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ORIGINAL INVESTIGATION

The $GABA_B$ receptor positive allosteric modulator ASP8062 reduces operant alcohol self-administration in male and female Sprague Dawley rats

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Abstract

Rationale Pre-clinical evidence implicates the GABAergic system in mediating the reinforcing effects of alcohol and offers a therapeutic target for alcohol use disorder (AUD). The orthosteric $GABA_B$ receptor agonist baclofen decreases alcohol self-administration in animals and alcohol use in humans; however side effects limit its utility. Pre-clinical evidence shows positive allosteric $GABA_B$ receptor modulators also decrease alcohol self-administration without untoward side effects.

Objectives We assessed the impact of the novel $GABA_B$-positive allosteric modulator ASP8062 and baclofen on operant alcohol self-administration and their potential non-specific effects.

Methods The effects of ASP8062 (1 – 10 mg/kg, PO) and baclofen (0.3 – 3 mg/kg, IP) were evaluated in male and female rats lever pressing for alcohol (10%, w/v) under a fixed ratio 2 schedule of reinforcement. On the fourth consecutive day of vehicle, ASP8062 or baclofen administration, active and inactive lever presses, reinforcers earned, head entries, and estimated alcohol consumed were analyzed. Locomotor activity was assessed in separate groups of rats following dosing.

Results Both ASP8062 and baclofen decreased alcohol self-administration and amount consumed (g/kg) in male and female rats. ASP8062 decreased operant alcohol self-administration to a greater extent in male rats, whereas baclofen was more efficacious in female rats. ASP8062 did not alter locomotor activity in either sex, whereas baclofen (3.0 mg/kg) decreased activity in male rats yet (1.0 mg/kg) increased activity in female rats.

Conclusions ASP8062 decreases alcohol reinforcement like baclofen but without non-specific effects which are influenced by sex. Results support further development of ASP8062 as a potential treatment for AUD in humans.

Keywords Alcohol use Disorder · Operant responding · Sex differences · Ethanol · Repeated administration · Oral alcohol

Introduction

An estimated 140 million Americans (aged 12 and older) regularly used alcohol in 2018 (SAMHSA 2019). Of this population, 83.7 million misused alcohol through binging and heavy drinking. Excessive alcohol misuse is associated with 90,000 deaths annually and approaches $250 billion in estimated losses per year (Sacks et al. 2015; Stahre et al. 2014). Alcohol-related disorders such as alcohol use disorder (AUD) are chronic relapsing disorders associated with compulsion to consume alcohol leading to loss of control, negative affective states, and eventual withdrawal syndrome (DSM-5 2013). Available FDA-approved pharmacotherapies for the treatment of AUD have proven inadequate and new treatments with greater efficacy, compliance, and better side effect profiles are needed.

Despite decades of research, the precise mechanism of action within the central nervous system (CNS) that relates to alcohol reinforcement is still unknown. Evidence does indicate however that alcohol acts upon the mesocorticolimbic system that mediates the reinforcing effects of most compounds associated with substance use disorders (SUD) in humans. A broad literature demonstrates the effects of alcohol on neurotransmitter systems within mesocorticolimbic circuitry including dopamine (DA) (Sulzer 2011), glutamate

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(Alasmari et al. 2018), and Gamma-aminobutyric acid (GABA) (Hillmer et al. 2015), among others. The GABA system is of particular interest in the development of potential pharmacotherapies for AUD. GABA is the primary inhibitory neurotransmitter in the CNS and modulates neural activity generally through two classes of ionotropic ($GABA_A$ and $GABA_C$) and metabotropic ($GABA_B$) receptors and their isoforms (Filip et al. 2015). Compounds that target the $GABA_B$ receptor consistently alter alcohol-related behaviors in animals. Numerous studies demonstrate the ability of the orthosteric full agonist baclofen to alter behavior in rodent models of AUD. Indeed, baclofen attenuates acquisition of voluntary and operant oral alcohol consumption (Anstrom et al. 2003; Colombo et al. 2002; Stromberg 2004), lever pressing for alcohol under fixed (FR) and progressive ratio (PR) schedules (Liang et al. 2006; Maccioni et al. 2008, 2005, 2012; Quintanilla et al. 2008; Walker and Koob 2007), and responding in models of relapse (Colombo et al. 2003; Vengeliene et al. 2018) and reinstatement (Maccioni et al. 2008). Additional evidence shows baclofen attenuates signs of alcohol withdrawal syndrome (AWS) such as anxiety and seizures in rats with prolonged alcohol exposure (Colombo et al. 2000; File et al. 1991, 1992; Knapp et al. 2007). The aforementioned studies achieved significant effects at doses of baclofen that did not induce overt non-specific effects (e.g., sedation, motor impairment) providing support for clinical studies. Initial human case study reports indicated baclofen may be efficacious in treating AWS and AUD in humans (Agabio and Colombo 2014). Follow-up randomized placebo-controlled studies however showed mixed results and significant side effects. A recent meta-analysis examined twelve randomized controlled trials (1128 participants) and found no efficacy for baclofen over placebo and noted prominent adverse events (depression, vertigo, somnolence, numbness, and muscle rigidity) (Minozzi et al. 2018). As noted by previous research (Vengeliene et al. 2018), it is intriguing that non-specific effects were not reported in the baclofen pre-clinical literature. That baclofen was administered acutely (instead of repeatedly) in most of these studies may have contributed to a lack of detecting adverse effects in animals.

Positive allosteric modulators (PAMs) of the $GABA_B$ receptor are a new class of agents that have garnered much interest as potential treatments for various SUDs, in particular for AUD (Filip et al. 2015; Maccioni and Colombo 2019). PAMs of the $GABA_B$ receptor do not have agonist activity on their own but bind to an allosteric site on the receptor different than that of endogenous GABA (Urwyler 2011). Binding to this site increases the potency and efficacy of endogenous GABA at its usual orthosteric recognition site (Gjoni et al. 2006). This unique mechanism of action suggests GABAergic PAMs could show greater efficacy and lead to fewer side effects and less tolerance compared with orthosteric GABA agonists like baclofen. Indeed, numerous studies demonstrate that PAMs of the $GABA_B$ receptor consistently decrease alcohol-associated behaviors in rodents (Agabio and Colombo 2014) such as operant alcohol self-administration (Augier et al. 2017; Liang et al. 2006; Loi et al. 2013; Maccioni et al. 2009, 2017, 2008, 2019, 2010, 2012; Vengeliene et al. 2018) suggesting GABAergic PAMs may serve as potential therapeutic agents for AUD.

A novel $GABA_B$ PAM (ASP8062) has recently been characterized in in vitro and in vivo assays demonstrating high selectivity and potency by increasing the affinity and efficacy of GABA binding to $GABA_B$ receptors (Murai et al. 2019). Orally administered ASP8062 results in appreciable levels in plasma and brain in rats. Clinical studies assessed the tolerability and pharmacokinetics of ASP8062 in two Phase I clinical trials (Walzer et al. 2020). Single ($N = 56$, males), and multiple ascending (14 days, $N = 56$, 32 males, 24 females) oral dosing studies of ASP8062 showed detectable compound in CSF, a half-life of 40 – 50 h, and no evidence of significant treatment emergent adverse effects. Based on growing evidence that $GABA_B$ PAMs are well tolerated in humans and decrease alcohol self-administration in animals, the present study was designed to assess the impact of repeated ASP8062 on maintenance of operant alcohol self-administration in male and female Sprague Dawley rats as compared with baclofen. Baclofen is known to disrupt behavior, so potential non-specific effects on gross locomotor activity were also assessed for both compounds.

Materials and methods

All procedures were approved by the University of Houston Institutional Animal Care and Use Committee in accordance with the National Institutes of Health Guidelines (ILAR 2011).

Animals

A total of 14 male and 13 female Sprague Dawley rats (Charles River, Wilmington, MA) were used for the alcohol self-administration studies. Separate groups of rats (male = 55, female = 55) were used to assess the impact of repeated vehicle (male = 10, female = 10) and three different doses of ASP8062 (male, 1 mg/kg = 10, 3 mg/kg = 10, 10 mg/kg = 1 0; female, 1 mg/kg = 10, 3 mg/kg = 10, 10 mg/kg = 10) and baclofen (male, 0.3 mg/kg = 5, 1 mg/kg = 5, 3 mg/kg = 5; female, 0.3 mg/kg = 5, 1 mg/kg = 5, 3 mg/kg = 5) tested on locomotor activity in open field chambers. Rats were initially housed 3 – 5 to a cage in polypropylene cages housed in circular towers (Animal Care Systems, Inc, Centennial, CO) located within a temperature- and humidity-controlled vivarium that was maintained on a

12:12 light/dark cycle (lights on at 6 AM). Rats weighed about 250 – 350 gm at the start of the experiment and were at least 100 days old. Food and water were available ad libitum throughout the study. To facilitate alcohol self-administration, rats were exposed to alcohol in vapor chambers (La Jolla Alcohol Research, La Jolla, CA) for 6 weeks prior to operant training using a chronic intermittent alcohol exposure (i.e., alcohol vapors were on for 14 h and off for 10 h, 5 days per week) (Gilpin et al. 2008; Haile and Kosten 2017).

Drugs

Alcohol (ethyl alcohol, 190 Proof, USP grade, Koptec, King of Prussia, PA) was diluted to 10% (with RO water, w/v) and made available for consumption via standard operant chambers (described below). ASP8062 was prepared as a suspension in methyl cellulose (0.5%, Sigma Aldrich, St. Louis, MO), and baclofen was dissolved in sterile saline. The three doses of ASP8062 (1, 3, and 10 mg/kg) and baclofen (0.3, 1, and 3 mg/kg, Sigma Aldrich, St. Louis, MO) were prepared fresh for each test day in a volume of 1 ml. Methyl cellulose or ASP8062 was administered intra-gastrically (*per os*, PO) and baclofen via intraperitoneal (IP) injection 30 min prior to beginning the test sessions. Each dose of test drug was administered for four consecutive days in a randomized order within test drug across rats with at least 3 – 7 days intervening days between dose administrations. The effects of baclofen on alcohol self-administration were tested after all doses of ASP8062 were tested.

Behavioral apparatus

Ten, standard operant chambers (Coulbourn Instruments, Holliston, MA) enclosed in sound-attenuating cubicles (Coulbourn Habitest isolation cubicle) were used in the present study. Each chamber was equipped with two levers located on either side of an access area into which a dipper (0.1-mL capacity) could protrude. Prior to activation, the dipper was maintained in a small reservoir of alcohol. Infrared sensors located in the dipper access area tabulated numbers of head entries. A house light, a dipper access area light, and two sets of three, colored cue lights, one above each lever, were located within the operant chamber. Stimulus parameters and data tabulation were programmed using Graphic State Notation (version 4.0).

The open field chambers consisted of 17.08" L × 17.08″ W × 11.92″ H Plexiglas walls connected by metal brackets. Infra-red emitters and detectors line the base of the walls to detect movement. Data were collected using a computer and specialized software (MED Associates, St Albans, VT).

Operant self-administration training and testing

Training sessions (60 min) began with the illumination of the house light and, initially, two non-contingent dipper presentations (primes) for 10 s. The dipper access light was illuminated for the entire length of the dipper presentation time. Dipper presentation times were gradually reduced (10 > 5 > 3 s) over subsequent weeks of training, based on each animal's performance, until they were 3 s in duration. Three cue lights were illuminated above both the active and inactive levers. When the rat pressed the active lever, the house light would turn off, dipper would protrude, and the access area light and the triple cue light above the levers went off. Presses on the inactive lever had no programmed consequences. Once a rat emitted 20 or more active lever presses with 20% variability or less in response levels over 2 days, the ratio requirement was raised to fixed-ratio 2 (FR2), the schedule used for the rest of the training. Stable response levels under the FR2 schedule ($\leq$20% variability over 2 days) under 3 s time of the dipper presentation were required for the animal to move into the testing phase.

Test sessions (60 min) were conducted over 4 consecutive days in which test drug was administered. Each dose of test drug and vehicle was administered 30 min prior to the test sessions on each of the four consecutive days.

Open field test-locomotor activity

Different groups of rats were assigned to a specific drug and dose to assess potential non-specific effects on locomotor activity. Similar to tests performed in the self-administration studies, saline, ASP8062, and baclofen were administered 30 min before rats were placed into the open field chambers and activity recorded for 30 min over 5 consecutive days.

Statistical analysis

Statistical analysis was conducted using SAS version 9.4 (SAS Institute). Self-administration data (active lever presses, inactive lever presses, reinforcers earned, and head entries) were initially analyzed using repeated measures ANOVA with drug dose and sex as primary factors and day as the repeating factor with an additional control for test dose order within drug. Data from tests performed on day 4 of drug treatment were used for further analysis since maximal effects of both ASP8062 and baclofen occurred on this day. For day 4, data from the self-administration tests were analyzed separately by sex using repeated-measure ANOVAs with the primary factor of drug dose (ASP8062 0, 1, 3, 10 mg/kg or baclofen 0, 0.3, 1, 3 mg/kg) and controlling for test dose order within drug. Data on alcohol intake (g/kg) were analyzed separately by sex in a similar manner. Data on total distanced traveled in (cm) in the open field tests

were also analyzed separately by sex using ANOVA with the primary factors of drug dose (ASP8062 0, 1, 3, 10 mg/kg or baclofen 0, 0.3, 1, 3 mg/kg). This model was also adjusted by the baseline total distance traveled for each animal.

For all analyses, significant main effects were followed by post hoc pairwise multiple comparison analysis (false-discovery rate (FDR) nethod between doses and Dunnett's test comparisons to vehicle. Post hoc comparisons with p values less than 0.05 were considered significant and those with p values less than 0.10 were considered to trend towards significance.

Results

Alcohol self-administration

Four male and 3 female rats failed to acquire the operant and were excluded from the study leaving $N = 10$ per sex. For those rats that did acquire, active lever presses were significantly greater than inactive lever presses following administration of vehicle in both males ($F_{(1,9)} = 140.57$, $P < 0.001$) and females ($F_{(1,9)} = 312.60$, $P < 0.001$) indicating alcohol was acting as a reinforcer and controlling contingent behavior of both male (Figs. 1a – c) and female (Figs. 2a – c) rats.

Effects of ASP8062 and Baclofen across days

Active lever presses emitted following dosing across days showed a significant interactive effect between drug dose and day for ASP8062 ($F(9287) = 3.61$, $P < 0.001$) and baclofen ($F(9287) = 2.45$, $P < 0.05$). A significant interaction was also noted between sex and drug dose for both compounds [ASP8062: ($F(3287) = 3.03$, $P < 0.05$); baclofen: ($F(3287) = 3.07$, $P < 0.05$)]. The largest effect of study drug for both compounds was seen on day 4, when differences between active study drug and the vehicle were largest. Due to the differences seen between sexes and the largest study effect being observed on day 4, subsequent analyses were separated by sex and used only data on day 4.

ASP8062 tests male rats

Active and inactive lever presses following vehicle and test doses of ASP8062 for male rats are presented in Fig. 1a. Active lever presses emitted following dosing revealed a significant main effect for drug dose ($F_{(3, 35)} = 14.06$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated a significant decrease in active lever presses for all test doses compared with vehicle ($P < 0.001$). No significant differences were noted between any of the ASP8062 test doses; however, there were trends towards significance between ASP8062 10 mg/kg vs ASP8062 1 mg/kg ($P = 0.06$) and vs 3 mg/kg ($P = 0.09$).

Analysis of the number of inactive lever presses across drug doses revealed no significant differences across tests ($F_{(3, 35)} = 1.72$, $P > 0.05$).

Reinforcers earned (dipper presentations) following vehicle and test doses for male rats are presented in Fig. 1bb. The number of dipper presentations earned following dosing with ASP8062 revealed a significant main effect for drug dose ($F_{(3, 35)} = 13.23$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in dipper presentations for all test doses compared with vehicle ($P < 0.01 - 0.001$). A significant decrease was also found between ASP8062 1 mg/kg vs ASP8062 10 mg/kg doses ($P < 0.05$). There was a trend towards significance for fewer dipper presentations between ASP8062 3 mg/kg and ASP8062 10 mg/kg ($P = 0.09$).

Head entries following vehicle and ASP8062 test doses for male rats are presented in Fig. 1c. Analysis of number of head entries following dosing revealed a significant main effect for drug dose ($F_{(3, 35)} = 18.72$, $P < 0.001$). Post hoc analysis following the main effect of drug dose revealed significant decreases in head entries for all test doses compared with vehicle ($P < 0.01 - 0.001$). Significant decreases were also found between ASP8062 10 mg/kg vs ASP8062 1 mg/kg ($P < 0.01$) and vs ASP8062 3 mg/kg ($P < 0.05$).

Baclofen tests male rats

Active and inactive lever presses following vehicle and test doses of baclofen for male rats are presented in Fig. 1d. Active lever presses emitted following dosing revealed a significant main effect for drug dose ($F_{(3, 35)} = 40.73$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in active lever presses for both baclofen 1.0 mg/kg and 3.0 mg/kg compared with vehicle ($P < 0.001$). Significant differences were found between the two highest baclofen test doses ($P < 0.001$) and baclofen 0.3 mg/kg, with the fewest active lever presses seen at the baclofen 3.0 g/kg test dose followed by baclofen 1.0 mg/kg.

Analysis of the number of inactive lever presses across drug doses revealed a trend toward significant differences across tests ($F_{(3, 35)} = 5.50$, $P = 0.08$). Post hoc analysis following the main effect of drug dose indicated a significant decrease in number of inactive lever presses between vehicle and baclofen 3.0 mg/kg ($P < 0.05$).

Reinforcers earned (dipper presentations) following vehicle and baclofen test doses for male rats are presented in Fig. 1s. Dipper presentations earned following dosing revealed a significant main effect for drug dose ($F_{(3, 35)} = 9.71$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in dipper

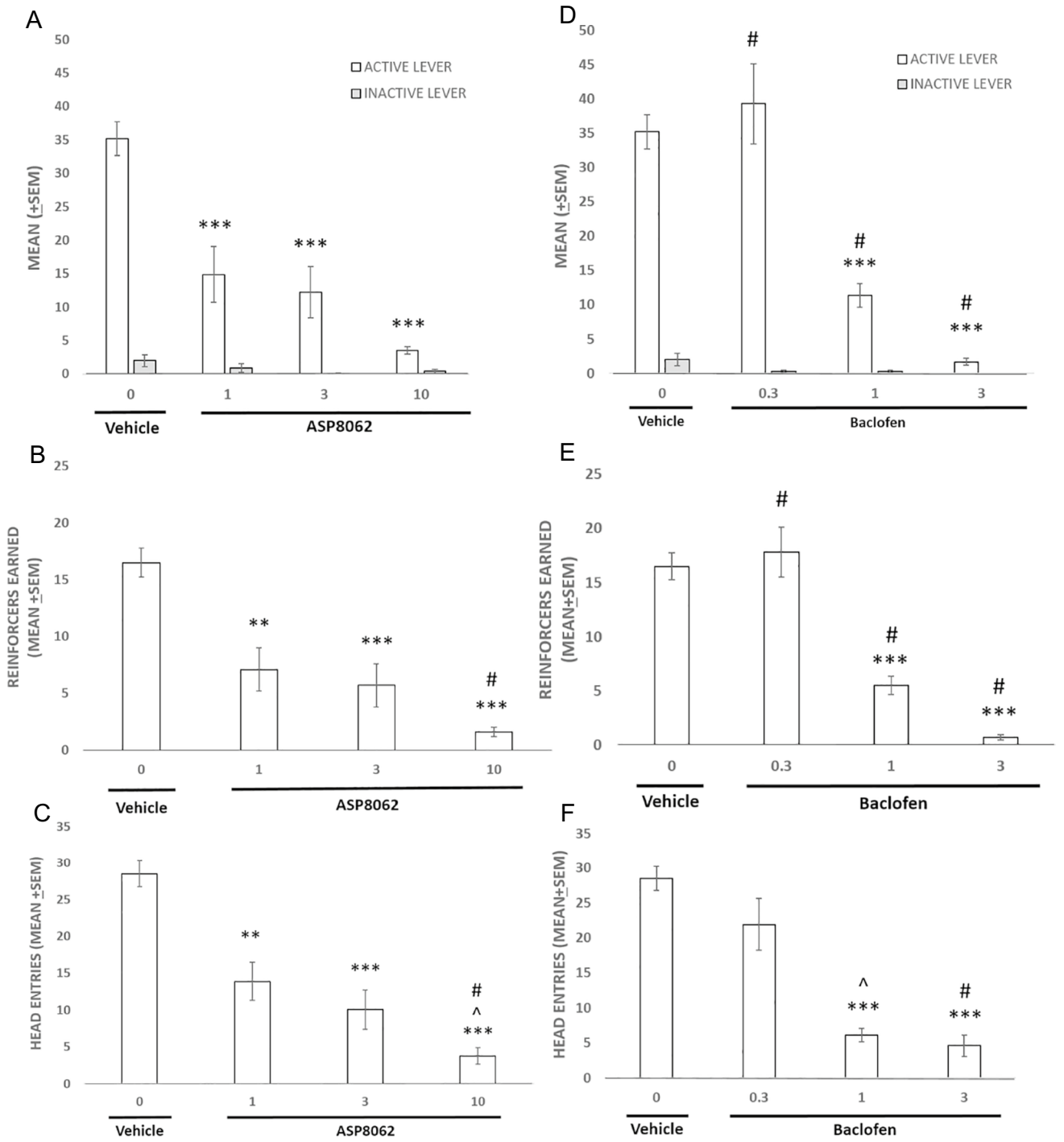
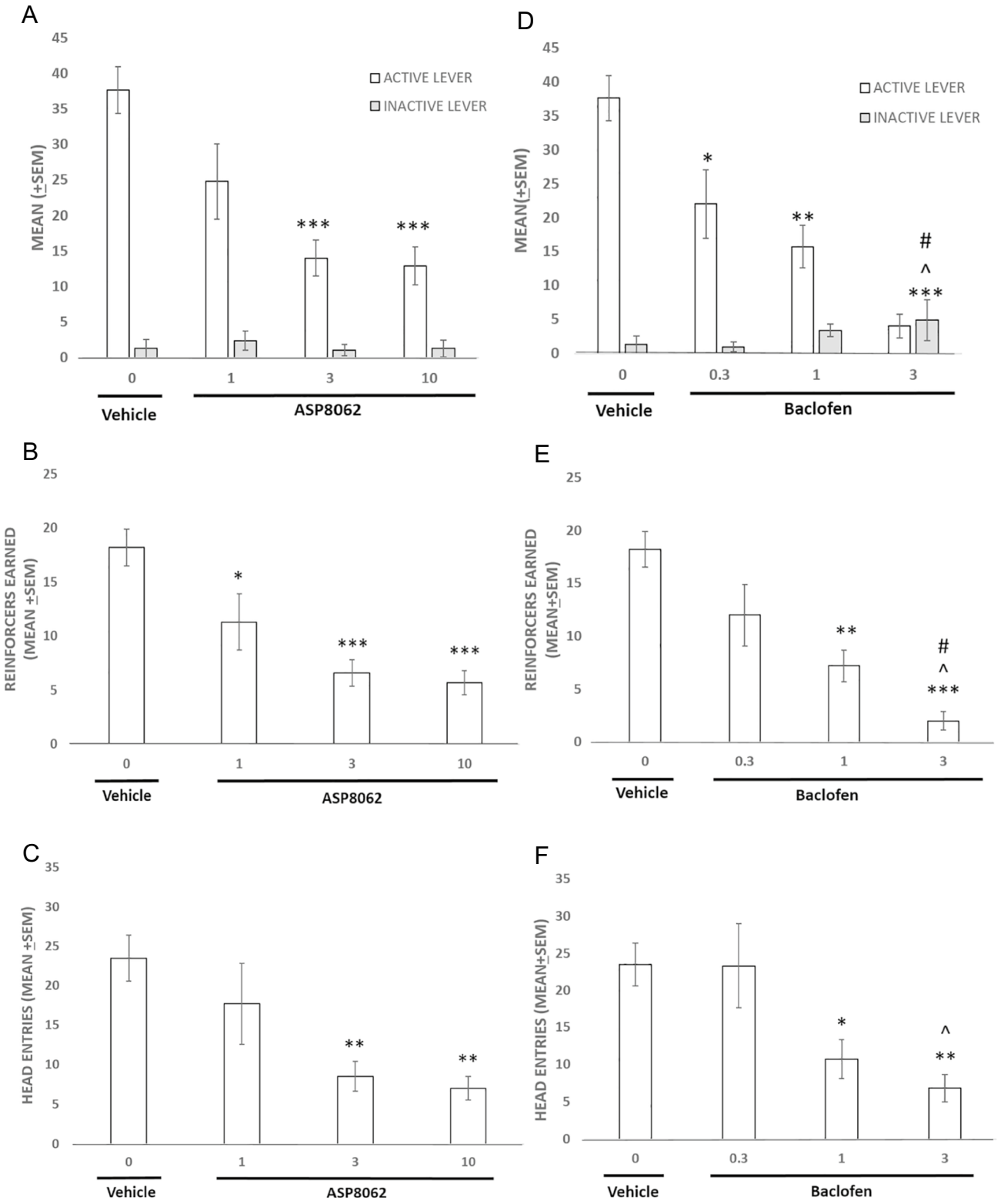


Fig. 1 Effects of ASP8062 on operant self-administration of 10% alcohol under a fixed ratio (FR2) schedule of reinforcement in daily 60 min sessions, in male rats. The effect of ASP8062 on (**a**) active and inactive lever presses, (**b**) reinforcers earned, (**c**) head entries and baclofen, (**d**) active and inactive lever presses, (**e**) reinforcers earned, (**f**) head entries. Data are presented as mean (±SEM) ($N=10$ per group). Statistical differences: (**a**) vs. Vehicle *** ($P<0.001$); (**b**) vs. Vehicle ** ($P<0.01$) *** ($P<0.001$), 1 mg/kg vs. 10 mg/kg # ($P<0.05$); (**c**) vs. Vehicle ** ($P<0.01$) *** ($P<0.001$), vs. ASP8062 10 mg/kg, 1 mg/kg # ($P<0.05$) and 3 mg/kg ^ ($P<0.001$); (**d**) vs. Vehicle *** ($P<0.001$). All baclofen dose comparisons # ($P<0.001$), (**e**) vs. Vehicle ***($P<0.001$). All baclofen dose comparisons # ($P<0.001$), (**f**) vs. Vehicle *** ($P<0.001$), vs. baclofen 0.3 mg/kg, 1 mg/kg ^($P<0.001$) and 3 mg/kg # ($P<0.001$)



presentations for both 1.0 mg/kg and 3.0 mg/kg baclofen compared with vehicle ($P < 0.001$). Significant differences were noted between the two highest baclofen test doses ($P < 0.001$) and baclofen 0.3 mg/kg, with the fewest dipper presentations seen in the baclofen 3.0 g/kg test dose followed by baclofen 1.0 mg/kg.

Number of head entries following vehicle and baclofen test doses for male rats are presented in Fig. 1f. Head entries

◂**Fig. 2** Effects of ASP8062 on operant self-administration of 10% alcohol under a fixed ratio (FR2) schedule of reinforcement in daily 60-min sessions, in female rats. The effect of ASP8062 on (**a**) active and inactive lever presses, (**b**) reinforcers earned, (**c**) head entries and baclofen (**d**) active and inactive lever presses, (**e**) reinforcers earned, (**f**) head entries. Data are presented as MEAN (±SEM) ($N=10$ per group). Statistical differences: (**a**) vs. Vehicle *** ($P<0.001$); (**b**) vs. Vehicle, * ($P<0.05$), *** ($P<0.001$); (**c**) vs. Vehicle, ** ($P<0.01$); (**d**) vs. Vehicle * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), baclofen 3.0 mg/kg vs 0.3 mg/kg ^ ($P<0.001$) and 1.0 mg/kg # ($P<0.001$); (**e**) vs. Vehicle, ** ($P<0.01$), ***($P<0.001$), 3.0 mg/kg vs 0.3 mg/kg ^ ($P<0.001$) and 1.0 mg/kg # ($P<0.001$); (**f**) vs. Vehicle * ($P<0.05$), ** ($P<0.01$), 3.0 mg/kg vs 0.3 mg/kg ^ ($P<0.05$)

following dosing revealed a significant main effect for drug dose ($F_{(3, 35)}=25.16$, $P<0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in head entries for both 1.0 mg/kg and 3.0 mg/kg baclofen compared with vehicle ($P<0.001$). Significant differences were also revealed between 0.3 mg/kg compared to 1.0 mg/kg and to 3.0 mg/kg baclofen ($P<0.001$), with fewer head entries seen in the baclofen 1.0 mg/kg and 3.0 mg/kg doses.

ASP8062 tests female rats

The number of active and inactive lever presses following vehicle and ASP8062 test doses for female rats are presented in Fig. 2a. Active lever presses emitted following ASP8062 dosing revealed a significant main effect for drug dose ($F_{(3, 35)}=9.15$, $P<0.001$). Post hoc analysis of number of active lever presses following the main effect of drug dose indicated significant decreases in active lever presses for ASP8062 3 mg/kg and 10 mg/kg compared with vehicle ($P<0.001$). No significant differences were noted between any of the ASP8062 test doses; however, there was a trend towards significance for fewer active lever presses between 1 mg/kg and 3 mg/kg ($P=0.052$) and 10 mg/kg ($P=0.05$).

Analysis of the numbers of inactive lever presses across drug doses revealed no significant differences across groups ($F_{(3, 35)}=0.44$, $P>0.05$).

Reinforcers earned (dipper presentations) following vehicle and ASP8062 test doses for female rats are presented in Fig. 2b. Dipper presentations earned following dosing revealed a significant main effect for drug dose ($F_{(3, 35)}=9.96$, $P<0.001$). Post hoc analysis of number of dipper presentations following the main effect of drug dose indicated significant decreases in dipper presentations for all test doses compared with vehicle ($P<0.05-0.001$). No significant differences were noted between any of the ASP8062 test doses; however, there were trends towards significant decreases in dipper presentations between 1 mg/kg compared to both 3 mg/kg ($P=0.06$) and 10 mg/kg ($P=0.055$).

Head entries following vehicle and ASP8062 test doses for female rats are presented in Fig. 2c. Head entries following dosing revealed a significant main effect for drug dose ($F_{(3, 35)}=5.36$, $P<0.01$). Post hoc analysis of head entries following the main effect of drug dose indicated significant decreases in head entries for both 3 mg/kg and 10 mg/kg compared with vehicle ($P<0.01$). No significant differences were noted for any of the comparisons between ASP8062 test doses.

Baclofen tests female rats

Active and inactive lever presses following vehicle and baclofen test doses for female rats are presented in Fig. 2d. Active lever presses emitted following dosing revealed a significant main effect for drug dose ($F_{(3, 35)}=21.49$, $P<0.001$). Post hoc analysis following the main effect of drug dose revealed significant decreases in active lever presses for all baclofen doses compared to vehicle ($P's<0.05–0.001$). Significant decreases in active lever presses were also found between 3.0 mg/kg compared with both 0.3 mg/kg and 1.0 mg/kg ($P<0.001$).

Analysis of inactive lever presses revealed no significant differences across doses ($F_{(3, 35)}=1.44$, $P>0.05$).

Dipper presentations (reinforcers earned) following vehicle and test doses for female rats are presented in Fig. 2e. Dipper presentations earned following baclofen dosing revealed a significant main effect for drug dose ($F_{(3, 35)}=18.50$, $P<0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in dipper presentations for both 1.0 mg/kg and 3.0 mg/kg compared with vehicle ($P<0.01-0.001$). Significant decreases in dipper presentations were also found between 3.0 mg/kg compared with both baclofen 0.3 mg/kg ($P<0.001$) and 1.0 mg/kg ($P<0.01$).

Head entries following vehicle and baclofen test doses for female rats are presented in Fig. 2f. Head entries following dosing revealed a significant main effect for drug dose ($F_{(3, 35)}=7.04$, $P<0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in head entries for both 1.0 mg/kg and 3.0 mg/kg compared with vehicle ($P<0.05-0.01$). A significant decrease in head entries was also found between 3.0 mg/kg and 0.3 mg/kg ($P<0.05$). There was a trend towards a significantly fewer head entries between 1.0 mg/kg and 0.3 mg/kg ($P=0.07$).

Estimated alcohol consumed

Total alcohol (g/kg) intake was estimated for each rat at baseline and following test doses of ASP8062 and baclofen based on the number of reinforcers earned and volume of alcohol consumed. The amount of alcohol self-administered at baseline significantly differed between male and female rats ($F_{(1, 18)}=18.86$, $P<0.001$), with female rats consuming more alcohol than male rats.

ASP8062 tests male rats

Estimated total alcohol intake (g/kg) following vehicle and test doses of ASP8062 for male rats are presented in Fig. 3a. Alcohol intake following dosing revealed a significant main effect for drug dose ($F_{(3, 27)} = 17.74$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated a significant decrease in alcohol intake for all test doses compared with vehicle ($P < 0.001$). A significant decrease in alcohol intake was noted between 1 mg/kg and 10 mg/kg ($P < 0.05$). A trend towards a significant decrease in alcohol consumption was revealed between 3 mg/kg and 10 mg/kg ($P = 0.09$).

Baclofen tests male rats

Estimated total alcohol intake (g/kg) following vehicle and test doses of baclofen for male rats are presented in Fig. 3b. Alcohol intake following dosing revealed a significant main effect for drug dose ($F_{(3, 27)} = 33.14$, $P < 0.001$). Post hoc analysis following the main effect of drug dose revealed significant decreases in alcohol intake following 1.0 mg/kg and 3.0 mg/kg compared with vehicle ($P < 0.001$). Significant differences were noted between all three baclofen test doses ($P < 0.05 - 0.001$), with the least alcohol intake observed at the 3.0 mg/kg test dose.

ASP8062 tests female rats

Estimated total alcohol intake (g/kg) following vehicle and test doses of ASP8062 for female rats are presented in Fig. 3c. Alcohol intake following dosing revealed a significant main effect for drug dose ($F_{(3, 27)} = 10.66$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in alcohol intake for all test doses compared with vehicle ($P < 0.05 - 0.001$). There were no significant differences revealed across any ASP8062 test doses; however, there was a trend towards a significant decrease in alcohol consumption between 1 mg/kg and 10 mg/kg ($P = 0.08$).

Baclofen tests female rats

Estimated alcohol intake (g/kg) following vehicle and test doses of baclofen for female rats are presented in Fig. 3D. Alcohol intake following dosing revealed a significant main effect for drug dose ($F_{(3, 27)} = 13.69$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated significant decreases in alcohol intake for both 1.0 mg/kg and 3.0 mg/kg compared with vehicle as well as between 0.3 mg/kg and 3.0 mg/kg ($P < 0.001$). There was a trend towards significant decreased alcohol intake between 1.0 mg /kg with both 0.3 mg/kg ($P = 0.09$) and 3.0 mg/kg ($P = 0.07$).

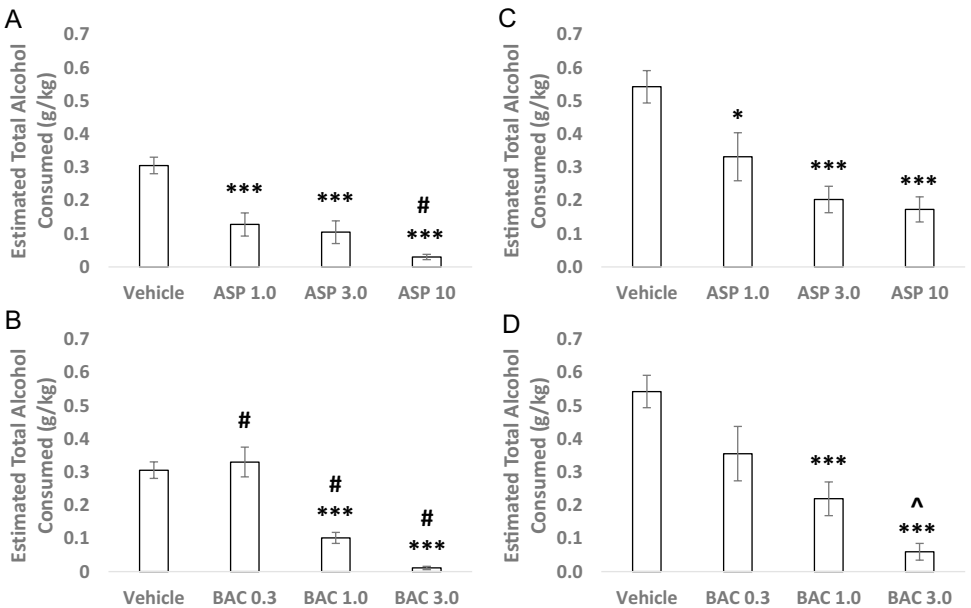


Fig. 3 sEffects of chronic ASP8062 and baclofen on estimated total alcohol consumed (g/kg) in male (**a**) [ASP8062] and (**b**) [baclofen]) and female (**c**) [ASP8062] and (**d**) [baclofen]) Sprague Dawley rats trained to lever press for oral alcohol (10%, vol/vol) under a fixed ratio (FR2) schedule of reinforcement in daily 60 min sessions. Data are presented as MEAN (±SEM) (N = 10 per group). Statistical differences: (**a**) vs. Vehicle vs all ASP8062 doses ***($P < 0.001$), 1 mg/kg vs 10 mg/kg #($P < 0.05$); (**b**) vs. Vehicle *** ($P > 0.001$), All baclofen dose comparisons #($Ps < 0.001$); (**c**) vs. Vehicle * ($P < 0.05$), ***($P < 0.001$); (**d**) vs. Vehicle ***($P < 0.001$), vs. baclofen 3.0 mg/kg vs. 0.3 mg/kg ^($P < 0.01$)

Open field: ASP8062 and Baclofen in male rats

Distance traveled (cm) following vehicle or test doses of ASP8062 for male rats are presented in Fig. 4a. Distance traveled following dosing revealed no significant main effect for drug dose ($F_{(3, 35)} = 0.57$, $P > 0.05$).Total distance traveled (cm) following vehicle or test doses of baclofen for male rats are presented in Fig. 4b. Distance traveled following dosing revealed a significant main effect for drug dose ($F_{(3, 20)} = 17.42$, $P < 0.001$). Post hoc analysis following the main effect of drug dose indicated a significant decrease in total distance traveled for baclofen 3.0 mg/kg compared with vehicle ($P < 0.001$). Significant decreases in total distance traveled were observed following 3.0 mg/kg compared with other baclofen test doses ($P < 0.001$).

Open field: ASP8062 and baclofen in female rats

Distance traveled (cm) following vehicle or test doses of ASP8062 for female rats are presented in Fig. 4c. Distance traveled following dosing revealed no significant main effect for drug dose ($F_{(3, 35)} = 0.93$, $P > 0.05$). Total distance traveled (cm) following vehicle or test doses of baclofen for female rats are presented in Fig. 4d. Analysis of the total distance traveled across drug doses revealed a significant increase in total distance traveled between vehicle and baclofen 1.0 mg/kg ($P < 0.05$).

Discussion

Results from the present series of studies show both the allosteric $GABA_B$ PAM ASP8062 and the orthosteric $GABA_B$ agonist baclofen reduced operant alcohol self-administration and amount consumed in both male and female Sprague Dawley rats. ASP8062-induced decreases in alcohol self-administration occurred at doses devoid of any non-specific effects in both sexes as evidenced by open field tests. In contrast, baclofen dramatically decreased general activity at the highest dose tested in male rats. Unexpectedly, baclofen significantly increased total activity in female rats, yet this same dose decreased alcohol self-administration. This study is one of the few studies to directly compare the effects of a $GABA_B$ PAM to baclofen in rats that orally self-administer alcohol and assess nonspecific effects of both drugs on locomotor activity. It is also the first study to assess ASP8062 on oral alcohol self-administration in outbred Sprague Dawley rats of both sexes that suggest its clinical

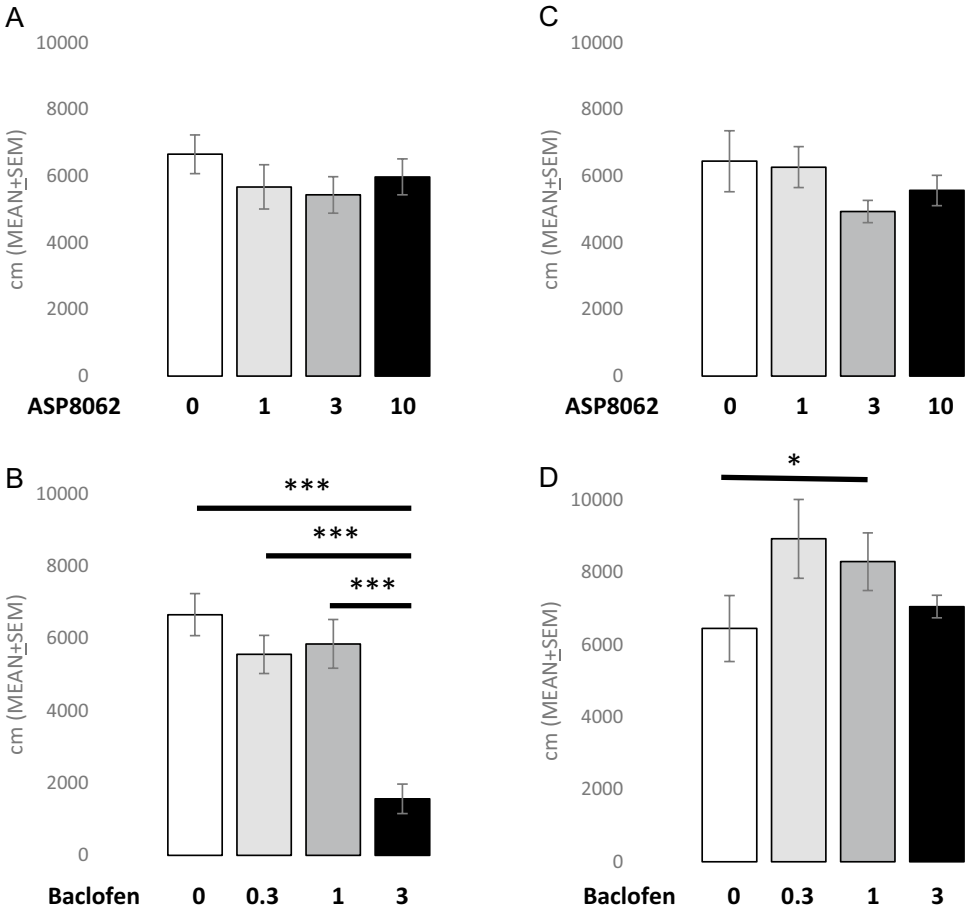


Fig. 4 Effects of repeated ASP8062 and baclofen on total distance traveled (cm) in male (**a** and **b**) and female rats (**c** and **d**) Sprague Dawley rats following ASP8062 or baclofen in the open field test. Data are presented as MEAN(± SEM) (N = 5–10/ group). Numbers are drug doses on a mg/kg basis delivered PO (ASP8062) or IP (Baclofen). Statistical differences (**b**) vs. 3.0 mg/kg *** ($P < 0.001$); (**d**) vs. 0 and 1 mg/kg * ($P < 0.05$)

utility for treating AUD may generalize across genetic background and be effective for both males and females. The study is also unique since the test drugs were administered over multiple days that mimic dosing in humans. Finally, that ASP8062 has been tested in human Phase 1 and 2 clinical trials highlights the immediate translational impact this compound may possess.

Results with ASP8062 presented herein add to the growing number of $GABA_B$ PAM compounds shown to decrease alcohol self-administration and other alcohol-associated behaviors in rats (Augier et al. 2017; Liang et al. 2006; Loi et al. 2013; Maccioni et al. 2009, 2017, 2008, 2019, 2010, 2012; Vengeliene et al. 2018). For example, acute administration of the PAM CGP7930 (3-(3',5'-Di-tert-butyl-4'-hydroxy)phenyl-2,2-dimethylpropanol) significantly decreased lever pressing for alcohol (10%) under an FR3 requirement at the highest dose tested (20 mg/kg, IP) in male inbred alcohol-preferring iP rats (Liang et al. 2006). We found 4 days of ASP8062 administration leads to significant decreases in responding for alcohol in both male and female Sprague Dawley rats at lower doses (1, 3, and 10 mg/kg, PO) suggesting greater potency compared with CGP7930 although there are procedural differences between studies. Other results from that same study showed acute administration of baclofen at 3 mg/kg, but not 2 mg/kg, decreased alcohol responding, whereas we showed decreased lever pressing after administering doses of 1 and 3 mg/kg for 4 days. However, as elaborated below, the higher dose of baclofen produced non-specific effects (e.g., decreased locomotor activity, sedation) as measured with the open field test. In contrast to the present study, the highest dose of baclofen (3 mg/kg) tested in the referenced study did not alter locomotor activity. Differences in experimental outcomes may be due to a number of factors such as sex and rat strain (iP vs. Sprague Dawley) utilized between studies.

Maccioni et al. compared the effects of both PAM GS39783 (N, N'-Dicyclopentyl-2-methylsulfanyl-5-nitropyrimidine-4, 6-diamine) and baclofen (1 and 3 mg/kg, IP) on operant alcohol self-administration under a progressive ratio (PR) schedule of reinforcement in male Sardinian alcohol-preferring rats (sP) (Maccioni et al. 2008). Results showed GS39783 decreased total number of lever presses for alcohol (15% v/v) across a broad dose range (25 – 100 mg/kg, PO) and did not affect responding for sucrose. Baclofen also significantly decreased number of lever presses at both doses tested; however the highest dose (3 mg/kg) significantly decreased the number of lever responses for sucrose. Consonant with the present study decreases in lever responding for alcohol and sucrose in the aforementioned study are likely due to the non-specific effects of baclofen. An extensive follow-up study assessed GS39783 and baclofen on operant alcohol self-administration in three different alcohol-preferring rat strains under FR4 and PR schedules of reinforcement (Maccioni et al. 2012). The authors again demonstrated that GS39783 significantly decreases alcohol self-administration under a PR schedule of reinforcement and extended this effect to two other alcohol-preferring rat strains (P and AA rats) although the potency of GS39783 differed across strains. Strain differences in potency of GS39783 may relate to significant baseline differences of alcohol self-administration (Maccioni et al. 2012). The impact of baclofen on lever pressing for alcohol also differed by strain in that 1.7 and 3 mg/kg significantly decreased lever pressing for alcohol in P rats, whereas only 3 mg/kg was effective in sP and AA rats, a dose that also significantly decreased lever pressing for food in all strains. In contrast to the present study however, we found that compared to vehicle pretreatment, 1 mg/kg baclofen does produce decreases on self-administration measures in both male and female rats. This discrepancy is likely due to the strain of rats used (inbred vs. outbred). Inbred alcohol-preferring strains develop tolerance to alcohol's effects that is mediated through GABAergic circuitry (Colombo et al. 2006; Kemppainen et al. 2010) and also have altered $GABA_B$ receptor function (Castelli et al. 2005). Indeed, the EC_{50} for baclofen-stimulated [^{35}S] GTPγS in limbic areas was 125% greater in sP rats compared with non-alcohol preferring strains suggesting compromised $GABA_B$ receptor function (Castelli et al. 2005). Compromised $GABA_B$ receptor function would likely necessitate greater amounts of baclofen needed to decrease intake in an alcohol-preferring strain suggesting a shift in the dose response function of baclofen to the right. Although speculative, that sP rats have altered $GABA_B$ receptor function and are resistant to the non-specific effects of baclofen may explain divergent findings between studies.

Consistent with the growing literature on the effects of PAMs on alcohol's action in rodents, acute dosing with the PAM COR659 [methyl2-(4chlorophenylcarboxamido)-4-ethyl-5-methylthiophene-3carboxylate] decreased operant self-administration (FR4 and PR schedules) of alcohol (15% v/v) in sP rats (Maccioni et al. 2017). Doses of COR659 that significantly decreased lever pressing for alcohol however also decreased breaking points for sucrose and self-administration of a chocolate solution (FR10) suggesting non-specific effects although higher doses did not impact open field activity. In the present study, none of the doses of ASP8062 efficacious for reducing lever pressing for alcohol produced non-specific effects in either male or female rats suggesting decreases were a result of ASP8062 acting specifically on alcohol reinforcement circuitry in the CNS. The lack of effects of ASP8062 on locomotor activity is consistent with results from a previous study that utilized rotarod to measure motor coordination at doses higher (30 mg/kg, PO) than those tested in the present study (Murai et al. 2019). In one

of the few studies conducted using only female rats (sP) the effects of the PAM CMPPE (2-{1-[2-(4-chlorophenyl)-5-methylpyrazolo[1,5-a]pyrimidin-7-yl]-2-piperidinyl ethanol) were assessed on operant alcohol self-administration under FR5 and PR schedules of reinforcement (Maccioni et al. 2019). CMPPE significantly decreased responding for alcohol under both schedules and did not alter lever pressing for food. Overall, results from the present study and others further support the notion that $GABA_B$ receptors play a critical role in alcohol reinforcement and may provide a potential pharmacotherapeutic target for the treatment of AUD.

A strength of the present study is that we employed both male and female rats. There are clear sex differences in response to many substances associated with SUDs in humans (Becker and Koob 2016). Further, sex differences in alcohol reinforcement are also apparent in outbred (Nieto and Kosten 2017; Randall et al. 2017) and inbred (Cailhol and Mormède 2001; Loi et al. 2014; Lorrai et al. 2019; Moore and Lynch 2015) rats. Although experimental parameters vary across studies, in general, female rats consume more alcohol on a g/kg basis than male rats (Loi et al. 2014; Lorrai et al. 2019; Nieto and Kosten 2017; Randall et al. 2017). We also observe that female rats self-administered greater amounts of estimated alcohol consumption on a g/kg basis compared with males but at much lower levels compared with inbred alcohol-preferring strains. More importantly are potential sex differences in response to pharmacotherapies for treating AUD in humans. In general, evidence indicates that compounds that are either presently being used to treat AUD or are in development appear to be more effective in reducing alcohol consumption in male rats compared with females and ASP8062 fits this profile (Moore and Lynch 2015; Nieto et al. 2018). Indeed, compared with vehicle, we found that ASP8062 significantly decreased all measures of operant alcohol self-administration at all doses tested in male rats (Figs. 1a – c). In contrast, only the two highest doses of ASP8062 (3 and 10 mg/kg) significantly decreased alcohol-related measures in female rats, and the highest dose was not more efficacious than the intermediate dose (Figs. 2a – c). Contradictory to our study however is a recent report that found no sex difference in the efficacy of PAM GS39783 on operant alcohol self-administration in sP rats (Lorrai et al. 2019). This study also showed comparable efficacy of naloxone to decrease alcohol self-administration in both sexes. Further, we have shown this sex-dependent effect extends to naltrexone, a drug indicated for the treatment of AUD in humans (Nieto et al. 2018). Evidence from the present study shows differing effects of ASP8062 on alcohol self-administration in male and female outbred Sprague Dawley rats. Thus it appears the discrepancy between studies may, in part, be explained by again the particular rat strain being employed. Nevertheless, our results are consistent with the majority of previous studies showing sex-dependent effects in amount of alcohol consumed and responses to pharmacotherapies targeting AUD.

To our knowledge, the present study is the first to assess baclofen's effects on operant alcohol self-administration in both male and female Sprague Dawley rats. Results show baclofen produced significant decreases on all alcohol self-administration measures tested in male rats, consistent with numerous prior studies in male inbred and outbred rat strains (Anstrom et al. 2003; Colombo et al. 2003; Liang et al. 2006; Maccioni et al. 2005; Stromberg 2004; Walker and Koob 2007). These effects however were not dose-dependent in that the lowest dose of baclofen did not decrease any alcohol self-administration measures in male rats in the present study. In contrast, baclofen produced classic dose-dependent responses in female rats decreasing active lever presses and amount of alcohol consumed. Maximal decreases on all measures occurred following administration of the highest dose of baclofen (3 mg/kg) in both sexes. Differences in the potency of baclofen to reduce alcohol self-administration is corroborated by a study showing divergent effects on acquisition of cocaine self-administration between sexes (Campbell et al. 2002). That is, daily administration of baclofen (2.5 mg/kg) attenuated, to a greater degree, acquisition of cocaine self-administration in female rats compared to male outbred Wistar rats. Why baclofen is more efficacious at decreasing alcohol self-administration (and other substances) in female compared to male rats is unknown but may relate to pharmacokinetic variation between sexes. A broad literature shows pharmacokinetic differences between male and female Sprague Dawley rats in that females have slower clearance rates and longer half-lives of numerous substances (Milesi-Halle et al. 2005; Peckham and Traynor 2006; Tseng et al. 2004), and these pharmacokinetic differences are also present in humans (Franconi and Campesi 2014). Whether baclofen's sex-dependent effects seen in the present study is due to pharmacokinetic factors is unknown and deserves further study.

Sex differences were also seen in some non-specific behaviors after baclofen administration. Substantial decreases in active lever presses were associated with concomitant increases in lever pressing on the inactive lever in female rats following the highest dose of baclofen. Enhanced responses on the inactive lever may reflect a non-specific effect of the highest baclofen dose. To rule out potential non-specific effects we assessed locomotor activity after repeated ASP8062 and baclofen. The highest dose of baclofen significantly decreased distance traveled in the open field tests in male rats which is consistent with other studies showing non-specific effects (e.g., sedation, hypo-motility) on general activity (Agmo and Tarasco 1985; Paredes and Agmo 1989; Perdona et al. 2011) as well as on complex behaviors (Anstrom et al. 2003; Maccioni et al. 2005; Vengeliene

et al. 2018). In contrast, the highest dose of baclofen had no effect on locomotor activity in female rats. That the low and intermediate dose of baclofen increased activity in the open field test in female rats was unexpected and difficult to explain. Further, the intermediate baclofen dose significantly decreased all self-administration measures (Figs. 2d – e) and estimated total alcohol consumed (Fig. 3d). Additional studies are needed to replicate and examine this sex-dependent effect of low-dose baclofen on locomotor activity in female rats. Notably, none of the doses of ASP8062 tested in the present study affected activity or inactive lever presses in either sex.

One key characteristic of our study is repeated dosing which is more translatable to the human condition where multiple doses of a particular medication are administered over many weeks or years depending upon the disease state. There is a paucity of studies assessing the effects of repeated administration of PAMs on alcohol self-administration. Of note, Maccioni et al. assessed the impact of the PAMs GS39783 [N, N'-Dicyclopentyl-2-methylsulfanyl-5-nitropyrimidine-4, 6-diamine] and *rac*BHFF [(R,S)-5, 7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one] on alcohol (15%) self-administration in male sP rats under a FR4 schedule of reinforcement (Maccioni et al. 2015). In that study, GS39783 (50 mg/kg) administered over 10 consecutive days decreased lever pressing for alcohol but not sucrose. The same dose of PAM *rac*-BHFF administered over five consecutive days also decreased alcohol consumption but also decreased sucrose self-administration. Results are congruent with a previous study showing a wide dose range of repeated *rac*-BHFF decreased alcohol self-administration in male sP rats (Loi et al. 2013). Results from the present study are consistent with the aforementioned studies in that following four consecutive days of ASP8062 various measures of alcohol self-administration behavior and total alcohol consumed were significantly decreased in male and female rats. These results are also congruent with a previous study assessing ASP8062 that also employed repeated administration (PO, 6 days) showing efficacy in an animal model of fibromyalgia (Murai et al. 2019).

Results from the present study support clinical development of ASP8062. For example, translation of doses (Reagan-Shaw et al. 2008) of ASP8062 that showed efficacy in decreasing alcohol reinforcement in rats in the present study (e.g., 1 mg = 12.97 mg in 80 kg human and 3 mg/kg = 38.91 mg/80 kg human) are within the dose range (0.3-70 mg/ dose) assessed in two Phase 1 safety and pharmacokinetic clinical studies conducted in healthy individuals (Walzer et al. 2020). Outcomes from both studies showed ASP8062 to be safe, well tolerated, exhibit a desirable side effect profile, engender adequate CNS penetration and impressive elimination half-life (40 – 50 h). In addition, a Phase 1 randomized, placebo-controlled, crossover clinical trial was recently completed assessing the potential interaction between ASP8062 and alcohol in healthy adult subjects ($N = 20$) (NCT04003402). Whether results from that interaction study warrant testing in individuals with AUD is presently unknown.

In summary, the goal of the present study was to assess the impact of repeated administrations of the $GABA_B$ PAM ASP8062 compared to the orthosteric agonist baclofen on operant alcohol self-administration in male and female Sprague Dawley rats. We showed that ASP8062 reduced measures of alcohol self-administration and amount consumed in both sexes however the effects were more robust in male rats. In contrast, baclofen had a greater effect in female rats compared to males on all self-administration measures. Additional sex-dependent effects were apparent in locomotor activity tests whereby baclofen decreased activity in male rats and increased activity in female rats. Overall, the data support further development of ASP8062 as a potential treatment for AUD.

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In conducting research using animals, the investigator adhered to the laws of the USA and regulations of the Department of Agriculture. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense.

Declarations

Conflict of interest The Authors claim no conflicts of interest.

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