Delving into the reducing effects of the $GABA_B$ positive allosteric modulator, KK-92A, on alcohol-related behaviors in rats

Paola Maccioni[a,§], Katarzyna Kaczanowska[b,§], Carla Lobina[a], Laura Regonini Somenzi[a], Valentina Bassareo[c], Gian Luigi Gessa[a], Harshani R. Lawrence[d], Patricia McDonald[e,*,†], Giancarlo Colombo[a,†]

[a]Neuroscience Institute, Section of Cagliari, National Research Council of Italy, Monserrato (CA), Italy; [b]Department of Chemistry, Scripps Research, La Jolla, CA, USA; [c]Department of Biomedical Sciences, University of Cagliari, Monserrato (CA), Italy; [d]Chemical Biology Core, Moffitt Cancer Center, Tampa, FL, USA; [e]Department of Cancer Physiology, Moffitt Cancer Center, Tampa, FL, USA

[§]These authors have contributed equally to this work and share first authorship
[†]These authors have contributed equally to this work and share senior authorship

Corresponding author:
Giancarlo Colombo
Neuroscience Institute, Section of Cagliari
National Research Council of Italy
S.S. 55, km. 4,500
I-09042 Monserrato (CA)
Italy
Phone: +39 070 675-4342
E-mail: giancarlo.colombo@cnr.it

*Present address:
Lexicon Pharmaceuticals
The Woodlands, TX, USA

Abstract

Recent studies have demonstrated the ability of the positive allosteric modulator (PAM) of the $GABA_B$ receptor ($GABA_B$ PAM), KK-92A, to suppress operant alcohol self-administration and reinstatement of alcohol seeking in selectively bred Sardinian alcohol-preferring (sP) rats. The present study was designed to scrutinize the suppressing effects of KK-92A on alcohol-related behaviors; to this end, four separate experiments were conducted to address just as many new research questions, some of which bearing translational value. Experiment 1 found that 7-day treatment with KK-92A (0, 5, 10, and 20 mg/kg, i.p.) effectively reduced alcohol intake in male sP rats exposed to the homecage 2-bottle "alcohol (10% v/v) *vs* water" choice regimen with 1-hour/day limited access, extending to excessive alcohol drinking the ability of KK-92A to suppress operant alcohol self-administration. Experiment 2 demonstrated that the ability of KK-92A to reduce lever-responding for alcohol was maintained also after acute, intragastric treatment (0, 20, and 40 mg/kg) in female sP rats trained to lever-respond for 15% (v/v) alcohol under the fixed ratio 5 schedule of reinforcement. In Experiment 3, acutely administered KK-92A (0, 5, 10, and 20 mg/kg, i.p.) dampened alcohol seeking behavior in female sP rats exposed to a single session under the extinction responding schedule. Experiment 4 used a taste reactivity test to demonstrate that acute treatment with KK-92A (0 and 20 mg/kg, i.p.) did not alter either hedonic or aversive reactions to a 15% (v/v) alcohol solution in male sP rats, ruling out that KK-92A-induced reduction of alcohol drinking and self-administration could be due to alterations in alcohol palatability. Together, these results enhance the behavioral pharmacological profile of KK-92A and further strengthen the notion that $GABA_B$ PAMs may represent a novel class of ligands with therapeutic potential for treating alcohol use disorder.

Key-words: KK-92A; Positive allosteric modulation of the $GABA_B$ receptor; 2-Bottle choice alcohol drinking; Operant alcohol self-administration; Extinction responding for alcohol; Alcohol taste and palatability.

Introduction

Research work conducted over the last 15 years has led to positive allosteric modulators (PAMs) of the $GABA_B$ receptor ($GABA_B$ PAMs) being deemed a potentially effective pharmacotherapy for alcohol use disorder (AUD). Investigations performed to date have indeed demonstrated, without exception, the ability of all tested $GABA_B$ PAMs (namely: CGP7930, GS39783, BHF177, *rac*-BHFF, ADX71441, COR659, CMPPE, ORM-27669, and ASP8062) to suppress multiple alcohol-motivated behaviors, including excessive alcohol drinking, binge-like drinking, relapse-like drinking, operant oral alcohol self-administration, cue- and stress-induced reinstatement of alcohol seeking, alcohol-induced hyperlocomotion, and alcohol-induced conditioned place preference, in rats and mice (for review, see Maccioni & Colombo, 2019; Augier, 2021; Holtyn & Weerts, 2022; Li & Slesinger, 2022; Nieto et al., 2022). $GABA_B$ PAMs do not directly activate $GABA_B$ receptors (as baclofen and all orthosteric agonists do), but potentiate the receptor activation induced by endogenous GABA or exogenously added agonists, thus limiting their action only at those synapses at which and when GABA has been released (see Urwyler, 2011; Urwyler, 2016). This use-dependent mechanism of action results in a number of potential advantages, including minimal off-target side-effects and limited receptor desensitization (see Urwyler 2011; Urwyler, 2016). Currently on-going clinical studies testing the frontline $GABA_B$ PAM, ASP8062, on alcohol craving and consumption in subjects with moderate-to-severe AUD (ClinicalTrials.gov, 2022; Ito et al., 2022) will soon reveal whether, and to what extent, these promising preclinical data translate to AUD patients.

KK-92A is one of the most recently synthesized $GABA_B$ PAMs (Li et al., 2017). In close agreement with the multiple and consistent existing literature data on $GABA_B$ PAMs (see above), its acute and repeated administration suppressed operant oral alcohol self-administration and cue-induced reinstatement of alcohol seeking in selectively bred Sardinian alcohol-preferring (sP) rats (Maccioni et al., 2021; Maccioni et al., 2022). The suppressing effects of KK-92A on alcohol self-administration occurred under both fixed ratio (FR; measure of the reinforcing properties of alcohol) and progressive ratio (PR; measure of the motivational properties of alcohol) schedules of reinforcement. All these effects arose at doses of KK-92A remarkably lower than those inducing hypolocomotion and sedation (Maccioni et al., 2021); comparison of doses inducing the “desired” pharmacological effects (i.e., reduction of alcohol self-administration) and “unwanted” adverse effects (i.e., reduction of spontaneous locomotor activity) resulted in a therapeutic index higher than 8 (Maccioni et al., 2021). Finally, pretreatment with KK-92A synergistically potentiated the effect of baclofen on alcohol self-administration in sP rats: combination of *per se* totally ineffective doses of both compounds produced indeed a marked reduction in lever-responding for alcohol (Maccioni et al., 2021). Together, these data depict KK-92A as a promising agent for AUD treatment, thus rendering further scrutiny of its pharmacological profile a compelling research issue.

Based on this premise, the present study was designed to address the following 4 research questions. First, does the ability of KK-92A to suppress alcohol self-administration and reinstatement of alcohol seeking extend to excessive alcohol drinking? To address this research question, KK-92A was administered repeatedly to sP rats exposed to a singular procedure of voluntary alcohol drinking comprised of daily 1-hour drinking sessions occurring at the end of the dark phase of the light/dark cycle (Experiment 1). Under this experimental procedure, sP rats consume large and intoxicating amounts of alcohol (Colombo et al., 2014), providing therefore a model of excessive alcohol drinking deemed highly suitable to address this first research question. Second, is the ability of KK-92A to suppress alcohol self-administration maintained after *per os* treatment? To address this research

question, that bears evident translational relevance, KK-92A was administered intragastrically to sP rats exposed to the conventional FR schedule of reinforcement (Experiment 2), i.e. the same experimental procedure previously used to disclose the suppressing effect of intraperitoneally administered KK-92A on alcohol self-administration (Maccioni et al., 2021; Maccioni et al., 2022). Third, does the ability of KK-92A to suppress the motivational properties of alcohol under the PR schedule of reinforcement extend to an extinction responding (ER) procedure? The motivational properties of alcohol constitute the animal correlate of human craving for alcohol (see Markou et al. 1993). Their suppression is therefore a key element in the pharmacological profile of a compound with therapeutic potential for AUD. Experiment 3 intended to assess KK-92A effect in an experimental procedure known to measure the appetitive strength of alcohol independent of alcohol consumption (Samson et al., 2003). Indeed, and at variance with the PR schedule of reinforcement in which alcohol is still available (although at a progressively increasing response requirements) likely influencing lever-responding, under ER schedule alcohol is absent, thus providing an unequivocal measure of persistence of alcohol seeking behavior (see Markou et al., 1993). Fourth, does KK-92A alter alcohol palatability? Alteration of chemosensory properties of alcohol, and eventually increase of its aversive gustatory attributes, might be a means by which, in addition to reduction of the reinforcing and motivational properties of alcohol, KK-92A reduces alcohol drinking and self-administration. To address this research question, Experiment 4 employed the conventional taste reactivity procedure (see Grill and Norgren, 1978; Kiefer, 1995) to assess whether treatment with KK-92A produced any distortion of both hedonic and aversive attributes of water, sucrose, quinine, and – indeed – alcohol taste in sP rats.

Materials and Methods

The experimental procedures employed in the present study fully complied with European Directive no. 2010/63/EU and subsequent Italian Legislative Decree no. 26, March 4, 2014, on the “Protection of animals used for scientific purposes”.

Animals

Rats of the sP line (bred at Neuroscience Institute, National Research Council of Italy, Section of Cagliari, Monserrato, Italy), belonging to 118th and 119th generations, were used. Rats were 50-days-old and alcohol-naive at the start of each experiment. Experiments 1 and 4 employed male rats. Experiments 2 and 3 employed female rats. Rats were housed singly (Experiments 1 and 4) or 3 per cage (Experiments 2 and 3) in standard plastic cages with wood chip bedding. The animal facility was under an inverted 12:12-hour light-dark cycle (lights on at 7:00 p.m.), at a constant temperature of 22 ± 2°C and relative humidity of approximately 60%. Standard rat chow (Mucedola, Settimo Milanese, Italy) and tap water were always available in the homecage, except as noted below. Rats were extensively habituated to handling, intraperitoneal injections, and intragastric infusions (the latter limited to rats allocated to Experiment 2).
Each single experiment used an independent set of rats.

Drugs

KK-92A was synthesized in gram-scale with >99% purity (as determined by HPLC) in the Chemical Biology Core laboratory at Moffitt Cancer Center, Tampa, FL, USA, according to the procedure described in detail by Li et al. (2017). The chemical analysis (^{1}H and ^{13}C NMR, HPLC-MS) of in-house synthesized KK-92A matched the reported data [for structure and chemical analysis, see Maccioni et al. (2021)].

For *in vivo* assessment, KK-92A was dissolved in a mixture containing dimethyl sulfoxide, polysorbate 80, and distilled water (ratio of the mixture components: 5:10:85). KK-92A was administered intraperitoneally (injection volume: 2 ml/kg) 30 min before the start of (i) each daily drinking session (Experiment 1), (ii) ER session (Experiment 3), and (iii) taste reactivity test (Experiment 4). KK-92A was administered intragastrically [by means of a flexible, plastic gavage needle (Instech Laboratories, Plymouth Meeting, PA, USA); infusion volume: 2 ml/kg] 30 min before the start of the self-administration session (Experiment 2). In Experiments 1 and 3, KK-92A was tested at doses of 0, 5, 10, and 20 mg/kg; this dose-range was chosen as to be identical to that previously tested on alcohol self-administration and reinstatement of alcohol seeking in sP rats (Maccioni et al., 2021; Maccioni et al., 2022) as well as on nicotine self-administration and reinstatement of nicotine seeking in Wistar rats (Li et al., 2017). In Experiment 2, KK-92A was tested at the doses of 0, 20, and 40 mg/kg; this dose-range was chosen on the basis of the results of preliminary experiments aimed at identifying behaviorally effective doses of intragastrically administered KK-92A in rats. In Experiment 4, KK-92A was tested at the doses of 0 and 20 mg/kg; the latter was chosen as being the most effective dose tested in any “alcohol” experiment in sP rats (Maccioni et al., 2021; Maccioni et al., 2022; Experiments 1 and 3 of the present study).

Experimental procedures

Alcohol drinking (Experiment 1)

This experiment employed *n*=32 male rats.

Rats were initially exposed to the homecage 2-bottle “alcohol (10% v/v) *vs* water” choice regimen with unlimited access for 24 hours/day and 14 consecutive days (Phase 1). Daily alcohol intake initially averaged approximately 4.0 g/kg, then rose to and stabilized at 5.0-5.5 g/kg [i.e., the daily amount of alcohol usually consumed by sP rats when exposed to this basic drinking regimen (see Colombo et al., 2006)].

Rats were then deprived of alcohol for 3 consecutive days, with water as the sole liquid available. Subsequently, rats were exposed to the above 2-bottle choice regimen in daily 1-hour drinking sessions occurring during the last hour of the dark phase; this regimen was maintained for 16 consecutive days (Phase 2). The choice of planning the drinking session at the last hour of the dark phase was based on the notion that sP rats consume large and intoxicating amounts of alcohol when the limited-access drinking session occurs at the end of the dark phase (Colombo et al., 2014).

The first 5 drinking sessions of Phase 2 served as “acclimatization” period to the limited-access regimen. Sessions 6 and 7 constituted the pre-treatment period. The following 7 drinking sessions were devoted to testing the effect of repeated treatment with KK-92A on alcohol intake (treatment period). Specifically, rats were divided into 4 groups (*n*=8), matched for alcohol intake over the 2 drinking sessions of the pre-treatment period, and treated with one of the 4 doses of KK-92A (0, 5, 10, and 20 mg/kg, i.p.). The last 2 drinking sessions served as post-treatment period. Drinking sessions of pre- and post-treatment were preceded by administration of KK-92A vehicle to all rats.

In both Phases 1 and 2, bottles were refilled every day with fresh solution and their left-right positions interchanged daily to avoid development of position preference. Alcohol and water intake was monitored by weighing the bottles (0.01-g accuracy) immediately after lights on (Phase 1) or immediately before and immediately after each drinking session (Phase 2). Possible fluid spillage was calculated by using bottles filled with 10% (v/v) alcohol and water and positioned in empty cages interspersed in the cage rack; mean spilt volumes were subtracted before data analysis.
Data on alcohol and water intake (expressed in g/kg pure alcohol and ml/kg, respectively) during the 2-session pre-treatment period, the 7-session treatment period, as well as the 2-session post-treatment period were evaluated by separate 2-way [treatment (KK-92A dose) or group (when referring to the pre- and post-treatment periods); time (drinking session)] ANOVAs with repeated measures on the factor "time", followed by Tukey's test for *post hoc* comparisons.

Alcohol self-administration (Experiment 2)

This experiment employed *n*=45 female rats.
Rats were initially exposed to the homecage 2-bottle "alcohol (10% v/v) *vs* water" choice regimen with unlimited access for 24 hours/day over 10 consecutive days, according to the procedure described in detail elsewhere (e.g.: Maccioni et al., 2015).
Subsequently, rats were introduced into the operant chambers and trained to lever-respond for alcohol. Setting-up of operant chambers (Med Associates, St. Albans, VT, USA) has been described in detail elsewhere (e.g.: Maccioni et al., 2015). Briefly, each chamber was equipped with 2 retractable response levers (connected to 2 syringe pumps located outside the chamber), one dual-cup liquid receptacle, 2 stimulus lights (mounted above each lever), and one tone generator. Achievement of the response requirement had the following consequences: activation of alcohol or water pumps, delivery of 0.1 ml fluid, illumination of the stimulus light for the time period of fluid delivery, and activation of the tone generator.
Self-administration sessions lasted 30 min (with the sole exception of the first session, that lasted 120 min) and were conducted 5 days per week. Rats were water-deprived exclusively during the 12 hours prior to the first self-administration session. Rats were initially exposed to an FR 1 (FR1) schedule of reinforcement for 10% alcohol (v/v) for 4 self-administration sessions. FR was then progressively increased to FR5 over 4 self-administration sessions. In Sessions 9 and 10, the alcohol solution was presented at a final concentration of 15% (v/v). Rats were then exposed to 4 self-administration sessions during which the water lever alone or alcohol lever alone was available every other day; water and alcohol were available on FR1 and FR5, respectively. From then onwards, both levers were concomitantly available (maintenance phase) for a total of 20 self-administration sessions conducted with FR5 and FR1 on the alcohol and water lever, respectively.
The test session occurred the day after completion of the maintenance phase, lasted 30 min, and was identical to those of the maintenance phase (FR5 and FR1 on the alcohol and water lever, respectively). Rats were divided into 3 groups of *n*=15, matched for the number of responses on the alcohol lever over the last 3 sessions of the maintenance phase, and treated with one of the 3 doses of KK-92A (0, 20, and 40 mg/kg, i.g.).
Measured variables were (i) number of responses on each lever, (ii) amount of self-administered alcohol (expressed in g/kg pure alcohol), estimated from the number of earned reinforcers and assuming that each reinforcer was entirely consumed, and (iii) latency (expressed in s) to the first alcohol reinforcer. Data on number of responses on the alcohol lever, amount of self-administered

alcohol, and latency to the first alcohol reinforcer were statistically evaluated by 1-way ANOVA, followed by Tukey's test for *post hoc* comparisons. Data on number of responses on the water lever were statistically evaluated by Kruskal-Wallis test.

Alcohol seeking (Experiment 3)

This experiment employed *n*=64 female rats.
Rats were initially exposed to the homecage 2-bottle "alcohol (10% v/v) *vs* water" choice regimen and then trained to lever-respond for alcohol (15% v/v; FR5) and water (FR1) according to the procedures described above (see Experiment 2).
The test session occurred the day after completion of the maintenance phase and lasted 60 min. In this session (named ER session), lever-responding for either alcohol or water was never reinforced. Rats were divided into 4 groups of *n*=16, matched for the number of responses on the alcohol lever over the last 3 sessions of the maintenance phase, and treated with one of the 4 doses of KK-92A (0, 5, 10, and 20 mg/kg, i.p.).
Measured variables were (i) number of responses (defined ER) on each lever and (ii) latency (expressed in s) to the first response on the alcohol-associated lever. Data on each variable were statistically evaluated by Kruskal-Wallis test, followed by Dunn's test for *post hoc* comparisons.

Alcohol taste reactivity (Experiment 4)

This experiment employed *n*=12 male rats.
Rats were initially exposed to the homecage 2-bottle "alcohol (10% v/v) *vs* water" choice regimen with unlimited access for 24 hours/day over 10 consecutive days. Over this time period, rats were exposed to daily, brief sessions of habituation to stay inside the hemispheric arena subsequently used for assessment of taste reactions (see below).
The taste reactivity test was conducted according to the procedure previously described by Bassareo and coworkers (Bassareo et al., 2003; Vargiu et al., 2021), with slight modifications. The experiment started with a surgery session for positioning of an oral catheter. Surgery was performed the day after completion of the alcohol-drinking phase. The custom-made, oral catheter comprised of a 2 cm-long, 22-gauge stainless steel needle and connected polyethylene tubing (the length of which was adjusted to each rat). At the side opposite to needle insertion, the tubing ended with a plastic, perforated circular disk. Rats were anesthetized with an isoflurane-oxygen mixture (isoflurane concentration: 5% and 2.5% in induction and maintenance phase, respectively), using a conventional rodent anesthesia machine (Kent Scientific Corporation; Torrington, CT, USA). Once anesthetized, rats were implanted with the oral catheter. Specifically, the catheter was inserted at the level of the first molar and passed along the skull. The catheter portion ending with the needle was fixed to the skull with dental cement. The catheter portion ending with the disk adhered to the wall of the oral cavity. Rats were then allowed to fully recover from surgery for 4 consecutive days.
The taste reactivity session took place on the 5th day after surgery. Thirty min after KK-92A treatment (0 and 20 mg/kg, i.p.), rats were positioned inside the hemispheric arena (made of transparent plexiglass, having a diameter of 50 cm, and firmly hung on the ceiling, so to be easily accessible for complete visual inspection of rat behavior). The oral catheter was connected to an infusion pump and the rat left undisturbed for 2 min. The tested solution was then pumped inside the oral cavity at a constant rate of 0.5 ml/min; infusion lasted 2 min. Tested solutions were: water; alcohol (15% v/v)

in water; sucrose (0.3 M) in water; quinine hydrochloride (0.0005 M) in water. Alcohol concentration was selected as being that used in all experiments testing KK-92A effect on operant oral alcohol self-administration (Maccioni et al., 2021; Maccioni et al., 2022; Experiment 2 of the present study). Sucrose and quinine concentrations were chosen from literature data demonstrating their ability to produce clear hedonic and aversive reactions, respectively (e.g.: Kiefer et al., 1995). All 4 tastants were tested in sequence in each rat. The sequence started with water infusion in all rats; alcohol, sucrose, and quinine solutions were then tested in a *random* order. A "washout" water infusion, lasting 2 min, was interposed between infusions of alcohol, sucrose, and quinine solutions. The entire sequence of infusions lasted 12 min. The rat behavior was recorded by 2 investigators blinded to the treatment group to which each rat was allocated. Recordings lasted for the entire 2-min period of each infusion.

Three different classes of affective taste-reactivity patterns were scored: hedonic (positive, ingestive), aversive (negative), and neutral. Hedonic reactions included lateral tongue protrusions, rhythmic tongue protrusion, and paw licks; aversive reactions included gapes, chin rubs, face washing, forelimb flails, paw tread, and locomotion; neutral reactions included rhythmic mouth movements and passive drip of the solution (Grill and Norgren, 1978). Reactions were scored according to the scale depicted by Bassareo and coworkers (Bassareo et al., 2003; Vargiu et al., 2021). Specifically, each lateral and rhythmic tongue protrusion, gape, chin rub, forelimb flail, and paw tread was counted as a yes-or-no event and assigned 1 point; all other events were assigned 1 point if their duration lasted between 1 and 5 s, and 2 points if their duration lasted more than 5 s.

Data on scores of hedonic and aversive reactions were evaluated by separate 2-way [treatment (KK-92A dose); tastant] ANOVAs with repeated measures on the factor "tastant".

Results

Alcohol drinking (Experiment 1)

ANOVA indicated no significant effect of group [$F(3,28)=0.01$, $P>0.05$] and time [$F(1,28)=3.74$, $P>0.05$], and no significant interaction [$F(3,28)=0.20$, $P>0.05$], on alcohol intake in male sP rats over the 2 drinking sessions of the pre-treatment period (Fig. 1). As specified above, these 2 drinking sessions served to combine 4 rat groups with virtually identical alcohol intake.

ANOVA indicated highly significant effects of treatment [$F(3,28)=24.89$, $P<0.0001$] and time [$F(6,168)=7.19$, $P<0.0001$], and a significant interaction [$F(18,168)=1.67$, $P<0.05$], on alcohol intake over the 7-session treatment period (Fig. 1). Mean alcohol intake in vehicle-treated rats averaged 0.95-1.25 g/kg, suggestive of a consumption of relatively high doses of alcohol. Repeated, intraperitoneal treatment with 10 and 20 mg/kg KK-92A resulted in a decrease in alcohol intake in most drinking sessions. Compared to vehicle treatment, magnitude of the overall reducing effect on alcohol intake averaged approximately 15%, 30%, and 45% in the rat groups treated with 5, 10, and 20 mg/kg KK-92A, respectively.

ANOVA indicated no effect of group [$F(3,28)=1.02$, $P>0.05$] and time [$F(1,28)=1.28$, $P>0.05$], and no significant interaction [$F(3,28)=0.62$, $P>0.05$] on alcohol intake in sP rats over the 2 drinking sessions of post-treatment period (Fig. 1).

No difference in water intake was recorded over the 2-session pre-treatment period [$F_{group}(3,28)=0.49$, $P>0.05$; $F_{time}(1,28)=0.24$, $P>0.05$; $F_{interaction}(3,28)=1.46$, $P>0.05$] (Table 1). ANOVA

indicated a significant effect of time [F(6,168)=4.55, *P*<0.0005], but not of treatment [F(3,28)=1.02, *P*>0.05], and no significant interaction [F(18,168)=1.01, *P*>0.05], on water intake over the 7-session treatment period. Water intake was relatively low and erratic among drinking sessions as well as KK-92A doses (Table 1). No difference in water intake was recorded over the 2-session post-treatment period [$F_{group}(3,28)=1.43$, *P*>0.05; $F_{time}(1,28)=0.04$, *P*>0.05; $F_{interaction}(3,28)=2.60$, *P*>0.05] (Table 1).

Alcohol self-administration (Experiment 2)

Acute, intragastric treatment with KK-92A reduced both number of lever-responses for alcohol [F(2,42)=5.21, *P*<0.01] (Fig. 2a) and amount of self-administered alcohol [F(2,42)=4.75, *P*<0.05] (Fig. 2b) in female sP rats. *Post hoc* test indicated that statistical significance was reached, in both variables, by treatment with 40 mg/kg KK-92A (*P*<0.01). The magnitude of the reducing effect of 40 mg/kg KK-92A on number of lever-responses for alcohol and amount of self-administered alcohol averaged approximately 40%. Conversely, treatment with KK-92A did not alter latency to the first alcohol reinforcer [F(2,42)=0.87, *P*>0.05] (Fig. 2c).
Lever-responding for water was negligible (averaging <5 in all 3 rat groups) and not altered by treatment with KK-92A [F(2,42)=0.60, *P*>0.05] (data not shown).

Alcohol seeking (Experiment 3)

Acute, intraperitoneal treatment with KK-92A reduced ER for alcohol in female sP rats [F(3,60)=14.85, *P*<0.005] (Fig. 3a). *Post hoc* test indicated that statistical significance was reached by treatment with 20 mg/kg KK-92A (*P*<0.005). The magnitude of the decreasing effect of 20 mg/kg KK-92A on ER for alcohol averaged approximately 45%. In addition, treatment with KK-92A increased latency to the first response on the alcohol-associated lever [F(3,60)=10.11, *P*<0.05] (Fig. 3b). *Post hoc* test indicated that statistical significance was again reached by treatment with 20 mg/kg KK-92A (*P*<0.005) (Fig. 3b). Latency to the first response on the alcohol-associated lever in the rat group treated with 20 mg/kg KK-92A was approximately 3 times higher than that recorded in vehicle-treated rat group.
ER for water was low (averaging <4 in all 4 rat groups) and not altered by treatment with KK-92A [F(3,60)=4.61, *P*>0.05] (data not shown).

Alcohol taste reactivity (Experiment 4)

ANOVA indicated a highly significant effect of tastant [F(3,66)=57.35, *P*<0.0001], but no effect of treatment [F(1,22)=0.05, *P*>0.05], and no significant interaction [F(3,66)=0.18, *P*>0.05], on score of hedonic reactions in male sP rats treated acutely and intraperitoneally with KK-92A and then exposed to the taste reactivity test (Fig. 4a). Scores differed largely among tastants, with tastants ranking in the following order: sucrose > alcohol >> water > quinine. However, treatment with 20 mg/kg KK-92A did not alter the score of any tastant.
As regards score of aversive reactions, ANOVA indicated a highly significant effect of tastant [F(3,66)=16.04, *P*<0.0001], but no effect of treatment [F(1,22)=0.42, *P*>0.05], and no significant interaction [F(3,66)=0.46, *P*>0.05] (Fig. 4b). Scores differed largely among tastants, with tastants ranking as follows: quinine >> water > alcohol > sucrose. Again, treatment with 20 mg/kg KK-92A was ineffective on the score of each tastant.

Discussion

The present study was designed to scrutinize the pharmacological profile of the $GABA_B$ PAM, KK-92A, contributing additional lines of experimental evidence to the recently discovered ability of acute and repeated treatment with KK-92A to suppress operant oral alcohol self-administration and cue-induced reinstatement of alcohol seeking in alcohol-preferring rats (Maccioni et al., 2021; Maccioni et al., 2022). Very briefly, the results collected in the present study indicated that (i) treatment with KK-92A reduced voluntary alcohol intake in a rat model of excessive alcohol drinking (Experiment 1), (ii) the ability of KK-92A to decrease operant oral alcohol self-administration was maintained also when KK-92A was given intragastrically (Experiment 2), (iii) the ability of KK-92A to weaken the appetitive strength of alcohol extended to ER schedule (Experiment 3), and (iv) treatment with KK-92A did not alter taste perception of different tastants, including alcohol (Experiment 4).

The results of Experiment 1 constitute the first line of evidence on the ability of KK-92A to reduce alcohol drinking. Use of an experimental procedure characterized by voluntary consumption of high, psychopharmacologically meaningful amounts of alcohol confers additional relevance to the collected results. During the 7-day treatment period, overall alcohol drinking was virtually halved by treatment with 20 mg/kg KK-92A, suggestive of a remarkable efficacy of KK-92A in reducing excessive alcohol drinking. The reducing effect of KK-92A on alcohol intake was relatively constant over the treatment period (net of daily fluctuations that often occur in procedures of repeated alcohol drinking), suggestive of a limited development of tolerance. This aspect is somewhat different from the partial tolerance to the reducing effect of repeatedly administered KK-92A on operant alcohol self-administration (Maccioni et al. 2022). In an attempt to explain this apparent discrepancy, a prudent approach would suggest that the above-mentioned, relatively unstable baseline of daily alcohol intake tended to mask the development of some degree of tolerance to the reducing effect of KK-92A, especially at the highest dose tested, on alcohol drinking. Alternatively, we might hypothesize that repeated treatment with KK-92A impacted differently on the neural substrates underlying alcohol drinking and alcohol self-administration. After completion of treatment with KK-92A, alcohol intake returned immediately to control values; because of the relatively short half-life of KK-92A [1.8 hours after intraperitoneal administration in rats (Dr. Michael Cameron, personal communication)], plasma levels of KK-92A were likely equal to 0 at the time of the first drinking session of post-treatment period, thus explaining the lack of any carry-over effect of KK-92A on alcohol intake 24 hours after its last injection. The results of Experiment 1 extend to KK-92A the ability of the $GABA_B$ PAMs, CGP7930, GS39783, *rac*-BHFF, ADX71441, and COR659, to reduce alcohol intake in rodent models of excessive alcohol drinking, including sP rats (Orrù et al., 2005; Loi et al., 2013; Hwa et al., 2014; Lorrai et al., 2022).

Repeated treatment with KK-92A did not alter water intake, suggesting that the reducing effect of KK-92A was specific to alcohol intake. This conclusion should however be considered with due caution, as water intake was likely too low and erratic to serve as a reliable term for specificity assessment. Conversely, specificity of the reducing effect of KK-92A on alcohol intake is more properly demonstrated by comparison of the results of Experiment 1 with those of a previous study indicating that intraperitoneally administered doses of KK-92A equal to or lower than 40 mg/kg were devoid of any decreasing effect on spontaneous locomotor activity in sP rats (Maccioni et al., 2021), thus ruling out that the reducing effect on alcohol intake exerted by doses of KK-92A ranging between

5 and 20 mg/kg (i.e., the dose-range tested in the present study) could be secondary to motor-incoordination and sedation.

The results of Experiment 2 demonstrate that the ability of KK-92A to reduce operant alcohol self-administration is maintained also after intragastric treatment, thus enriching the pharmacological profile of KK-92A with an attribute of remarkable translational value. Since all previous *in vivo* studies on KK-92A tested the effects of its intraperitoneal administration (Li et al., 2017; Maccioni et al., 2021; Maccioni et al., 2022), the results of Experiment 2 constitute the very first line of experimental evidence on KK-92A efficacy after intragastric treatment. Analysis of cumulative response patterns (Fig. 2d) indicates that treatment with KK-92A did not affect the onset of lever-responding (accordingly, latency to the first alcohol reinforcer did not differ between vehicle- and KK-92A-treated rat groups); however, (i) a less steep curve (suggestive of a reduced frequency in lever-responding for alcohol) was observed in the rat group treated with 40 mg/kg KK-92A, and (ii) lower *plateau* values (suggesting that fewer ratios were completed before lever-responding for alcohol ended) were observed in the rat groups treated with 20 and 40 mg/kg KK-92A.

Experiment 2 was ultimately designed as a proof-of-evidence experiment. Accordingly, it assessed a relatively narrow dose-range of KK-92A as it was aimed at disclosing, just as a simple yes-or-no research question, presence of the pharmacological effect. Future experiments will test higher doses of KK-92A and will more properly assess potency, efficacy, and dose-response relationship of the reducing effect of intragastrically administered KK-92A on alcohol self-administration and, possibly, other alcohol-related behaviors. These additional experiments will also reveal whether higher doses of KK-92A impact on onset of, or urge for, lever-responding for alcohol, as predictable on the basis of the results demonstrating the ability of intraperitoneally administered KK-92A to dramatically prolong the latency to commencing lever-responding for alcohol (Maccioni et al., 2021).

With the caution needed when comparing two independent studies (although conducted using an identical experimental protocol and rats of the same line), the results of Experiment 2 suggest that – in terms of magnitude – the reducing effect of intragastric treatment with 40 mg/kg KK-92A was comparable to that exerted by intraperitoneal treatment with 10 mg/kg KK-92A (Maccioni et al., 2021); this comparison is indicative of high bioavailability of oral KK-92A, likely comprising high rates of intestinal absorption and limited sensitivity to hepatic first-pass metabolism. Finally, the results of Experiment 2 extend to KK-92A the ability of the $GABA_B$ PAMs, CGP7930, GS39783, BHF177, *rac*-BHFF, ADX71441, and ASP8062, to reduce alcohol drinking and operant alcohol self-administration in rodents, including sP rats, after intragastric treatment (e.g.: Orrù et al., 2005; Maccioni et al., 2009; Maccioni et al., 2010; Maccioni et al., 2012; Hwa et al., 2014; Haile et al., 2021).

The results of Experiment 3 demonstrate that treatment with KK-92A dampened alcohol seeking behavior in sP rats exposed to ER schedule. These results extend and confirm previous data on the ability of KK-92A to reduce the motivational properties of alcohol in sP rats exposed to the PR schedule of reinforcement (Maccioni et al., 2021). In ER schedule, lever-responding is never reinforced, irrespective of the number of responses on the lever, thus providing a measure of the appetitive strength of alcohol devoid of any sustaining effect that each alcohol reinforcer may provide (Samson et al., 2003). This is actually the aspect that mainly differentiates ER schedule from PR schedule, in which alcohol is always available over the session (although its attainment requires progressively increasing response requirements). More broadly, this ability of isolating and measuring the sole motivational component of lever-responding for alcohol may render, depending upon the study objectives, ER schedule complementary to or even preferable over the more widely used PR schedule. In this perspective, it is worth noting that ER is pharmacologically manipulable

and has predictive validity as measure of craving for alcohol (see Markou et al., 1993); more specifically, ER-based procedures have repeatedly been used to evaluate the effect of a variety of drugs, including naltrexone and baclofen, on the persistence of alcohol-seeking behavior in rodents (e.g.: Bienkowski et al., 1999; Colombo et al., 2003; Vengeliene et al., 2008).

Beside reducing ER for alcohol, treatment with KK-92A remarkably increased latency to the first response on the alcohol-associated lever, suggesting a reduced urge to seek alcohol. All these effects are effectively recapitulated in the graph depicting cumulative response patterns over the ER session (Fig. 3c): treatment with 10 and 20 mg/kg KK-92A resulted in less steep curves and lower *plateau* values of lever-responding.

The motivational properties of alcohol, measured by PR and ER schedules, represent validated animal models of human craving for alcohol (see Markou et al., 1993). The ability of KK-92A to effectively diminish breakpoint for alcohol under PR schedule (Maccioni et al., 2021) and ER for alcohol (Experiment 3 of the present study) is thus suggestive of the *anti*-craving potential of KK-92A. Lessening the motivational properties of alcohol appears to be a valuable feature of the $GABA_B$-PAM class in its entirety: previous studies reported indeed the decreasing effects of GS39783, BHF177, ADX71441, COR659, and CMPPE on breakpoint for alcohol in rodents, including sP rats (e.g.: Maccioni et al,. 2009; Maccioni et al., 2012; Maccioni et al., 2017; Maccioni et al., 2019; Augier et al., 2017).

Experiment 4 used the taste reactivity method to assess whether KK-92A altered alcohol palatability. In the taste reactivity method, a small amount of a given fluid is pumped directly into the rat's oral cavity. The resulting, multiple orofacial movements are recorded and categorized as hedonic or aversive reactions. Hedonic reactions are indicative of taste acceptance and fluid ingestion; conversely, aversive reactions are indicative of distaste and fluid removal from the oral cavity (Grill and Norgren, 1978). The results of Experiment 4 indicate that treatment with KK-92A, tested at the dose (20 mg/kg) found to be most effective in reducing several alcohol-related behaviors in sP rats (Maccioni et al., 2021; Maccioni et al., 2022; Experiments 1 and 3 of the present study), did not alter – even minimally – both hedonic and aversive reactions induced by the intra-oral infusion of a 15% (v/v) alcohol solution.

These data are of some relevance in the preclinical characterization of the pharmacological profile of KK-92A as they tend to rule out that reduction of alcohol drinking (Experiment 1 of the present study) and operant alcohol self-administration (Maccioni et al. 2021; Maccioni et al., 2022) was even partially related to any disturbance in alcohol palatability. This issue is not at all trivial, as naltrexone – one of the few medications currently approved for AUD treatment (see Jonas et al., 2014; Reus et al., 2018) – has repeatedly been reported to increase the taste aversiveness of alcohol solutions in rats (e.g.: Hill & Kiefer 1997; Ferraro et al., 2002; Hill et al., 2010). This shift in alcohol palatability likely contributes, together with its central reward-suppressing effects, to the reducing effect of naltrexone on several alcohol-seeking and -taking behaviors in rodents. At the clinical level, cases of naltrexone-induced distortion of the taste of alcoholic beverages (e.g., beer taste becoming "weird", "strange", or "soapy"), and thus leading to drinking cessation, have occasionally been reported (e.g.: Davidson et al., 1999). Conversely, if theoretically transposed to humans, treatment with KK-92A should reduce alcohol drinking solely because of its suppressing effects on alcohol-related reinforcing and motivational properties, with no contribution of gustatory factors.

$GABA_B$ receptor is one of the several receptor systems underlying the cellular mechanisms of taste perception. Multiple lines of experimental evidence have indeed demonstrated, in both rats and mice, that activation of $GABA_B$ receptors located in taste buds inhibited cell-to-cell communication among

taste receptor cells, thus dampening taste reception and taste-induced signaling (Cao et al., 2009; Starostik et al., 2010; Dvoryanchikov et al., 2011). Accordingly, administration of relatively high doses of baclofen impaired sensory ability and decreased gustatory discrimination in rats (Wilson et al., 2011), and baclofen is listed among the prescription drugs that may produce taste disorders in humans (see Doty et al., 2008).

In keeping with the notion that $GABA_B$ receptors are involved in the "taste" neural system, Experiment 4 also investigated the possible altering effect of KK-92A on palatability of sucrose and quinine solutions. Although this investigation was limited to a single concentration of each tastant, thus indicating a need for more in-depth analyses before drawing definitive conclusions, the collected results suggested that neither sweet nor bitter taste was affected by treatment with KK-92A. Although preliminary, these data constitute – to our knowledge – the results of the very first investigation on the ability of a $GABA_B$ PAM to possibly alter taste perception.

The present study employed both male (used in Experiments 1 and 4) and female (used in Experiments 2 and 3) rats. This choice was dictated by practical reasons, including (i) suitability of female sP rats for commercially available, standard operant chambers (in contrast with the unfitness of male sP rats, that weigh in excess of 800 g at the end of the long period of time required by operant training and testing) (Lorrai et al., 2019), and (ii) use of as many rats as possible from each single litter, in compliance with ethical requirements and the "Reduction" principle of the Three Rs guidelines. In other words, use of both sexes was intended solely as a convenient, procedural advantage and not to investigate possible sex similarities or differences in KK-92A efficacy on alcohol-related behaviors in sP rats. This latter, important outcome is deferred to future experiments testing male and female sP rats concurrently and under the same experimental procedure, thus being able to assess experimentally and systematically the degree of generalizability to male sP rats of data collected in female sP rats, and *vice versa*.

In conclusion, the results of the present study expand the behavioral pharmacological profile of KK-92A, with a specific focus on its ability to weaken multiple alcohol-related behaviors in a rat model of excessive alcohol drinking and strong motivation to seek for and consume alcohol. These data also add further strength to the notion that $GABA_B$ PAMs may represent a novel class of ligands with therapeutic potential for AUD treatment.

Declarations of interest

None.

Acknowledgements

The authors are grateful to Mrs. Carla Acciaro for animal breeding and care, and to Dr. Michael Cameron, Senior Director of Drug Metabolism and Pharmacokinetics (DMPK) UF Scripps, FL, USA, for guidance on drug administration. The authors also acknowledge the Moffitt Chemical Biology Core which is supported by the National Cancer Institute (Award No. P30-CA076292) as a Cancer Center Support Grant. The synthesis of KK-92A was in part funded by NIH/NCI grant (to H.R.L.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Augier, E., Dulman, R. S., Damadzic, R., Pilling, A., Hamilton, J. P., & Heilig M. (2017). The $GABA_B$ positive allosteric modulator ADX71441 attenuates alcohol self-administration and relapse to alcohol seeking in rats. *Neuropsychopharmacology*, *42*, 1789-1799.
https://doi.org/10.1038/npp.2017.53

Augier, E. (2021). Recent advances in the potential of positive allosteric modulators of the $GABA_B$ receptor to treat alcohol use disorder. *Alcohol and Alcoholism*, *56*, 139-148.
https://doi.org/10.1093/alcalc/agab003

Bassareo, V., De Luca, M. A., Aresu, M., Aste, A., Ariu, T., & Di Chiara, G. (2003). Differential adaptive properties of accumbens shell dopamine responses to ethanol as a drug and as a motivational stimulus. *European Journal of Neuroscience*, *17*, 1465-1472.
https://doi.org/10.1046/j.1460-9568.2003.02556.x

Bienkowski, P., Kostowski, W., & Koros, E. (1999). Ethanol-reinforced behaviour in the rat: effects of naltrexone. *European Journal of Pharmacology*, *374*, 321-327.
https://doi.org/10.1016/S0014-2999(99)00245-9

Cao, Y., Zhao, F. L., Kolli, T., Hivley, R., & Herness, S. (2009). GABA expression in the mammalian taste bud functions as a route of inhibitory cell-to-cell communication. *Proceedings of the National Academy of Sciences of the USA*, *106*, 4006-4011.
https://doi.org/10.1073/pnas.0808672106

ClinicalTrials.gov,
https://clinicaltrials.gov/ct2/show/NCT05096117?term=ASP8062&draw=2&rank=5

Colombo, G., Vacca, G., Serra, S., Brunetti, G., Carai, M. A. M., & Gessa, G. L. (2003). Baclofen suppresses motivation to consume alcohol in rats. *Psychopharmacology*, *167*, 221-224.
https://doi.org/10.1007/s00213-003-1397-y

Colombo, G., Lobina, C., Carai, M. A. M., & Gessa, G. L. (2006). Phenotypic characterization of genetically selected Sardinian alcohol-preferring (sP) and -non preferring (sNP) rats. *Addiction Biology*, *11*, 324-338.
https://doi.org/10.1111/j.1369-1600.2006.00031.x

Colombo, G., Maccioni, P., Acciaro, C., Lobina, C., Loi, B., Zaru, A., Carai, M. A. M., & Gessa, G. L. (2014). Binge drinking in alcohol-preferring sP rats at the end of the nocturnal period. *Alcohol*, *48*, 301-311.
https://doi.org/10.1016/j.alcohol.2014.02.001

Davidson, D., Palfai, T., Bird, C., & Swift, R. (1999). Effects of naltrexone on alcohol self-administration in heavy drinkers. *Alcoholism: Clinical and Experimental Research*, *23*, 195-203.
https://doi.org/10.1111/j.1530-0277.1999.tb04099.x

Doty, R. L., Shah, M., & Bromley, S. M. (2008). Drug-induced taste disorders. *Drug Safety*, *31*, 199-215.
https://doi.org/10.2165/00002018-200831030-00002

Dvoryanchikov, G., Huang, Y. A., Barro-Soria, R., Chaudhari, N., & Roper, S. D. (2011). GABA, its receptors, and GABAergic inhibition in mouse taste buds. *Journal of Neuroscience*, *31*, 5782-5791.
https://doi.org/10.1523/JNEUROSCI.5559-10.2011

Ferraro, F. M. 3rd, Hill, K. G., Kaczmarek, H. J., Coonfield, D. L., & Kiefer, S. W. (2002). Naltrexone modifies the palatability of basic tastes and alcohol in outbred male rats. *Alcohol*, *27*, 107-114.
https://doi.org/10.1016/s0741-8329(02)00220-3

Grill, H. J., & Norgren, R. (1978). The taste reactivity test. I. Mimetic responses to gustatory stimuli in neurologically normal rats. *Brain Research*, *143*, 263-279.
https://doi.org/10.1016/0006-8993(78)90568-1

Haile, C. N., Carper, B. A., Nolen, T. L., & Kosten, T. A. (2021). The $GABA_B$ receptor positive allosteric modulator ASP8062 reduces operant alcohol self-administration in male and female Sprague Dawley rats. *Psychopharmacology*, *238*, 2587-2600.
https://doi.org/10.1007/s00213-021-05881-0

Hill, K. G., & Kiefer, S. W. (1997). Naltrexone treatment increases the aversiveness of alcohol for outbred rats. *Alcoholism: Clinical and Experimental Research*, *21*, 637-641.
https://doi.org/10.1111/j.1530-0277.1997.tb03815

Hill, K. G., Sable, H. J., Ferraro, F. M., & Kiefer, S. W. (2010). Chronic naltrexone treatment and ethanol responsivity in outbred rats. *Alcoholism: Clinical and Experimental Research*, *34*, 272-279.
https://doi.org/10.1111/j.1530-0277.2009.01090.x

Holtyn, A. F., & Weerts, E. M. (2022). $GABA_B$ receptors and alcohol use disorders: Preclinical studies. *Current Topics in Behavioral Neurosciences*, *52*, 157-194.
https://doi.org/10.1007/7854_2020_178

Hwa, L. S., Kalinichev, M., Haddouk, H., Poli, S., & Miczek, K. A. (2014). Reduction of excessive alcohol drinking by a novel $GABA_B$ receptor positive allosteric modulator ADX71441 in mice. *Psychopharmacology*, *231*, 333-343.
https://doi.org/10.1007/s00213-013-3245-z

Kiefer, S. W. (1995). Alcohol, palatability, and taste reactivity. *Neuroscience and Biobehavioral Reviews*, *19*, 133-141.
https://doi.org/10.1016/0149-7634(94)00027-x

Kiefer, S. W., Badia-Elder, N., & Bice, P.J. (1995). Taste reactivity in high alcohol drinking and low alcohol drinking rats. *Alcoholism: Clinical and Experimental Research*, *19*, 279-284.

https://doi.org/10.1111/j.1530-0277.1995.tb01503.x

Ito, M., Spence, A., Blauwet, M. B., Heo, N., Goldwater, R., Maruff, P., & Marek, G. J. (2022). A phase 1 study to assess potential interaction between ASP8062 and alcohol in healthy adult subjects. *Journal of Psychopharmacology*, *36*, 756-767.
https://doi.org/10.1177/02698811211058967

Jonas, D. E., Amick, H. R., Feltner, C., Bobashev, G., Thomas, K., Wines, R., Kim, M. M., Shanahan, E., Gass, C. E., Rowe, C. J., & Garbutt, J.C. (2014). Pharmacotherapy for adults with alcohol use disorders in outpatient settings: a systematic review and meta-analysis. *Journal of the American Medical Association*, *311*, 1889-1900.
https://doi.org/10.1001/jama.2014.3628

Li, X., Sturchler, E., Kaczanowska, K., Cameron, M., Finn, M. G., Griffin, P., McDonald, P., & Markou, A. (2017). KK-92A, a novel $GABA_B$ receptor positive allosteric modulator, attenuates nicotine self-administration and cue-induced nicotine seeking in rats. *Psychopharmacology*, *234*, 1633-1644.
https://doi.org/10.1007/s00213-017-4594-9

Li, X., & Slesinger, P. A. (2022). $GABA_B$ receptors and drug addiction: Psychostimulants and other drugs of abuse. *Current Topics in Behavioral Neurosciences*, *52*, 119-155.
https://doi.org/10.1007/7854_2020_187

Loi, B., Maccioni, P., Lobina, C., Carai, M. A. M., Gessa, G. L., Thomas, A. W., Malherbe, P., & Colombo, G. (2013). Reduction of alcohol intake by the positive allosteric modulator of the $GABA_B$ receptor, *rac*-BHFF, in alcohol-preferring rats. *Alcohol*, *47*, 69-73.
https://doi.org/10.1016/j.alcohol.2012.11.002

Lorrai, I., Contini, A., Gessa, G. L., Mugnaini, C., Corelli, F., Colombo, G., & Maccioni, P. (2019). Operant, oral alcohol self-administration: Sex differences in Sardinian alcohol-preferring rats. *Alcohol*, *79*, 147-162.
https://doi.org/10.1016/j.alcohol.2019.04.003

Lorrai, I., Shankula, C., Gaytan, J. M., Kawamura, T., Maccioni, P., Mugnaini, C., Corelli, F., Gessa, G. L., Sanna, P. P., & Colombo, G. (2022). Reducing effect of the novel positive allosteric modulator of the $GABA_B$ receptor, COR659, on binge-like alcohol drinking in male mice and rats. *Psychopharmacology*, *239*, 201-213.
https://doi.org/10.1007/s00213-021-06022-3

Maccioni, P., Carai, M. A. M., Kaupmann, K., Guery, S., Froestl, W., Leite-Morris, K. A., Gessa, G.L., & Colombo, G. (2009). Reduction of alcohol's reinforcing and motivational properties by the positive allosteric modulator of the $GABA_B$ receptor, BHF177, in alcohol-preferring rats. *Alcoholism: Clinical and Experimental Research*, *33*, 1749-1756.
https://doi.org/10.1111/j.1530-0277.2009.01012.x

Maccioni, P., Thomas, A. W., Carai, M. A. M., Gessa, G. L., Malherbe, P., & Colombo, G. (2010). The positive allosteric modulator of the $GABA_B$ receptor, *rac*-BHFF, suppresses alcohol self-administration. *Drug and Alcohol Dependence*, *109*, 96-103.
https://doi.org/10.1016/j.drugalcdep.2009.12.019

Maccioni, P., Zaru, A., Loi, B., Lobina, C., Carai, M. A. M., Gessa, G. L., Capra, A., Mugnaini, C., Pasquini, S., Corellim F., Hyytiä, P., Lumeng, L., & Colombo, G. (2012). Comparison of the effect of the $GABA_B$ receptor agonist, baclofen, and the positive allosteric modulator of the $GABA_B$ receptor, GS39783, on alcohol self-administration in three different lines of alcohol-preferring rats. *Alcoholism: Clinical and Experimental Research*, *36*, 1748-1766.
https://doi.org/10.1111/j.1530-0277.2012.01782.x

Maccioni, P., Vargiolu, D., Thomasm A. W., Malherbe, P., Mugnaini, C., Corelli, F., Leite-Morris, K. A., Gessa, G. L., & Colombo, G. (2015). Inhibition of alcohol self-administration by positive allosteric modulators of the $GABA_B$ receptor in rats: Lack of tolerance and potentiation of baclofen. *Psychopharmacology*, *232*, 1831-1841.
https://doi.org/10.1007/s00213-014-3815-8

Maccioni, P., Colombo, G., Lorrai, I., Zaru, A., Carai, M. A. M., Gessa, G. L., Brizzi, A., Mugnaini, C., & Corelli, F. (2017). Suppressing effect of COR659 on alcohol, sucrose, and chocolate self-administration in rats: Involvement of the $GABA_B$ and cannabinoid CB_1 receptors. *Psychopharmacology*, *234*, 2525-2543.
https://doi.org/10.1007/s00213-017-4644-3

Maccioni, P., Fara, F., Lorrai, I., Acciaro, C., Mugnaini, C., Corelli, F., & Colombo, G. (2019). Suppressing effect of CMPPE, a new positive allosteric modulator of the $GABA_B$ receptor, on alcohol self-administration and reinstatement of alcohol seeking in rats. *Alcohol*, *75*, 79-87.
https://doi.org/10.1016/j.alcohol.2018.05.015

Maccioni, P., & Colombo, G. (2019). Potential of $GABA_B$ receptor positive allosteric modulators in the treatment of alcohol use disorder. *CNS Drugs*, *33*, 107-123.
https://doi.org/10.1007/s40263-018-0596-3

Maccioni, P., Kaczanowska, K., Lawrence, H., Yun, S., Bratzu, J., Gessa, G. L., McDonald, P., & Colombo, G. (2021). The novel positive allosteric modulator of the $GABA_B$ receptor, KK-92A, suppresses alcohol self-administration and cue-induced reinstatement of alcohol seeking in rats. *Frontiers in Cell and Developmental Biology*, *9*, 727576.
https://doi.org/10.3389/fcell.2021.727576

Maccioni, P., Kaczanowska, K., McDonald, P., & Colombo, G. (2022). Development of partial tolerance to the suppressing effect of the positive allosteric modulator of the $GABA_B$ receptor, KK-92A, on alcohol self-administration in rats. *Alcohol and Alcoholism*, *57*, 706-711.
https://doi.org/10.1093/alcalc/agac026

Markou, A., Weiss, F., Gold, L. H., Caine, S. B., Schulteis, G., & Koob, G. F. (1993). Animal models of drug craving. *Psychopharmacology*, *112*, 163-182.
https://doi.org/10.1007/BF02244907

Nieto, A., Bailey, T., Kaczanowska, K., & McDonald, P. (2022). $GABA_B$ receptor chemistry and pharmacology: Agonists, antagonists, and allosteric modulators. *Current Topics in Behavioral Neurosciences*, *52*, 81-118.
https://doi.org/10.1007/7854_2021_232

Orrù, A., Lai, P., Lobina, C., Maccioni, P., Piras, P., Scanu, L., Froestl, W., Gessa, G. L., Carai, M. A. M., & Colombo, G. (2005). Reducing effect of the positive allosteric modulators of the $GABA_B$ receptor, CGP7930 and GS39783, on alcohol intake in alcohol-preferring rats. *European Journal of Pharmacology*, *525*, 105-111.
https://doi.org/10.1016/j.ejphar.2005.10.005

Reus, V. I., Fochtmann, L. J., Bukstein, O., Eyler, A. E., Hilty, D. M., Horvitz-Lennon, M., Mahoney, J., Pasic, J., Weaver, M., Wills, C. D., McIntyre, J., Kidd, J., Yager, J., & Hong, S. H. (2018). The American Psychiatric Association Practice Guideline for the pharmacological treatment of patients with alcohol use disorder. *American Journal of Psychiatry*, *175*, 86-90.
https://doi.org/10.1176/appi.ajp.2017.1750101

Samson, H. H., Czachowski, C. L., Chappell, A., & Legg, B. (2003). Measuring the appetitive strength of ethanol: use of an extinction trial procedure. *Alcohol*, *31*, 77-86.
https://doi.org/10.1016/j.alcohol.2003.09.002

Starostik, M. R., Rebello, M. R., Cotter, K. A., Kulik, A., & Medler, K. F. (2010). Expression of GABAergic receptors in mouse taste receptor cells. *PLoS One*, *5*, e13639.
https://doi.org/10.1371/journal.pone.0013639

Urwyler, S. (2011). Allosteric modulation of family C G-protein-coupled receptors: from molecular insights to therapeutic perspectives. *Pharmacological Reviews*, 63, 59-126.
https://doi.org/10.1124/pr.109.002501

Urwyler, S. (2016). Allosteric modulators: the new generation of $GABA_B$ receptor ligands. In $GABA_B$ Receptor, G. Colombo ed. (Cham, Switzerland: Springer International Publishing), pp. 357-375.
https://doi.org/10.1007/978-3-319-46044-4_18

Vargiu, R., Broccia, F., Lobina, C., Lecca, D., Capra, A., Bassareo, P. P., & Bassareo, V. (2021). Chronic Red Bull consumption during adolescence: Effect on mesocortical and mesolimbic dopamine transmission and cardiovascular system in adult rats. *Pharmaceuticals*, *14*, 609.
https://doi.org/10.3390/ph14070609

Vengeliene, V., Kiefer, F., & Spanagel, R. (2008). D-cycloserine facilitates extinction of conditioned alcohol-seeking behaviour in rats. *Alcohol and Alcoholism*, *43*, 626-629.
https://doi.org/10.1093/alcalc/agn067

Wilson, G. N., Biesan, O. R., Remus, J. L., & Mickley, G. A. (2011). Baclofen alters gustatory discrimination capabilities and induces a conditioned taste aversion (CTA). *BMC Research Notes*, *4*, 527.
https://doi.org/10.1186/1756-0500-4-527

Table 1 – Effect of repeated treatment with the positive allosteric modulator of the $GABA_B$ receptor, KK-92A, on water intake in male Sardinian alcohol-preferring (sP) rats.

KK-92A (mg/kg)	Daily drinking sessions of pre-treatment period		Daily drinking sessions of treatment period							Daily drinking sessions of post-treatment period	
	-2	-1	1	2	3	4	5	6	7	+1	+2
0	0.71 ± 0.40	0.25 ± 0.08	0.20 ± 0.05	0.37 ± 0.13	0.46 ± 0.08	0.38 ± 0.06	0.46 ± 0.07	0.68 ± 0.15	0.69 ± 0.04	0.40 ± 0.04	0.50 ± 0.07
5	0.20 ± 0.08	0.70 ± 0.55	0.24 ± 0.07	0.58 ± 0.36	0.47 ± 0.10	0.40 ± 0.07	0.67 ± 0.15	0.95 ± 0.23	0.98 ± 0.09	1.74 ± 0.71	0.76 ± 0.28
10	2.78 ± 1.22	0.73 ± 0.28	0.38 ± 0.10	0.39 ± 0.08	0.34 ± 0.17	1.11 ± 0.69	0.64 ± 0.07	0.63 ± 0.10	0.66 ± 0.09	0.66 ± 0.11	1.37 ± 0.62
20	0.20 ± 0.05	0.36 ± 0.09	0.19 ± 0.08	0.32 ± 0.08	0.40 ± 0.15	0.27 ± 0.10	0.46 ± 0.09	0.56 ± 0.12	1.08 ± 0.33	0.48 ± 0.07	0.49 ± 0.13

Alcohol (10%, v/v) and water were offered under the homecage 2-bottle choice regimen in daily drinking sessions of 1 hour occurring at the last (12^{th}) hour of the dark phase of the daily light/dark cycle. KK-92A was administered intraperitoneally 30 min before the start of 7 consecutive drinking sessions (treatment period). KK-92A vehicle was administered to all rats 30 min before the start of the 2 drinking sessions of pre- and post-treatment periods. Water intake is expressed in ml/kg. Each value is the mean ± S.E.M. of *n*=8 rats.

Figure captions

Fig. 1 – Effect of repeated treatment with the positive allosteric modulator of the $GABA_B$ receptor, KK-92A, on alcohol intake in male Sardinian alcohol-preferring (sP) rats. Alcohol (10%, v/v) and water were offered under the homecage 2-bottle choice regimen in daily drinking sessions of 1 hour occurring at the last (12^{th}) hour of the dark phase of the daily light/dark cycle. KK-92A was administered intraperitoneally 30 min before the start of 7 consecutive drinking sessions (treatment period). KK-92A vehicle was administered to all rats 30 min before the start of the 2 drinking sessions of pre- and post-treatment periods. Alcohol intake is expressed in g/kg. Each bar is the mean ± S.E.M. of *n*=8 rats. ✶: *P*<0.05 in comparison to the rat group treated with 0 mg/kg KK-92A in the same drinking session (Tukey's test).

Fig. 2 – Effect of acute treatment with the positive allosteric modulator of the $GABA_B$ receptor, KK-92A, on number of lever-responses for alcohol (panel a), amount of self-administered alcohol (panel b), latency to the first alcohol reinforcer (panel c), and cumulative response pattern of alcohol self-administration (panel d) in female Sardinian alcohol-preferring rats. Rats were initially trained to lever-respond for oral alcohol [15% v/v in water; fixed ratio (FR) 5 (FR5)] and water (FR1) in daily 30-min self-administration sessions. Once lever-responding had stabilized, rats were tested with KK-92A under the same FR schedule of reinforcement. KK-92A was administered intragastrically 30 min before the start of the self-administration session. Amount of self-administered alcohol is expressed in g/kg. Latency to the first alcohol reinforcer is expressed in s. In panel d, the self-administration session was divided into 30 intervals of 1 min each. Each bar or point is the mean ± SEM of *n*=15 rats. ✶: *P*<0.01 in comparison to the rat group treated with 0 mg/kg KK-92A (Tukey's test).

Fig. 3 – Effect of acute treatment with the positive allosteric modulator of the $GABA_B$ receptor, KK-92A, on extinction responding (ER) for alcohol (panel a), latency to the first response on the alcohol-associated lever (panel b), and cumulative response pattern on the alcohol-associated lever (panel c) in female Sardinian alcohol-preferring rats. Rats were initially trained to lever-respond for oral alcohol [15% v/v in water; fixed ratio (FR) 5 (FR5)] and water (FR1) in daily 30-min self-administration sessions. Once lever-responding had stabilized, rats were tested with KK-92A under the ER schedule (i.e., lever-responding for either alcohol or water was not reinforced). KK-92A was administered intraperitoneally 30 min before the start of the ER session. Latency to the first response on the alcohol-associated lever is expressed in s. In panel c, the ER session was divided into 60 intervals of 1 min each. Each bar or point is the mean ± SEM of *n*=16 rats. ✶: *P*<0.005 in comparison to the rat group treated with 0 mg/kg KK-92A (Dunn's test).

Fig. 4 – Effect of acute treatment with the positive allosteric modulator of the $GABA_B$ receptor, KK-92A, on score of hedonic (panel a) and aversive (panel b) reactions to water and acqueous solutions of alcohol (15% v/v), sucrose (0.3 M), and quinine hydrochloride (0.0005 M) in male Sardinian alcohol-preferring rats exposed to a taste reactivity test. Solutions were pumped into the oral cavity by a permanent, implanted catheter. Orofacial movements were recorded and scored over the 2-min period of infusion of each tastant. Each bar is the mean ± SEM of *n*=12 rats.