$mGlu_2$ and $mGlu_3$ receptor negative allosteric modulators affect the discriminative stimulus effects of alcohol

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Abstract

All drugs with the potential for addiction liability, including alcohol, produce distinct interoceptive stimulus effects that influence drug intake. Our lab has shown involvement of Group II metabotropic receptors ($mGlu_{2/3}$) in the expression of alcohol interoceptive effects using a nonspecific $mGlu_{2/3}$ agonist and antagonist. Furthermore, the anterior insular cortex (aIC) is a hub for interoceptive processing, including the interoceptive effects of alcohol. Therefore, the goal of the present work was to assess the independent functional contribution of $mGlu_2$ and $mGlu_3$, and the role of $mGlu_3$ in the aIC, in the interoceptive effects of alcohol. As such, male, Long-Evans rats were trained to discriminate the stimulus effects of alcohol (2 g/kg, i.g.) from water (i.g.) using an operant, 2-lever drug discrimination procedure. To assess involvement of $mGlu_2$ and $mGlu_3$ in the discriminative stimulus effects of alcohol, rats were administered the $mGlu_2$ negative allosteric modulator (NAM, VU6001966; 0, 3, 6 mg/kg, i.p.) and the $mGlu_3$-NAM (VU6010572; 0, 3, 6, 12 mg/kg, i.p.) before alcohol (2 g/kg, i.g.). $mGlu_2$-NAM (6 mg/kg) and $mGlu_3$-NAM (12 mg/kg) decreased alcohol-appropriate lever responses without affecting response rate, reflecting diminished interoceptive alcohol sensitivity. $mGlu_3$-NAM microinjection (3 µg) into the aIC before alcohol decreased response rate to such an extent that alcohol-appropriate lever responding could not be determined. As such, $mGlu_3$-NAM was tested in the aIC before water administration to determine if VU6010572 substitutes for alcohol. $mGlu_3$-NAM VU6010572 (3 µg) in the aIC produced partial alcohol-like stimulus effects without affecting response rate. These data indicate that the newly developed $mGlu_2$ and $mGlu_3$ NAMs can modulate interoceptive effects of alcohol. The opposing effects of systemic vs. microinjected (aIC) $mGlu_3$-NAM on alcohol interoceptive effects may reflect differences in

receptor binding, a functionally relevant metabolite, and/or brain region differences in receptor function.

Keywords: VU6010572, VU6001966, operant drug discrimination, interoceptive alcohol sensitivity, insular cortex, mGluR3, mGluR2

Introduction

All drugs that have the potential to produce substance use disorder, including alcohol, produce distinct interoceptive effects that influence subsequent drug taking by signaling satiety or driving more intake [1, 2]. As such, the mechanisms underlying the interoceptive processing of alcohol are an important and clinically relevant research avenue. Animals can be trained to discriminate the interoceptive effects of alcohol from vehicle using an operant drug discrimination procedure in which alcohol is trained as a discriminative stimulus. The operant drug discrimination procedure used in the present work has been used for several decades to evaluate the discriminative stimulus/interoceptive effects of many different drugs of abuse, including alcohol [3-10].

Metabotropic glutamate (mGlu) receptors are GPCR's activated by glutamate. There are three groups of 8 total mGlu receptors, Group I (mGlu1 and mGlu5), Group II ($mGlu_2$ and $mGlu_3$), and Group III (mGlu4, mGlu6, mGlu7, mGlu8). Group I receptors are predominantly expressed at the postsynaptic site and are coupled to Gq proteins, whereas Group II and III receptors are considered mostly presynaptic and coupled to Gi proteins [11, 12]. mGlu receptors are promising targets for their potential in the treatment of alcohol use disorder (AUD), with both preclinical and clinical studies implicating these receptors in alcohol-related adaptations and AUD [11, 13-17]. Numerous studies have shown that manipulation of mGlu receptors (Group I and Group II) are effective at reducing drinking and reinstatement of alcohol seeking [18-21], as well as being involved in the interoceptive effects of alcohol [22-24]. In humans, AUD was associated with an increase in mGlu5 radioligand binding using positron emission tomography [25]. Furthermore, single nucleotide polymorphisms (SNPs) of the mGlu3 gene have been identified as highly associated with alcohol use disorder in a Genome Wide Association Study

(GWAS) [26]. Prior studies from our lab show that $mGlu_{2/3}$ receptor agonism and antagonism disrupt interoceptive sensitivity to alcohol [23, 24]. However, at the time, pharmacological limitations did not allow for the evaluation of the specific role of $mGlu_2$ vs. $mGlu_3$ receptors. This obstacle has recently been overcome by the development of highly selective $mGlu_2$ and $mGlu_3$ negative allosteric modulators (NAMs) [27, 28]. Both NAMs show blood-brain barrier permeability, high specificity for their respective receptors and both in vitro and in vivo efficacy [27, 28]. These are the first studies to evaluate these drugs on the discriminative stimulus effects of alcohol.

Because $mGlu_{2/3}$ antagonism attenuated the discriminative stimulus effects of alcohol [24], we hypothesized that negative allosteric modulation of $mGlu_2$ and $mGlu_3$ using VU6001966 and VU6010572, respectively would also attenuate interoceptive effects of alcohol. Therefore, the functional role of $mGlu_2$ and $mGlu_3$ receptors in the interoceptive effects of alcohol was assessed. The alcohol training dose of 2 g/kg was chosen because the contribution of glutamatergic signaling in the discriminative stimulus of alcohol appears to be greater at doses higher than 1.5 g/kg [4, 8]. We hypothesized that both $mGlu_2$ and $mGlu_3$ NAM would attenuate the discriminative stimulus effects of alcohol potentially through increasing excitatory drive [29] and therefore blunting the ability of alcohol to decrease excitation in the brain [1]. Because the anterior insular cortex (aIC) has been identified as an important region in the expression of the interoceptive effects of alcohol [30, 31], we next evaluated the effects of $mGlu_3$-NAM in the aIC before alcohol. Follow up experiments were conducted to test for alcohol substitution of $mGlu_3$-NAM in the aIC. Together, these experiments test the hypothesis that $mGlu_2$ and $mGlu_3$ receptors are involved in the expression of the interoceptive stimulus effects of alcohol.

Methods

Animals

Male, Long-Evans rats (N=10; Envigo, Indianapolis, IN) were used for all experiments. Rats arrived at 7 weeks and were housed individually. The vivarium was maintained on a 12-hr light/dark cycle and all experiments were conducted during the light cycle. All rats were handled for at least 1 minute daily for 1 week prior to beginning discrimination training. Food intake was restricted to maintain body weight (325-340 g). Rats had ad libitum access to water. Rats were under continuous care and monitoring by veterinary staff from the Division of Comparative Medicine at UNC Chapel Hill. All procedures were conducted in accordance with the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines.

Operant Drug Discrimination

Apparatus - The chambers (Med Associates, Georgia, VT; measuring 31 × 32 × 24 cm) used for drug discrimination training and testing have been detailed elsewhere [31, 32]. Briefly, two retractable levers were on each side (left or right) of a liquid dipper receptacle on the right-side wall of the chamber. One stimulus light was located above each lever. Completion of a fixed ratio 10 (FR10) schedule of reinforcement on the appropriate lever activated a dipper that presented 0.1 mL of sucrose (10% w/v) for 4 seconds. Chambers were inside of a sound-attenuating cubicle with an exhaust fan. Med Associates program was used to control sessions and record data.

Discrimination Training

Training sessions were identical to [32] and similar to our previous work [22, 24, 30, 31, 33-39]. Briefly, training sessions took place each day from Monday-Friday. Rats were administered alcohol (2 g/kg, i.g.) or water and immediately placed in the chamber for a 20-min timeout

period before the session started. Once the session started, both the right and left levers were introduced into the chambers and the houselights was illuminated to indicate the beginning of the training session. During a water training session, completion of FR10 on the water-appropriate lever resulted in sucrose presentation. In contrast, on alcohol training sessions, completion of FR10 on the alcohol-appropriate lever resulted in sucrose presentation. For each session, responding on the inappropriate lever was obtained but did not produce a programmed consequence. To prevent lever selection bias, the alcohol- and water-appropriate levers were counterbalanced across rats. Training days varied on a double alternating schedule (alcohol, alcohol, water, water,…). Testing began when the following performance criteria were met: >80% appropriate lever responses for at least 8 out of 10 consecutive days for the first reinforcer and for the entire session.

Discrimination Testing

Test sessions were identical to training sessions except that they were 2-min in duration and FR10 on either lever resulted in sucrose delivery to prevent lever selection bias and to allow for quantification of response rate. Upon meeting criteria for training sessions, 4 separate test sessions were conducted to obtain an alcohol dose response curve (0.3, 1.0, 2.0, 2.5 g/kg). Doses were counterbalanced across days and interspersed with training sessions. Identical to training sessions, a 20-min delay period elapsed from alcohol administration to the start of the test session (2 min).

Drug Testing

Experiment 1: Effect of systemic $mGlu_2$ *and* $mGlu_3$ *NAM on alcohol discriminative stimulus.*

Rats were injected with the $mGlu_3$-NAM VU6010572 (0, 3, 6 mg/kg, i.p.) or $mGlu_2$-NAM VU6001966 (0, 3, 6 mg/kg, i.p.) 25 mins before alcohol (2 g/kg, i.g.) administration and the start

of a test session. 45 mins elapsed from the time the drug was administered to the start of the operant test session (25-min drug pretreatment + 20-min alcohol pretreatment). dAs such, 4 rats were tested on both mGlu2-NAM and mGlu3-NAM. The $mGlu_3$-NAM was tested first and then the $mGlu_2$-NAM was tested. For each drug, doses administered were counterbalanced across days and all rats received all doses. Drug testing days were interspersed with training sessions. Because 6 mg/kg VU6010572 did not produce an effect on alcohol-appropriate lever responding or response rate, a follow-up test was conducted using a higher dose (12 mg/kg) of $mGlu_3$-NAM.

Experiment 2: Effect of $mGlu_3$-NAM in the aIC on alcohol discriminative stimulus.

Following the conclusion of the systemic drug tests, rats (n=7) were used to test the effects of VU6010572 in the aIC. Rats were anesthetized with an isoflurane/oxygen combination and secured in a stereotaxic apparatus. Bilateral guide cannulae (26 gauge, Plastics One) were implanted to terminate above the aIC (AP: +2.5 mm, ML: +4.0 mm, DV: -4.6 mm) [40]. Cannulae were secured using stainless steel screws and dental cement (Lang Dental, NY) that was adhered to the skull. A stylet (28 gauge stainless-steel, PlasticsOne, Roanoke, VA) was inserted into the cannulae to prevent clogging and infection. All rats received analgesia (Banamine, 2.5 mg/kg, s.c.) 24 hrs prior to surgery, the day of surgery, and 24 hrs following surgery. Rats were allowed to recover for at least one week before resuming discrimination training.

For the testing, rats received bilateral microinjections of $mGlu_3$-NAM (VU6010572, 1.5 µg, 3 µg) or vehicle (1:1 45% β-cyclodextrin: aCSF) into the aIC. One rat died following a gavage and another had a clogged cannula and could not be tested, resulting in sample size of 5 for the microinjection experiment. A Hamilton syringe was connected to polyethylene tubing, which connected to an injector at the other end. Injectors (33 gauge, Plastics One) extended 2

mm below the guide cannulae and were used to deliver the drug. A pump (Harvard Apparatus) was used to deliver the drug on each side at a rate of 0.5 µL/min over a period of 1 min, then followed by a 2 min diffusion period. Rats were awake and freely moving during the microinjections. Following the diffusion period, rats received water or alcohol (2 g/kg) gavage and underwent a test session.

Following the completion of testing, rats were anesthetized with isoflurane prior to perfusion with 0.1 M PBS solution (4 C, pH = 7.4) and 4 % paraformaldehyde (PFA; 4 C, pH=7.4). Brains were extracted and stored in 4% PFA solution for 24 hrs at 4° C. Next, brains were rinsed with 0.1 M PBS solution and then transferred to a 30% sucrose in 0.1 M PBS solution. Using a freezing microtome, 40-µm sections were collected for cannulae verification using cresyl violet staining. All 5 rats tested showed correct cannulae placement for the aIC as shown in Figure 3.

Drugs

Alcohol (95% (v/v), Pharmaco-AAPER, Shelbyville, KY) was diluted with tap water to make a 20% alcohol solution for all experiments. $mGlu_2$-NAM (VU6001966) was purchased from Tocris Bioscience (Bristol, UK). $mGlu_3$-NAM (VU6010572) was synthesized by RTI. For systemic injections in Experiment 1, both drugs were dissolved completely in 45% β-cyclodextrin at a concentration of 3 or 6 mg/mL. For the aIC microinjections in Experiment 2, VU6010572 was dissolved completely in a 1:1 mixture of 45% β-cyclodextrin and aCSF at a concentration of 3 or 6 µg/µL. Because 0.5 µL was injected into both sides of the aIC, 1.5 and 3 µg were delivered per side using the 3 and 6 µg/µL stock solutions, respectively.

Data Analysis

For all experiments, response accuracy was evaluated as the percentage of alcohol-appropriate lever response upon delivery of the first sucrose reinforcer (i.e., the first 10 lever responses). Full expression of the discriminative effects of alcohol was defined as ≥80% alcohol-appropriate responses. Partial substitution was defined as >40% and <80% alcohol-appropriate responding. No substitution was defined as <40% alcohol-appropriate responding. The response rate was calculated as total lever responses per min. If a rat did not complete an FR10, that animal was removed from the response accuracy analysis, but still included in the response rate analysis [31]. For all read-out measures, a repeated measures one-way ANOVA was used with drug dose as the within-subjects factor. For the alcohol dose response curve (Fig. 1), Tukey's multiple comparisons were conducted to compare each dose to all other doses of alcohol. For the drug pretreatment experiments (Fig. 2 and Fig. 3), Dunnett's multiple comparison tests were conducted to compare each drug dose to vehicle. Effect sizes for all statistically significant results were calculated as eta squared (η^2). For reference, a large effect size is defined as η^2 = 0.1379 or greater, a medium effect size is η^2 = 0.0588 or greater and a small effect size is η^2 = 0.009 or less [41]. $P \leq 0.05$ was set for significance.

Results

Confirmation of discriminative stimulus control

All 10 rats responded > 80% on the alcohol-appropriate lever at the training dose (2 g/kg), demonstrating full substitution for the training dose. A significant main effect of alcohol dose was observed (Fig. 1A; $F(1.48, 13.36) = 27.44$, $p < 0.0001$, $\eta 2 = 0.60$). 0.3 g/kg differed from 2 g/kg and 2.5 g/kg ($p < 0.05$). 1 g/kg differed from 2 g/kg ($p < 0.05$). 1 g/kg alcohol showed partial substitution for the training dose. 2.0 and 2.5 g/kg alcohol showed full substitution for the

training dose. Alcohol dose did not affect response rate (Fig. 1B). These data show a dose-dependent increase in alcohol appropriate lever responding, demonstrating discriminative stimulus control of alcohol on behavioral responding.

Systemic $mGlu_2$-NAM and $mGlu_3$-NAM attenuate the discriminative stimulus effects of alcohol

The $mGlu_2$-NAM (VU6001966) significantly reduced alcohol-appropriate responses ($F(1.02, 5.14) = 7.17$, $p = 0.04$, $\eta^2 = 0.52$, Fig. 2A), with a reduction at 6 mg/kg compared to vehicle ($p = 0.05$). Alcohol-appropriate responses at 3 mg/kg $mGlu_2$-NAM did not differ from vehicle ($p > 0.05$) and showed full substitution for the training dose. No effect of drug treatment was found on response rate (Fig. 2B).

The $mGlu_3$-NAM also significantly reduced alcohol-appropriate responses ($F(1.92, 9.61) = 6.72$, $p = 0.01$, $\eta^2 = 0.46$, Fig. 2C), with a reduction at the 12 mg/kg dose relative to vehicle ($p = 0.04$). In contrast, responses at 3 and 6 mg/kg did not differ from vehicle ($p > 0.05$). Drug treatment at all doses tested did not affect response rate (Fig. 2D). These data show that $mGlu_2$ and $mGlu_3$ receptors are involved in the expression of the discriminative stimulus effects of alcohol. Next, we sought to followed up by testing the effects of these NAMs in the aIC specifically on alcohol discrimination.

$mGlu_3$-NAM in the aIC produces partial alcohol-like stimulus effects

The $mGlu_3$-NAM was tested first and given the change in testing plan (as will be discussed), this required 6 microinjections. As such, it was not feasible to test the $mGlu_2$-NAM in the aIC. Administration of $mGlu_3$-NAM into the aIC prior to alcohol (2 g/kg) resulted in the majority of rats not completing an FR10 such that they could not be included in the response accuracy analysis (Fig. 3A), making it not possible to do a proper analysis for the accuracy measure. This was evident in the significant reduction in response rate (Fig, 3B, $F(1.35, 5.42) = 5.60$, $\eta^2 = 0.42$,

p=0.05). Post hoc tests showed a significant decrease in response rate in the 3 µg group compared to vehicle ($p<0.05$), but not vehicle compared to 1.5 µg ($p>0.05$). Because this $mGlu_3$-NAM pretreatment appeared to produce motor-impairing effects, the NAM was tested prior to water administration to test for substitution for the alcohol training dose. Interestingly, the $mGlu_3$-NAM microinjected into the aIC produced partial alcohol-like stimulus effects (Fig. 3C). There was a significant main effect of drug dose on alcohol appropriate lever responses ($F(1.01, 4.05) = 12.22$, $p = 0.02$, $\eta 2 = 0.71$), with increased alcohol-appropriate responding at the 3 µg dose compared to vehicle ($p<0.05$), but not in the 1.5 µg dose compared to vehicle ($p>0.05$). Of the 5 rats tested, 2 rats showed full substitution (>80% response accuracy), 2 rats showed partial substitution (between 80% and 40% response accuracy), and 1 rat displayed no substitution (<40% accuracy) for the alcohol training dose (2 g/kg). There was also a significant reduction in response rate ($F(1.24, 4.97) = 11.48$, $p = 0.01$, $\eta^2 = 0.15$), but post-hoc tests did not show an effect of $mGlu_3$-NAM on response rate at either dose tested compared to vehicle. Together, these data show that microinjection of $mGlu_3$-NAM into the aIC partially substitutes for the interoceptive effects of alcohol (2 g/kg) and acts synergistically with alcohol to produce motor-impairing effects.

Discussion

These data show that $mGlu_2$-NAM (6 mg/kg, i.p.) and $mGlu_3$-NAM (12 mg/kg, i.p.) pretreatment attenuated the discriminative stimulus effects of 2 g/kg alcohol without affecting response rate. Furthermore, $mGlu_3$-NAM (3 µg) microinjected into the aIC partially substituted for the interoceptive effects of alcohol, whereas in combination with alcohol (2 g/kg, i.g.) there was a reduction in response rate compared to alcohol alone. As such, these data are the first to

demonstrate individual involvement of $mGlu_2$ and $mGlu_3$ receptors in alcohol's interoceptive stimulus.

$mGlu_2$ and $mGlu_3$ activation attenuate presynaptic neurotransmitter release, decreasing excitatory drive [12]. Both the $mGlu_2$ and $mGlu_3$ NAMs have been shown to enhance c-Fos expression and increase excitatory currents on pyramidal PFC neurons [29]. Furthermore, alcohol exerts its interoceptive stimulus effects mainly through inhibition of excitatory (NMDA) and potentiation of inhibitory ($GABA_{A)}$ receptors, resulting in diminished excitation in the brain [1, 4, 8, 10, 39, 42-44], probably in cortical regions that integrate interoceptive signals. Therefore, the ability of $mGlu_2$ and $mGlu_3$ NAMs to block the interoceptive effects of alcohol may be due to the ability of these drugs to counteract the effects of alcohol on diminished neuronal excitation – i.e., through increasing neuronal excitation. Another interpretation is that $mGlu_2$ and $mGlu_3$ NAMs may produce their own interoceptive effects distinct from alcohol, which then interfere with the interoceptive stimulus of alcohol. This interpretation is supported by data showing that both $mGlu_{2/3}$ agonism and antagonism disrupt expression of the discriminative stimulus of alcohol [23, 24], as well as the data shown here demonstrating that $mGlu_3$-NAM in the aIC partially substitutes for alcohol. If $mGlu_2$ and $mGlu_3$ NAMs do produce their own distinct interoceptive stimulus effects, this may have implications for abuse liability of these drugs. Future experiments should be conducted to determine if these drugs can be trained as discriminative stimuli. Regardless of the precise mechanism(s), these studies demonstrate that systemically administered $mGlu_2$ and $mGlu_3$ NAMs may be used to attenuate discriminative stimulus effects of alcohol.

It is interesting that $mGlu_3$-NAM required twice the dose of $mGlu_2$-NAM to produce similar effects on alcohol's discriminative stimulus. Upon comparison of the pharmacological

properties of these drugs, literature shows that the $mGlu_2$-NAM has a larger K_p value and a smaller IC_{50} value compared to $mGlu_3$-NAM [27, 28], indicating that $mGlu_2$-NAM has greater blood brain barrier permeability and functional strength on receptor inhibition compared to $mGlu_3$-NAM. Additionally, while $mGlu_2$ receptors are predominantly presynaptically located, $mGlu_3$ receptors are also highly expressed on post-synaptic terminals as well as in astrocytes [12, 45, 46]. Furthermore, $mGlu_2$ and $mGlu_3$ receptors have also been shown to produce distinct effects on circuit and behavioral outcomes [29, 45, 47, 48]. These differences between $mGlu_2$ and $mGlu_3$ NAM pharmacological properties and differences in $mGlu_2$ and $mGlu_3$ receptor function may contribute to the dose discrepancy observed here.

Interestingly, while systemic administration of $mGlu_3$-NAM attenuated the interoceptive effects of alcohol, microinjection of $mGlu_3$-NAM into the aIC produced partial alcohol-like interoceptive stimulus effects. Given that in the aIC the $mGlu_3$-NAM prior to alcohol produced a significant impairment in rats completing the first FR, we hypothesized that the NAM may be potentiating the effects of alcohol. This prompted the strategy to test the NAM alone (prior to water) to determine if was producing some alcohol-like effects and indeed partial substitution for the 2 g/kg training dose was observed. Therefore, it will be important for future experiments to test whether $mGlu_3$-NAM in the aIC potentiates the interoceptive stimulus effects of a low dose (e.g., 0.5 g/kg) alcohol. Additionally, follow up experiment should also evaluate the effects of $mGlu_2$-NAM in the aIC as limitations on the number of microinjections precluded $mGlu_2$-NAM testing here.

The discrepancy between systemic and microinjection results raises the possibility that $mGlu_3$ receptors may play different brain region-specific roles in their relationship to alcohol interoceptive effects. $mGlu_3$ receptors in excitatory neurons are predominantly thought to be

presynaptically located but have also been shown to be postsynaptically located on pyramidal neurons in the prelimbic cortex (PrL) [45, 47]. They have also been found in glial cells [46, 49]. Furthermore, $mGlu_2$ and $mGlu_3$ receptors form functional hetero or homo dimers, and the composition of which affect function [12]. As such, there may be brain region differences in composition of the dimerized $mGlu_2$/$mGlu_3$ receptor complex. As such, brain region differences that may exist in cell-type expression and dimerization may in part account for differing $mGlu_3$ receptor function on alcohol interoceptive effects when administered systemically vs. aIC microinjection. In addition, this discrepancy may arise from differences in receptor binding from microinjection vs. systemic injection. Because the drug does not need to bypass the blood-brain barrier for the microinjections, more receptors may be bound by the NAM for microinjections than for systemic injections. Finally, these differences may arise from functionally relevant metabolites of the $mGlu_3$-NAM when administered systemically. The pretreatment time for the systemic injections was 45-min while the operant tests were started immediately after microinjections. As such, it is possible that a metabolite generated from systemic administration of $mGlu_3$-NAM played some role in the discrepancy observed here.

These are the first studies to demonstrate direct involvement of both $mGlu_2$ and $mGlu_3$ receptors in the interoceptive effects of alcohol. Additionally, the higher potency of $mGlu_2$ compared to $mGlu_3$-NAM is consistent with prior literature about their pharmacological profile. The differing effects of $mGlu_3$-NAM when microinjected into the aIC vs. systemic injection suggests the possibility of brain region and cell-type localization differences in how $mGlu_3$ interacts with alcohol. Important follow up experiments will evaluate the role of these receptors on alcohol interoceptive stimulus in female rats, as only male rats were used in this study. These data add to a growing literature on the involvement of $mGlu_2$ and $mGlu_3$ receptors in alcohol-

related behaviors [11]. Finally, $mGlu_2$ and $mGlu_3$ NAMs may be therapeutic for AUD through blocking the interoceptive effects of alcohol.

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Conflicts of interest: none.

Data Availability Statement: Data available on request from the authors.

Figure Captions

Figure 1 – A timeline of the alcohol administration and operant discrimination testing for the alcohol dose response curve is displayed above Fig. 1 data. Following acquisition training with the training dose of 2 g/kg alcohol, a dose-response curve was conducted to confirm stimulus control by alcohol. (A) Alcohol-appropriate lever responding dose-dependently increased as a function of alcohol dose. #1 g/kg v. 2 g/kg. *2.0 and 2.5 v. 0.3 g/kg. (B) Alcohol dose did not affect response rate. Dotted lines at 80% indicate full substitution for the 2 g/kg alcohol training dose. *$p \leq 0.05$

Figure 2 – A timeline for the $mGlu_2$ and $mGlu_3$ NAM systemic injection experiments is displayed above figure 2. (A) $mGlu_2$-NAM VU6001966 attenuated alcohol-appropriate lever responses of 2 g/kg alcohol. (B) $mGlu_2$-NAM did not affect response rate. (C) $mGlu_3$-NAM VU6010572 attenuated alcohol-appropriate lever responses of 2 g/kg alcohol. (D) $mGlu_3$-NAM did not affect response rate. Dotted lines at 80% indicate full substitution for the 2 g/kg alcohol training dose. *$p \leq 0.05$

Figure 3 – A timeline for the experiment of $mGlu_3$-NAM microinjection into the anterior insular cortex (aIC) is displayed above figure 3. (A) $mGlu_3$-NAM microinjected into the aIC prior to alcohol administration (2 g/kg, i.g.) did not affect alcohol-appropriate lever responses, but (B) diminished response rate to the extent that accuracy analysis could not be properly determined. (C) $mGlu_3$-NAM tested prior to water administration partially substituted for the interoceptive effects of the alcohol training dose. (D) $mGlu_3$-NAM in the aIC prior to water administration showed a main effect on response rate but did not show post-hoc differences between doses. *$p \leq 0.05$

Figure 1

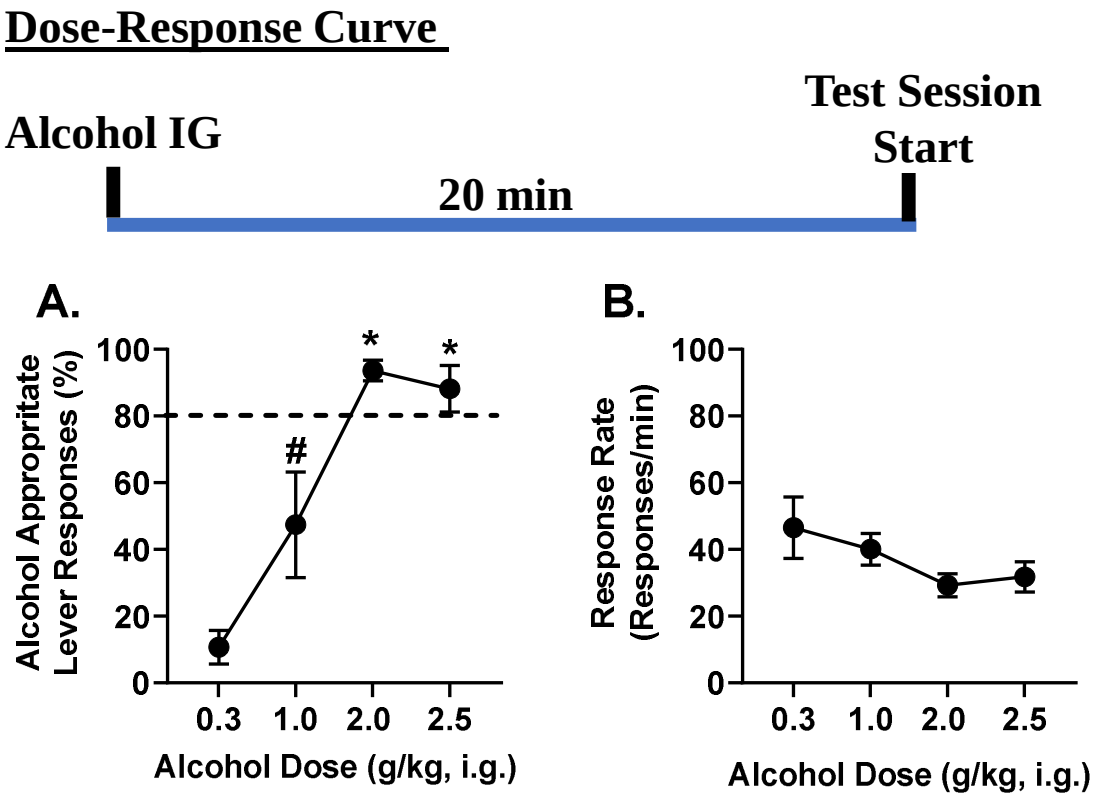

Dose-Response Curve
Alcohol IG
Test Session Start
20 min
A.
Alcohol Appropriate Lever Responses (%)
100
80
60
40
20
0
*
*
#
0.3
1.0
2.0
2.5
Alcohol Dose (g/kg, i.g.)
B.
Response Rate (Responses/min)
100
80
60
40
20
0
0.3
1.0
2.0
2.5
Alcohol Dose (g/kg, i.g.)

Figure 2

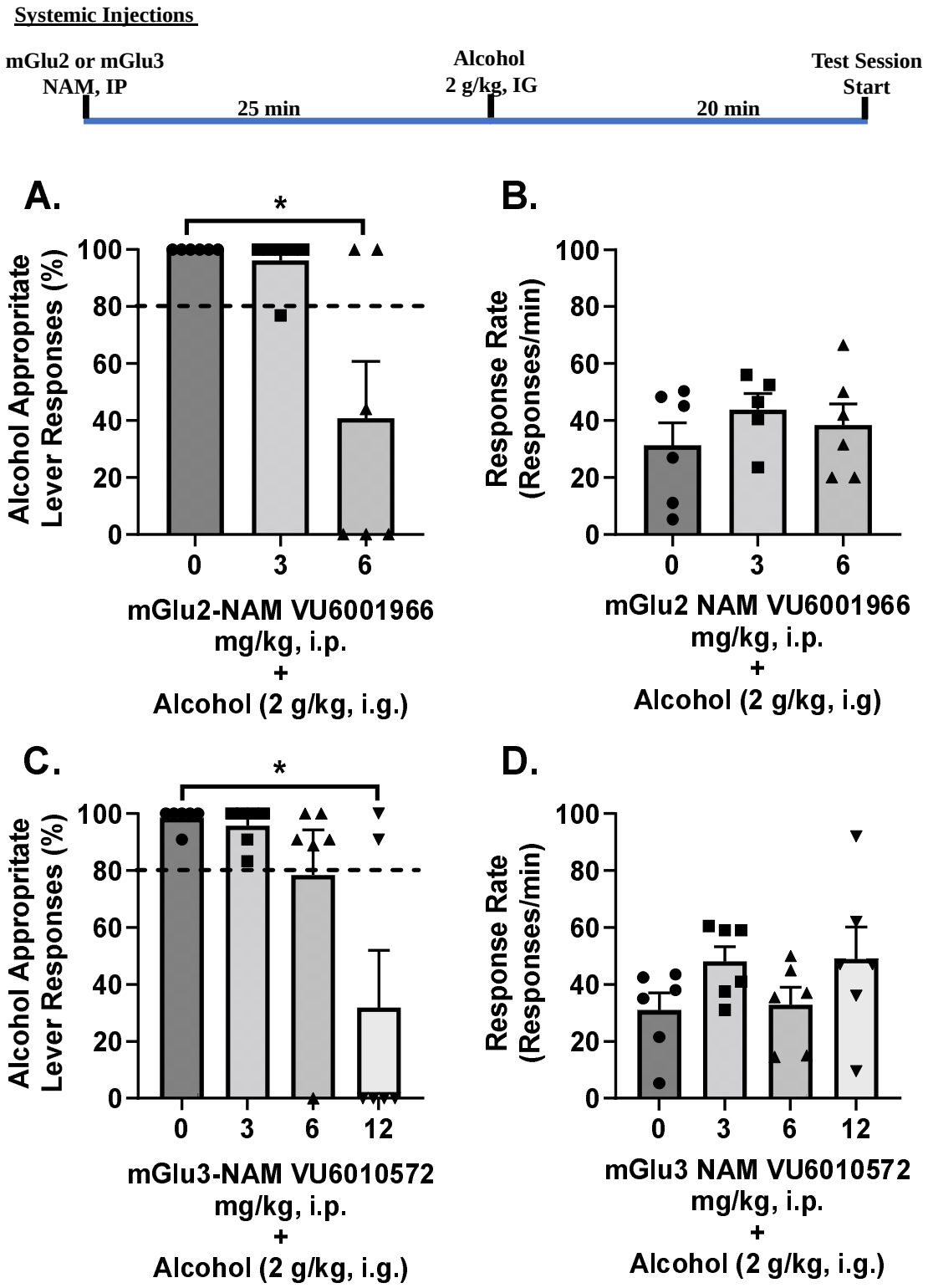
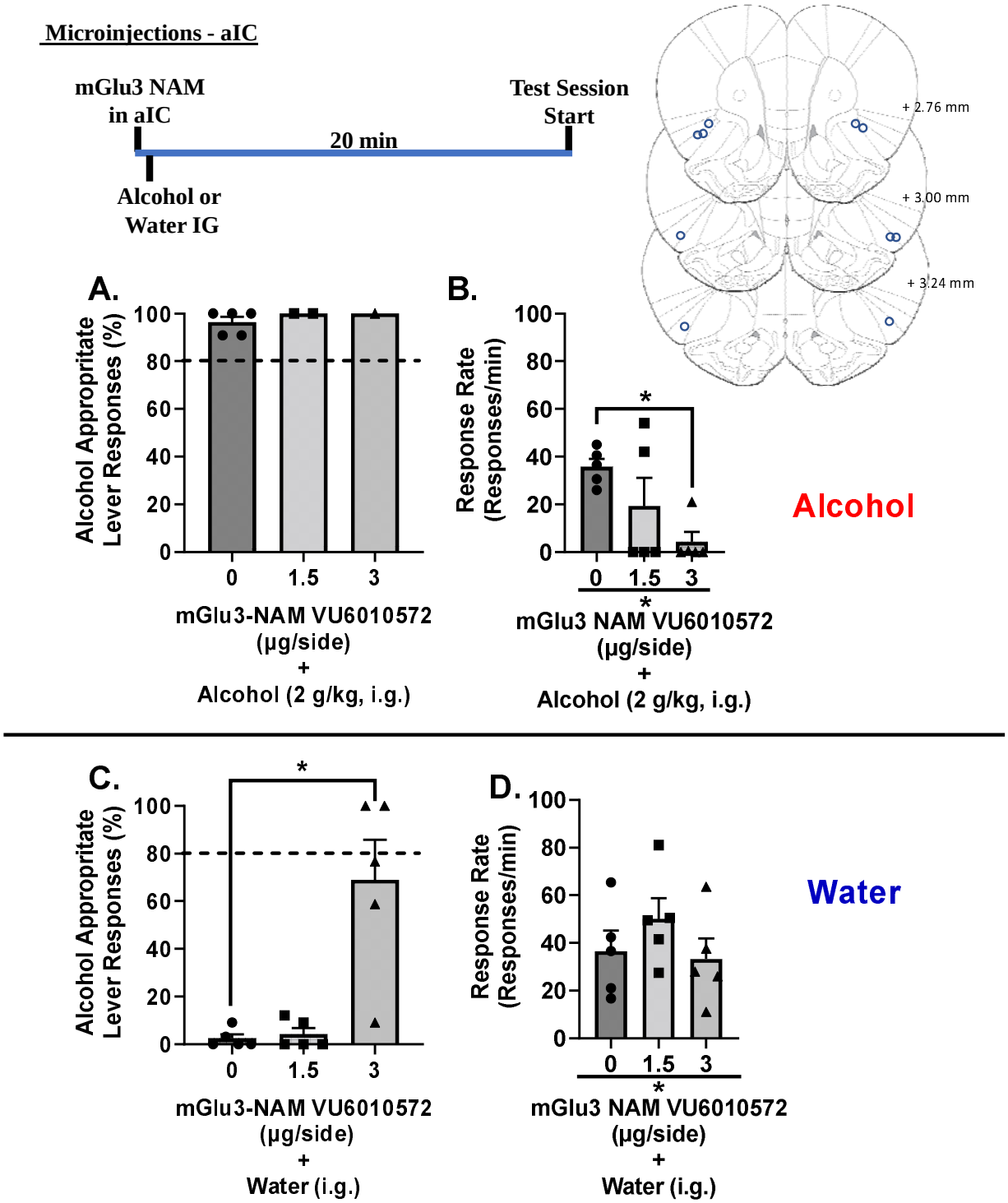

Systemic Injections
mGlu2 or mGlu3 NAM, IP
25 min
Alcohol 2 g/kg, IG
20 min
Test Session Start
A.
*
Alcohol Appropriate Lever Responses (%)
100
80
60
40
20
0
0
3
6
mGlu2-NAM VU6001966 mg/kg, i.p. + Alcohol (2 g/kg, i.g.)
B.
Response Rate (Responses/min)
mGlu2 NAM VU6001966 mg/kg, i.p. + Alcohol (2 g/kg, i.g)
C.
*
12
mGlu3-NAM VU6010572 mg/kg, i.p. + Alcohol (2 g/kg, i.g.)
D.
mGlu3 NAM VU6010572 mg/kg, i.p. + Alcohol (2 g/kg, i.g.)

Figure 3


Microinjections - aIC
mGlu3 NAM in aIC
20 min
Test Session Start
Alcohol or Water IG
+ 2.76 mm
+ 3.00 mm
+ 3.24 mm
A.
Alcohol Appropriate Lever Responses (%)
mGlu3-NAM VU6010572 (µg/side) + Alcohol (2 g/kg, i.g.)
B.
Response Rate (Responses/min)
mGlu3 NAM VU6010572 (µg/side) + Alcohol (2 g/kg, i.g.)
Alcohol
C.
Alcohol Appropriate Lever Responses (%)
mGlu3-NAM VU6010572 (µg/side) + Water (i.g.)
D.
Response Rate (Responses/min)
mGlu3 NAM VU6010572 (µg/side) + Water (i.g.)
Water