by offering a novel role for enhanced AMPAR signaling in modulating cue-induced alcohol-seeking. That is, enhanced AMPAR activity may have bolstered the ability of alcohol cues to promote alcohol-seeking behavior. Interestingly, previous studies show that a history of excessive alcohol consumption increases purported conditioned responses to alcohol cues in humans (Drummond, 2000; Papachristou et al., 2012). Accordingly, the aniracetam-induced potentiation of alcohol cue salience to promote alcohol-seeking could have been a major contributing factor in influencing the observed increase in alcohol self-administration. These data are of particular importance given that the present results suggest a role for enhanced AMPAR activity in modulating *both* heightened alcohol self-administration *and* relapse to alcohol-seeking. The findings suggest that enhanced AMPAR signaling could be involved in promoting vulnerability to relapse episodes.

The present data highlight the role of increased glutamate transmission in relapse-like behavior. Indeed, glutamate levels have recently been shown to be elevated in the nucleus accumbens and amygdala during alcohol-seeking behavior using a cue-induced reinstatement procedure (Gass et al., 2011). These findings are intriguing and suggest that aniracetam may have potentiated an already-heightened glutamatergic state during reinstatement; a possibility that will be interesting for future work to address. To date, it is not known if P-rats differentially express basal levels of AMPARs as compared to other strains, which could influence pharmacological response to aniracetam and affect behavioral outcomes. An assessment of AMPAR expression across strains could provide additional understanding of AMPAR sensitivity to aniracetam treatment. Future experiments using AMPAR positive modulators and antagonists targeting AMPARs in specific limbic regions that regulate reward and drug-seeking could provide critical information about the neuronal circuitry involved in modulating increased alcohol self-administration and potentiated reinstatement. Interestingly, the present data parallel other studies investigating the role of AMPAR neurotransmission in modulating relapse to other drugs of abuse, particularly cocaine. Indeed, microinjections of AMPA agonists in the nucleus accumbens have been shown to reinstate cocaine-seeking behavior (Cornish and Kalivas, 2000); strongly suggesting that AMPAR signaling may regulate drug-seeking across a variety of drugs of abuse.

In conclusion, the present work provides strong evidence for the involvement of enhanced AMPAR activity in modulating both increased alcohol self-administration and potentiated relapse-like behavior. These data suggest that enhanced synaptic strength (particularly enhanced AMPAR signaling) may be one of the key pathophysiological changes that produces a behavioral phenotype similar to that of an alcoholic (i.e, increased drinking and susceptibility to relapse). A better understanding of how enhanced glutamate activity at AMPARs plays a role in alcohol abuse disorders might be important in elucidating the underlying mechanisms of alcoholism and for the development of glutamate-based therapeutics aimed at treating alcohol abuse disorders.

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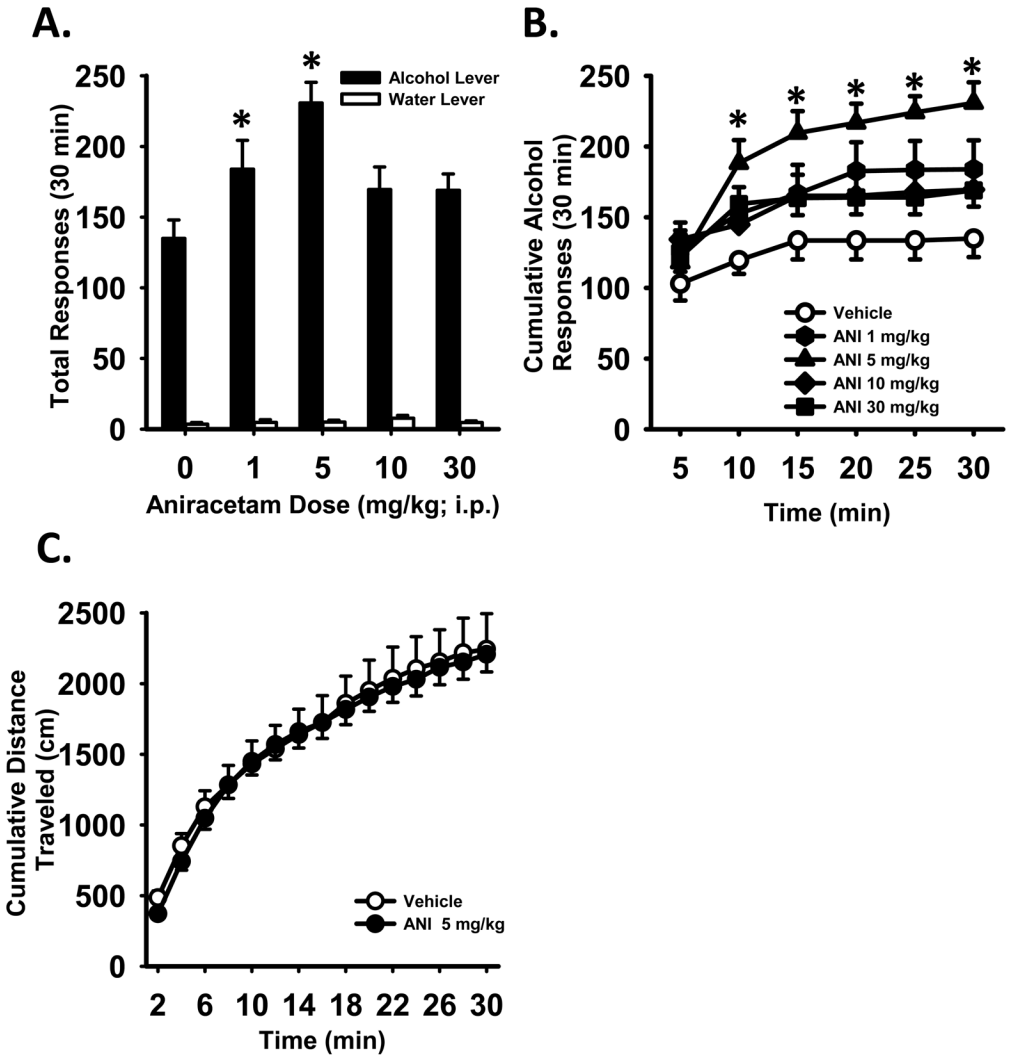

Figure 1. Positive modulation of AMPA receptors potentiates alcohol self-administration

(**A**) Pretreatment with aniracetam (ANI; 1 and 5 mg/kg, i.p.) significantly increased total alcohol- but not water-reinforced responses during operant self-administration sessions in P-rats (n=9) trained to self-administer alcohol (15%, v/v) vs. water. (**B**) Increased cumulative alcohol-reinforced responses became apparent early within the session and remained elevated throughout the entire session after aniracetam pretreatment (5 mg/kg). (**C**) Spontaneous locomotor activity in an open field was not altered in P-rats pretreated with aniracetam (5 mg/kg). Graphed values are expressed as mean ± s.e.m. *$p<0.05$ vs. vehicle (Tukey post hoc).

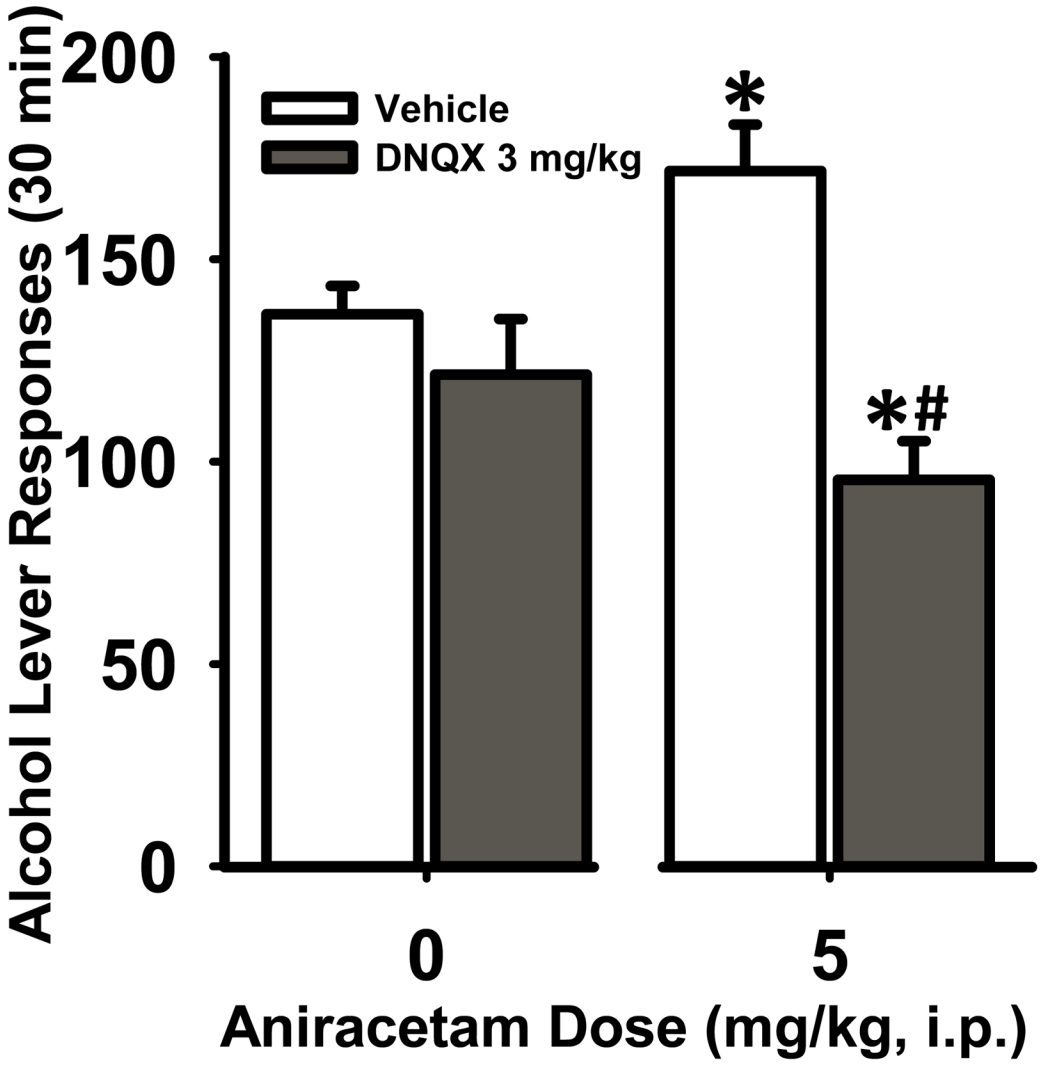

Figure 2. Antagonism of AMPA receptors by DNQX pretreatment blocks aniracetam-induced increased alcohol self-administration

Administration of the AMPA receptor antagonist, DNQX, significantly reversed aniracetam-induced increased alcohol lever responding in P-rats (n=9) trained to self-administer alcohol (15%, v/v). Graphed values are expressed as mean ± s.e.m. * indicates significant changes relative to pretreatment with vehicle (dH2O) + vehicle (0.5% CMC). # indicates significant changes relative to pretreatment with vehicle (dH2O) + 5 mg/kg aniracetam (Tukey post hoc).

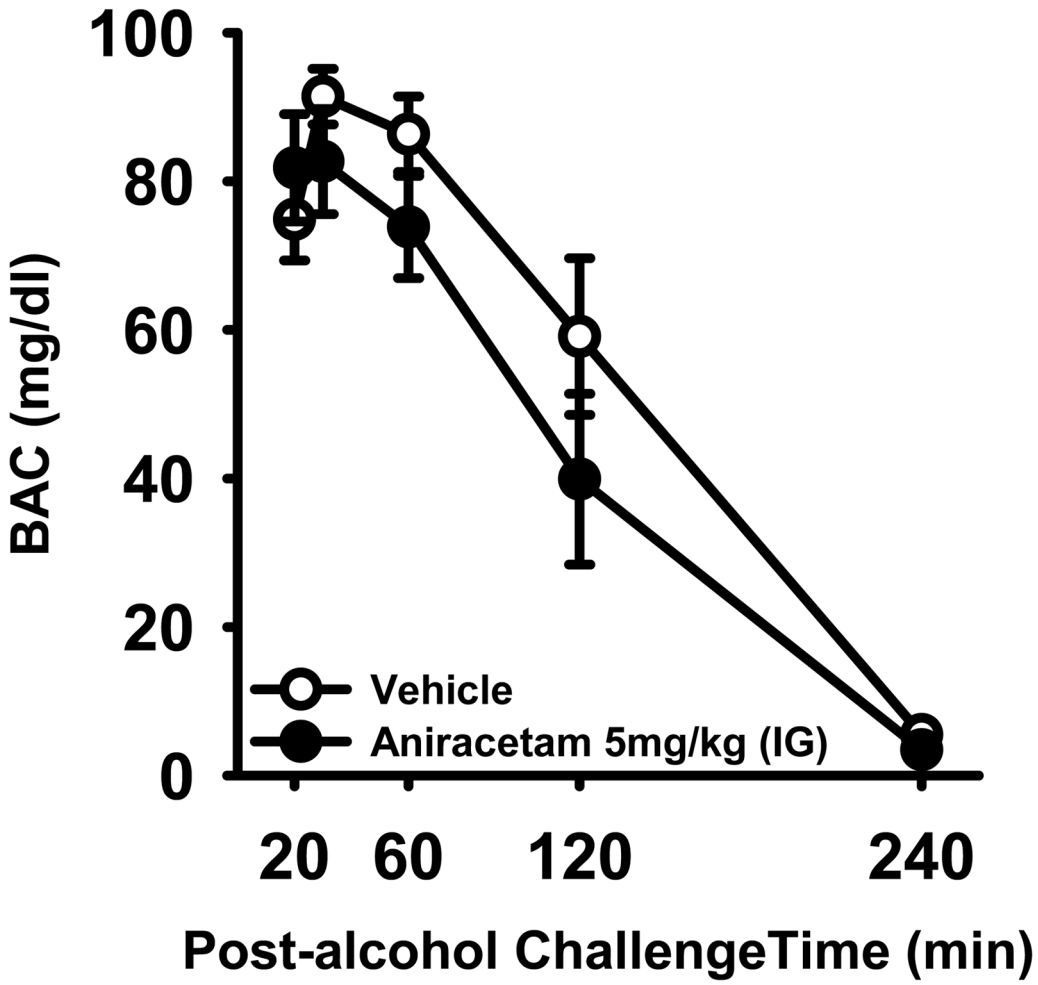

Figure 3. Alcohol clearance is not altered by aniracetam pretreatment

Pretreatment with aniracetam (5 mg/kg; n=6) did not affect blood-alcohol content (BAC) across time after administration of alcohol (1 g/kg; IG) vs. vehicle treated P-rats (n=6). Graphed values are expressed as mean ± s.e.m..

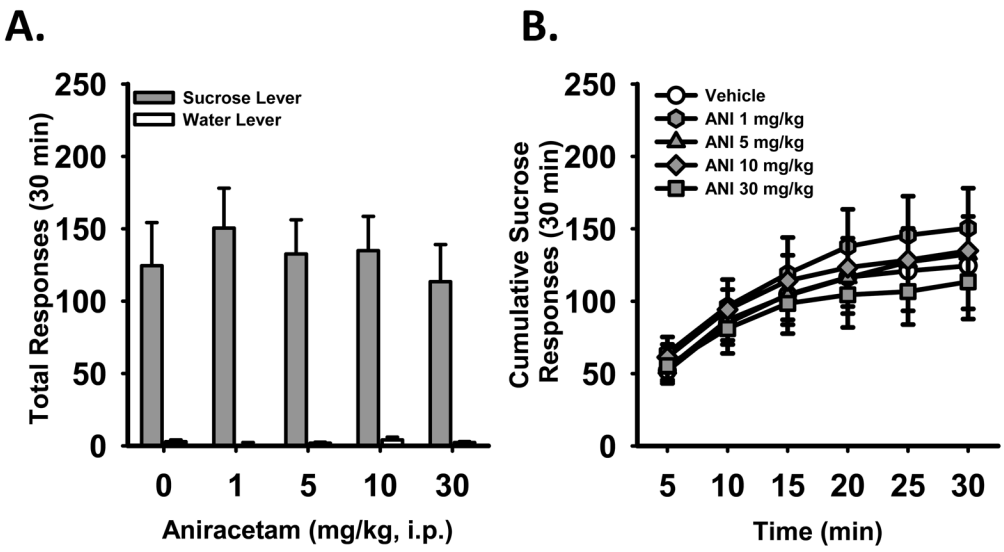

Figure 4. Positive modulation of AMPA receptors does not alter sucrose self-administration

(**A**) Pretreatment with aniracetam (ANI) did not alter total sucrose- or water-reinforced responses during operant self-administration sessions in P-rats (n=11) trained self-administer sucrose (0.8%, w/v) vs. water. (**B**) Aniracetam pretreatment did not alter the pattern of sucrose-reinforced responses across the course of the self-administration session. Graphed values are expressed as mean ± s.e.m.

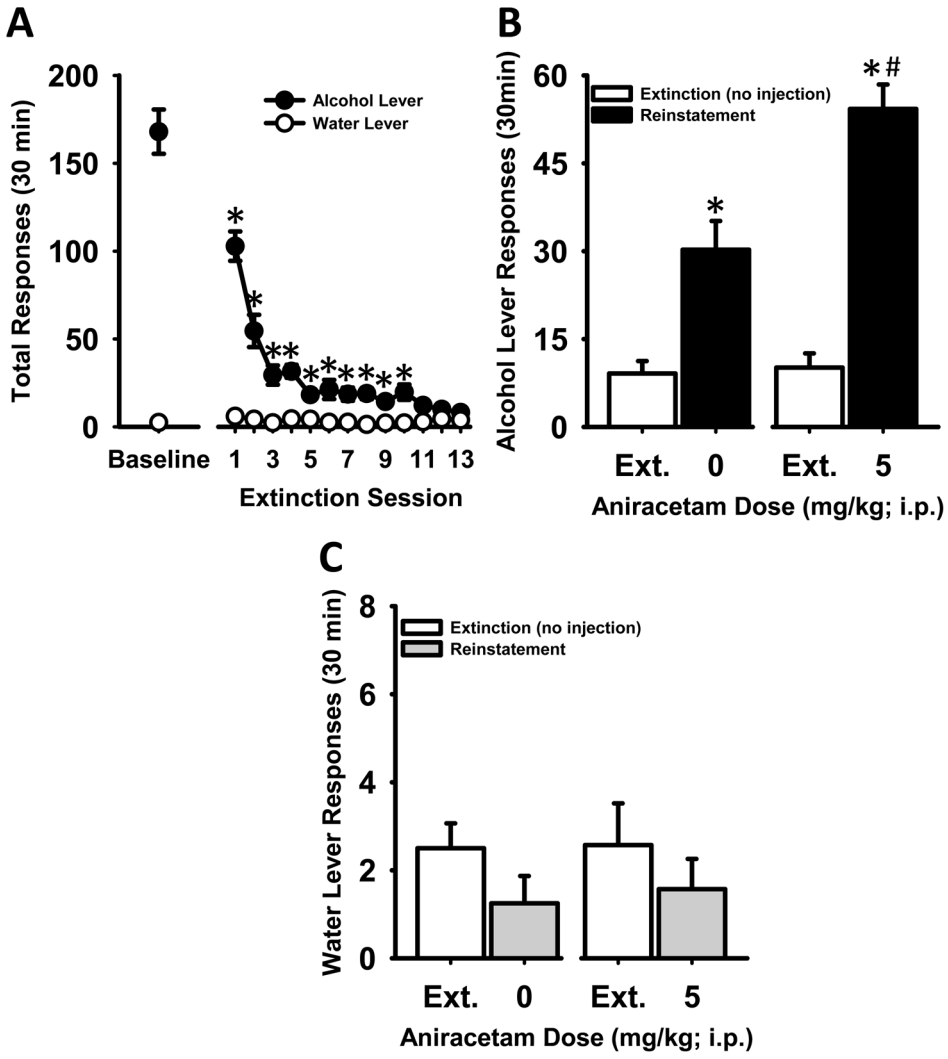

Figure 5. Positive modulation of glutamate activity at AMPA receptors potentiates cue-induced reinstatement of alcohol seeking behavior in P-rats

(**A**) Removal of response contingencies significantly reduced alcohol-associated lever responding over a period of 13 consecutive days during the extinction phase (*p<0.05 vs water lever). (**B**) Responding on the alcohol-associated lever was potentiated (n=7–8 per group) after pretreatment with aniracetam (5mg/kg) during a cue-induced reinstatement session. (*p<0.05 vs. Extinction; # p<0.05 vs. Reinstatement (vehicle)). Lever responding resulted in the illumination of an alcohol-associated cue light, but alcohol was not available during this test session. (**C**) Responding on the water-associated lever remained unaltered after aniracetam pretreatment during the same reinstatement test session. Water lever responding resulted the illumination of a water-associated cue light, but water was not available during reinstatement testing. Graphed values are expressed as mean ± s.e.m7. (Tukey post hoc).

Table 1

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

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

Resp, Responses

Table 2

Alcohol clearance parameters (mean ± SEM) after aniracetam pretreatment.

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