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Selective enhancement of alcohol aversion by nicotinic acetylcholine receptor drugs

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Abstract

The prevalence of alcohol use disorders (AUDs) has steadily increased in the United States over the last 30 years. Alcohol acts on multiple receptor systems including the nicotinic acetylcholine receptors (nAChRs), which are known to mediate alcohol consumption and reward. We previously reported that the preclinical drug sazetidine-A, a nAChR agonist, reduces alcohol consumption without affecting nicotine consumption in C57BL/6J mice. Here, we found that sazetidine-A enhances alcohol aversion without affecting the expression or acquisition of conditioned alcohol reward in C57BL/6J mice. Microinjection of sazetidine-A into the ventral midbrain targeting the ventral tegmental area (VTA) reduced binge alcohol consumption, implicating the neurocircuitries in this region in mediating the effects of sazetidine-A. Furthermore, sazetidine-A-induced reduction in alcohol consumption was mediated by non-α4 containing nAChRs, as sazetidine-A reduced binge alcohol consumption in both α4 knock-out and wild-type mice. Finally, we found that in mice pre-treated with sazetidine-A, alcohol induced *Fos* transcript within *Th*-expressing but not *Gad2*-expressing neurons in the VTA as measured by increased *Fos* transcript expression. In summary, we have identified a previously unknown role for non-α4 nAChRs in alcohol aversion that likely mediates the reduction in alcohol consumption induced by sazetidine-A. Elucidating the identity of non-α4 nAChRs in alcohol aversion mechanisms will provide a better understanding the complex role of nAChRs in alcohol addiction and potentially reveal novel drug targets to treat AUDs.

Introduction

Alcohol use disorders (AUD) have steadily increased in the United States, along with associated increases in high-risk drinking and mortality [1]. An estimated 30 million Americans met criteria for an AUD in 2013, an increase of 12 million cases compared with the previous decade [1]. Currently, there are only 3 approved pharmacotherapies for AUD (naloxone, acamprosate, disulfiram), all with variable success rates [2], which highlights the need to further understand how alcohol interacts with other receptor systems and identify new drug targets.

The neuronal nicotinic acetylcholine receptors (nAChRs) play an important role in mediating the rewarding properties of alcohol. The nAChRs are pentameric cation channels located on presynaptic terminals and cell bodies of neurons, thus modulating cell excitability and neurotransmitter release [3, 4]. There are 11 nAChR subunits found in the brain (α2-7, α9-10, β2-4) that can combine to form multiple nAChR subtypes, each with its own unique receptor properties such as different ligand binding affinities, calcium permeability and expression patterns [3]. Alcohol does not directly agonize nAChRs, but modulates nAChR functional activity [5] and increases the release of acetylcholine (ACh), the endogenous ligand for nAChRs [6]. Both nAChR antagonists such as mecamylamine (a non-specific antagonist) and partial agonists such as varenicline (an α4β2 partial agonist) reduce alcohol consumption in rodents, illustrating the complex involvement of nAChRs in mediating alcohol consumption [7–9]. Varenicline has been tested in human trials for alcohol dependence with mixed results, with some studies reporting reduced alcohol consumption [10–12] but not others [13, 14].

The preclinical drug sazetidine-A initially agonizes and then desensitizes α4β2, α3β4*, α6* and α7 nAChR subtypes (*denotes additional subunits in the nAChR pentamer) [15–18]. We previously reported an alcohol-specific effect of sazetidine-A in

mice; a peripheral injection reduced continuous and binge alcohol consumption in male and female C57BL/6J mice without affecting the consumption of water, saccharin, or nicotine [19]. Here, we investigated the mechanism by which sazetidine-A reduces alcohol consumption. We report that sazetidine-A selectively enhances alcohol aversion without affecting the expression or acquisition of alcohol reward, and identify a previously unknown role for non-α4 containing nAChRs in alcohol aversion mechanisms.

Materials and Methods

Animals and Drugs

Eight week old adult male C57BL/6J mice (Jackson Laboratory, Sacramento, CA) acclimated to our facility for a minimum of six days before behavioral experiments. Heterozygous α4 nAChR subunit knock-out (KO) breeder pairs were provided by Dr. Jerry Stitzel and α4 nAChR knock-out and their wild-type (WT) littermates were bred on site. All mice were group housed in standard cages under a 12-hour light/dark cycle until the start of behavioral experiments, when they were individually housed. All animal procedures were performed in accordance with the Institutional Animal Care and Use Committee at the University of Minnesota, and conformed to NIH guidelines (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2010). Alcohol (Decon Labs, King of Prussia, PA) was mixed with tap water or saline to 20% v/v. Sazetidine-A dihydrochloride was purchased from Tocris Bioscience (Bio-techne, Minneapolis, MN), and made fresh for each experiment by dissolving in saline. All peripheral injections were administered intraperitoneally and all experiments were performed in separate groups of drug naïve mice.

Ventral tegmental area (VTA) microinfusion and binge alcohol consumption in male C57BL/6J mice.

Male C57BL/6J mice were anesthetized with ketamine/xylazine (80:10 mg/kg *i.p.*) and implanted with 26 gauge stainless steel guide cannula (Plastics One, Roanoke, VA) targeting the VTA (-3.2mm AP, +/- 0.5mm ML, and -4.96 mm ventral to the surface according to the Paxinos and Watson brain atlas). The cannulae were fixed with dental adhesive (Geristore, DenMat, Lompoc, CA). Mice then recovered for 3 weeks in their home cage. One week prior to the binge consumption procedure, mice were habituated to handling and received 3 saline infusions at a volume of 0.5 µL, at a rate of 0.25 µL/min.

For the binge drinking-in-the-dark (DID) procedure, mice were presented with a bottle of 20% alcohol for 2 hours, starting 3 hours into the dark cycle for 4 consecutive days, based on Rhodes, et al. [20]. The water bottle was removed only when the alcohol bottle was present, but was freely available at all other times. On Day 5, mice were infused with either saline (1µL) or sazetidine-A (1µg) at a rate of 0.25 µL/min, 20 minutes prior to the presentation of the alcohol bottle for 4 hours. Alcohol consumption was calculated by volume, and spillage or evaporation was controlled for by a bottle in an empty control cage. After the completion of the experiment, injection sites were visualized by infusing 1µL of ink. Mice with injections outside of the VTA were excluded from the analysis.

Binge alcohol consumption in female α4 wild-type and knock-out mice

Binge alcohol consumption was performed as described above with the following modifications: Mice were habituated to three *i.p.* injections of saline (10µL/g) one week prior to the experiment. Mice were presented with a bottle of 20% alcohol for 2 hours, starting 2 hours in the dark cycle for 3 consecutive days. On Day 4, mice were injected

with either saline or sazetidine-A (1mg/kg *i.p.*) 1 hour prior to the presentation of the alcohol bottle for 4 hours.

Expression and acquisition of alcohol conditioned place preference (CPP)

Male C57BL/6J mice were tested in an unbiased alcohol CPP procedure modified from Cunningham, et al. [21]. The chamber apparatus contained a standard two-compartment place preference insert with different floor textures (Med Associates, St. Albans, VT). The procedure consisted of one habituation session, ten conditioning sessions, one preference test (Test 1), and one test session with sazetidine-A pre-treatment (Test 2). For the habituation session, mice were injected with saline and placed in the apparatus with access to both chambers for 30 minutes, and the baseline time spent in each chamber was recorded. For the conditioning sessions, alcohol (2g/kg *i.p.*) or an equivalent volume of saline was injected and mice were immediately confined to one chamber for 5 minutes. The next day, mice received the opposite injection paired with the alternate chamber, and the pairings were alternated each day for 10 total sessions. Control mice received saline paired with both sides. For the first preference test (Test 1), mice were injected with saline and had access to both sides for 30 minutes. The alcohol CPP index was calculated as the time spent in the alcohol-paired chamber during test day minus time spent in that same chamber on habituation day. The next day, all alcohol-conditioned mice that had a positive CPP index were randomly divided into 2 groups that were pre-treated with sazetidine-A (1mg/kg, *i.p.*) or saline 1 hour prior to a second 30 min preference test (Test 2).

To test the effect of sazetidine-A on the acquisition of alcohol CPP, mice underwent a similar procedure described above except that mice were pre-treated with sazetidine-A (1mg/kg, *i.p.*) or saline, 1 hour before the alcohol-paired session. For the saline-paired sessions, mice received a pre-treatment saline injection. The alcohol CPP

index was calculated as the time spent in the alcohol-paired chamber during test day minus time spent in that same chamber on habituation day.

Alcohol conditioned place aversion (CPA)

Male mice were tested in an unbiased alcohol CPA procedure, which was similar to the alcohol CPP procedure except that the mice received an injection of alcohol (2g/kg, *i.p.*) or saline immediately upon removal from the chamber. A second experiment testing the aversive effects of mecamylamine was conducted in a different cohort of mice. Mecamylamine (2 or 3mg/kg *i.p.*) or saline was injected 20 minutes prior to the second aversion test (Test 2). The alcohol CPA index was calculated was calculated similarly to the alcohol CPP index.

Place conditioning with sazetidine-A

Male C57BL/6 mice were tested in a place conditioning procedure consisting of one habituation session, ten daily conditioning sessions and one test. For the habituation session, mice were injected with saline and had access to both chambers for 30 minutes. For the conditioning sessions, mice were treated with sazetidine-A (1mg/kg *i.p.*) or saline 1 hour prior to the 30-minute conditioning session. The next day, mice received the opposite injection paired with the alternate chamber, and the pairings were alternated each day. Control mice received saline paired with both sides. For the test session, mice were injected with saline and had access to both chambers for 30 minutes. The conditioning index was calculated similar to the CPP index.

Alcohol loss-of-righting reflex (LORR)

Male C57BL/6J mice were pre-treated for 1 hour with sazetidine-A (1mg/kg, *i.p.*) or saline. Mice were then injected with 4g/kg alcohol *i.p.* and placed on their backs,

alone, in clean cages. Righting reflex was considered lost when the mouse failed to right itself for at least 30 seconds after administration of the alcohol injection. The mouse was considered to have recovered the righting reflex when it could right itself three times within 30 seconds.

Locomotor activity

Male C57BL/6J mice were pre-treated with sazetidine (1mg/kg, *i.p.*) or saline for 1 hour and then placed in a locomotor chamber (Med Associates, St. Albans, VT) for 1 hour. Total distance traveled, measured via infrared beam breaks, was measured in 5 minute time blocks and compared across the two treatment groups.

c-Fos immunohistochemistry

To compare the effect of sazetidine-A on alcohol-induced c-Fos expression in the VTA, male C57BL/6J mice divided into four treatment groups: 1) saline pre-treatment for 1 hour, then injected with alcohol (saline-alcohol group), 2) sazetidine-A pre-treatment for 1 hour, then injected with alcohol (SAZ-alcohol group), 3) sazetidine-A pre-treatment for 1 hour, then injected with saline (SAZ-saline group), and 4) injected with saline during both timepoints (saline-saline group). All injections were *i.p.* with sazetidine-A at 1mg/kg and alcohol at 2g/kg. Ninety minutes after the last injection, mice were anesthetized with sodium pentobarbital and transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed and post-fixed in PFA and dehydrated in sucrose prior to freezing. Each brain was cut into 30 µm sections on a cryotstat (ThermoFischer). The expression of c-Fos in the VTA was detected using a 1:1000 dilution of a polyclonal anti-cFos primary antibody (EMD Millipore Corp, cat# PC05), a 1:50 dilution of a biotin labeled goat anti-rabbit secondary antibody (BD Biosciences, cat# 550338), followed by avidin/biotin peroxidase detection (Vectastain, Vector Laboratories).

Quantification of c-Fos expression in slices was performed based on a modification of a previously described procedure [22, 23]. Briefly, sections containing the VTA were imaged on a Nikon SMZ1270 with a 6X objective lens. Image analysis and c-Fos quantification was performed using the NIH freeware program ImageJ. A total of 5-7 sections were collected per mouse. The VTA was outlined bilaterally on the images by an experimenter blinded to the treatment groups using the ImageJ Region of Interest tool, and the number of c-Fos+ cells for each section was counted automatically using histogram-based thresholding (RenyiEntropy). The average number of c-Fos+ cells per VTA section were averaged for all mice per group and compared across treatments.

Fluorescent in situ hybridization (FISH)

To compare the effect of alcohol and sazetidine-A on *Fos* transcript expression within neuronal subpopulations, mice were pre-treated with saline for 1 hour, followed by an injection of 1) saline (saline-saline), 2) alcohol (saline-alcohol) or 3) sazetidine-A (saline-SAZ). A fourth group was pre-treated with 1mg/kg sazetidine-A for 1 hour and then injected with 2g/kg alcohol (SAZ-alcohol group). Mice were sacrificed and brains were removed, snap frozen in isopentane, and sectioned on a cryostat (HM 525 NX, ThermoFischer) into 16 µM sections. Sections were adhered to Superfrost® Plus slides, kept at -20°C for 60 minutes to dry and stored at -80°C until use. Sections were fixed with 4% PFA for 1 hour and processed for RNAScope (Advanced Cell Diagnostics) multichannel FISH according to manufacturer instructions for fluorescent multiplex assays. Sections were counterstained with DAPI for 20 seconds at room temperature, coverslipped with Prolong Gold Antifade (ThermoFisher Scientific), and stored at 4°C. Probes for detection of specific targets *(Fos, Th*, *Gad2*) were purchased from Advanced Cell Diagnostics (ACD; http://acdbio.com/). Separate slices were processed for *Fos*+*Th*, and *Fos*+*Gad2*.

FISH image acquisition and analysis

Sections containing the VTA were imaged on a Keyence BZ-X700 epifluorescent microscope at 20X magnification. Images for each channel were obtained in z-stack and stitched using Keyence analysis software, and all images were acquired and processed in the same manner. Multichannel images were opened and all channels (including DAPI) were overlaid. A fixed area was imposed around the VTA based on stereotaxic landmarks to establish regional boundaries. Cells within the boundaries of the VTA were considered positive for each transcript when at least 5 puncta were observed within each cell, and cell counts were tracked by a blinded researcher using the Cell Counter plugin in ImageJ v1.52e [24]. 3-4 mice for each treatment group were analyzed, with 4 slices (2 *TH+Fos* and 2 *GAD+Fos*) per mouse.

Statistical Analysis

All analyses were calculated using Prism 8.0 (GraphPad, La Jolla, CA). Data were tested for normality and variance, and outliers were detected using the Grubb's test. Welch's corrections were used if variances were unequal. Establishment of alcohol preference or aversive conditioning was analyzed using one-sample t-tests. Comparison across treatment groups was analyzed using Student's *t*-tests or one-way ANOVA followed by multiple comparisons test (Dunnett's multiple comparison tests or unpaired *t*-tests with Welch's corrections). Comparison of data across time used two-way repeated measures ANOVA followed by Tukey's multiple comparisons tests.

Results

Microinjection of sazetidine-A into the ventral midbrain reduced alcohol consumption. We tested whether administration of sazetidine-A into the ventral midbrain targeting the VTA would reduce acute binge alcohol consumption in a modified drinking-in-the-dark (DID) procedure. Mice microinjected with 1mg/kg sazetidine-A consumed significantly less alcohol during the binge session compared with mice infused with saline (**Fig. 1**, unpaired t-test, t=2.965, P=0.02).

Sazetidine-A enhanced alcohol aversion without affecting alcohol reward. To determine the behavioral mechanism by which sazetidine-A reduced alcohol consumption, we first tested the effect of sazetidine-A on the expression of alcohol reward using conditioned place preference (CPP). Male C57BL/6J mice were conditioned with 2g/kg *i.p.* alcohol injections, which produced significant preference for the alcohol-paired chamber compared with saline conditioning (**Fig. 2A**, Student's t-test between saline and alcohol groups: t=2.675, P=0.01; one-sample t-test compared with an index of zero: saline group t=1.384, P=0.19; alcohol group t=3.123, P=0.004). The next day, when mice were pre-treated with sazetidine-A (1mg/kg *i.p.*) or saline 1 hour prior to a second alcohol preference test, we found no significant difference in the alcohol CPP indices (**Fig. 2B**, t=0.191, P=0.85), indicating that sazetidine-A does not impact the expression of alcohol CPP.

We then tested whether sazetidine-A impaired the acquisition of alcohol reward with a separate group of mice that were trained in an alcohol CPP procedure in which sazetidine-A (1mg/kg) or saline was injected 1 hour prior to each alcohol conditioning session. We found no significant difference in alcohol CPP index on test day between the sazetidine-A and saline pre-treated groups, indicating that sazetidine-A does not impact the acquisition of alcohol CPP (**Fig. 2C**, t=0.26, P=0.79; one-sample t-test

compared with an index of zero: saline pre-treated group: t=3.106, P=0.01; sazetidine-A pre-treated group: t=2.250, P=0.05).

We then tested whether sazetidine-A affected the expression of alcohol aversion using conditioned place aversion (CPA). Male C57BL/6J mice were conditioned with 2g/kg alcohol injections, which produced significant aversion for the alcohol-paired chamber compared with saline conditioning (**Fig. 2D**, one-sample t-test compared with an index of zero: saline group t=1.18, P=0.25; alcohol group t=4.47, P=0.001). The next day, when mice were pre-treated with sazetidine-A (1mg/kg) or saline, we found that sazetidine-A enhanced alcohol CPA (**Fig. 2E**, t=2.28, P=0.03).

Since sazetidine-A initially activates and then desensitizes nAChRs, we determined whether nAChR activation or desensitization was important for the enhancement of alcohol aversion by testing mecamylamine, a non-specific nAChR antagonist, on the expression of alcohol aversion. Alcohol aversion conditioning produced significant aversion in all groups of mice (**Fig. 2F**, one-sample t-test compared with an index of zero: saline group t=5.68, P<0.0001; mec 2 mg/kg group t=5.28, P=0.0005, mec 3 mg/kg group t=5.61, P=0.0003). The next day, pre-treatment with mecamylamine 20 minutes prior to a second aversion test enhanced aversion at the 3 mg/kg dose, but not the 2 mg/kg dose, compared with the saline pre-treated group (**Fig. 2G**, one-way ANOVA F(2,32)=6.38, P=0.005). These results indicate that antagonism of nAChRs, rather than activation of nAChRs, mediates enhanced alcohol aversion.

Sazetidine-A did not require the α4 nAChR subunit to reduce binge alcohol consumption. To begin determining which nAChR subunits were important for the actions of sazetidine-A, we tested the effect of sazetidine-A in mice that lack the α4 nAChR subunit in the DID procedure. As we previously showed that sazetidine-A could reduce alcohol consumption in both sexes [19], we used female α4 nAChR WT and KO

mice in this experiment. We found that sazetidine-A (1mg/kg) reduced alcohol consumption in both α4 WT and KO mice, as there was a main effect of drug treatment with no effect of genotype or an interaction between drug treatment and genotype (**Fig. 3A**, 2-way ANOVA $F_{interaction}(1,27)=0.281$, $P=0.60$; $F_{treatment}(1,27)=13.08$, $P=0.001$; $F_{genotype}(1,27)=0.004$, $P=0.95$). Thus, this data suggest that sazetidine-A reduces alcohol consumption by acting on non-α4* nAChRs.

Sazetidine-A enhanced the sedative-hypnotic effects of alcohol. We tested whether sazetidine-A affected acute alcohol-induced sedation using the loss-of-righting-reflex (LORR) test. Pre-treatment with sazetidine-A (1mg/kg) for 1 hour enhanced alcohol LORR duration produced by 4 g/kg alcohol compared with saline pre-treatment (**Fig. 3B**, $t=2.55$, $P=0.04$).

Sazetidine-A treatment does not affect place conditioning or locomotor activity. All our alcohol consumption and conditioning experiments used a 1 hour pre-treatment of 1mg/kg sazetidine-A. We tested whether this dose and timing of sazetidine-A had any rewarding or aversive effects alone in place conditioning, and whether it affected locomotor activity. We found that 1mg/kg sazetidine-A produced no reward or aversion and was similar to the conditioning produced by saline (**Fig. 3C**, $t=0.559$, $P=0.59$). We found no main effect of drug treatment on locomotor activity as measured by beam breaks (**Fig. 3D**, 2-way RM ANOVA $F_{interaction}(11,154)=4.75$, $P=0.07$; $F_{treatment}(1,154)=0.89$, $P=0.22$; $F_{time}(11,154)=48.80$, $P<0.0001$).

Sazetidine-A induced neuronal activation in the VTA primarily in TH-expressing neurons. Pre-treatment with sazetidine-A (1mg/kg) prior to a 2g/kg alcohol injection produced an increase in c-Fos+ cells in the VTA compared with saline pre-treatment

prior to alcohol injection, as detected by immunohistochemical DAB staining (one-way ANOVA with Welch's correction F(3,11)=4.34, *P*=0.03; multiple comparisons test sal/alc versus SAZ/alc: **P*=0.046, **Fig. 4**). There was no significant difference in c-Fos+ cells between the alcohol and saline treatment conditions (*P*=0.48 for sal/sal versus sal/alc). Sazetidine-A pre-treatment followed by saline injection increased c-Fos+ cells compared with saline pre-treatment (^*P*=0.03 for sal/sal versus SAZ/sal), and also produced greater c-Fos+ cells compared with saline pre-treatment followed by alcohol injection (^*P*=0.02 for sal/alc versus SAZ/sal). Together, these data show that the increase in c-Fos expression in the VTA was primarily due to sazetidine-A.

We performed a separate experiment to determine whether the sazetidine-A induced c-Fos expression occurred in dopamine (DA) or γ-aminobutyric acid (GABA) neurons in the VTA. Fluorescence *in situ* hybridization (RNAScope) was used to identify *Fos* transcript expression in VTA cells that express tyrosine hydroxylase (*Th*), a common transcript in DA neurons, or glutamate decarboxylase 2 (*Gad2*), a transcript expressed in GABA neurons **(Fig. 5A)**. Pre-treatment with sazetidine-A (1mg/kg) prior to a 2g/kg alcohol injection produced an increase in *Fos* expression in *Th*-, but not *Gad2*-expressing neurons, compared with saline pre-treatment (one-way ANOVA F(3,11)=19.53, *P*=0.0001; Tukey's multiple comparisons test ****P*<0.001 for sal/alc versus SAZ/alc, **Fig. 5B-C**). There was no significant increase in the number of *Fos*-expressing *Th*-positive cells between the saline, alcohol and sazetidine-A only treated groups, suggesting that the increase in *Fos* expression in *Th*-expressing neurons is not due to either sazetidine-A or alcohol alone (Tukey's multiple comparisons tests, *P*>0.05 between sal/sal, sal/alc and sal/SAZ). *Fos* expression in *Gad2*-expressing neurons did not change across treatment conditions (one-way ANOVA F(3,11)=1.356, *P*=0.31, **Fig. 5D-E**). These data demonstrate that pre-treatment with sazetidine-A followed by alcohol injection selectively increased *Fos* expression in *Th*-expressing neurons of the VTA.

Discussion

In this study, we determined that sazetidine-A, which we previously showed reduced alcohol consumption in mice [19], enhances alcohol aversion without affecting alcohol reward. The results of this study suggest a previously unknown role for nAChRs in mediating alcohol aversion. Pharmacological manipulation of nAChRs has been shown to reduce alcohol consumption in animal models, as we and others have shown that mecamylamine, varenicline and sazetidine-A reduce alcohol consumption in rodents despite their different mechanisms of actions and nAChR subtype targets [7–9, 19]. Varenicline is best known as a partial agonist at α4β2 receptors, but *in vitro* studies show that it is also a partial agonist at α3* and α6* receptors and a full agonist at α7 receptors [16, 25–27]. *In vitro* studies show that sazetidine-A is an agonist at α4β2, α3β4*, α6* and α7 nAChRs [15–18], thus sazetidine-A and varenicline share similar nAChR subtype specificity but have differing potencies [15]. While varenicline requires the α4 nAChR subunit to reduce alcohol consumption [28], we found that sazetidine-A decreased binge alcohol consumption in both α4 KO and WT mice, indicating that sazetidine-A does not require the α4 subunit to decrease alcohol consumption. A study by Turner and colleagues also demonstrated dissociable effects of varenicline and sazetidine-A in nicotine withdrawal in mice [29]. Together, these data suggest that despite the similarity in nAChR targets *in vitro*, sazetidine-A and varenicline likely act through different nAChR subtypes *in vivo* to produce distinct behavioral effects.

A decrease in alcohol consumption may be due to a decrease in alcohol reward and/or an increase in alcohol aversion. We found no effect of sazetidine-A on the expression or acquisition of alcohol CPP in male C57BL/6J mice. In contrast, the non-specific nAChR antagonist mecamylamine has been shown to inhibit the acquisition and

expression of alcohol CPP in mice when administered intracerebroventricularly [30], which suggests that antagonism of nAChRs reduces alcohol reward. Interestingly, varenicline has been shown to have no effect on alcohol CPP in mice [31], similar to our findings with sazetidine-A. Notably, the role of nAChRs in alcohol aversion has not been previously explored in pre-clinical models. Here, we found that sazetidine-A and mecamylamine both enhanced the expression of alcohol aversion. As sazetidine-A initially activates and then desensitizes nAChRs [15–18], and we showed that both sazetidine-A and mecamylamine enhance alcohol aversion, we speculate that reduced nAChR function, either through desensitization or antagonism, mediates the enhanced alcohol aversion. Overall, these data demonstrate an important role for nAChRs in alcohol aversion, and suggest that enhancing alcohol aversion can be one mechanism by which nAChR drugs mediate a decrease in alcohol consumption. Interestingly, the opiate antagonist naloxone, a clinically approved medication for alcohol dependence, has also been shown to have no effect on expression of alcohol CPP, yet enhances expression of alcohol CPA [32]. Therefore, developing additional pharmacological agents that increase alcohol aversion, rather than decrease alcohol reward, may be a potentially useful and viable strategy to reduce alcohol consumption.

nAChRs involvement in alcohol aversion

We found that sazetidine-A injected directly into the VTA was able to reduce binge alcohol consumption in C57BL/6 mice, implicating this region in mediating the effects of sazetidine-A. The VTA is a heterogeneous structure that mediates reward and aversion, and contains DA, GABA and glutamate neurons [33, 34]. Optogenetic activation of VTA GABA neurons and inhibition of VTA DA neurons produces conditioned place aversion [35].

We found that pre-treatment with sazetidine-A followed by an injection of alcohol produced c-Fos expression in the VTA, and further examination of transcript expression showed that the increase in *Fos* occurred in *Th*, and not *Gad2*, expressing neurons. Based on these findings, we speculate that sazetidine-A agonism of nAChRs produces neuronal activation in *Th*-expressing cells, which then leads to the induction of *Fos* expression. This would be followed by prolonged sazetidine-A induced nAChR desensitization, as sazetidine-A is cleared slowly from the brain and can occupy brain nAChRs for ~8 hours [36]. Since nAChR desensitization is thought to decrease neuronal activity [37, 38], sazetidine-A-induced desensitization may lead to decreased activity of putative VTA DA neurons, thus enhancing alcohol aversion and reducing alcohol consumption. Since we showed that sazetidine-A did not require the α4 nAChR subunit to reduce alcohol consumption, our data suggest that non-α4 nAChRs on *Th*-expressing neurons are important in sazetidine-A mediated alcohol aversion. We speculate that α6 may be involved, as sazetidine-A also acts on α6 nAChRs and this subunit has been implicated in alcohol consumption and alcohol-induced DA neuron activity [15, 16, 39–44].

Implications

In summary, we found that sazetidine-A, a nAChR agonist and desensitizer, enhanced the expression of alcohol aversion without affecting the expression or acquisition of alcohol conditioned place preference. The enhanced alcohol aversion was likely mediated by desensitization of nAChRs, as mecamylamine, a nAChR antagonist, also enhanced alcohol aversion. Lastly, our data suggest that sazetidine-A induced *Fos* transcript expression in *Th*-expressing neurons in the VTA via a non-α4 nAChR mechanism. We speculate that the increase in alcohol aversion may be a major driving force behind the reduction in alcohol consumption we observed after treatment with

sazetidine-A. Further elucidating the role of nAChRs in alcohol aversion mechanisms may identify novel drug targets for the development of pharmacotherapies to treat alcohol use disorder.

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Figure legends

Fig. 1. *Microinfusion of sazetidine-A into the ventral tegmental area reduces binge alcohol consumption.* **(A)** Coronal mouse brain atlas diagrams from Paxinos and Watson (2001) showing the confirmed injection sites in mice infused with sazetidine-A (1 µg/µL, red) or saline (1 µL, black). **(B)** Average alcohol consumption on binge day in mice pre-treated with saline or sazetidine-A 20 min prior to alcohol access. *P=0.02, male C57BL/6J mice n=5-6 per group, mean+SEM.

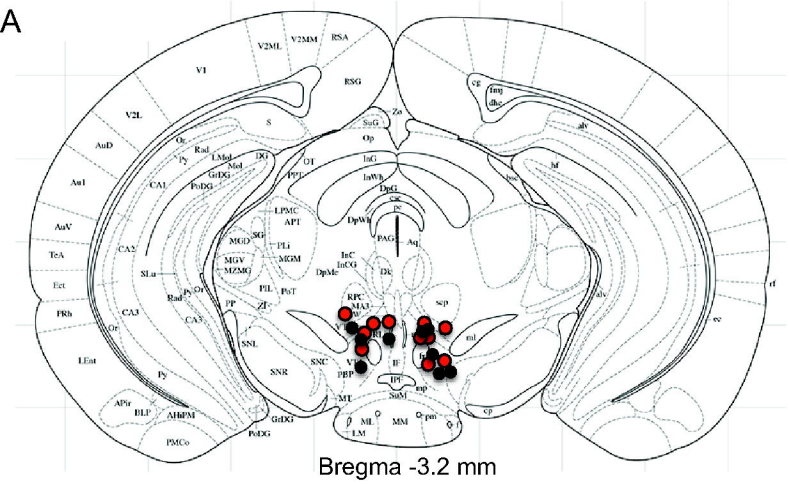
Fig. 2. *Sazetidine-A enhanced expression of alcohol aversion without affecting alcohol reward.* **(A)** Conditioning with 2 g/kg alcohol resulted in preference for the alcohol-paired chamber (Test 1), whereas conditioning with saline produced no significant preference. ***P*<0.05 using a one-sample *t*-test compared with a CPP index of zero, *n*=30 alcohol, *n*=11 saline. **(B)** Pre-treatment with sazetidine-A (1 mg/kg *i.p.*) 1h prior to the second test day (Test 2) did not produce significant alcohol CPP versus saline pre-treated mice, *n*=13-14 per group. **(C)** There was no difference in the CPP index between mice pre-treated with sazetidine-A (1 mg/kg *i.p.*) or saline prior to each alcohol conditioning session, *n*=10 per group. **(D)** Aversion conditioning with 2 g/kg alcohol resulted in aversion to the alcohol-paired chamber (Test 1), whereas conditioning with saline produced no significant aversion. ****P*=0.0001 using one-sample *t*-test compared with a CPA index of zero, *n*=30 alcohol, *n*=18 saline. **(E)** Pre-treatment with sazetidine-A (1 mg/kg *i.p.*) 1h prior to the second test day (Test 2) enhanced alcohol aversion. **P*=0.03 compared to the saline group, *n*=9-11 per group. **(F)** Aversion conditioning with 2 g/kg alcohol produced equivalent alcohol CPA in our groups prior to mecamylamine or saline injections. **(G)** Pre-treatment with mecamylamine at 3 mg/kg *i.p.* 20 min prior to the second test day (Test 2) enhanced alcohol aversion. **P*<0.05 compared to saline group by Dunnett's post-hoc test, *n*=10-15 per group. All conditioning indices are in minutes, all mice used were male C57BL/6J, all data shown as mean ± SEM.

Fig. 3. *Effect of sazetidine-A on binge alcohol consumption and acute behaviors.* **(A)** Sazetidine-A (1 mg/kg *i.p.*) reduced binge alcohol consumption in both wild-type and α4 knock-out female mice. *n*=7-8 per group. ANOVA $F_{treatment}(1,27)=13.08$, ***P*=0.001. **(B)** Average LORR duration from mice pre-treated with sazetidine-A (1 mg/kg, *i.p.*) was greater than mice pre-treated with saline. **P*=0.04 compared with saline treatment, *n*=7-8 per group. **(C)** Place conditioning with sazetidine-A only using the same dose and timing as for he alcohol pre-treatment experiments (1 mg/kg, *i.p.*, 1h) was not significantly different from saline conditioning, *n*=7-8 per group. **(D)** Average ambulatory distance in mice after injection with sazetidine-A (1 mg/kg, *i.p.*, 1h) was not significantly different from the saline treated group, *n*=8 per group. All data shown as mean ± SEM.

Fig. 4. *c-Fos expression in the VTA after acute alcohol and sazetidine-A injections.* **(A)** Representative images of one half of the VTA with c-Fos+ cells detected by immunohistochemistry. Scale bar = 250 µm. **(B)** Average c-Fos+ cells per VTA section was higher in alcohol-injected mice pre-treated with sazetidine-A compared with saline pre-treatment (*P=0.046 for SAZ/alc versus sal/alc). Sazetidine-A injection increased c-Fos expression compared with saline and alcohol injections (^P<0.05 for SAZ/sal versus sal/sal and sal/alc). n=11 mice for sal/sal, n=5-6 mice for all other groups, all data shown as mean ± SEM.

Fig. 5. *Sazetidine-A pre-treatment followed by alcohol injection induced Fos expression in Th-expressing VTA neurons.* **(A)** Representative images of *Fos* (white, Cy5) expression in *Th*- (green, mCherry) or*Gad2*-positive neurons (red, mCherry) in the VTA. Scale bar = 100 µm. *1,2*: magnification of *Th* and *Gad2* SAZ/alc. **(B-C)** The number of *Fos*-expressing *Th*-positive neurons was increased in the VTA of alcohol-injected mice pre-treated with sazetidine-A compared with saline pre-treatment (***$P<0.001$ for SAZ/alc versus sal/alc), and compared with groups that received only saline or sazetidine-A (***$P<0.001$ for SAZ/alc versus sal/sal and sal/SAZ). **(D-E)** *Fos*-expressing *Gad2*-positive neurons was similar between all treatment groups. n=3-4 mice per group, mean ± SEM for **B, D** and averaged group data for **C, E**.

A



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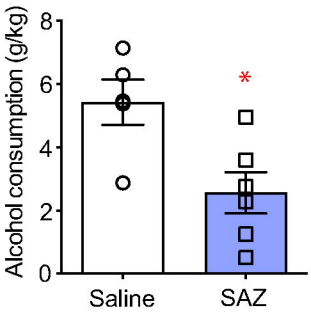
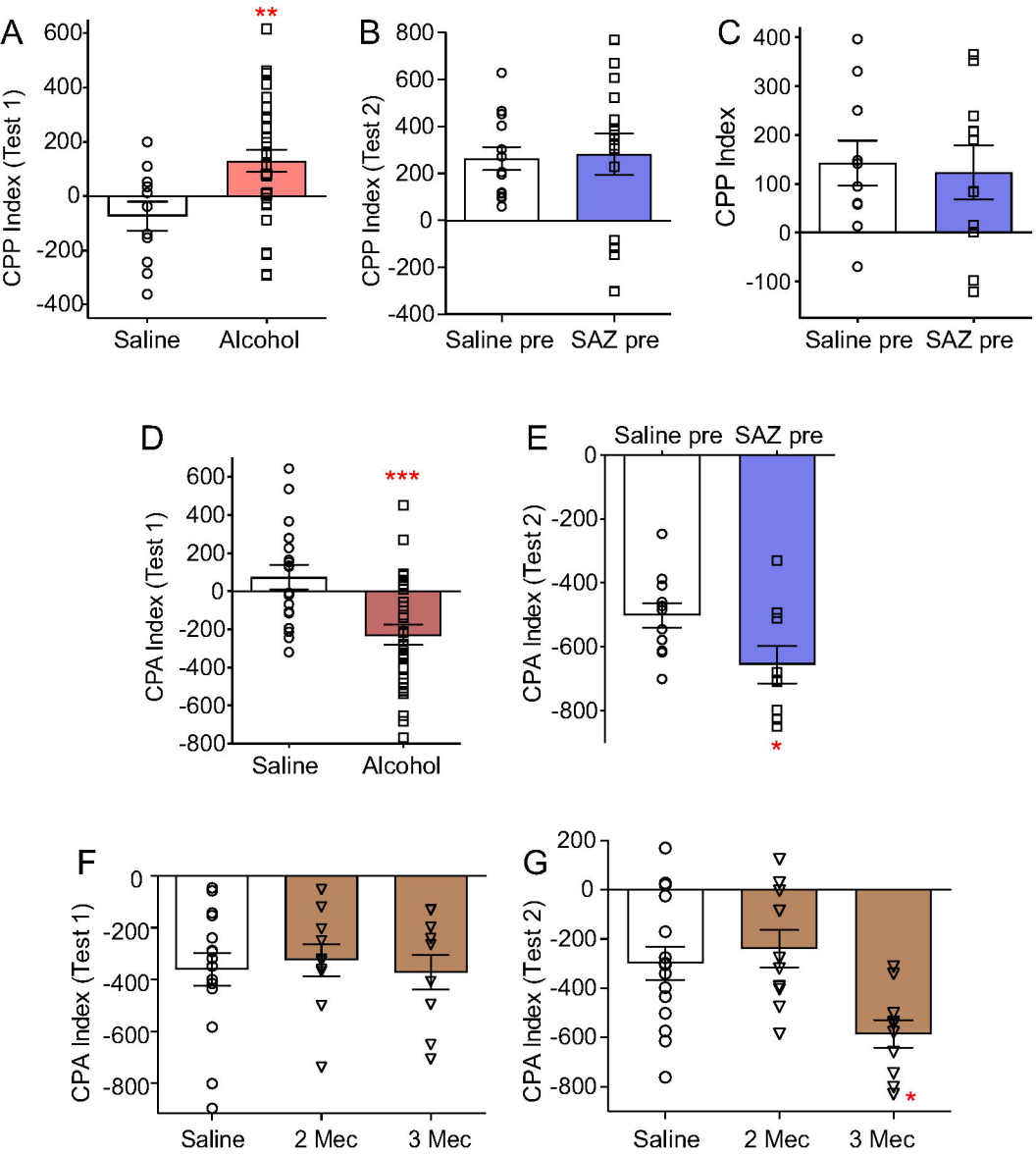
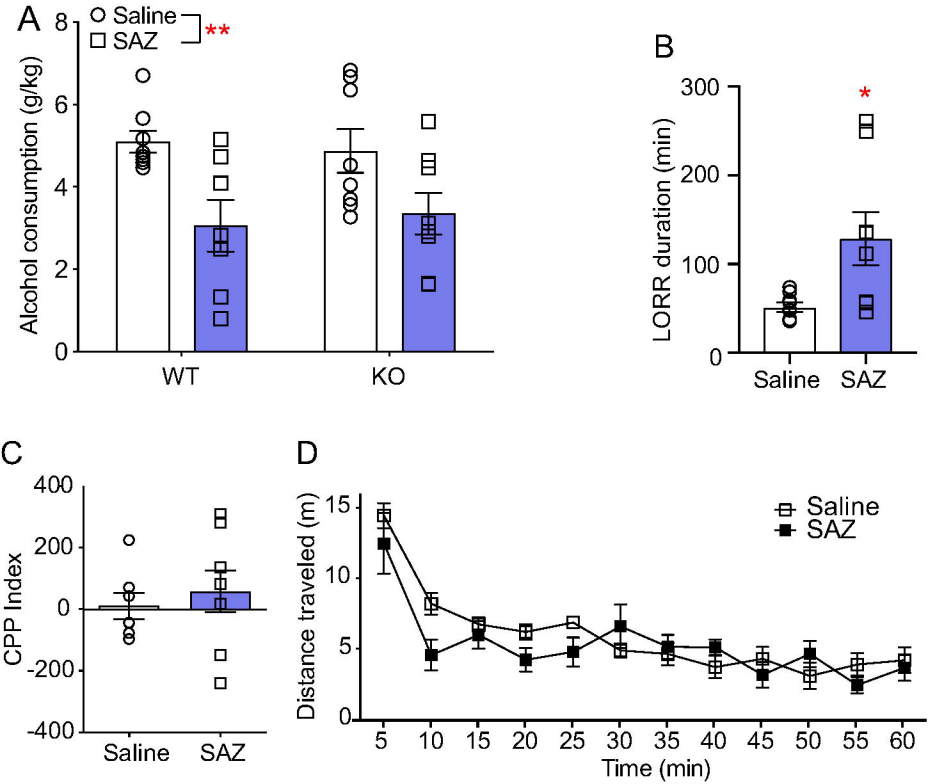
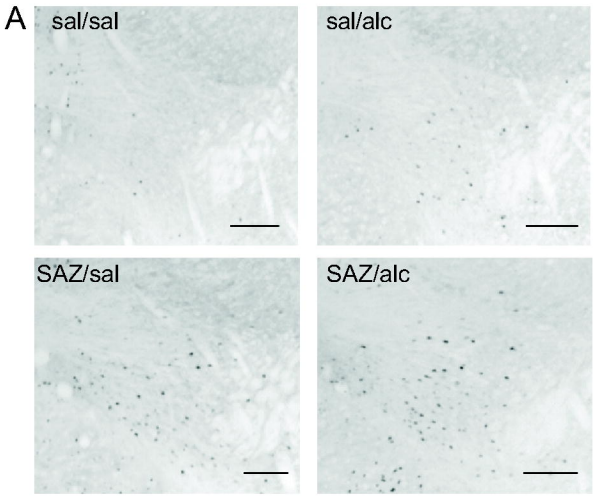
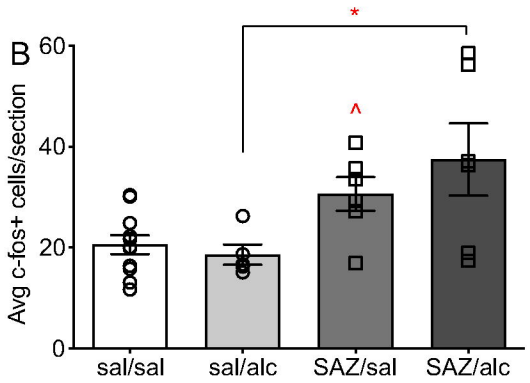
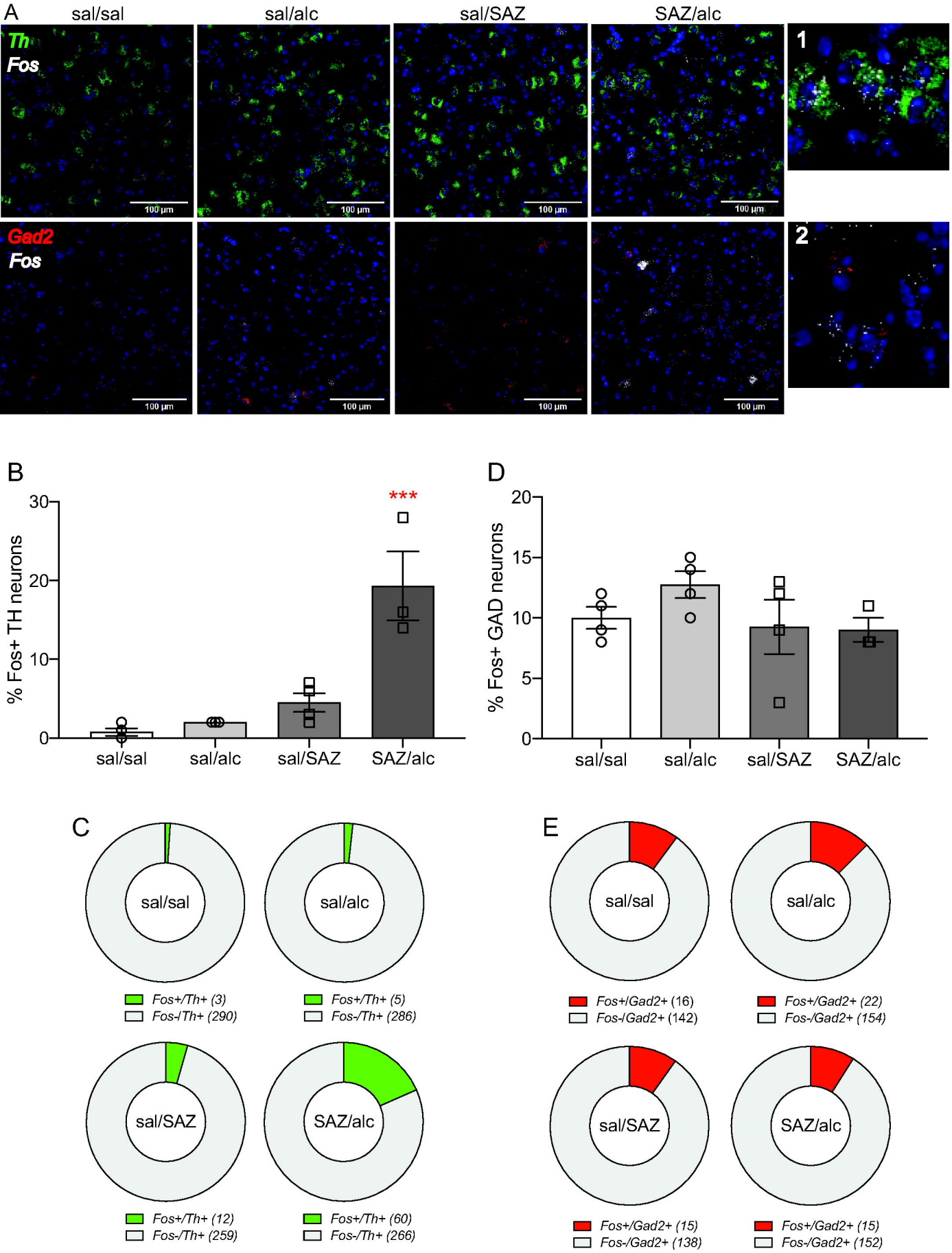



A
CPP Index (Test 1)
600
400
200
0
-200
-400
**
Saline
Alcohol
B
CPP Index (Test 2)
800
600
400
200
0
-200
-400
Saline pre
SAZ pre
C
CPP Index
400
300
200
100
0
-100
Saline pre
SAZ pre
D
CPA Index (Test 1)
600
400
200
0
-200
-400
-600
-800
***
Saline
Alcohol
E
Saline pre
SAZ pre
CPA Index (Test 2)
0
-200
-400
-600
-800
*
F
CPA Index (Test 1)
0
-200
-400
-600
-800
Saline
2 Mec
3 Mec
G
CPA Index (Test 2)
200
0
-200
-400
-600
-800
*
Saline
2 Mec
3 Mec


A
Saline
SAZ
**
Alcohol consumption (g/kg)
0
2
4
6
8
WT
KO
B
*
LORR duration (min)
0
100
200
300
Saline
SAZ
C
CPP Index
400
200
0
-200
-400
Saline
SAZ
D
Saline
SAZ
Distance traveled (m)
0
5
10
15
5
10
15
20
25
30
35
40
45
50
55
60
Time (min)


A
sal/sal
sal/alc
SAZ/sal
SAZ/alc


B
Avg c-fos+ cells/section
60
40
20
0
*
^
sal/sal
sal/alc
SAZ/sal
SAZ/alc


A
sal/sal
sal/alc
sal/SAZ
SAZ/alc
Th
Fos
Gad2
Fos
1
2
100 µm
B
% Fos+ TH neurons
***
sal/sal
sal/alc
sal/SAZ
SAZ/alc
D
% Fos+ GAD neurons
C
Fos+/Th+ (3)
Fos-/Th+ (290)
Fos+/Th+ (5)
Fos-/Th+ (286)
Fos+/Th+ (12)
Fos-/Th+ (259)
Fos+/Th+ (60)
Fos-/Th+ (266)
E
Fos+/Gad2+ (16)
Fos-/Gad2+ (142)
Fos+/Gad2+ (22)
Fos-/Gad2+ (154)
Fos+/Gad2+ (15)
Fos-/Gad2+ (138)
Fos+/Gad2+ (15)
Fos-/Gad2+ (152)