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THE ROLE OF BETA- AND ALPHA-ADRENERGIC RECEPTORS ON ALCOHOL DRINKING

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Abstract

Alcohol Use Disorders (AUD) is characterized by compulsion-like alcohol drinking (CLAD), where intake despite negative consequences can be a major clinical obstacle. With few treatment options available for AUD, there is a significant need for novel therapies. The noradrenergic system is an important hub for regulating stress responses and maladaptive drives for alcohol. Studies have shown that drugs targeting α1 adrenenergic receptors (ARs) may represent a pharmacological treatment for pathological drinking. However, the involvement of β ARs for treating human drinking has received scant investigation, and thus we sought to provide pre-clinical validation for possible AR utility for CLAD by analyzing whether β AR antagonists propranolol (β1/2), betaxolol (β1), and ICI, 118 551 (β2) impacted CLAD and alcohol-only drinking (AOD) in male Wistar rats. We found that the highest dose of propranolol tested systemically (10mg/kg) reduced alcohol drinking, while 5mg/kg propranolol reduced drinking with a trend to impact CLAD more than AOD, and with no effects of 2.5mg/kg. Betaxolol (2.5mg/kg) also decreased drinking, while ICI 118.551 had no effects. Also, while AR compounds might have utility for AUD, they can also lead to undesirable side effects. Here, a combination of ineffective doses of propranolol and prazosin reduced both CLAD and AOD. Finally, we investigated the effect of propranolol and betaxolol in two brain areas related to pathological drinking, the anterior insula (aINS) and medial prefrontal cortex (mPFC). Surprisingly, propranolol (1–10μg) in aINS or mPFC did not affect CLAD or AOD. Together, our findings provide new pharmacological insights into noradrenergic regulation of alcohol consumption, which may inform AUD therapy.

1. INTRODUCTION

Alcohol Use Disorder (AUD) ranks among the most prevalent mental disorders and is characterized by compulsive heavy alcohol use and loss of control over alcohol intake, with

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5. Authorship contribution

TDOS, and FWH designed the experiments, analyzed the data, and wrote a first draft of the manuscript; TDOS, SW and SK performed the experiments; all authors edited the manuscript and approved the final version.

Disclosure

All authors declare no competing interests.

negative consequences on both physical and mental health (Larimer *et al.*, 1999; Koob *et al.*, 2010; Hopf *et al.*, 2014; Carvalho *et al.*, 2019; G.B.D. Collaborators, 2020; Epstein, 2020). Also, the compulsion for alcohol, where consumption persists despite negative consequences, can often relate to negative affect and activation of brain stress systems (Koob *et al.*, 2014). In addition, currently approved medications for AUD show modest therapeutic efficacy, and there is a critical need for novel therapies, especially drugs that are already FDA approved and could be quickly repurposed (Spanagel, 2009; Kranzler *et al.*, 2018; Downs *et al.*, 2022). However, even with robust evidence for an important relation between AUD and stress activation, there are no approved medication targeting the brain stress system to modulate excessive alcohol drinking (Haass-Koffler *et al.*, 2018; Downs *et al.*, 2022; Varodayan *et al.*, 2022).

The noradrenergic system is an important hub in the stress response system (Valentino *et al.*, 2008; Downs *et al.*, 2022, see Discussion), as well as maladaptive drives for alcohol (Vazey *et al.*, 2018). Preclinical and clinical studies have shown that functional inhibition of α1 adrenergic receptors (AR) may represent a therapeutic target for AUD (Simpson *et al.*, 2018; Sinha *et al.*, 2021; Sinha *et al.*, 2022). In humans, recent studies found that the α1 AR antagonist prazosin reduces drinking and craving in patients with more severe alcohol withdrawal symptoms (Sinha *et al.*, 2021), and decreases alcohol withdrawal effects on the prefrontal-striatal activation (Sinha *et al.*, 2022). In parallel, we used an animal model for compulsion-like alcohol drinking (CLAD), where animals will tolerate alcohol adulterated with quinine levels that are strongly avoided in water (Hopf *et al.*, 2010, Lei *et al.*, 2016, Sneddon *et al.*, 2019; Katner *et al.*, 2022, reviewed in Radke *et al.*, 2021), including in our recent findings in Wistar rats where quinine 10mg/L decreased water intake >70% (De Oliveira Sergio *et al.*, biorxiv) (similar to Domi *et al.*, 2021, see Discussion). Also, our recent findings show that systemic administration of prazosin reduces both alcohol-only drinking (AOD) and CLAD in male rats (De Oliveira Sergio *et al.*, 2021). We also examined the impact of prazosin in the anterior insula (aINS), a key regulator of emotional states and a strong contributor to many aspects of addiction and emotion in humans and rodents (Centanni *et al.*, 2021; Sommer *et al.*, 2022). Interestingly, prazosin injection into aINS also decreases both CLAD and AOD, while, in contrast, aINS projections to the Locus Coeruleus area mediate CLAD but not AOD (or sweet fluid intake) (De Oliveira Sergio *et al.*, 2021). Thus, while α1 AR modulation can influence both CLAD and AOD, some aspects of noradrenergic signaling may be more selective for challenge-resistant action. Similarly, we previously found that CLAD but not AOD requires aINS inputs to striatal areas (Seif *et al.*, 2013), as well as striatal inputs from mPFC (another area that can regulate drinking, e.g. Linsenbardt et al., 2019; Barbier et al., 2021; Dao et al., 2021), and compulsion-like responding for alcohol activates a similar insular circuit in heavy drinking humans (Grodin *et al.*, 2018) (see also Arcurio *et al.*, 2015), validating the importance of these circuits for at least some aspects of problem drinking in humans.

The involvement of β ARs for AUD has received limited study (Haass-Koffler *et al.*, 2018; Downs *et al.*, 2022). Early clinical studies found that non-selective β AR antagonists propranolol and atenolol can reduce symptoms related to alcohol withdrawal and cravings (Carlsson *et al.*, 1971; Zilm *et al.*, 1975; Horwitz *et al.*, 1989; Bailly *et al.*, 1992). In rodents, propranolol (but not nadolol) reduces alcohol consumption in alcohol-dependent

rats, suggesting that central (but not peripheral) β ARs are involved in regulating alcohol consumption (Gilpin *et al.*, 2010). Furthermore, a higher dose of propranolol decreases alcohol intake in dependent P rats during early withdrawal (Rasmussen *et al.*, 2014).

Given that compulsion-like drives can be an important feature of AUD, the likely relation of CLAD with stress, and our previous work showing α1 AR importance for CLAD and AOD, we hypothesized here that β AR signaling would also regulate CLAD and AOD. We first investigated if systemic administration of propranolol (2.5, 5 and 10mg/kg), the β1 AR antagonist betaxolol (2.5 and 5mg/kg), or the β2 ARs antagonist ICI 118, 551 (1 mg/kg), would alter alcohol intake. Further, while clinical and preclinical studies show the beneficial effects of α1 and β antagonists on AUD, these compounds can also lead to undesirable side effects on blood pressure and cardiovascular system (Vazey *et al.*, 2018; Downs *et al.*, 2022). Thus, any strategy lowering the dose of these compounds to reduce drinking could have broad utility. Importantly, we found that co-administering ineffective doses of propranolol and prazosin reduced CLAD and AOD. Finally, we also investigated the potential role of β AR signaling in aINS and mPFC, two brain areas related to AUD and CLAD, through local injection of propranolol and betaxolol. Together, our results provide pharmacological and neurocircuit insights into a novel treatment strategy (prazosin+propranolol) that already is FDA-approved and could be used for AUD treatment.

2. MATERIALS AND METHODS

All experimental procedures were conducted in accordance with the Guide for Care and Use of Laboratory animals provided by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of Indiana University. All efforts were undertaken to reduce the number of animals needed and to minimize pain and suffering.

2.1 Subjects and Alcohol Drinking Methods

Male Wistar rats (Envigo) 45–50 days old were singly housed with *ad libitum* food and water. After two weeks of acclimatization to the vivarium, rats had access to alcohol (20% v/v diluted in water) in the intermittent two-bottle choice paradigm (IA2BC) (with the second bottle containing water). Briefly, three times a week (starting Monday, Wednesday and Friday), rats had an 18–24hr period where alcohol was available concurrently with water. The alcohol and water bottle positions were alternated across days to prevent a position bias. Several studies showed that Wistar and other outbred rat strains need at least 3 months of IA2BC to develop CLAD (Hopf *et al.*, 2010; Seif *et al.*, 2013; Seif *et al.*, 2015; Spoelder *et al.*, 2015; Spoelder *et al.*, 2017). Thus, we allowed rats ~3 months of IA2BC, at which point rats were shifted to limited daily access two-bottle choice (LDA), with 20 min access to 20% alcohol or water Monday through Friday. After at least 2–3wk LDA, rats had 2–3 alcohol-quinine sessions (with 10mg/L quinine) to habituate to the novelty of quinine in alcohol, and then were returned to AOD. These methods are as we previously published (Hopf *et al.*, 2010; Seif *et al.*, 2013; Seif *et al.*, 2015; Darevsky *et al.*, 2019; Darevsky *et al.*, 2020; De Oliveira Sergio *et al.*, 2021).

One week before starting the experimental sessions rats were gently handled by the experimenter, once a day, for ~5 min, to familiarize them with the experimental conditions

and to reduce non-specific stress responses (and see Section 2.4). Experimental days were generally carried out twice per week, with at least one day of AOD between test sessions; all other alcohol drinking days in the week involved alcohol-only.

One concern is that the 10mg/L dose of quinine might be too low, and that inability of this quinine level to reduce alcohol intake might reflect it's failure to reduce consumption more generally. While we did not test quinine sensitivity in water in the present study, studies from our lab and others have confirmed that rodents strongly avoid quinine in water at quinine doses that do not reduce alcohol drinking (Hopf *et al.*, 2010, Lei *et al.*, 2016, Sneddon *et al.*, 2019; Katner *et al.*, 2022, reviewed in Radke *et al.*, 2021). In addition, we (De Oliveira Sergio *et al.*, biorxiv) and other recent work (Domi *et al.*, 2021, Suppl.Fig.5E) found that quinine-water intake with 10mg/L is reduced 70–75% relative to water alone. These findings concur that this quinine dose greatly reduces water intake, suggesting that alcohol drinking rodents can sense and avoid quinine when in water, while the willingness to continue drinking alcohol adulterated with quinine suggests that they their responding is aversion-resistant (compulsion-like) for alcohol (reviewed in Radke *et al.*, 2021).

2.2 Cannulas implantation and drugs infusion

After 3–4 weeks in LDA and 2–3 quinine sessions for habituation, surgery was performed to bilaterally implant guide cannulae (Plastics One; 26 gauge) aimed 1mm above the aINS (AP +2.8, ML ± 4.8, and DV −4.7 mm) or mPFC (AP +3.2, ML ± 1.2, and DV −3.0 mm with a 10° angle) (as in Seif *et al.*, 2013; Seif *et al.*, 2015; De Oliveira Sergio *et al.*, 2021). After 7 days of recovery rats returned to LDA and had 2–3 quinine sessions again. Animals were then randomized to receive drug or vehicle during experimental sessions. As described in (De Oliveira Sergio *et al.*, 2021), during a test session, the injection needle was connected to a 10μl microsyringe (Hamilton 701-RN, USA) through a polyethylene tube. The injection needle (Plastics One; 33 gauge) was lowered to reach 1 mm below the lower end of the cannulae. To control the volume and duration of injections, a digital syringe pump (KD Scientific Inc., USA) was programmed to inject a volume of 0.6 μl at 0.2 μl/min of drugs. To reduce reflux, needle was left in place for 1 min before removal.

2.3 Reagents.

Propranolol hydrochloride was obtained from Tocris. Prazosin hydrochloride, Betaxolol hydrochloride and ICI 118,551 hydrochloride were all from Sigma-Aldrich (USA). All drugs were dissolved in sterile saline (0.9%) except prazosin that was dissolved in sterile water. The references for the doses chosen for each compound are described in Supplemental Table 1. All drugs were prepared at the same day minutes before beginning of experiments.

2.4 Overview of experimental design during drinking sessions.

For all studies (systemic or central), animals were exposed to each drinking condition (alcohol-only and quinine-alcohol) and pharmacological treatments (drug vs vehicle) using a within-subject design. Experimental groups were randomized across animals before the beginning of the test sessions. Also, all conditions were balanced to assure that some rats in each experimental condition were tested on same test day. For all systemic administrations

(i.p), rats were habituated to handling by experimenter, and with 1–3 sessions of habituation to i.p. vehicle injection before test sessions (rats received more than one i.p. vehicle habituation sessions until alcohol drinking was not reduced after such injections). This prior habituation with the vehicle is important to reduce stress during the injection procedure. Supplemental Table 2 summarizes the different cohorts of rats used. In study 1, propranolol or vehicle were injected 20 minutes before drinking test sessions at 2.5mg/kg (*n*=10; Fig. 1A), 5mg/kg (*n*=16; Fig. 1B) or 10mg/kg (n=10; Fig. 1C). Some animals were tested with the three doses. Only for propranolol 5mg/kg, a second cohort with 6 rats were added to the experiment. Supplemental materials show a schematic timeline for the propranolol experiments (Suppl.Fig.1A), to show how multiple doses of propranolol were administered within the same rats across time, and how a second cohort was added to test 5mg/kg propranolol.

Another cohort of animals were injected with betaxolol or vehicle at 2.5mg/kg (n=14; Fig. 1D), 5mg/kg (n=15; Fig. 1E) and ICI 118,551 or vehicle 1mg/kg (n=15; Fig. 1F) 30 minutes before the drinking sessions. Betaxolol 5mg/kg and ICI were tested in the same rat, with the order of vehicle, BTX (5 mg/kg) and ICI randomized across rats. For co-administration of an ineffective dose of prazosin (0.25mg/kg) and an ineffective dose of propranolol (2.5mg/kg) rats were first injected with prazosin or vehicle (water), and 10 minutes later to propranolol or vehicle (saline). 20 minutes after the last injection, rats went to drinking session (n=13; Fig. 3B).

For bilateral intra-aINS administration, propranolol or vehicle was tested at 0.5 and 2ug/0.6ul (n=7; Fig. 4A) or 5 and 10ug/0.6ul (n=9; Fig. 4B) 10 minutes before drinking session, and betaxolol or vehicle at 307ng/0.6ul 30 minutes before drinking sessions (*n*=9; Fig. 4C). For bilateral intra-mPFC administration, cannulae and injections were targeted to prelimbic cortex (following previous studies from our, Seif *et al.*, 2013, 2015, and other labs), although we cannot rule out some impact in infralimbic. Propranolol or vehicle was tested at 0.5 and 5ug/0.6ul (n=7; Fig. 5A) 10 minutes before the drinking session. To test the effect of intra-mPFC betaxolol and prazosin, rats were bilaterally injected with 307ng/0.6ul of betaxolol or vehicle (*n*=6; Fig. 5B), or 0.3ug/0.6ul of prazosin or vehicle (*n*=7; Fig. 5C) 30 minutes before drinking sessions.

2.5 Statistics and Data Analysis

Alcohol consumption was determined through changes in bottle weight before and after a drinking session and converted to grams ethanol/kilograms body weight. Concurrent water intake was determined by changes in bottle weights before and after a drinking session and expressed in ml consumption/kilograms body weight. Statistical comparisons were primarily performed in a within-subject manner. Data were mostly analyzed by one- or two-way ANOVA with repeated-measures, while some comparisons used paired t-test or correlation. Statistical analysis was performed using GraphPad Prism or SPSS. All data are shown as mean ± SEM. Also, we describe some potential differences as trends, since such differences would not be considered significant with correction for multiple comparisons, but we include them as information for the reader.

We also examined the effects of drug treatment by normalizing drinking level during drug treatment to the corresponding vehicle session, yielding a percent change in drinking with drug treatment, as we have done before (Seif *et al.*, 2013, 2015; Lei *et al.*, 2019; De Oliveira Sergio *et al.*, 2021). In particular, to further examine drinking changes, across groups and for correlations, we determined the impact of a given treatment using log[100 × (intake during drug treatment)/(intake during vehicle)], as we previously used (detailed in Lei et al., 2019). Log value of 2 (log[100]) indicates no effect of drug on alcohol drinking relative to it's matched vehicle condition, while values lower than 2 indicate a decrease in drinking level with drug. One additional possible concern is that shifts in basal drinking level would impact the change in firing with drug. However, across our many studies, we have found that some treatments reduce drinking level while others don't, irrespective of variation in basal intake (e.g. Seif *et al.*, 2013, Wegner *et al.*, 2019). In addition, we have correlated basal intake with the percent change in drinking with drug, and, e.g. in De Oliveira Sergio *et al.* (2021), systemic prazosin causes a greater percent reduction in CLAD drinking in higher-drinking individuals, while prazosin reduction in drinking level does not correlate with basal AOD intake level. Thus, having somewhat lower basal intake does not impede ability to meaningfully assess the change in drinking with treatment. For this reason, we have found that drug-related percent change in drinking (vs vehicle) to be a valuable measure that is robust to differences in basal intake across conditions that we examine.

Finally, we largely do not examine drug effects on concurrent water intake, since water intake in the 20min sessions is quite low, as we observed in previous studies (e.g. De Oliveira Sergio et al., 2021). Nonetheless, we did examine concurrent water data for Fig. 3F for comparison and information purposes only.

3. Results

3.1 Effect of $\beta_{1/2}$ ARs antagonists on AOD and CLAD

We first tested if systemic administration of a nonselective β ARs antagonist propranolol, at the doses of 2.5, 5, and 10mg/kg, would affect CLAD and/or AOD. Our results showed that 2.5mg/kg of propranolol had no effect on CLAD or AOD [Fig. 1A, n=10; two-way ANOVA; $F_{(treatment;1,9)}=0.563$, $p=0.472$; $F_{(drinking\text{-}condition;1,9)}=0.238$, $p=0.637$; $F_{(interaction;1,9)}=0.066$, $p=0.802$]. Results for propranolol 5mg/kg also reduced drinking levels, with a significant effect of treatment and drinking condition, although no interaction [Fig. 1B, n=16; $F_{(treatment;1,15)}=9.150$, $p=0.009$; $F_{(drinking\text{-}condition;1,15)}=4.878$, $p=0.043$; $F_{(interaction;1,15)}=0.469$, $p=0.504$]. In addition, systemic administration of propranolol 10mg/kg had a significant effect of treatment [Fig. 1C, n=10; two-way ANOVA; $F_{(treatment;1,9)}=26.517$, $p<0.001$; $F_{(drinking\text{-}condition;1,9)}=0.057$, $p=0.816$; $F_{(interaction;1,9)}=0.250$, $p=0.629$]. Together, these data suggest that β-ARs could regulate both AOD and CLAD.

Since propranolol acts through β1 and β2 ARs, we next evaluated if these dose-dependent effects of propranolol on CLAD and AUD could be related to a differential activation of these two ARs. Specifically, we systemically injected betaxolol, a β1 ARs antagonist, or ICI 118,551, a β2 ARs antagonist, and measured AOD and CLAD intake. Our results showed that betaxolol 2.5mg/kg decreased alcohol drinking, with a trend for greater impact

on CLAD [Fig. 1D, n=14; two-way ANOVA; $F_{(treatment;1,13)}$=3.578, p=0.081; $F_{(drinking-condition;1,13)}$=6.341, p=0.025; $F_{(interaction;1,13)}$=4.498, p=0.054]. Betaxolol 5mg/kg was tested in the same rats on a different day as 1 mg/kg of ICI 118,551, with the same vehicle (n=14), and these tests had a significant effect of treatment and drinking condition [Fig. 1E, two-way ANOVA; $F_{(treatment;2,26)}$=5.258, p=0.012; $F_{(drinking-condition;1,13)}$=7.046, p=0.020; $F_{(interaction;2,26)}$=0.241, p=0.788]. In a preliminary attempt to examine the effects of betaxolol 5 mg/kg or ICI 118,551alone, we ran separate two-way ANOVAs, which showed a significant effect of treatment and drinking condition for betaxolol [$F_{(treatment;1,13)}$=7.950, p=0.014; $F_{(drinking-condition;1,13)}$=14.373, p=0.002; $F_{(interaction;1,13)}$=0.057, p=0.815] but not ICI 118,551 [$F_{(treatment;1,13)}$=2.557, p=0.134; $F_{(drinking-condition;1,13)}$=2.591, p=0.131; $F_{(interaction;1,13)}$=0.203, p=0.659]. Taken together, our findings suggest that the effects of propranolol on CLAD are through β2 rather than β1 ARs.

Studies above indicate that propranolol and BTX could reduce alcohol drinking. Thus, we next examined the results by normalizing drinking level during drug treatment to the corresponding vehicle session, as we have done before (Seif *et al.*, 2013, 2015; Lei *et al.*, 2019; Wegner *et al.*, 2019; De Oliveira Sergio *et al.*, 2021) (and see Methods). With this analysis, we found a difference in the impact of 5 mg/kg propranolol on CLAD vs AOD (Fig. 1G, paired t-test t(15)=2.455, p=0.027) but not propranolol 10mg/kg (Fig. 1G, $t_{(9)}$=1.113, p=0.294); betaxolol 2.5mg/kg also showed a difference between CLAD and AOD percent decrease in drinking (Fig.1H, $t_{(13)}$ = 2.24, p=0.043). However, none of these patterns would survive multiple corrections, and should be considered trends.

We next examined the relationship between the impact of propranolol changes AOD and CLAD versus basal drinking levels. We previously showed that the α1 ARs antagonist (prazosin) reduces drinking in both AOD and CLAD, but the changes in AOD with prazosin are unrelated to changes in CLAD, suggesting different α1 ARs-regulated pathways for AOD and CLAD (De Oliveira Sergio et., 2021). In addition, prazosin changes in drinking were related to basal drinking level for CLAD (bigger change in higher basal drinkers) but not AOD. For these analyses, we determined the drinking change with drug versus it's matched vehicle session using log[100 × (intake during drug treatment)/(intake during vehicle)], as we have done previously (De Oliveira Sergio *et al.*, 2021; detailed in Lei et al., 2019). A dashed line indicates where drug intake was not different from vehicle, and values below the dashed line indicate that alcohol drinking under drug exposure was reduced relative to vehicle treatment.

Here, we found a related pattern for propranolol and betaxolol as we previously observed for prazosin. In particular, there was no correlation between 5mg/kg propranolol change in AOD versus change in CLAD (Fig. 2A, R^2=0.078, $F_{(1,14)}$=1.197, p=0.292). Similarly, there were no relations between CLAD changes and AOD changes for 10 mg/kg propranolol (Fig. 2B, R^2=0.372, $F_{(1,8)}$=0.892, p=0.373) or 2.5 mg/kg betaxolol (Fig. 2C, R^2=0.009, $F_{(1,12)}$=0.113, p=0.743). We then examined whether drug-related changes in drinking correlated with basal intake levels. For propranolol 5mg/kg, the drug-related change in firing had a trend relationship with basal AOD intake (Fig. 2D, R^2=0.245, $F_{(1,14)}$=4.559, p=0.051), and the propranol change in CLAD was significant negatively correlated with basal CLAD drinking levels (Fig. 2E, R^2=0.247, $F_{(1,14)}$=4.614, p=0.049), similar to what we reported for prazosin

(De Oliveira Sergio *et al.*, 2021), and suggesting a greater propranolol 5mg/kg effect in higher alcohol drinkers. For 10 mg/kg, there was no relation between basal intake and change in AOD (Fig. 2F, R^2=0.000, $F_{(1,8)}$=0.007, p=0.935) or CLAD (Fig. 2G, R^2=0.003, $F_{(1,8)}$=0.026, p=0.875). Similarly, changes in drinking with 2.5 mg/kg betaxolol were not related to basal AOD (Fig. 2H, R^2=0.084, $F_{(1,12)}$=1.121, p=0.311) or basal CLAD (Fig. 2I, R^2=0.003, $F_{(1,8)}$=0.039, p=0.846) drinking. Finally, we found that the AOD change and CLAD change with 5 mg/kg betaxolol were not correlated (Fig. 2J, R^2=0.006, $F_{(1,12)}$=0.075, p=0.789), while changes in drinking with 5 mg/kg betaxolol were significantly related to basal AOD (Fig. 2K, R^2=0.400, $F_{(1,12)}$=8.004, p=0.015) or basal CLAD (Fig. 2L, R^2=0.616, $F_{(1,12)}$=19.26, p<0.001) drinking.

3.2 Targeting α1 and β ARs together to regulate CLAD and AOD

Despite our findings showing the effects of α1 and β antagonists on CLAD and AOD, these compounds can also provoke a series of undesirable side effects (see Introduction). Thus, any strategy allowing lower the dose of these compounds to reduce drinking could have broad utility. We next investigated if combining a sub-therapeutic doses of these both compounds could affect CLAD and AOD intake. The results showing that combining 0.25mg/kg of prazosin with 2.5mg/kg of propranolol had main effects of treatment and drinking condition, [Fig. 3B, n=14; two-way ANOVA; $F_{(treatment;3,39)}$=7.753, p<0.001; $F_{(drinking\text{-}condition;1,13)}$=10.722, p=0.006; $F_{(interaction;3,39)}$=0.227, p=0.877]. Moreover, for prazosin+propranolol AOD changes were not correlated with CLAD changes (Fig. 3C, R^2=0.181, $F_{(1,12)}$=2.663, p=0.128). Also, basal intake was not correlated with change in drinking for AOD (Fig. 3D, R^2=0.166, $F_{(1,12)}$=2.405, p=0.146) however, changes in CLAD were significantly negatively correlated for basal CLAD intake (Fig. 3E, R^2=0.328, $F_{(1,12)}$=5.859, p=0.033). Further, the administration of prazosin+propranolol did not affect the concurrent water intake [Fig. 3F, n=14; two-way ANOVA; $F_{(treatment;3,39)}$=0.386, p=0.763; F(drinking-condition;1,13)=1.018, p=0.331; $F_{(interaction;3,39)}$=1.586, p= 0.208]. Water intake in the 20min sessions was quite low, as we observed in previous studies (De Oliveira Sergio *et al.*, 2021). Nonetheless, we did this concurrent water data for this particular comparison for information purposes only. Suppl.Fig.2 compares percent change in firing versus vehicle for prazosin+propranolol experiments with other experimental groups. Thus, our data suggest that the ineffective doses of prazosin+propranolol can be used for reducing CLAD and AOD intake.

3.3 Impacts of inhibiting β receptors in aINS on CLAD and AOD

In order to investigate the β ARs signaling in a brain area that regulates CLAD,we next evaluated if the effects of injection into aINS of propranolol or betaxolol. We used a widely utilized doses of intracranial propranolol or betaxolol (see Suppl. Table 2). Intra-aINS propranolol (0.5 and 2 μg/side) did not affect CLAD or AOD [Fig. 4A, n=7; two-way ANOVA; $F_{(treatment;2,12)}$=3.574, p=0.061; $F_{(drinking\text{-}condition;1,6)}$=0.565, p=0.481; $F_{(interaction;2,12)}$ =2.679, p=0.109; *post hoc* veh-vs- propranolol 0.5ug for AOD: p=0.836; CLAD p=0.508; p*ost hoc* veh-vs- propranolol 2ug for AOD: p = 0.940; CLAD: p=0.103]. In a separate cohort, propranolol (5 and 10 μg/side) did not impact CLAD or AOD [Fig. 4B, n=9; two-way ANOVA; $F_{(treatment;2,16)}$=1.785, p=0.200; $F_{(drinking\text{-}condition;1,8)}$=0.011, p=0.917; $F_{(interaction;2,16)}$=1.102, p=0.356]. Also, betaxolol (307ng/side) injection into

aINS did not affect CLAD or AOD [Fig. 4C, n=12; $F_{(treatment;1,11)}$=11.798, p=0.006; $F_{(drinkingcondition;1,11)}$=0.446, p=0.518; $F_{(interaction;1,11)}$ =1.922, p=0.193; *post hoc* veh-vs-betaxolol for AOD: p=0.469; CLAD: p=0.136]. Moreover, when we normalized drinking level to vehicle for each animal that received aINS injections of betaxolol we found a trend for greater change in CLAD versus AOD ($t_{(8)}$=2.229, p=0.074). Suppl. Fig.3 shows aINS histology, and Suppl. Fig. 4 shows the percent change in drinking with drug vs vehicle for intra-aINS studies. Together, our data suggests that aINS β AR signaling did not regulate CLAD or AOD.

3.4 Impacts of inhibiting β receptors in mPFC on CLAD and AOD

Since our data showed no effect of intra-aINS propranolol or betaxolol on alcohol drinking levels, we next examine the potential involvement of β ARs within mPFC (another region that can regulate CLAD, see Discussion). Our results showed that propranolol (0.5 or 5μg/side) did not impact CLAD or AOD **[**Fig. 5A, n=7; two-way ANOVA; $F_{(treatment;2,12)}$=0.487, p=0.626; $F_{(drinking-condition;1,6)}$=0.180, p=0.687; $F_{(interaction;2,12)}$=0.590, p=0.569**]**. The higher dose of propranolol (10μg) or betaxolol (307ng) also did not affect CLAD or AOD [Fig. 5B, n=6; two-way ANOVA; $F_{(treatment;2,10)}$=0.122, p=0.887; $F_{(drinking-condition;1,5)}$=12.944, p=0.016; $F_{(interaction;2,10)}$=0.489, p=0.627]. Based on the lack of effects of the β ARs antagonists into mPFC, we next investigated if the prazosin, an α1 adrenergic antagonist could affect CLAD or AOD in this structure. For this we used the same dose (0.3μg/side) used in our previous results showing that prazosin decreases AOD and CLAD when administrated into aINS (De Oliveira Sergio *et al.*, 2021). However, prazosin into mPFC did not affect CLAD or AOD [Fig. 5C, n=7; two-way ANOVA; $F_{(treatment;1,6)}$=2.002, p=0.207; $F_{(drinking-condition;1,6)}$=0.051, p=0.828; $F_{(interaction;1,6)}$=0.586, p=0.473]. Suppl. Fig.3 shows mPFC histology, and Suppl. Fig. 5 shows the percent change in drinking with drug vs vehicle for intra-aINS studies. Thus, these data suggest that mPFC β and α1 AR signaling did not regulate CLAD or AOD.

4. Discussion

Compulsion-like alcohol drinking, where consumption continues despite negative consequences, is a major obstacle to treating AUD, and is likely related to activation of the stress response system (Koob *et al.*, 2014; Carvalho *et al.*, 2019). Here we investigated the effect of β1/2 antagonists on CLAD and AOD in male rats through systemic administration and intracranial injections into aINS and mPFC, two brain areas related to the compulsion for alcohol in animals and humans. Based on our findings with systemic injections, we also investigated if co-administration of ineffective doses of β ARs antagonist propranolol and α1 ARs antagonist prazosin could affect CLAD and AOD intake.

We found that there was a dose dependent effect of propranolol on CLAD and AOD intake. While 2.5mg/kg of propranolol had no effects, the intermediate dose (5mg/kg) and higher dose (10mg/kg) significantly reduced alcohol drinking, with the intermediate dose having a trend for greater effect on CLAD. Interestingly, these same doses of propranolol decrease operant responding for alcohol reinforced in dependent rats, with only propranolol 10mg/kg affecting the consumption in non-dependent rats (Gilpin *et al.*, 2010). Further, in

alcohol-dependent P rats, 10 but not 5 mg/kg propranolol decrease alcohol intake during early withdrawal (Rasmussen *et al.*, 2014). It is possible that discrepancies in these results, relative to our findings here, is related to the alcohol model used and breed of the animals (P rats versus Wistar rats). Our findings also showed that CLAD drinking changes and AOD drinking changes with the highest dose of propranolol were not correlated, suggesting the presence of different β AR mechanisms that regulate CLAD and AOD. Similarly, our recent study (De Oliveira Sergio *et al.*, 2021) found that the systemic effects of the α1 ARs antagonist prazosin on CLAD versus AOD were not correlated. We speculate that noradrenergic modulation of CLAD and AOD could be regulated through different mechanisms, and future studies will be necessary to better understand these processes.

We next investigated if the dose dependent effect of propranolol on CLAD and AOD intake could be regulated by differential inhibition of β1 ARs and/or β2 ARs, through injection of the β1 antagonist betaxolol and the β2 antagonist ICI 118,551. To our knowledge this is the first study to investigate the effects of betaxolol and ICI 118,551 in animal models of AUD. Our results showed that ICI 118,551 had no effects. However, betaxolol (2.5mg/kg) did significantly reduce drinking, with a trend for a preferential effect on CLAD. Taken together, our findings suggest that propranolol regulation of alcohol intake could be related to an inhibition of β1 rather than β2 ARs. Betaxolol is considered a highly selective antagonist for β1 ARs (Tondo *et al.*, 1985; Satoh *et al.*, 1993) and ICI 118,551 a selective antagonist for β2 ARs (Nathanson, 1988; Willette *et al.*, 1999), and it is known that both ICI 118, 551 and betaxolol promptly cross the blood brain barrier (Swartz, 1998; Moresco *et al.*, 2000; Hare *et al.*, 2006). Prior findings investigating the effects of betaxolol and ICI 118, 551 in animal addiction models have produced mixed results. Systemic administration of betaxolol at 5mg/kg during early cocaine withdrawal decreases cocaine withdrawal-related anxiety-like behavior in rats, without affecting the locomotor activity (Rudoy *et al.*, 2007). However, betaxolol has no effect on cocaine self-administration (Wee *et al.*, 2008), and ICI 118,551 but not betaxolol (10mg/kg) blocks stress-induced reinstatement of cocaine in mice (Mantsch *et al.*, 2010). Furthermore, 20mg/kg betaxolol (but not lower doses) block reinstatement of cocaine induced by stress (Vranjkovic *et al.*, 2012). Interestingly, betaxolol and ICI alone have no effect on the compulsion-like behavior assessed by the nestlet shredding model, but the combination of both drugs decreases this compulsion-like behavior (as does propranolol at 10mg/kg) (Lustberg *et al.*, 2020), suggesting β2 importance for some compulsion-related conditions.

Despite the effects of propranolol and betaxolol on alcohol drinking, one significant challenge with ARs compounds is that they also regulate cardiovascular function, with potential for significant side effects (also, betaxolol is only FDA approved to treat an ophthalmic condition). Thus, the development of strategies that could decrease the doses of these drugs used could be an advantage for AUD pharmacotherapy. We thus investigated how combined inhibition of β ARs and α1 ARs, using ineffective doses of propranolol (2.5mg/kg) and prazosin (0.25mg/kg) in combination, could impact alcohol consumption. We found that combining the lower doses of propranolol and prazosin significantly reduced alcohol intake. Interestingly, changes on AOD and CLAD were not correlated, and the ability of this combined treatment to reduce CLAD was correlated with basal intake, with a greater impact in higher drinkers, as we observed here with 5mg/kg of propranolol and

BTX, and we showed in our previous study with 0.75mg/kg prazosin (De Oliveira Sergio *et al.*, 2021). In addition, the ineffective dose of prazosin used here (0.25mg/kg) also did not show effects in a model of self-administration of nicotine (Forget *et al.*, 2010). Further, one study combined lower, ineffective doses of propranolol and prazosin (10mg/kg and 1mg/kg, respectively), which decreased alcohol intake in dependent P rats during early withdrawal without affecting locomotion (Rasmussen *et al.*, 2014). Co-administration of prazosin and propranolol also decreases compulsion-like behavior in mice in the marble burying model, and the combination of each drug was more effective than each drug alone (Lustberg *et al.*, 2020). We found that combining prazosin and propranolol impacted both CLAD and AOD (Fig. 3, Suppl. Fig.1), supporting the possible use of combined therapy to reduce alcohol use disorder.

We then examined whether the systemic effects of propranolol and betaxolol could be mediated by two brain areas relate to CLAD, aINS and mPFC. However, all doses tested of intra-aINS propranolol and betaxolol did not affect CLAD or AOD. Furthermore, our findings showed no effect of propranolol or betaxolol into mPFC on CLAD or AUD. Similarly, 0.3μg of prazosin within mPFC did not affect CLAD or AOD intake, while a similar dose of prazosin within aINS decreases both CLAD and AOD (De Oliveira Sergio *et al.*, 2021). The aINS and mPFC receive noradrenergic projections from the Locus Coeruleus (Robertson *et al.*, 2013). Although we found no effects of intra-aINS propranolol, a prior study showed that propranolol at 2.5μg into aINS decreases incidental taste learning, but not aversive taste learning (Miranda *et al.*, 2008). Also, 5μg of propranolol in aINS decreases arousal-induced neophobia although the doses of 1 and 10μg have no effect (Rojas *et al.*, 2015). The same has been shown for mPFC, where propranolol control different responses. 5μg propranolol in mPFC affects aversive taste association as well as aversive retrieval, but not incidental taste memory formation (Reyes-Lopez *et al.*, 2010). Also, systemic administration of propranolol (10mg/kg) regulates the stress-induced changes, mediated by mPFC, that contribute to impaired fear extinction (Fitzgerald *et al.*, 2015).

Although, our data found no effects of propranolol on CLAD and AOD when injected direct into aINS or mPFC, a recent study showed that propranolol injection into central amygdala decreases alcohol intake only in dependent rats, while the prazosin injection in this area decreases alcohol intake only in non-dependent rats (Varodayan *et al.*, 2022). Also, propranolol in basolateral amygdala prevents reinstatement of alcohol seeking in rats (Chesworth *et al.*, 2018). Thus, we speculate that systemic effects of propranolol on alcohol intake could be mediated through amygdala regions, while the effects of prazosin, based on our recent study, could be mediated through aINS (De Oliveira Sergio *et al.*, 2021).

This study has some limitations. First, we only used male rats, and another recent study from our laboratory showed that males and females have different strategies on CLAD intake, with females being more persistent than males for alcohol (De Oliveira Sergio *et al.*, biorxiv). Thus, it would be of value to investigate the effect of these compounds in females. However, despite these limitations, the present findings provide new insights about the effect of the β ARs modulators on alcohol drinking, and, especially, the advantage of using lower doses of propranolol and prazosin in combination as a pharmacological strategy for AUD treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

6. Acknowledgements

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Highlights

- Systemic beta-adrenergic receptor inhibition reduces drinking through beta-1 receptors.
- Beta-receptor inhibition of drinking is larger in rats with higher basal drinking levels.
- Combining subthreshold doses of beta-receptor blocker (propranolol) and alpha1-receptor blocker (prazosin) decreases alcohol drinking.
- Effectiveness of combined lower doses may represent a novel therapeutic intervention with fewer side effects.
- Beta-receptor inhibition in the anterior insula surprisingly did not reduce alcohol intake.
- Beta- and alpha1-receptor inhibition in the medial prefrontal cortex surprisingly did not reduce alcohol intake.

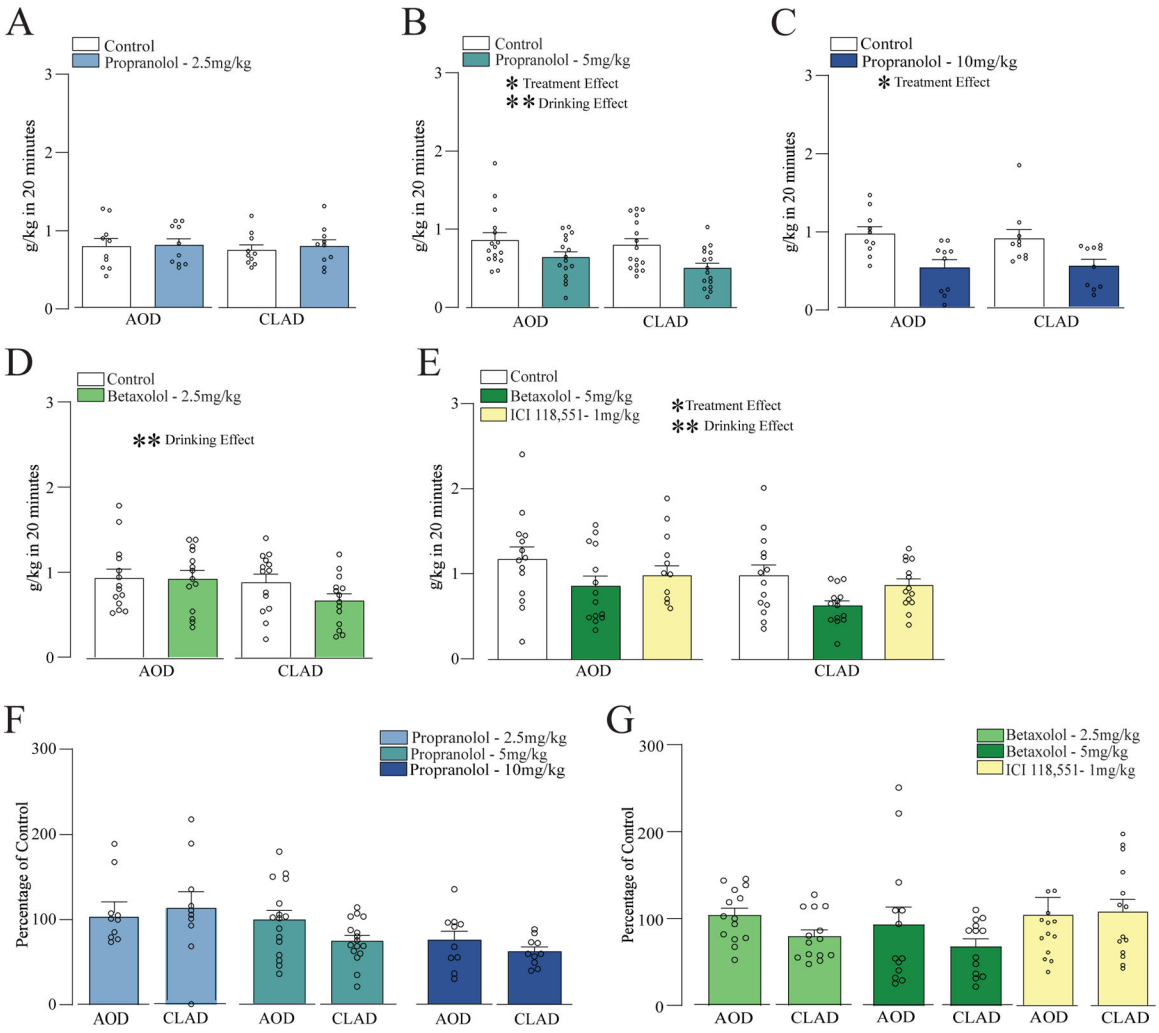

Figure 1. Dose dependent effects of inhibiting β ARs and β1 ARs with propranolol and betaxolol on alcohol drinking.

(A-C) Systemic administration of propranolol at **(A)** 2.5mg/kg, **(B)** 5mg/kg, and **(C)** 10mg/kg**. (D)** Systemic administration of β1 AR antagonist betaxolol at **(D)** 2.5mg/kg. **(E)** Systemic administration of betaxolol 5mg/kg or β2 ARs antagonist ICI 118,551. **(F)** Percentage change in drinking (relative to vehicle) for propranolol at 2.5, 5 and 10mg/kg. **(G)** Percentage of vehicle change for betaxolol 2.5, 5mg/kg and ICI 118,551. *,** $p<0.05$, $p<0.01$ for treatment or drinking condition effects, from two-way ANOVA.

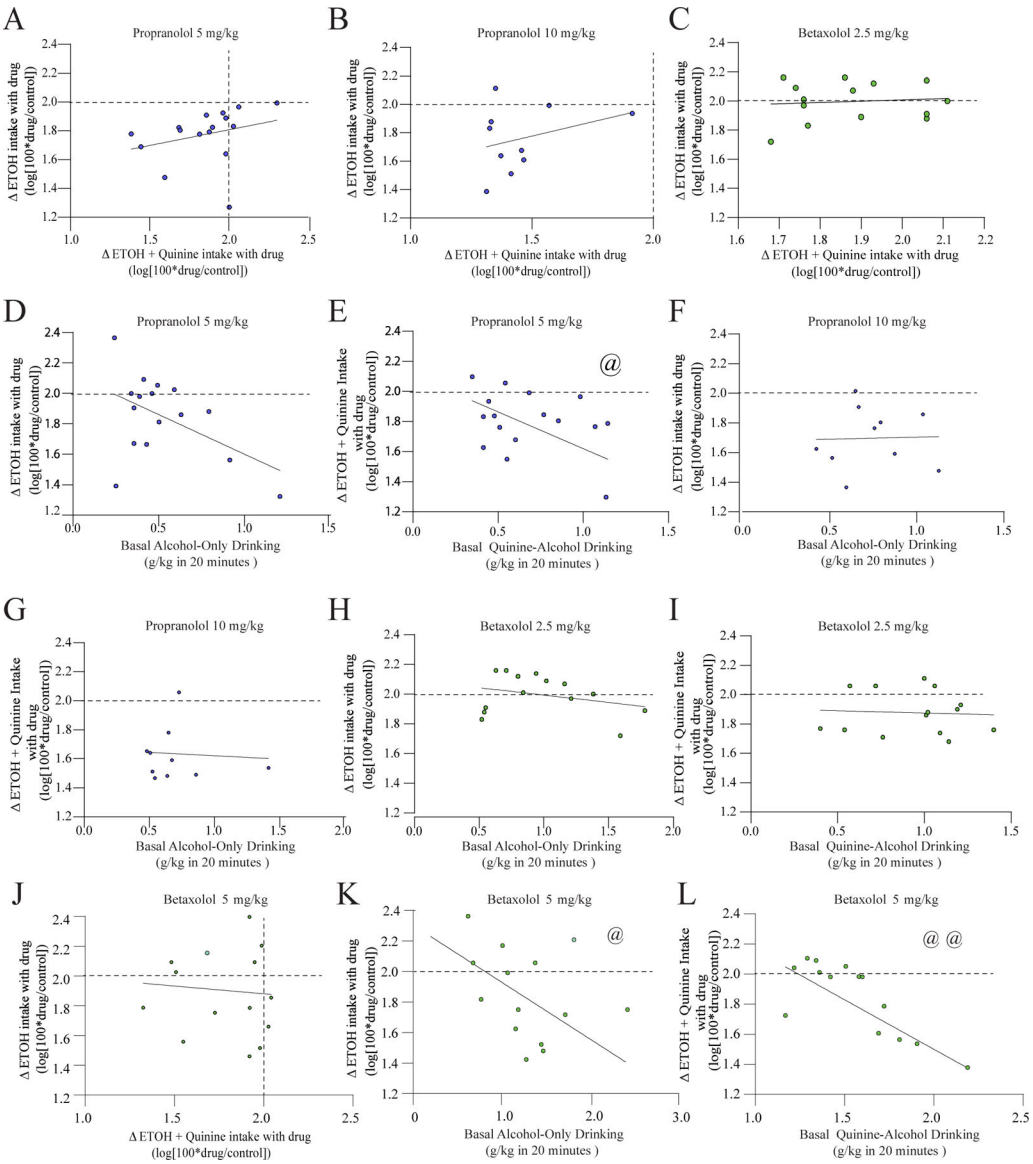

Figure 2. Correlations related to propranolol and betaxolol effects on drinking.

(A-C) Drug-related changes in AOD and changes in CLAD were not correlated for 5 mg/kg propranolol **(A)**, 10 mg/kg propranolol **(B)**, or 2.5 mg/kg betaxolol **(C)**. **(D,E)** Relation between changes in drinking with 5 mg/kg propranolol (y-axis) and basal intake levels (x-axis) for AOD **(D)** and CLAD **(E)**. **(F,G)** Relation between change in drinking with 10 mg/kg propranolol and basal intake levels for AOD **(F)** and CLAD **(G)**. **(H,I)** Relation between change in drinking with 2.5 mg/kg betaxolol and basal intake levels for AOD **(H)** and CLAD **(I)**. **(J)** Drug-related changes in AOD and changes in CLAD were not correlated for 5 mg/kg betaxolol. **(K,L)** Relation between change in drinking with 5 mg/kg betaxolol and basal intake levels for AOD **(K)** and CLAD **(L)**. A dashed line indicates where drug intake was not different from vehicle, and values below the dashed line indicate that alcohol drinking under drug exposure was reduced relative to vehicle treatment. @, @@ $p<0.05$, $p<0.01$ correlation.

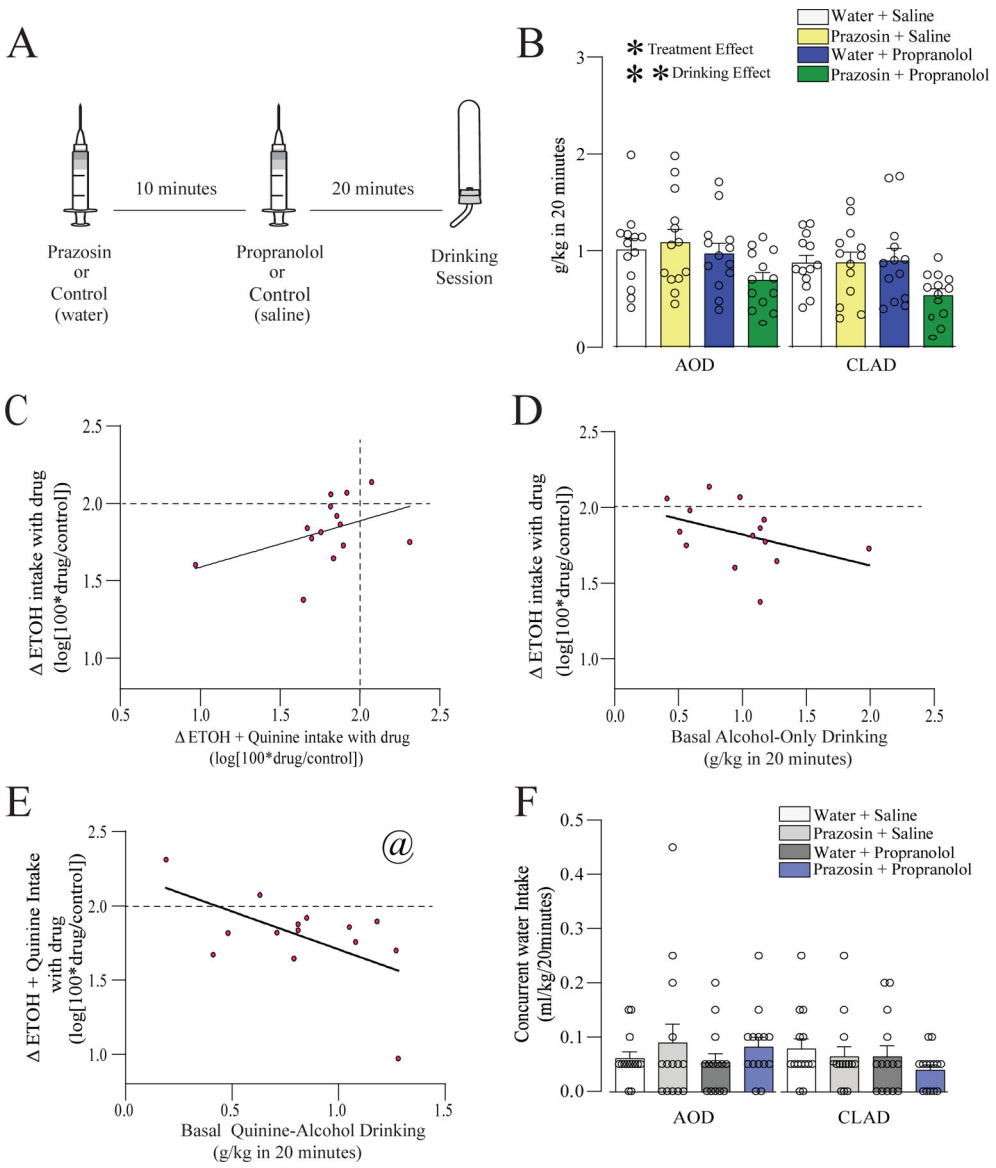

Figure 3. Co-administration of ineffective doses of prazosin and propranolol reduced AOD and CLAD.

(A) Schematic experimental timeline for co-administration of ineffective doses of prazosin and propranolol. Prazosin (0.25mg/kg) or vehicle was first injected, and 10 minutes animals were injected with propranolol (2.5mg/kg) or vehicle and then 20 minutes later were exposed to AOD or CLAD drinking. **(B)** Systemic administration of prazosin or propranolol alone did not affect AOD or CLAD. However, co-administration of these drugs together decreased both drinking conditions. **(C)** Prazosin+propranolol changes in AOD and CLAD were not correlated. **(D)** Changes in AOD were not correlated with the basal AOD. **(E)** Changes on CLAD were negatively correlated with basal quinine-alcohol drinking. **(F)** Co-administration of prazosin and propranolol did not change the concurrent water intake. A dashed line in **(C-E)** indicates where drug intake was not different from vehicle, and values below the dashed line indicate that alcohol drinking under drug exposure was reduced

relative to vehicle treatment. *,** $p<0.05$, $p<0.01$ for treatment or drinking condition effects, from two-way ANOVA. @ $p<0.05$ correlation.

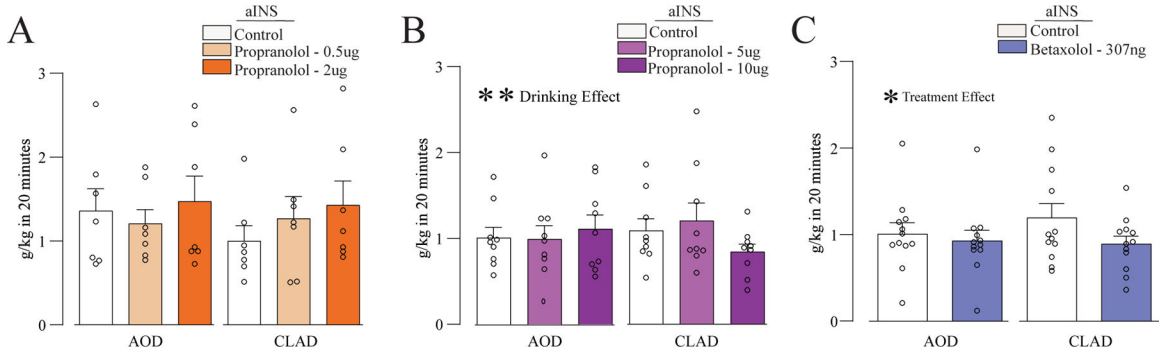

Figure 4. Inhibition of β ARs in aINS did not affect AOD or CLAD.

(A,B) Intra-aINS administration of propranolol (0.5, 2, 5, or 10μg) had no impact on alcohol intake. **(C)** Administration of β1 ARs antagonist betaxolol (307ng) into aINS had limited effects on CLAD (see Results). *,** $p<0.05$, $p<0.01$ for treatment or drinking condition effects, from two-way ANOVA.

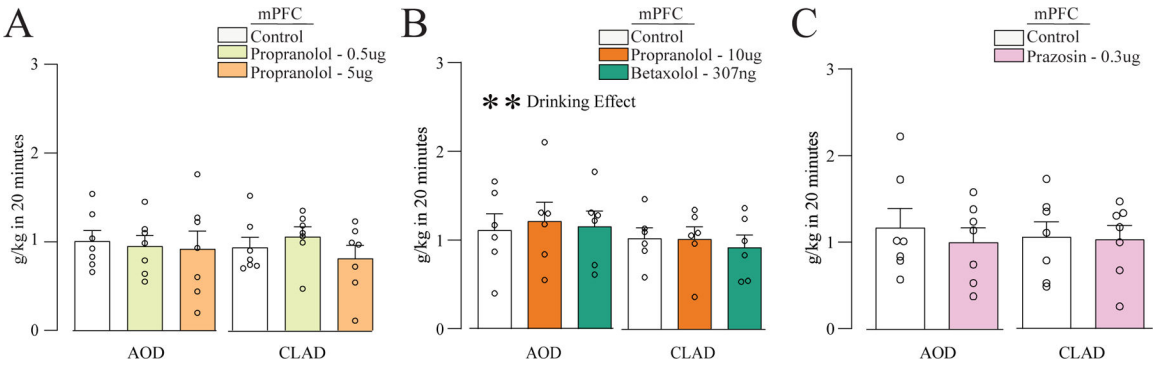

Figure 5. Inhibition of β and α1 ARs in mPFC did not affect AOD or CLAD.

Intra-mPFC injection of propranolol 0.5 or 5μg **(A)**, propranolol 10μg or betaxolol 307ng **(B)**, or prazosin 0.3μg **(C)**, had no impact on AOD or CLAD.