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α5GABA$_A$ subunit-containing receptors and sweetened alcohol cue-induced reinstatement and active sweetened alcohol self-administration in male rats

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Abstract

Rationale: GABA$_A$ receptors containing the α5 subunit (i.e., α5GABA$_A$) appear to be critically involved in the reinforcing and subjective effects of alcohol. Their role in alcohol relapse remains unknown.

Objectives: Pharmacological approaches were used to probe the role of α5GABA$_A$ receptors in alcohol seeking induced by re-exposure to an sweetened alcohol-paired cue, as well as in alcohol +sucrose vs. sucrose self-administration.

Methods: For reinstatement studies, rats were trained to self-administer alcohol under a fixed-ratio schedule in which responding was maintained by alcohol+sucrose deliveries and an alcohol-paired stimulus. Sweetened alcohol seeking was extinguished by eliminating solution deliveries and the sweetened alcohol-paired stimulus. During reinstatement tests, animals received pretreatments of an α5GABA$_A$ inverse agonist (L-655,708) or an agonist (QH-ii-066) prior to sessions in which presentation of the sweetened alcohol-paired stimulus was restored, but no solution was delivered. For self-administration studies, rats were trained to self-administer alcohol +sucrose or sucrose under a fixed-ratio schedule. Once stable, animals received pretreatments of QH-ii-066, L-655,708, the inverse agonist RY-023 or naltrexone.

Results: L-655,708 attenuated reinstatement of sweetened alcohol seeking by alcohol+sucrose-paired cues; whereas sweetened alcohol-seeking behavior was augmented by QH-ii-066, albeit at different doses in different rats. Both L-655,708 and RY-023 selectively reduced alcohol+sucrose vs. sucrose self-administration. In contrast, naltrexone reduced both alcohol+sucrose and sucrose self-administration; whereas QH-ii-066 enhanced sucrose self-administration only.

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Conflict of Interest.

On behalf of all authors, the corresponding author states that there is no conflict of interest.

Conclusions: α5GABA$_A$ receptors play a key role in the modulation of sweetened alcohol cue-induced reinstatement, as well as in alcohol+sucrose but not sucrose self-administration. Inverse agonist activity at α5GABA$_A$ receptors may offer a novel strategy for both reduction of problematic drinking and the prevention of relapse.

Keywords

Alcohol; Ethanol; Reinstatement; Self-administration; GABA$_A$ Receptors

On a global scale, alcohol use disorders (AUDs) result in significant health-related issues and are costly in terms of personal casualties and economic burden (cf. Rehm et al. 2009). The majority of the more than15 million Americans diagnosed with the disorder will experience episodes of relapse (cf. Grant et al. 2017). Patients report that relapse to renewed alcohol usage can be triggered by stressful events, re-exposure to alcohol itself, and re-exposure to environmental cues associated with past alcohol drinking (for review, see Weiss 2010). Medications that currently meet FDA approval for the treatment of AUDs are not universally effective at reducing the incidence of relapse. The investigation of novel receptor targets as modulators of alcohol relapse is needed to inform future drug development efforts aimed at improving relapse prevention strategies.

γ-aminobutyric acid (GABA)$_A$ receptors are significant mediators of the behavioral effects of alcohol due to alcohol's ability to potentiate the actions of GABA. These receptors are pentameric membrane bound proteins that contain subunits from >8 unique protein subfamilies (i.e., 6 α, 3 β, 3 γ, 1 δ, 1 ε, 1 θ, 1 π, and 3 ρ subunits), and are most commonly comprised of 2 α, 2 β and 1 γ2 subunit (Fritschy and Panzanelli 2014; McKernan and Whiting 1996; Rudolph and Möhler 2004). Mounting evidence suggests that specific GABA$_A$ receptor subtypes are involved in the sedative-motoric, reinforcing, and subjective effects of alcohol. However, the potential role of these receptors in alcohol relapse largely remains unknown.

Despite the fact that GABA$_A$ receptors containing the α5 subunit (α5GABA$_A$ receptors) comprise only a small proportion of native GABA$_A$ receptors found primarily in the ventral hippocampus and surrounding brain areas (Howell et al. 2000; Li et al. 2001; Sarantis et al. 2008; Sur et al. 1999; Uusi-Oukari and Korpi 2010), both genome-wide association studies and pre-clinical studies in laboratory animals point to these GABA$_A$ receptors as being particularly significant mediators of alcohol's abuse-related effects. For example, GABRA5, the gene that encodes the α5 subunit, has been associated with alcohol dependence in humans (Song et al. 2003). Furthermore, several psychiatric disorders (e.g., panic disorder, bipolar disorder, and unipolar depression) that share overlapping co-morbidity with AUDs are associated with polymorphisms for this receptor subtype (e.g., Hodges et al. 2014; López-León et al. 2008; Otani et al. 2005). Genetic studies in mice demonstrate that α5GABA$_A$ receptors can selectively regulate alcohol intake, as male α5 subunit knock-out mice consumed less alcohol than the wild-type in a preference test, while no differences were seen in preference for saccharine or quinine (Boehm et al. 2004). Pharmacological evidence also supports the involvement of this subtype in alcohol self-administration, as α5GABA$_A$ receptor selective inverse agonists reduce alcohol maintained responding across

multiple species/strains, including rats bred for high levels of alcohol drinking (June et al. 2001), an out-bred rat strain (McKay et al. 2004), and also rhesus monkeys (Rüedi-Bettschen et al. 2013). $\alpha 5GABA_A$ receptors also appear to play a role in the subjective/interoceptive effects of alcohol, as assessed by drug discrimination procedures. For instance, the $\alpha 5GABA_A$-preferring inverse agonist Ro 15–4513, blocked ethanol's discriminative stimulus effects in male (Rees and Balster 1998) and female (Middaugh et al. 1991) mice, male rats (Gatto and Grant 1997) and male and female cynomolgus monkeys (Helms et al. 2009). Moreover, the $\alpha 5GABA_A$-preferring agonist, QH-ii-066, substituted for alcohol in a discrimination procedure in squirrel monkeys; an effect that could be blocked by inverse agonists selective for the subtype (Platt et al. 2005).

The goal of the present study was to evaluate the contribution of $\alpha 5GABA_A$ receptors to alcohol relapse using a cue-induced reinstatement procedure in rats. Specifically, we determined the ability of the $\alpha 5GABA_A$-selective inverse agonist L-655,708 (Casula et al. 2001) to inhibit, and the $\alpha 5GABA_A$-preferring agonist QH-ii-066 (Huang et al. 1996) to augment, cue-induced sweetened alcohol seeking. We also extended our study to assess the selective modulation of active alcohol+sucrose versus sucrose only self-administration by the selective inverse agonists L-655,708 and RY-023 (June et al. 2001; Liu et al. 1996), compared to the preferring agonist QH-ii-066 and the opioid receptor antagonist naltrexone, an FDA-approved drug for the treatment of AUDs.

Materials and Methods

Subjects and apparatus

Adult male Sprague Dawley rats (Harlan, Indianapolis, IN; parametric/dose-response reinstatement study: N=9; repeated reinstatement study: N=6; alcohol self-administration: N=8–10 depending on drug pretreatment; sucrose self-administration: N=12 for all drug pretreatments), approximately 70 days old and weighing between 260–300 g at the start of the experiment, were pair-housed under a 12/12-hr light/dark cycle with food and water available *ad libitum*. All experiments were conducted during the light phase of the cycle. Although it is not unusual to conduct experiments with rats during the light phase (cf. Cannady et al. 2013), it should be noted that the light phase potentially may be more stressful for rodents. To assure that rats were habituated to the conditions of the study, they were handled for one week prior to the start of each experiment and were given sham injections for one week prior to the implementation of drug testing. All animals were maintained and experiments were conducted in accordance with the University of Mississippi Medical Center's Institutional Animal Care and Use Committee and were in accordance with the National Research Council's Guide for Care and Use of Laboratory Animals (eighth edition, 2011).

Self-administration sessions occurred in custom-made operant conditioning chambers (Gerbrands Corporation; Arlington, MA; h × w × l:19 cm × 23.5 cm × 22 cm). One wall of the chamber was equipped with two levers (one designated active, one designated inactive; Gerbrands Corporation), a stainless steel liquid reservoir located below each lever, and a white stimulus light located above each lever. A flashing, amber, jeweled stimulus light was mounted in the center of the operant conditioning panel, between the white stimulus lights.

Syringe pumps (Razel Scientific, St Albans, VT) controlled the delivery of the solutions from a 30 ml syringe, through an ~ 10 in. segment of polyethylene tubing connected to a metal spout that was fixed to the liquid reservoir. A Macintosh computer equipped with custom interface and software (Mac State) controlled all events in the experimental session and recorded data.

Cue-induced reinstatement of alcohol seeking

Rats were trained to self-administer sweetened alcohol using a standard sucrose-fading procedure (cf. Besheer et al. 2008; Samson 1986). Rats initially were trained to respond for a 10% (w/v) sucrose solution. Briefly, in the presence of a white light located above the active lever, the completion of every 2nd response (FR 2) on the active lever resulted in the delivery of 0.1 ml of solution and activation of the jeweled, flashing, amber cue-light (1.66 second duration) located in the center of the operant conditioning panel (i.e., the alcohol-paired cue). A 1 second time-out period followed the delivery of solution and its paired cue, during which all lights were off and responses had no scheduled consequences. Once responding stabilized for sucrose (no upward or downward trends in number of solution deliveries over 3 consecutive days), alcohol was gradually added to the sucrose and the sucrose was gradually decreased in the following sequence ("S" = sucrose, "E" = alcohol): 10S, 10S/2E, 10S/5E, 10S/10E, 5S/10E, 5S/15E, 2S/15E, 2S/10E. During training, subjects moved to the next step in the sequence when they met the *a priori* criterion of self-administering a dose of ≥ 0.5 g/kg alcohol. Additionally, at the start of each self-administration session, the equivalent of one delivery of solution (0.1 ml) was available in the liquid reservoir below the active lever. This amount of alcohol is not pharmacologically relevant and functioned to provide additional odor/taste cues (cf. Backstrom et al. 2004; Cannady et al. 2013). Self-administration training continued until the subjects self-administered a 2% sucrose/10 or 15% alcohol (w/v) solution (parametric/dose-response reinstatement study: n=5 and 4, respectively; repeated reinstatement study: n=4 and 2, respectively). The alcohol concentration was set for individual subjects as the concentration that maintained the highest intake in terms of dose. Self-administration sessions occurred 5 days per week, lasted 30 minutes, and continued until stability criteria (3 consecutive days with a self-administered dose > 0.5 g/kg with no upward or downward trends; cf. Besheer et al. 2013) were met.

Extinction training followed and continued until responding declined and stabilized at the *a priori* criterion of ≤ 10% of self-administration baseline. During extinction sessions, the light above the active lever was illuminated but lever presses had no scheduled consequences (i.e., no amber light flash, no pump operation, and no solution delivery). Cue-induced reinstatement tests followed. The operant conditioning chamber returned to the self-administration configuration (i.e., 0.1 ml of the sweetened alcohol solution was available in the liquid reservoir at the session's start), however, lever presses resulted only in the activation of the cue-light, as response-contingent delivery of the sweetened alcohol solution was omitted. Rats were pretreated with a dose of L-655,708 (0.3 – 5.6 mg/kg, i.p.) or QH-ii-066 (0.1 – 3 mg/kg, s.c.) in a counterbalanced order, 30 minutes prior to the reinstatement session. Doses, pretreatment time, and route of administration for each ligand were determined from the literature (cf. Navarro et al. 2002; Redrobe et al. 2012). After each test,

self-administration sessions recurred on the following day, initiating a repeat of the cycle. A within-subjects design was used such that all drug doses or vehicle were administered in a counterbalanced order across repeated reinstatement tests, to all subjects. The six rats assigned to "repeated reinstatement" study underwent repeated cycles of self-administration, extinction and cue-induced reinstatement. They received vehicle as pretreatment before each test session.

Active self-administration of alcohol+sucrose versus sucrose only

One group of rats was trained to self-administer alcohol+sucrose as described in the previous section using the same sucrose fading procedure and the same criterion. However, the final solution available for self-administration was 5% sucrose/10% alcohol (w/v). A second group was trained to self-administer a 5% (w/v) sucrose solution under the same FR 2 schedule of reinforcement. Self-administration sessions for both groups were 30 min in length and occurred 5 days per week. Drug testing began following acquisition of self-administration and stable responding (i.e., no upward or downward trends in number of solution deliveries over 3 consecutive days). Each dose of a given ligand was administered 30 minutes prior to the start of the session (i.p.: vehicle, L-655,708 [0.3 – 5.6 mg/kg], RY-023 [0.3 – 3 mg/kg], naltrexone [0.1 – 1 mg/kg]; s.c.: vehicle, QH-ii-066 [0.1 – 3 mg/kg]) for five consecutive days. Doses, pretreatment time, and route of administration for each ligand were determined from the literature (cf. Cook et al. 2005; Hay et al. 2013; Navarro et al. 2002; Redrobe et al. 2012). Three days of self-administration without pretreatment occurred between tests with different drugs/doses, and served as the baseline for subsequent tests. A within-subjects design was used such that drug doses or vehicle were administered in a counterbalanced order across multiple self-administration sessions to all subjects.

Drugs

Granulated sucrose (2%−10% w/v) was dissolved in warm tap water, and 190 proof ethyl alcohol (Ultra Pure, Darien, CT) was added to the solution, to a concentration of 10 or 15% w/v, resulting in a 10%−2% Sucrose/10% or 15% alcohol (w/v) solution. L-655,708 (Tocris Bioscience, Ellsville, MO) was dissolved in a 50% propylene glycol/50% sterile water solution. QH-ii-066 was synthesized at the University of Wisconsin-Milwaukee, as described in Huang et al. (2000) and dissolved in a 30% propylene glycol/ 70% 0.9% saline solution. RY-023 also was synthesized at the University of Wisconsin-Milwaukee as described previously (Huang et al. 2000) and dissolved in a 50% propylene glycol/50% sterile water solution. Naltrexone HCL (Sigma/RBI, St. Louis, MO) was dissolved in a 0.9% saline solution.

Statistical Analysis

All results are presented as mean ± S.E.M, and the alpha level was set at $p \leq 0.05$ for all tests, which were conducted using Graphpad Prism 7.02 software. Note that for all comparisons, a Geisser-Greenhouse correction was applied since sphericity of the data could not be assumed. Where relevant, alcohol intake was calculated based on weight and number of deliveries consumed during a 30 minute self-administration session (i.e., alcohol dose = [# reinforcers * 0.1 ml] * [concentration of alcohol (0.1 or 0.15 g/ml)]/body weight kg).

To determine whether the sweetened alcohol-paired cue light did, in fact, reliably reinstate alcohol+sucrose-seeking behavior, a one-way repeated measures ANOVA followed by Tukey's post hoc test for multiple comparisons was used to analyze active lever presses during self-administration, extinction and cue only-reinstatement tests. Note that a subset of rats (n=4) were tested for cue-induced reinstatement both before drug testing was initiated, and again after drug testing was completed, to ensure reproducibility of the effect (data not shown). For these rats, the average of the two tests was used for subsequent analysis. Additionally, a separate group of rats received repeated cue+vehicle tests. A one-way repeated measures ANOVA was used to analyze active lever presses across the four test sessions.

The active lever presses and alcohol intake (g/kg) associated with baseline self-administration phases between reinstatement tests with different doses of pretreatment drugs were analyzed with separate one-way repeated measures ANOVA. For reinstatement tests with drug pretreatments, data are presented as a percentage of responding for the cue alone and were analyzed with separate one-way repeated measures ANOVAs followed by Dunnett's multiple comparisons test, where each dose of drug was compared to cue alone (i.e., 100%).

For baseline measures associated with active self-administration of alcohol+sucrose or sucrose only, data were analyzed separately for each drug with one-way repeated measures ANOVA, followed by Dunnett's post-hoc test, where each drug dose was compared to vehicle. For self-administration following pretreatment with $\alpha 5GABA_A$ receptor-selective drugs, data from the last three days of testing with each drug/dose were converted to a percentage of baseline (i.e., the average of the three preceding days of baseline/no treatment self-administration) and then normalized to 100% (i.e., [(mean drug test – mean baseline)/(mean baseline)]*100 + 100). These data were analyzed separately for each drug with one-way repeated measures ANOVA, followed by Dunnett's post-hoc test, where each treatment was compared to baseline.

Results

Cue-induced reinstatement of sweetened alcohol seeking

It took subjects in the reinstatement experiment an average of 56.8 ± 4.0 days to complete the sucrose fading procedure, with different "steps" requiring different lengths of days to meet the criterion to move on to the next step (Table 1). Following successful sucrose fading, a 2% w/v sucrose/10 or 15% w/v alcohol solution maintained consistent rates of responding and alcohol intakes > 0.5 g/kg across the course of the study, depending upon the subject. Active lever presses during the last 3 sessions of self-administration prior to cue-alone tests ranged from 41.3 – 92.8 (group mean = 65.3 ± 5.6) and alcohol intakes ranged from 0.5 to 1.1 g/kg (group mean = 0.7 ± 0.05). During associated extinction sessions when responses had no programmed consequences, the number of responses per session declined to an average of 3.39 ± 0.48 (range = 1.5–6), meeting the *a priori* criterion of ≤ 10% of baseline responses. On average, it took the rats 5.8 ± 0.7 days (range: 3 – 9 days) to extinguish their behavior. During reinstatement tests when only response-contingent presentations of the sweetened alcohol-paired cue light were delivered, responding on the active lever increased

to 18.92 ± 1.39 (range = 11–26), with no concomitant increase in responding on the inactive lever (mean responses: 1.01 ± 0.35; data not shown). A repeated measures ANOVA indicated a significant difference for active lever presses between the three experimental conditions (Figure 1a; $F(1.125, 8.996) = 106.3$, $p<0.0001$). Tukey's multiple comparisons test revealed that active lever presses during the cue-only reinstatement test were significantly different from both extinction and self-administration (p's<0.0001) indicating that the sweetened alcohol-paired cue induced significant seeking behavior, but not to the level of active alcohol+sucrose self-administration. Importantly, cue-induced sweetened alcohol seeking appears to be repeatable and stable, in that there were no significant differences noted in active lever presses across 4 cycles of cue + vehicle tests (Figure 1b).

During the alcohol+sucrose self-administration phases preceding cue-induced reinstatement tests with different pretreatment doses of the α5GABA$_A$ receptor-selective inverse agonist L-655,708 or the α5GABA$_A$ receptor-preferring agonist QH-ii-066, it took an average of 6.2 ± 0.6 days for subjects to re-establish alcohol+sucrose self-administration that met stability and dose criteria. Importantly, when averages for the last 3 days of each self-administration phase were considered, no reliable differences were found for either baseline active lever presses or alcohol intake (data not shown). These results indicate that sweetened alcohol self-administration behavior was stably maintained over the course of the study. During extinction phases that preceded tests with the inverse agonist and agonist, it took an average of 3.7 ± 0.6 days for behavior to decline to criterion level. On reinstatement test days, L-655,708 dose-dependently inhibited cue-induced alcohol+sucrose-seeking behavior (Figure 2a; $F(2.278, 18.23) = 5.252$, $p < 0.05$). Dunnett's post hoc test indicated that the two highest doses tested (3.0 and 5.6 mg/kg) were reliably different from baseline ($p = 0.0139$ and $p = 0.0001$, respectively) and reduced sweetened alcohol seeking by more than 50%. Additionally, inactive lever presses were unchanged by L-655,708 and remained low on test days (mean responses: 0.53 ± 0.22; data not shown).

Although QH-ii-066 enhanced cue-induced reinstatement of sweetened alcohol seeking in the majority of rats, the enhancement varied in magnitude (115 – 309% of cue + vehicle) and occurred at different doses in individual rats (e.g., maximal enhancement by QH-ii-066 observed at: 0.1 mg/kg = 1 rat; 0.3 mg/kg = 4 rats; 1 mg/kg = 3 rats; 3 mg/kg = 1 rat). Thus, when analyzed as a group, QH-ii-066 failed to significantly alter cue-induced reinstatement (Figure 2b). Nor did the administration of QH-ii-066 alter inactive lever responding (mean responses: 0.57 ± 0.22; data not shown).

Active self-administration of alcohol+sucrose or sucrose only solutions

It took subjects in the alcohol+sucrose self-administration group an average of 46.4 ± 2.6 days for rats to complete the sucrose fading procedure, with different "steps" requiring different lengths of days to meet the criterion to move on to the next step (Table 1). The sucrose only self-administration group also began training with a 10% w/v sucrose solution which was then faded almost immediately to 5% w/v sucrose. In the sucrose-maintained group, testing began after 21 days of self-administration. In the different groups and at the final training concentrations, active self-administration was considered stable if sweetened alcohol intake was maintained at pharmacologically-relevant levels (i.e., > 0.5 g/kg; cf.

Besheer et al. 2013) and number of sucrose deliveries showed no upward or downward trend. Stable levels of sweetened alcohol intake ranged from 0.47–1.59 g/kg, and stable sucrose-maintained responding ranged from 96–504 lever presses/session. For the alcohol +sucrose self-administration group, baseline self-administration was stable across the course of the study with no significant differences for alcohol dose or baseline active lever presses (data not shown). By and large, the same was true for the sucrose only self-administration group. No reliable differences were evident in active lever presses associated with drug tests for L-655,708, naltrexone, or QH-ii-066 (data not shown). The baseline number of active lever presses associated with RY-023 in this group did produce a significant ANOVA (RY-023: $F(1.869, 18.69) = 5.564$, $p < 0.05$). A Tukey's post-hoc test revealed that the difference lie between the 0.3 and 1 mg/kg dose (data not shown).

Figure 3 shows the modulation of active alcohol+sucrose vs. sucrose only self-administration by ligands selective for α5GABA$_A$ receptors in comparison to naltrexone. The inverse agonist L-655,708 significantly attenuated the self-administration of alcohol +sucrose, without similarly altering sucrose consumption (Figure 3a, black circles vs. white squares; alcohol group: $F(2.875, 25.88) = 3.653$; $p < 0.05$). Dunnett's post hoc test comparing sweetened alcohol intake after pretreatment to intake after vehicle revealed that the 5.6 mg/kg dose of L-655,708 significantly reduced consumption to 63% of baseline ($p < 0.005$).

A second inverse agonist selective for α5GABA$_A$ receptors, RY-023, significantly and dose-dependently decreased alcohol+sucrose consumption (Figure 3b; $F(2.037, 14.26) = 4.218$, $p < 0.05$) to 60% of baseline at the 1 mg/kg dose ($p < 0.005$), and to 53% of baseline at the 3 mg/kg dose ($p < 0.05$) while having no systematic effect on sucrose only consumption (Figure 3b). As might be expected for an approved pharmacotherapy, all doses of naltrexone significantly reduced alcohol+sucrose consumption (Figure 3c; $F(1.472, 10.3) = 24.06$, $p < 0.0005$) to 36–53% of baseline depending on the particular dose (p's < 0.0005 for all doses). Somewhat unexpectedly, though, sucrose only consumption also was reduced by naltrexone ($F(1.73, 19.03) = 10.54$, $p < 0.005$), but not to the same extent as alcohol (i.e., naltrexone reduced sucrose intake to 62–80% of baseline, depending on the dose). Specifically, significant reductions were observed at the 0.1 and 1 mg/kg dose ($p < 0.005$ and $p < 0.0005$ respectively). In contrast to results with the inverse agonists and naltrexone, QH-ii-066 had no reliable effect on alcohol+sucrose consumption (Figure 3d). It did, however, significantly increase sucrose only consumption by 90% over baseline levels ($F(2.314, 25.46) = 11.91$, $p < 0.0005$) at the 3 mg/kg dose ($p < 0.005$).

Discussion

A number of studies have established that GABA$_A$ receptor subtypes are involved differentially in alcohol-related behaviors. This literature suggests that α5GABA$_A$ receptors are important mediators of the reinforcing and interoceptive/discriminative stimulus effects of alcohol, yet their role in alcohol relapse has not been investigated. Here, we demonstrate that α5GABA$_A$ receptors can modulate sweetened alcohol-seeking behavior, as evidenced by both the dose-dependent blockade of sweetened alcohol cue-induced reinstatement by the selective inverse agonist L-655,708, as well as the augmented cue-induced reinstatement

(albeit at different doses in different rats) by the α5GABA$_A$-preferring agonist QH-ii-066. It is important to note, however, that these reinstatement studies did not include explicit evaluation of reinstatement after a vehicle pretreatment. Although we don't believe that vehicle would have significantly increased or decreased responding compared to cue alone (cf. Figure 1b), there is the possibility that additional or different doses would have emerged as being significant. Thus, the lack of vehicle must be considered a limitation of the current study

Additionally, we show that α5GABA$_A$ receptor-selective inverse agonists selectively reduced active alcohol+sucrose self-administration to levels approaching those induced by the FDA-approved anti-alcohol medication naltrexone. Unlike naltrexone, though, the inverse agonists had no effect on active sucrose only self-administration.

GABA$_A$ receptors expressing the α5 subunit (i.e., α5GABA$_A$ receptors) comprise only a small proportion of native GABA$_A$ receptors and are found primarily in the hippocampus (Sarantis et al. 2008; Sur et al. 1999; Uusi-Oukari and Korpi 2010) and adjacent regions (Howell et al. 2000; Li et al. 2001). Within the hippocampus, α5GABA$_A$ receptors are expressed predominantly within the ventral CA1 and CA3 regions which send projections to brain areas associated with alcohol reward (e.g., nucleus accumbens, ventral tegemental area, amygdala, and hypothalamus; Amaral and Witter 1989; Groenwegen et al. 1987; Janak and Chaudhri 2010; Kelly and Domesick 1982; Luo et al. 2011; Sarantis et al. 2008; Sesack and Grace 2010). Recent evidence suggests that specific GABA-mediated circuits between the ventral CA1 and CA3 regions and the nucleus accumbens core regulate approach-avoidance behavior to a stimulus that is associated simultaneously with positive and negative valences (e.g., alcohol). Specifically, the ventral CA3 to nucleus accumbens core regulates approach behavior and the ventral CA1 to nucleus accumbens core regulates avoidance behavior (Schumacher et al. 2018). Importantly, approach-avoidance behavior has been shown to be dysregulated by exposure to drugs of abuse (Hamel et al. 2017; Nguyen et al. 2015; Schumacher et al. 2018). Interestingly, clinicians are targeting approach biases in alcoholics with behavioral therapies in an attempt to shift patients to avoidance biases (den Uyl et al. 2016). Based on these findings, one could speculate that it may be modulation of the GABAergic hippocampal – nucleus accumbens circuitry by the α5GABA$_A$ ligands that underlies the effects of these drugs on both sweetened alcohol seeking and alcohol+sucrose taking behaviors. Taking this idea a step further, α5GABA$_A$ receptor inverse agonists might be predicted to facilitate the shift to an avoidance bias.

Another potential mechanism by which the inverse agonists may be exerting their effect, especially in the context of the reinstatement procedure, is via enhancement of cognition. There is significant evidence that α5GABA$_A$ receptors play a role in cognitive processes (Collinson et al. 2002; Mohler and Rudolph 2017; Prut et al. 2010) and that inverse agonists at this subtype are nootropic (Atack et al. 2006). For example, L-655,708 has been shown to enhance performance, not only during acquisition but in a probe trial as well, in rats in a Morris water maze task (Atack et al. 2006). Similar behavioral results have been found with other inverse agonists selective for this receptor subtype (cf. Atack 2010; Knust et al. 2009). L-655,708 also has been shown to enhance deficits found in long-term potentiation (LTP) in the ventral hippocampus, while having no effect on LTP in the dorsal hippocampus (Pofantis

and Papatheodoropoulos 2014), again implicating the ventral region of the hippocampus as the mediator of the cognitive-enhancing effects associated with $\alpha5GABA_A$ receptor inverse agonists. It is a possibility, then, that through its cognition-enhancing properties L-655,708 facilitated the extinction of the sweetened alcohol-paired cue during reinstatement tests, enhanced the acquisition of a new context, and allowed for the association between sweetened alcohol and the paired stimulus light to be broken more quickly (i.e., the animals learned more rapidly that the presentation of the stimulus light no longer was predictive of sweetened alcohol delivery).

Regardless of the precise manner in which inverse agonists selective for $\alpha5GABA_A$ receptors reduce sweetened alcohol-seeking and drinking, they appear to be a promising target for potential AUD pharmacotherapies. The inverse agonists L-655,708 and RY-023 were similar to the approved pharmacotherapy naltrexone in that they significantly reduced alcohol+sucrose self-administration. Interestingly, and unlike naltrexone, the effects of the inverse agonists were selective for sweetened alcohol-maintained behavior compared to sucrose only-maintained behavior. These findings are in concordance with other studies in both rats and monkeys showing that inverse agonists targeting this subtype preferentially attenuate self-administration of alcohol vs. other non-drug reinforcers (e.g., sucrose, saccharin, water; Cook et al. 2005; June et al. 2001; McKay et al. 2004; Rüedi-Bettschen et al. 2013), and further suggest that the present results suggest a specific effect of the inverse agonists on the alcohol component of the alcohol+sucrose solution. The lack of effect of L-655,708 and RY-023 on sucrose only self-administration suggests that motoric effects are not confounding the findings; an interpretation that would be in agreement with other published data showing that motor-impairing effects of these drugs emerge only at higher doses (e.g., Cook et al. 2005). In contrast, that naltrexone decreases responding for both alcohol+sucrose and sucrose solutions may reflect nonspecific rate suppression (e.g., Hay et al. 2013; Shelton and Grant 2001) and/or inhibition of the rewarding effects of both solutions (e.g., Langlenen et al. 2012). Either of these outcomes is not ideal.

In each experiment, the effects of the $\alpha5GABA_A$ receptor-preferring agonist QH-ii-066 also were evaluated. In reinstatement studies, it was hypothesized that QH-ii-066 would augment cue-induced reinstatement. QH-ii-066 has been shown to share discriminative stimulus properties with alcohol (Platt et al. 2005), and therefore may act as a contextual cue associated with the subjective experience of the drug. Indeed, cue-induced reinstatement was enhanced by QH-ii-066; however, the maximal enhancement varied and occurred at different doses in different animals. The reasons contributing to the individual sensitivity to QH-ii-066 are not known. $\alpha5GABA_A$ receptors have been shown to be upregulated in response to chronic alcohol consumption in rodents (Charlton et al.1997), an effect also found in post-mortem tissue from human alcoholics (Jin et al. 2012). At least in rats, these changes appear to be time-, and hence cumulative dose-, dependent. In the present study, rats in the reinstatement group ranged in their daily sweetened alcohol intake raising the possibility that these differences in intake were sufficient to induce different degrees of upregulation of $\alpha5GABA_A$ receptors. Further correlational analysis, though, fails to support this idea. It may be that intakes were too low or the chronic period of self-administration too short to induce changes in receptor expression. Alternatively, it may simply be that sharing discriminative stimulus effects with alcohol is not sufficient to augment cue-induced reinstatement. Note

that in self-administration studies, it was hypothesized that QH-ii-066 would enhance alcohol+sucrose, but not sucrose only, self-administration as has been shown in monkeys (cf. Rüedi-Bettschen et al. 2013). In fact, we observed the opposite in rats. QH-ii-066, at the highest dose only, enhanced sucrose self-administration; no dose altered alcohol+sucrose self-administration. Again, the reasons underlying these differences are not clear. It could simply reflect differences between species. Alternatively, it may be that the procedure used in the rats was not sensitive enough to detect any modulation.

The experiments described herein are the first, to our knowledge, to explore the role of $\alpha5GABA_A$ receptors in alcohol+sucrose relapse. The data support the hypothesis that $\alpha5GABA_A$ receptors are critical mediators of the abuse-related effects of alcohol, including sweetened alcohol cue-induced relapse. Moreover, these findings suggest that $\alpha5GABA_A$ receptor inverse agonists have the potential to have an improved side effect profile (i.e., apparent selective effects against alcohol in some cases) compared to currently in-use pharmacotherapies.

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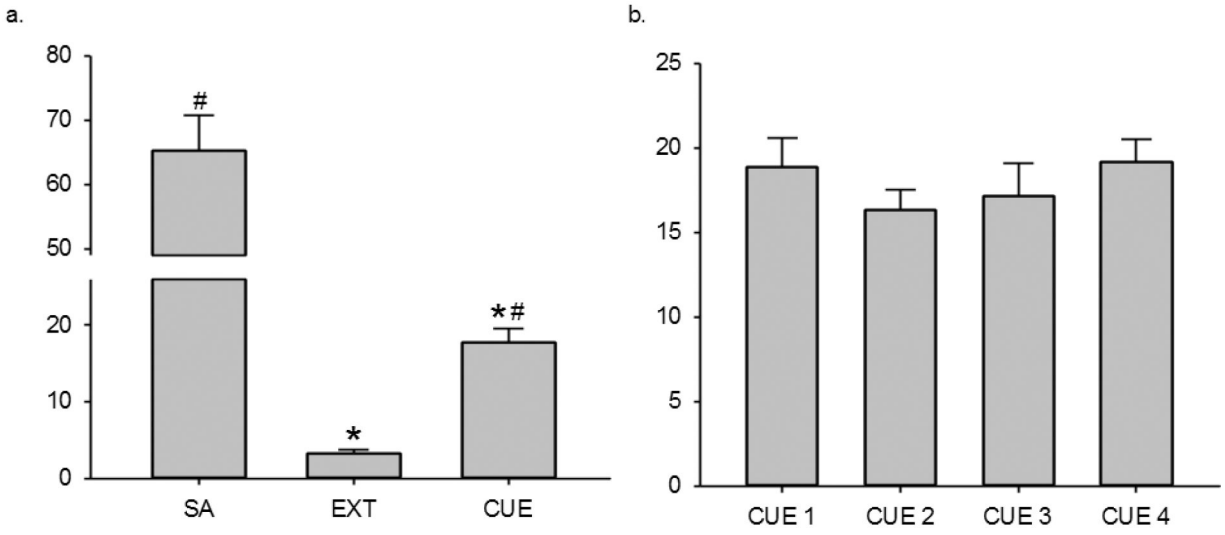

Figure 1.

Active lever presses during a) baseline self-administration (last 3 days of self-administration; "SA"), extinction (final day; "EXT"), and cue-reinstatement test ("CUE"; N=9), and b) repeated cue tests (N=6). Data are means ± S.E.M. # indicates $p < 0.05$ compared to extinction, * indicates $p < 0.05$ compared to self-administration.

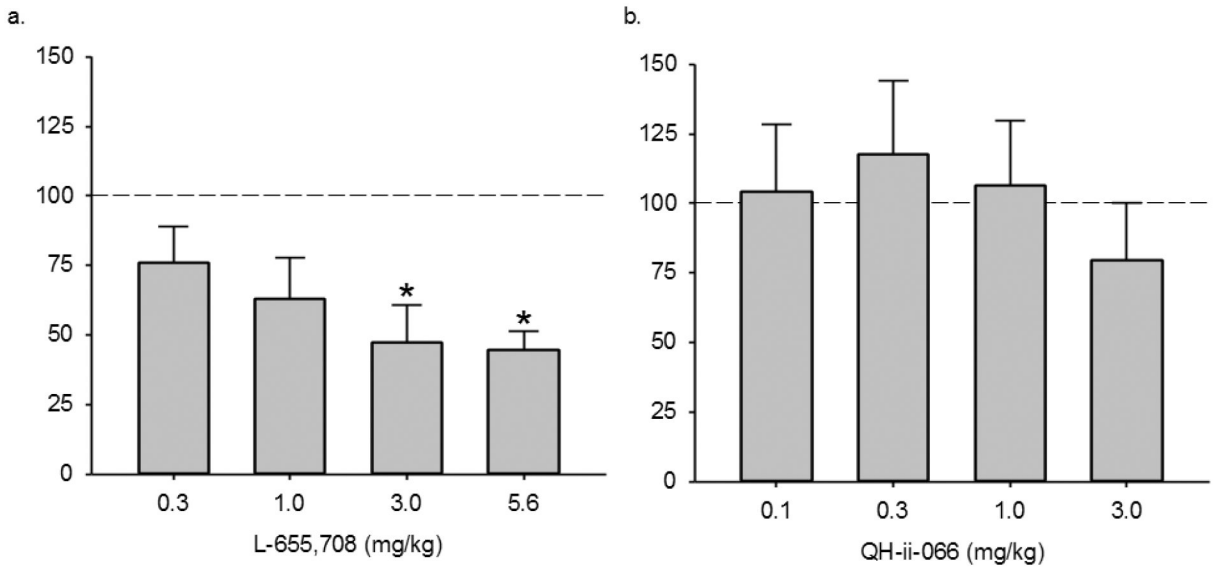

Figure 2.

Modulation of sweetened alcohol cue-induced reinstatement by a) L-655,708 (N=9) and b) QH-ii-066 (N=9). Data are means ± S.E.M. *indicates $p < 0.05$ compared to baseline.

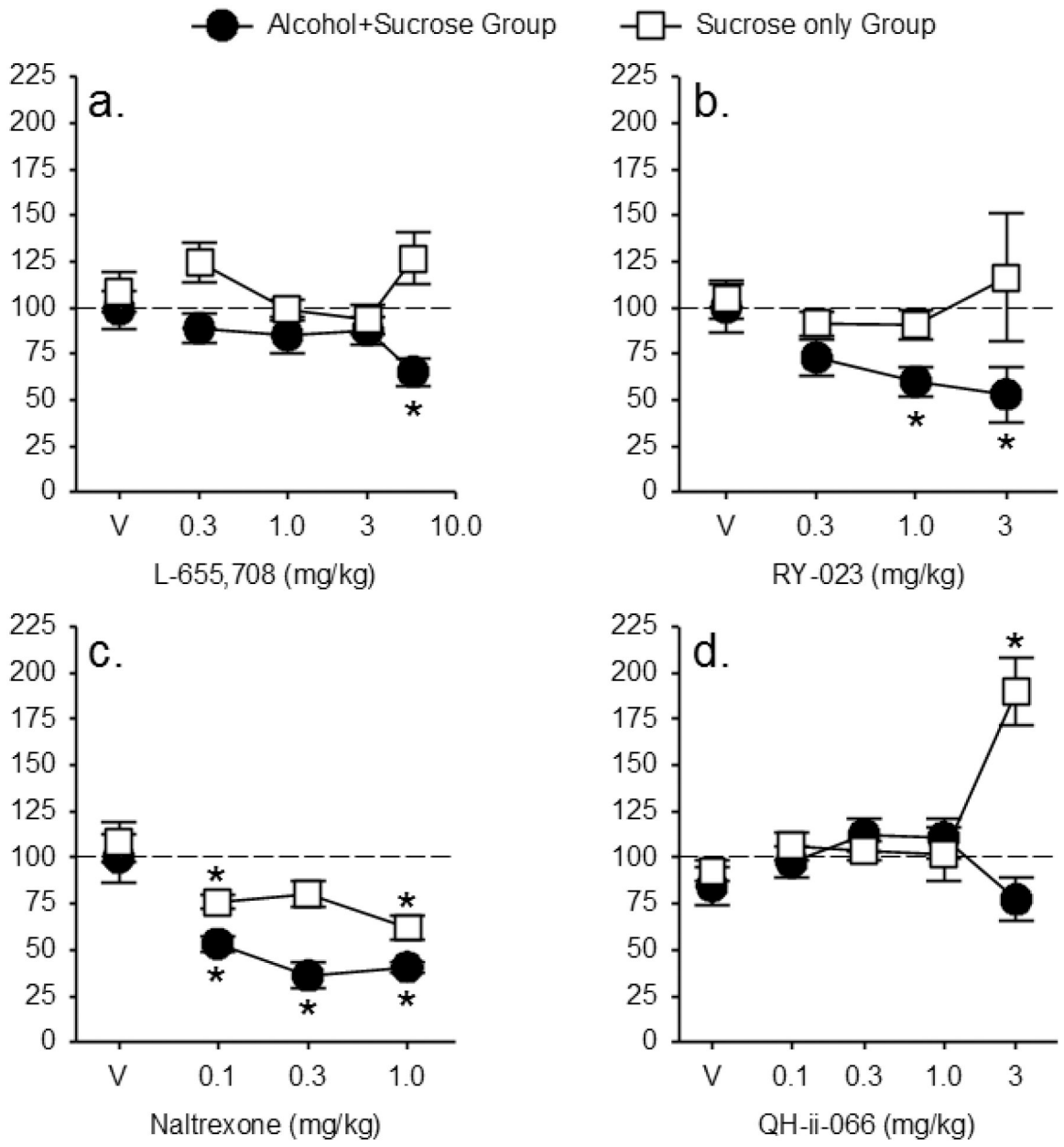

Figure 3.

Modulation of active alcohol+sucrose (black circles) or sucrose only (white squares) self-administration by a) L-655,708 (N=9–12), b) RY-023 (N=8–11), c) naltrexone (N=8–12), and d) QH-ii-066 (N=9–12). Data are means ± S.E.M. of the last 3 days of self-administration at each pretreatment dose. “V” = vehicle. * indicates $p < 0.05$ compared to baseline.

TABLE 1

Days to meet criteria and complete each step during sucrose fading in rats.

Step*	Range	Average (± SEM)
REINSTATEMENT:		
10S	9 – 13	10.8 ± 0.8
10S/2E	1 – 3	2.3 ± 0.3
10S/5E	1 – 3	1.8 ± 0.4
10S/10E	2 – 11	4.0 ± 1.5
5S/10E	3 – 27	11.5 ± 3.47
5S/15E	3 – 14	6.2 ± 1.7
2S/15E	9 – 30	20.2 ± 3.2
2S/10E	7 – 12	8.7 ± 1.1
Total	39 – 64	56.8 ± 4.0
SELF-ADMINISTRATION:		
10S	2 – 17	11.0 ± 1.4
10S/2E	1 – 4	2.1 ± 0.2
10S/5E	1 – 12	2.9 ± 0.9
10S/10E	1 – 17	5.0 ± 1.4
5S/10E	9 – 44	26.8 ± 3.4
Total	33 – 62	46.4 ± 2.6

* "S" = sucrose (% w/v); "E" = alcohol (% w/v)