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Differential effects of ghrelin antagonists on alcohol drinking and reinforcement in mouse and rat models of alcohol dependence

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Abstract

An effort has been mounted to understand the mechanisms of alcohol dependence in a way that may allow for greater efficacy in treatment. It has long been suggested that drugs of abuse seize fundamental reward pathways and disrupt homeostasis to produce compulsive drug seeking behaviors. Ghrelin, an endogenous hormone that affects hunger state and release of growth hormone, has been shown to increase alcohol intake following administration, while antagonists decrease intake. Using rodent models of dependence, the current study examined the effects of two ghrelin receptor antagonists, [D^{Lys}3]-GHRP-6 (DLys) and JMV2959, on dependence-induced alcohol self-administration. In two experiments adult male C57BL/6J mice and Wistar rats were made dependent via intermittent ethanol vapor exposure. In another experiment, adult male C57BL/6J mice were made dependent using the intragastric alcohol consumption (IGAC) procedure. Ghrelin receptor antagonists were given prior to voluntary ethanol drinking. Ghrelin antagonists reduced ethanol intake, preference, and operant self-administration of ethanol and sucrose across these models, but did not decrease food consumption in mice. In experiments 1 and 2, voluntary drinking was reduced by ghrelin receptor antagonists, however this reduction did not persist across days. Despite the transient effects to ghrelin antagonists, the drugs had renewed effectiveness following a break in administration as seen in experiment 1. The results show the ghrelin system as a potential target for studies of alcohol abuse. Further research is needed to determine the central mechanisms of these drugs and their influence on addiction in order to design effective pharmacotherapies.

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Keywords

Ghrelin; Ethanol Vapor; Intragastric Infusions; Two-Bottle Choice; Self-Administration

1. Introduction

Alcohol dependence is a disorder that affects millions of people worldwide. Current treatments for dependence include both behavioral approaches and pharmacological agents such as naltrexone and acamprosate. However, most treatments have drawbacks and issues with compliance. Thus, novel treatments are being developed and researched with the intention of improving effectiveness. Interests have turned to the gut hormone ghrelin, a gastric peptide that plays a key role in hunger and growth regulation through actions on the growth hormone secretagogue receptors [GHSR] (Kojima et al, 1999). Attention has been focused on how the ghrelin system affects use and abuse of addictive substances.

The ghrelin system appears to be highly associated with alcohol dependence in humans. Humans diagnosed with alcohol dependence show decreased plasma and fundic ghrelin levels at the onset of alcohol withdrawal when compared to matched healthy controls (Badaoui et al, 2008). In contrast, alcoholics show elevated circulating ghrelin levels after 30 days of abstinence (Kim et al, 2013), suggesting a compensatory increase in ghrelin production during recovery from withdrawal. Furthermore, there is a positive correlation between ghrelin levels and alcohol craving (Addolorato et al, 2006; Leggio et al, 2012). Congruently, intravenous ghrelin increases alcohol craving scores in alcoholics (Leggio et al, 2014). Oral consumption of alcohol exerts an acute inhibitory effect on ghrelin secretion in non-dependent men and women (Calissendorff et al, 2005; 2012). Despite ample evidence of interaction between the ghrelin system and alcohol consumption, testing of ghrelin receptor antagonists is still in early stages of development.

In rodent models, central administration of ghrelin increases alcohol consumption (Jerlhag et al, 2009) and peripheral administration induces conditioned place preference (CPP) and increases dopamine levels the nucleus accumbens (Jerlhag, 2008), suggesting that ghrelin influences alcohol consumption via reward-related circuitry. Compounds that block the ghrelin receptor GHSR1a generally show effects opposite to those of ghrelin agonists on alcohol drinking and reward. For example, the small molecule ghrelin receptor antagonist JMV2959 decreased ethanol intake (Jerlhag et al, 2009) and blocked ethanol-induced CPP (Bahi et al, 2013). Similarly, Kaur and Ryabinin (2010) found that the peptide-based ghrelin antagonist, [DLys3]-GHRP-6 (DLys), decreased ethanol intake and preference, while also blocking ethanol induced c-Fos immunoreactivity in the Edinger-Westphal nucleus. Models using ghrelin knockout mice have seen a reduction in alcohol-induced reactions, which include decreased locomotor effects and reduced accumbal dopamine release following alcohol administration (Jerlhag et al, 2011).

Studies that utilize ghrelin receptor antagonists to assess effects on alcohol drinking have generally tested acute effects in non-dependent animals. Dependence models of alcohol consumption may be better suited for understanding the role ghrelin plays in the processes that contribute to alcoholism in humans. Recently, Suchankova and colleagues (2013)

addressed the topic of alcohol dependence by examining the alcohol deprivation effect with and without administration of JMV2959. They found that JMV2959 blocks the increased ethanol intake seen following reintroduction of ethanol. However, the effect of ghrelin antagonism in models of alcohol dependence needs further evaluation.

A number of alcohol dependence models using mice and rats have been developed (review; Knapp and Breese, 2012). Among them are studies using alcohol vapor or intragastric exposure. Continuous and intermittent ethanol vapor exposure has been shown to reliably produce signs of dependence (e.g., Schulteis et al, 1995; 1996; Macey et al, 1996; Becker et al, 2000), to increase alcohol drinking and operant self-administration of alcohol (e.g., Becker and Lopez, 2004; O'Dell et al, 2004; Finn et al, 2007; Gilpin et al, 2009), and to increase alcohol self-administration to alleviate withdrawal symptoms (Roberts et al, 1996) and during a period of protracted abstinence (Roberts et al, 2000). More recently, the intragastric alcohol consumption (IGAC) model has shown increased alcohol self-administration in dependent mice given ethanol access during acute alcohol withdrawal (Fidler et al, 2012; Cunningham et al, 2013).

The current study tested the ability of two ghrelin receptor antagonists (GHSR1a), DLys and JMV2959, to decrease alcohol drinking across three procedures: 1) alcohol drinking after chronic intermittent ethanol vapor exposure in C57BL/6J mice (Finn et al, 2007); 2) intragastric (IG) alcohol self-infusion after passive IG alcohol infusions in C57BL/6J mice (Fidler et al, 2012); and 3) progressive ratio (PR) operant alcohol self-administration in Wistar rats after chronic intermittent ethanol vapor (O'Dell et al., 2004). These subjects and models were chosen to provide a wide array of examinations to test the effectiveness of ghrelin antagonists and their interaction with alcohol drinking. The models used were developed at different times in different laboratories, and therefore, differ in many procedural parameters. Our main goal was to test the consistency of GHSR1a antagonists across these different models without attempting to match all the parameters. Mice were used in experiment 1 and 2 as a way of testing two different models of dependence, while rats were used in experiment 3 as a way of testing a different species with similar dependence model allowing for comparison to experiment 1. We believe that using both rats and mice would allow us to determine the effectiveness of ghrelin receptor antagonists across species, thereby enhancing the generalizability of our findings. The general hypotheses for these experiments was that both of the ghrelin antagonists would reduce the expected ethanol self-administration levels in dependent animals.

2. Methods

2.1. Animals

Male C57BL/6J mice (N=96) from Jackson Laboratories (Sacramento, CA) and male Wistar rats (N=12) from Charles River (Wilmington, MA) were used in these experiments. All mice and rats had *ad libitum* access to food and water, except for 16-23 hours before IG cannulation in experiment 2. The Oregon Health & Science University Institutional Animal Care and Use Committee (IACUC) approved all procedures for experiments 1 and 2. Baylor College of Medicine IACUC approved all procedures for experiment 3.

In experiment 1, one week after arrival (6-7 weeks old), acclimation to the environment, and switch to a 12 hour reverse light cycle (*off* at 08:00), mice were randomly assigned to one of six groups (n=8/group) in a 2×3 design. Mice were individually housed and a baseline of limited access (2hr) alcohol intake was established. Then separate groups of mice were exposed to either ethanol vapor or air vapor for one cycle (16 hours on / 8 hours off × 3 days), followed by 2 hour alcohol intake for 5 days, a second cycle of intermittent ethanol vapor or air exposure, and a final 5 days of 2 hour alcohol intake. Prior to each of the post-vapor alcohol intake days, subgroups of mice were pre-treated with saline, DLys (15 mg/kg), or JMV2959 (9 mg/kg).

In experiment 2, mice arrived at 8-9 weeks of age and were allowed to acclimate for at least 1 week before surgery. Mice were initially group housed (4/cage) on a 12 hour light/dark cycle (*off* at 19:00). After surgery and recovery, mice were singly housed in operant conditioning chambers for the rest of the experiment. Mice initially received passive-infusions of either ethanol or water over 10 days (n=12/group). Ethanol self-infusion was then measured daily for 23.5 hours after IG administration of either DLys (14 – 18.2 mg/kg) or saline.

In experiment 3, rats were split into two groups (n=6/group) and trained to lever press for solutions of ethanol or sucrose on a fixed ratio schedule. Rats weighed over 400 g at the start of the experiment and were housed individually on a 12 hour light cycle (*off* at 18:00). Following intermittent ethanol vapor exposure, rats were then administered DLys (0, 2, 4 mg/kg) or JMV2959 (0, 1, 2, 4 mg/kg) and operant self-administration of ethanol or sucrose was tested under a PR schedule in a within-subjects design.

2.2. Drugs

Two ghrelin receptor (GHSR1a) antagonists were used for these experiments: [DLys3]-GHRP-6 (Tocris Bioscience, Bristol, UK; Cat #1922) and JMV2959 (Aeterna Zentaris, Germany). In experiments 1 and 3, drugs were diluted in sterile saline (0.9% sodium chloride). In experiment 2, sterile water was used as the vehicle. All solutions were prepared fresh daily and different doses of drugs were used in mice and rats based on previously published experiments (Jerlhag et al, 2009; Kaur and Ryabinin, 2010; Gomez and Ryabinin, 2014).

In experiment 1, DLys and JMV2959 were injected intraperitoneally (i.p.) at 15 mg/kg and 9 mg/kg, respectively. Ethanol (95% pure) was diluted with tap water to 15% v/v and presented using 25 ml graduated cylinders capped with a sipper tube. An alcohol dehydrogenase inhibitor, pyrazole HCl (68.1 mg/kg) with a priming dose of ethanol (2 g/kg, 20% v/v ethanol solution) was used prior to vapor exposure of mice to allow for exposure to lower concentrations of vaporized alcohol and gain consistent blood ethanol concentrations (BECs) across subgroups.

In experiment 2, DLys was administered IG at a set dose of 400 nmol/0.3 ml per mouse (which translates to 14 – 18.2 mg/kg given the range of body weights) or vehicle (0.3 ml) 30 minutes before each daily session during the No-Choice and Choice phases when 20% ethanol (v/v in sterile water) was self-infused.

In experiment 3, rats were administered three doses of DLys (0, 2, 4 mg/kg) and four doses of JMV2959 (0, 1, 2, 4 mg/kg). Rats were given 0.1 mL of 10% ethanol or 2% sucrose per delivery during operant conditioning sessions.

2.3. Apparatus

In experiment 1, mice were housed in flow-through cages (3-4 per cage) in each vapor inhalation chambers (Flair Plastics, Portland, OR). Immediately before each vapor exposure, all mice were injected with pyrazole HCl (68.1 mg/kg). Pyrazole was diluted in 20% ethanol for the ethanol vapor group or saline for the air vapor group. The chambers were primed to have ethanol vaporized at 7-8 mg/L air and adjusted daily to let animals achieve a target BEC that ranged from 150-200 mg/dL following each 16 hours of ethanol vapor exposure. The amount of ethanol vapor in the inhalation chamber was calculated hourly using gas chromatography (6890N GC using a HP-PLOT Q column; Agilent Technologies, Wilmington, DE).

In experiment 2, mice were trained to self-administer solutions intragastrically in acrylic and aluminum chambers (20 × 20 × 22.5 cm) enclosed in individual laminated sound-attenuating boxes (61 × 40.6 × 55.9 cm) with ventilation fans. The apparatus allowed for ethanol or water to be pumped into the animal's stomach via a back mount tethered to tubing that carried fluid from two syringe pumps (Model A or Model R-E, Razel Scientific Instruments Inc., St. Albans, VT, USA). Each chamber was equipped with two retractable sipper tubes on one wall (ENV-252M, Med Associates, St. Albans, VT, USA) positioned 9.5 cm apart and 3.5 cm above the stainless steel mesh floor (18-ga. T304, Western Group, Portland, OR, USA). The sipper tubes were connected to lickometers (ENV-250B, Med Associates) interfaced to a computer that stored lick totals and number of infusions automatically every 5 min using LabVIEW™ 6.1 software (National Instruments, Austin, TX, USA). Additional apparatus details can be found elsewhere (Fidler et al., 2012).

In experiment 3, rats in the ethanol group were housed singly in a cubicle room equipped with an alcohol vapor inhalation system (La Jolla Alcohol Research, La Jolla, CA) in which alcohol vapors cycled on for 14 hours and off for 10 hours, 5 days per week for a total of 8 weeks prior to initial training. BECs achieved during the alcohol vapor period ranged between 95 to 125 mg/dL when blood samples were taken during the vapor exposure period. BECs were negligible when blood samples were taken 3 hours after the alcohol vapors were turned off when operant sessions took place.

Operant self-administration training and test sessions were conducted in standard operant chambers housed within sound-attenuating cubicles equipped with fans to further mask outside noise (Coulbourn Instruments, Whitehall, PA). The chambers were equipped with two retractable levers each with a triple cue light located above it. Between the levers was an access area in which a dipper (0.1 mL capacity) could protrude when activated. When not activated, the dipper was immersed in a solution reservoir located outside the operant chamber. A house light was located on the wall opposite to the levers and access area. Sessions began with the illumination of the house light and the protrusion of the levers. One or more depressions of the active lever could activate the dipper, illuminate the triple cue light and a light within the dipper access area, and turn off the house light. Both the triple

cue light and the dipper light remained on for the 3 seconds duration of the dipper presentation time. After this time, these lights were turned off and the house light was turned on. The access area was equipped with infrared sensors that allowed tabulation of head entries. A hardware and software system (Coulbourn Instruments; Graphic State Notation v. 1.014) was used to program stimulus parameters and tabulate data.

2.4. Surgery

The surgical procedure in experiment 2 was identical to that described previously (Fidler et al., 2012). Briefly, each mouse was anesthetized with isoflurane gas (5% loading dose, 2.5-3% maintenance) and subcutaneously administered the non-narcotic NSAID Carprofen (5 mg/kg) or Meloxicam (0.2 mg/kg). The stomach was externalized through an incision on the animal's left side caudal to the rib cage. The catheter was inserted into the stomach through a puncture in the stomach wall and secured in place. The stomach was then returned to the abdominal cavity, and the incision through the muscle and peritoneum was sutured. The back mount was inserted through a small incision on the back just posterior to the scapulae and manipulated to emerge from a hole anterior to the incision. The catheter was threaded subcutaneously to the back incision and attached to the back mount. The catheter was attached to the back mount and secured. The skin incisions on the top and side of the body were sutured, the back mount capped (303DCFT/1, Plastics One) and anesthesia was removed. Mice were individually housed and allowed 6-10 days to recover before the start of the experiment. They received a single dose of Meloxicam (0.2 mg/kg) 24 hours after surgery and they were infused with sterile water (0.2 ml) once daily to ensure catheter patency.

2.5. Experimental Procedures

2.5.1. Experiment 1—tested the effects of both ghrelin antagonists on voluntary alcohol drinking in mice made ethanol dependent by 2 weeks of voluntary drinking and two cycles of intermittent exposure to vapor chambers. A procedure similar to the one described previously was used (Finn et al, 2007). All mice were given access to 15% ethanol for 2 hours a day for 10 days (Mon-Fri) in a 2-bottle choice paradigm beginning 2 hours into the dark cycle to establish baseline data and also familiarize the mice to drinking from bottles (25 ml graduated cylinders with sipper tubes). The ethanol and water bottles were presented in a counterbalanced fashion in order to balance any effects of side preference. After baseline drinking was stable (<15% variability across days), mice were exposed to intermittent ethanol or air vapor for 3 days. Each day consisted of 16 hours in the assigned chamber (ethanol or air) and 8 hours in the air chamber, with the same light cycle used in the home cage. Prior to vapor exposure, mice were either injected with 2 g/kg ethanol plus pyrazole HCl (68 mg/kg; ethanol group) or 68 mg/kg pyrazole HCl in saline (air group). All mice were tail-nicked, however, blood samples were only collected for BEC analysis in mice exposed to ethanol vapor after the 16 hour exposure. Once mice completed vapor exposure they were returned to their home cage/room, with free access to food and water for 72 hours. Then, 2 hour limited access ethanol intake was measured for 5 days, and mice were injected each day with either saline, DLys, or JMV2959 at 30 minutes prior to ethanol access. Ethanol intake, ethanol preference, water intake, food intake, and body weights were collected. Using the same methods as above, another 3 day cycle of vapor exposure was

administered, which was then followed by another 5 days of 2 hour ethanol access and pre-treatment with saline, DLys, or JMV2959. Mice were euthanized immediately after the final drinking session and blood was collected for BEC analysis.

2.5.2. Experiment 2—tested the effects of DLys on IGAC in mice made dependent by passive exposure to IG ethanol. The IGAC procedure has been described previously (Fidler et al, 2012). The experiment was conducted in two replications of 24 mice each with all groups represented in each cohort. The experiment consisted of the following phases: (1) surgery; (2) recovery (6-10 days); (3) habituation (3 days); (4) passive infusions of ethanol or water (10 days); (5) no-choice self-infusion (2 days); and (6) choice self-infusion (5 days). Beginning with the first habituation day, animals were housed in the apparatus on a normal light cycle and lick data was collected for about 23.5 hours per day. Mice were weighed and manually infused with sterile water (0.2 ml) before every session to ensure catheter patency.

Mice were attached to the tether and given free access to food and two bottles of 0.2% w/v saccharin in tap water during habituation. The right and left bottles were available during alternate 30-min periods to ensure that mice encountered both bottles and to reduce the formation of side preferences. The bottle available first alternated over days and was counterbalanced across groups. No infusions were given in the chamber during this phase.

During the passive infusion phase, half of the mice were randomly assigned to receive passive IG infusions of ethanol to induce ethanol dependence (10% v/v in sterile water, Ethanol Group, n=24) and half were assigned to receive daily infusions of water (Water Group, n=24). Three infusions were delivered at a rate of 0.031 ml/min at equal intervals on each of 10 consecutive nights. The first daily infusion was given 280 min after mice were returned to the apparatus; subsequent infusions began at 340-min intervals. These times coincided approximately with the beginning, middle and end of the dark cycle. The duration of each infusion (35- 45 min) was varied individually for each mouse (based on body weight) to control ethanol dose. All mice were scheduled to receive the same total daily dose by weight, which was divided equally across the three infusions. Target ethanol doses for each infusion were 3.0, 3.5, 4.0, 4.5, 5.0, 5.25, 5.25, 5.5, 5.5 and 5.75 g/kg on passive days 1-10, respectively. These doses were previously shown to be effective for producing a later enhancement of IG ethanol self-administration in this strain (Fidler et al., 2012).

Level of intoxication was assessed 2 hours after onset of the last passive infusion each day using a brief visual observation procedure that has been described elsewhere (Fidler et al., 2012). Withdrawal severity (indexed by handling-induced convulsions, HICs) was assessed about 7.5 h after onset of the last daily infusion, as previously described (Metten and Crabbe, 2005). On the few occasions when mice still showed signs of intoxication at the time of withdrawal assessment (n=4), the first passive infusion during the next session was omitted to avoid overdosing.

During the no-choice self-infusion phase, half of the mice within each of the passive-phase groups were randomly assigned to receive a manual IG infusion of DLys (14 – 18.2 mg/kg, DLys, n=12/group) or sterile water (0.3 ml, Veh, n=12/group) 30 min before each of the two

no-choice infusion sessions. The first of these sessions began about 8 hours after onset of the final passive infusion. Mice received access to a single drinking tube that contained 0.05% w/v grape or cherry Kool-Aid (counterbalanced) and 0.2% w/v saccharin in tap water (S+). This tube was placed on the side that had been preferred during the habituation and passive phases. IG ethanol (20% v/v) infusions (0.05-0.07 g/kg, 0.129 ml/min) were contingent upon licking the S+ tube (fixed-ratio 10 schedule in which lick defines the operant response) up to a maximum limit of 1.5 g/kg/30 min. This dose limit was imposed to minimize the likelihood that a high-dose bout would induce a conditioned taste aversion to the S+ flavor. When the maximum number of infusions was reached, the S+ tube remained available, but no further infusions were given until the cumulative dose received during the previous 30 minutes fell below the 1.5 g/kg limit. The two no-choice days were included to ensure that all animals encountered the S+ flavor-ethanol contingency during acute ethanol withdrawal (Cunningham et al., 2013).

The choice self-infusion days were the same as the no-choice days except that a second drinking tube containing a second flavored Kool-Aid solution (S-) was available in addition to the S+ tube. Licks on the S- tube were paired with infusions of sterile water using the same response contingency (FR10), infusion rate, and limit as the S+ tube. Mice received a manual IG infusion of DLys or water 30 minutes before each of the five sessions.

2.5.3. Experiment 3—tested effects of both ghrelin antagonists on PR self-administration of alcohol or sucrose in rats previously made dependent by 8 weeks of exposure to intermittent alcohol vapors. A procedure similar to the one described previously was implemented (O'Dell et al, 2004). Initial training to lever press for solution delivery was conducted for 1 week immediately after vapor exposure. During this time, the levers remained retracted and sessions began with two dipper presentations (primes) and the onset of the cue and dipper lights and the offset of the house light. These dipper presentations were 15 seconds in duration with an inter-trial interval of 5 seconds. For the rest of the 30 minute session, a head entry into the dipper access area would trigger a dipper presentation of 15 seconds duration with the same stimulus conditions. During the next week, sessions began with the protrusion of the levers followed by two primes. Based on lever press performance of each rat, the dipper presentation time was gradually reduced until it was 3 seconds in duration, the duration used for the rest of the training and testing. Once a rat emitted at least 25 active lever presses and response levels were consistent (<20% variability) over 2 days, the response requirement was raised from one lever press (i.e., fixed ratio 1 or FR1) to FR2. Stable responding under the FR2 schedule during the 30 minute sessions was required to move into the test phase of the study.

The test sessions were 3 hours in length and were conducted no more than two times per week (Tuesdays and Fridays) with standard training sessions (FR2) performed on the non-test days. Response levels on non-test days were monitored to ensure they had returned to baseline. If necessary, additional training sessions were conducted until baseline responding was achieved. Both DLys and JMV2959 were administered i.p. 20 minutes prior to the start of the test sessions. Each antagonist was tested separately with a gap of two weeks between testing JMV2959 then DLys. The doses of each antagonist were systematically counterbalanced where all rats were given dose 0 mg/kg on the first day of testing followed

by similar numbers of combination orders for other doses on subsequent days for a total of 7 days of testing (i.e. dose (mg/kg) – 1-2, 1-4, 2-1, 2-4, 4-1, 4-2 for JMV2959; 2-4 and 4-2 for DLys). During test sessions, the schedule of reinforcement employed was a modified PR schedule as described previously (Kosten, 2011; Walker and Koob, 2007). Briefly, after each reinforcer delivery, the lever press requirement was raised according to this schedule: 1, 1, 2, 2, 3, 3, 4, 4, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, 15, 15, 18, 18, 21, 21, 24, 24, etc.

2.6. Statistics

In experiment 1, all measurements (ethanol intake, ethanol preference, water intake, and food intake) were assessed at three different time points: baseline, after 1st vapor exposure (test 1), and after 2nd vapor exposure (test 2). Baseline data were collapsed over the 10 days of consumption and analyzed by one-way ANOVA to determine any pre-existing differences in behavior. For each testing period, a three-way repeated measures ANOVA was used with vapor and drug as between-subjects factors and day as the within-subjects factor. Planned comparisons assessed the drug and vapor effects on day 1, as previous work determined this day to show the most robust effects (Gomez and Ryabinin, 2014). To determine any drug differences on day 1, a one-way ANOVA was used with post-hoc LSD. Differences in vapor exposure (ethanol vs. air) within drug groups were tested using a single-tailed independent samples t-test. Body weights and blood ethanol content during vapor exposure were each analyzed using three-way repeated measures ANOVAs.

In experiment 2, withdrawal severity (HIC) scores were analyzed using a Mann-Whitney U test. Ethanol self-infusion intake, number of bouts, bout size, and preference ratios were analyzed using three-way ANOVAs (Passive Group $\times$ Drug Group $\times$ Days). Post-hoc ANOVAs were run as needed to interpret findings. Finally, planned comparisons (one-way ANOVAs) were used to determine group differences on the first choice day.

In experiment 3, the measures obtained from the PR test sessions included those reflective of reinforcer-seeking (number of active lever presses, total number of head entries into the dipper area, and the highest ratio completed) and those reflective of reinforcer taking (number of dipper presentations and number of reinforcers retrieved). The number of reinforcers retrieved was measured as the number of head entries made into the dipper presentation when the dipper was activated. Inactive lever presses were also tabulated but had no programmed consequences. Data were analyzed using two-way repeated measures ANOVAs representing the between group factor of solution group (Ethanol vs. Sucrose) with repeated measures on dose. Separate analyses were performed for each test drug (DLys and JMV2959) because a different number of doses were tested. Significant main effects or interactions were followed by post-hoc comparisons (Newman-Keuls).

3. Results

3.1. Experiment 1: Chronic intermittent ethanol vapor exposure in mice

This experiment assessed effects of DLys and JMV2959 on alcohol consumption following two cycles of ethanol vapor exposure.

Ethanol Intake (Figure 1A and 1B): Baseline data showed no significant differences between groups [$F(5,39)=1.87$, $p=0.13$]. During test 1, a repeated measures ANOVA found a day by drug within-subjects interaction [$F(8,136)=6.99$, $p<0.0001$] and a between-subjects main effect of drug [$F(2,34)=15.61$, $p<0.0001$]. The vehicle group drank more ethanol than the JMV2959 and DLys groups. Planned comparisons on day 1 found no significant effect of vapor exposure, but a significant effect of drug [$F(2,34)=64.91$, $p<0.0001$] showing that the vehicle group drank more than the JMV2959 group, which in turn drank more than the DLys group. During test 2, there was a within-subjects interaction of day by drug [$F(8,136)=6.69$, $p<0.0001$] and between-subjects main effects of drug [$F(2,34)=7.22$, $p=0.002$] and vapor [$F(1,34)=5.07$, $p=0.031$]. The vehicle group drank more than the JMV2959 group which drank more than the DLys group. Examining day 1, we found the same pattern of ethanol consumption [Veh > JMV2959 > DLys; $F(2,34)=28.12$, $p<0.0001$]. We also saw an increase attributed to vapor exposure in the vehicle group, whereby mice exposed to ethanol vapor drank more than those exposed to air [$t(9)=2.02$, $p=0.04$, one-tailed]. No differences between vapor exposure and air exposure were seen in the JMV2959 or DLys groups.

Ethanol Preference (Figure 1C and 1D): Baseline data showed no significant pre-exposure differences between groups [$F(5,39)=0.39$, $p=0.85$]. Test 1 revealed a within-subjects day by drug interaction [$F(8,132)=14.46$, $p<0.001$] and a between-subjects effect of drug exposure [$F(2,33)=29.03$, $p<0.0001$]. There was no difference in preference between the vehicle and JMV2959 groups, but a significant decrease from control levels ($\sim 75\%$) was seen in the DLys group. Analysis of day 1 also showed a significant reduction of preference in the DLys group compared to the vehicle and JMV2959 groups [$F(2,33)=71.56$, $p<0.0001$]. For test 2, there was a significant within-subjects interaction of day by drug [$F(8,136)=12.77$, $p<0.0001$] and a between-subjects interaction of drug by vapor [$F(2,34)=3.62$, $p=0.038$]. Focus on day 1 found that both ethanol vapor exposed mice in the vehicle and JMV2959 groups preferred ethanol more so than the air vapor controls [$t(9)=2.09$, $p=0.03$; $t(11)=3.14$, $p=0.004$, one-tailed, respectively]. No such increase in ethanol vapor induced preference was seen in the DLys group, however, mice exposed to DLys did show significantly lower overall preference than the vehicle and JMV2959 groups [$F(2,34)=70.02$, $p<0.0001$].

Food Intake (Figure 1E and 1F): There were no differences between groups during baseline consumption of food [$F(5,34)=1.43$, $p=0.24$]. There was a significant reduction in within-subjects consumption across days during test 1 [$F(3,102)=18.31$, $p<0.0001$] and test 2 [$F(3,102)=13.81$, $p<0.0001$]. However, there were no between-subjects differences in food intake, suggesting drug and vapor exposure did not alter general food consumption behavior.

Throughout the experiment from baseline to completion there were no differences in mean body weights seen between groups (Body Weight (g) by vapor exposure - Air: Veh= 25.27 ± 0.45 , DLys= 24.69 ± 0.35 , and JMV2959= 23.99 ± 0.59 ; Ethanol: Veh= 25.26 ± 0.71 , DLys= 25.48 ± 0.61 , and JMV2959= 24.95 ± 0.66). There was a within-subjects effect of day during vapor chamber exposure showing a slight reduction in body weight from day 1 to day 4 [VC1 – $F(3,102)=49.91$, $p<0.0001$; VC2 – $F(3,102)=126.31$, $p<0.0001$]. During the vapor chamber exposure there were no between group differences in mean BECs assessed immediately after removal from ethanol vapor exposure (BEC (mg/dL): Veh= 1.97 ± 0.40 , DLys= 1.45 ± 0.14 , JMV2959= 1.42 ± 0.13).

3.2. Experiment 2: Intragastric infusion of ethanol or water in mice

This experiment assessed effects of DLys on ethanol self-infusion following repeated passive intragastric infusions of either ethanol or water. Only DLys was tested in this study, as experiment 1 and our previous study (Gomez and Ryabinin, 2014) indicated more specific effects of DLys on ethanol intake in mice.

Attrition—One mouse (Ethanol Group) died for unknown reasons during the passive phase. Catheter problems required the removal of two additional mice (one Ethanol, one Water). Data from all three mice were excluded from all analyses, leaving group sizes that ranged from 10-12. Data from the final three choice self-infusion days were excluded for one additional mouse (Water) that developed a leak in the back mount.

Withdrawal Severity—Consistent with previous findings (Fidler et al, 2012), this regimen of passive ethanol infusions produced dependence as indexed by greater HIC scores measured about 7.5 hours after the last infusion each day. Median HIC scores averaged over the last 2 days of the passive phase were 2 and 0 for the Passive Ethanol and Passive Water groups, respectively (Mann-Whitney $U=505$, $p<0.0001$). Although HIC scores were generally lower during the No-Choice phase, the Passive Ethanol group (0.5) still showed significantly higher ($U=356.5$, $p<0.02$) median HIC scores than the Passive Water group (0). Intoxication ratings were similar to those previously reported for C57BL/6J mice (Fidler et al, 2012) and are not reported here.

Ethanol Self Infusion—Mean ethanol intakes (g/kg/day based on the number of ethanol self-infusions) during the self-infusion sessions are depicted in Figures 2A and 2B for the non-dependent (Water) and dependent (Ethanol) groups, respectively. As in a previous study (Fidler et al, 2012), ethanol self-infusion was greater after 10 days of passive ethanol exposure than after passive water exposure. Interestingly, DLys pretreatment had no effect on dependent mice, but appeared to decrease ethanol self-infusion in non-dependent mice. Three-way ANOVA (Passive Group $\times$ Drug Group $\times$ Days) showed significant main effects of Passive Group [$F(1,40)=30.0$, $p<0.0001$] and Days [$F(6,240)=22.8$, $p<0.0001$], as well as a significant Passive Group $\times$ Days interaction [$F(6,240)=2.3$, $p<0.05$], reflecting the gradual convergence of the Ethanol and Water groups toward the end of testing. Planned comparisons on the first choice day (C1) showed that Water-DLys mice infused significantly less ethanol than Water-Veh mice [$F(1,20)=4.8$, $p<0.05$]. In contrast, there was no significant effect of DLys in Ethanol group mice.

Treatment effects on the daily patterns of ethanol intake were examined by analyzing the mean number of ethanol bouts/day (Figures 2C and 2D) and the mean bout size in g/kg (Figures 2E and 2F), where a bout was defined as ethanol intake in consecutive 5-min periods without a break greater than 5 min (Fidler et al, 2012). Three-way ANOVAs (Passive Group $\times$ Drug Group $\times$ Days) suggested that passive ethanol exposure increased ethanol intake by increasing both the number of bouts/day [Passive Group main effect: $F(1,40)=9.5$, $p<0.01$] and mean bout size [Passive Group main effect: $F(1,38)=15.1$, $p<0.001$]. The inhibitory effect of DLys on ethanol intake appeared primarily to reflect an antagonist-induced reduction in bout number as indicated by a significant Passive Group $\times$

Drug Group interaction [$F(1,40)=4.8$, $p<0.05$]. Post-hoc pairwise comparisons revealed a significant Passive Group difference in bout number for the DLys [dotted lines in Fig. 2C vs. 2D, $F(1,20)=9.4$, $p<0.01$], but not for the Veh groups (solid lines in Fig. 2C vs. 2D). Post-hoc comparisons of bout number between the Veh vs. DLys groups within the Water (Fig. 2C, $p=0.11$) and Ethanol (Fig. 2E, $p=0.27$) conditions, however, were not significant. The overall analysis of bout size yielded no significant interactions. Separate planned comparisons (Veh vs. DLys) conducted for each Passive Group on the first choice day (C1) showed no significant effects of DLys on bout number in either group. However, similar planned comparisons showed that DLys significantly reduced bout size on day C1 in the Water groups [$F(1,20)=5.2$, $p<0.05$], but not in the Ethanol groups.

Body weights were lower in ethanol infused mice at the onset of withdrawal, but quickly recovered over the next few days. There were no effects of the ghrelin antagonist. An overall repeated measures ANOVA across all 7 days of self-infusion yielded a significant Passive Group $\times$ Sessions interaction [$F(6,240)=27.1$, $p<0.001$]; separate two-way follow-up ANOVAs on each day yielded a significant Passive-Group main effect on each of the No-Choice days ($p's < 0.01$), but not on Choice days. The overall ANOVA yielded no significant main effect or interaction involving the Drug factor, indicating that the ghrelin antagonist had no effect on body weights.

Preference ratios were calculated for the choice sessions by dividing the number of licks on the S+ tube by the total number of licks on both tubes. All groups showed a mean preference for the S+ tube (i.e., ratios >0.5), but three-way ANOVA (Passive Group $\times$ Drug Group $\times$ Days) yielded only a significant main effect of Days [$F(4,160)=14.7$, $p<0.0001$]. Planned comparisons on the first choice day showed no significant Drug Group effect for either the Water or Ethanol groups. Overall group mean ratios ($\pm$ SEM) during the 5-day choice phase were not significant across days or group (Table 1).

3.3. Experiment 3: Chronic intermittent ethanol vapor exposure in rats

This experiment assessed effects of DLys and JMV2959 on PR responding for ethanol and sucrose following chronic intermittent ethanol vapor exposure in rats.

Numbers of inactive lever presses seen after administration of DLys and JMV2959 are shown in Table 1. The level of responding was low in both groups and did not differ. There was no effect of DLys on inactive lever press responding, but there was a significant effect of JMV2959 on inactive lever press responding [$F(3,36)=4.17$, $p<0.05$]. This may reflect the decrease in numbers of inactive presses after administration of the highest dose seen in both groups although none of the post-hoc comparisons reached significance.

Reinforcer-seeking measures – The effects of DLys and JMV2959 administration on numbers of active lever presses emitted are presented in Figure 3A for the sucrose group and 3B for the ethanol group. Comparison of the data in the two graphs shows that the numbers of active lever presses were greater for the Sucrose rats for both the DLys [$F(1,10)=13.71$, $p<0.005$] and the JMV2959 tests [$F(1,12)=86.08$, $p<0.00001$]. Significant decreases in lever press responses were seen after administration of both DLys [$F(2,20)=57.91$, $p<0.00001$] and JMV2959 [$F(3,36)=65.14$, $p<0.005$]. As seen in Figures 3A and 3B, greater reductions

in active lever press responses were seen at higher doses of both drugs in both reinforcer groups. Post-hoc comparisons revealed that active lever presses were reduced significantly in both the alcohol and sucrose groups at both doses of DLys compared to vehicle conditions, $p's < 0.05$. Active lever press responses were also decreased after administration of the 2 and 4 mg/kg doses of JMV2959 compared to vehicle conditions in both reinforcer groups, $p's < 0.05$. In addition to these significant main effects, the group by dose interactions were significant for both DLys [$F(2,20)=7.93$, $p<0.005$] and JMV2959 [$F(3,36)=5.91$, $p<0.005$] which may reflect the slightly greater effect of DLys at the higher dose in the Sucrose group compared to the Ethanol group and slightly greater effects of the JMV2959 at higher dose in the Ethanol group compared to the Sucrose group.

The total numbers of head entries into the dipper access area are shown in Figure 3C for Sucrose rats and in 3D for Ethanol rats. The Sucrose group emitted greater numbers of head entries than the Ethanol group under both the DLys [$F(1,10)=6.05$, $p<0.05$] and the JMV2959 tests [$F(1,12)=60.05$, $p<0.00001$]. Significant decreases in head entries were seen after administration of both DLys [$F(2,20)=13.15$, $p<0.0005$] and JMV2959 [$F(3,36)=40.58$, $p<0.0001$]. Post-hoc comparisons revealed that numbers of head entries were significantly lower after administration of the highest dose of DLys compared to vehicle conditions in both reinforcer groups, $p's < 0.05$. Head entries were reduced significantly after administration of the highest JMV2959 dose compared to vehicle conditions in both the Ethanol and Sucrose groups according to post-hoc comparisons, $p's < 0.05$. The ability of DLys to decrease the number of head entries did not differ by reinforcer group, but JMV2959 decreased head entries more in the Sucrose rats compared to the Ethanol rats particularly at the highest dose as supported by the significant group by dose interaction [$F(3,36)=9.28$, $p<0.0005$].

The final ratios completed after DLys and JMV2959 administrations are shown by reinforcer group in Table 2. Sucrose rats completed higher ratios compared to the Ethanol rats after both DLys [$F(1,10)=25.48$, $p<0.001$] and JMV2959 administrations [$F(1,12)=120.01$, $p<0.0001$]. Final ratios completed were reduced after both DLys [$F(2,20)=31.07$, $p<0.0001$] and JMV2959 [$F(3,36)=13.03$, $p<0.0001$]. Post-hoc comparisons revealed that the final ratios completed were lower after administration of the highest DLys dose compared to vehicle conditions in both reinforcer groups, $p's < 0.05$. Final ratios completed were also reduced significantly in the Ethanol group after administration of all JMV2959 doses compared to vehicle conditions and after administration of the 2 and 4 mg/kg JMV2959 doses in the Sucrose group, $p's < 0.05$. The effect of JMV2959 on final ratios completed did not differ by reinforcer group, but were reduced to a greater extent by DLys in the Sucrose group compared to the Ethanol group [$F(2,20)=5.72$, $p<0.05$].

Next, we examined the numbers of reinforcers delivered by reinforcer group after administration of DLys and JMV2959. Sucrose rats earned more delivered reinforcers (Figure 3E) than Ethanol rats (Figure 3F) after DLys [$F(1,10)=11.57$, $p<0.01$] and after JMV2959 administrations [$F(1,12)=92.39$, $p<0.0001$]. Both DLys [$F(2,20)=37.30$, $p<0.0001$] and JMV2959 [$F(3,36)=65.92$, $p<0.0001$] reduced the numbers of delivered reinforcers. Post-hoc comparisons revealed that the numbers of delivered reinforcers were lower after administration of the highest DLys dose compared to vehicle conditions in both

reinforcer groups, $p's < 0.05$. The number of earned (delivered) reinforcers was significantly lower after administration of the highest JMV2959 dose compared to vehicle conditions in the Ethanol group and after administration of both the 2 and 4 mg/kg JMV2959 dose in the Sucrose group, $p's < 0.05$. The reduction in earned reinforcers differed by dose between the two reinforcer groups for DLys as supported by the significant group by dose interaction [$F(2,20)=4.41$, $p < 0.05$], but this interaction was not significant for JMV2959. As seen in figures 3E and 3F, the significant interaction for DLys likely reflects the greater reduction in numbers of earned reinforcers at the highest dose in the Sucrose rats compared to the Ethanol rats.

The number of reinforcers retrieved after DLys and JMV2959 administrations are shown by reinforcer group in Table 3. Ethanol rats retrieved fewer reinforcers than Sucrose rats after administration of both DLys [$F(1,10)=20.18$, $p < 0.05$] and JMV2959 [$F(1,12)=71.81$, $p < 0.0001$]. Both DLys [$F(2,20)=40.39$, $p < 0.0001$] and JMV2959 [$F(3,36)=26.86$, $p < 0.0001$] reduced the numbers of retrieved reinforcers. Post-hoc comparisons revealed that the number of reinforcers retrieved was significantly less after administration of both doses of DLys relative to vehicle condition in the Ethanol group and decreased significantly after the highest dose of DLys compared to vehicle administration in the Sucrose group, $p's < 0.05$. Compared to vehicle, the number of retrieved reinforcers was significantly reduced after administration all JMV2959 doses in the ethanol reinforcer group, but only the 2 and 4 mg/kg JMV2959 doses for the sucrose reinforcer group, $p's < 0.05$. These drug effects did not differ by group; the group by dose interactions failed to reach significance for either drug.

4. Discussion

These results show that ghrelin receptor antagonists have the potential to reduce alcohol intake in dependent and non-dependent mice and rats, albeit in a transient fashion. The intent of experiment 1 was to test how ghrelin antagonists altered ethanol consumption following exposure to chronic intermittent ethanol vapor. In general, treatment with either DLys or JMV2959 reduced ethanol intake the first day after vapor exposure in both dependent and non-dependent mice. We found a difference in specificity between the two compounds in that DLys also reduced ethanol preference, while JMV2959 did not affect this measure. The effects on ethanol intake were transient, as changes in consumption or preference for ethanol normalized to control levels after 2-3 days of drug administration. Experiment 2 also showed that DLys reduced ethanol intake, but only in non-dependent mice. Finally, experiment 3 found a dose dependent reduction in lever pressing and intake for both ethanol and sucrose after injections with DLys or JMV2959 in rats previously made dependent by ethanol vapor exposure.

Experiment 1

Our findings support other studies showing that ghrelin receptor antagonists reduce ethanol intake and preference (Jerlhag et al, 2009; Kaur and Ryabinin, 2010; Suchankova et al, 2013). However, our data show that the effects of ghrelin receptor antagonists are only present on the first day of administration, which partially replicates a previous finding showing that mice with 24 hour access to ethanol, via two-bottle choice, only reduce intake/preference on the first day of DLys administration 4 hours after injection (Gomez and

Ryabinin, 2014). When injected with either DLys or JMV2959, mice in both air and ethanol vapor groups showed a decrease in two-bottle choice ethanol drinking. Although the effect was only present on the first day, the GHSR1a antagonists effectively reduced ethanol intake during Test 1 and 2, suggesting a tolerance effect with daily administration during each Test session, but no carryover effect after a break in drug administration. Suchankova and colleagues (2013) found no tolerance effect after repeated administrations of JMV2959, however, the difference may be due to use of rats instead of mice and an 8-week drinking experience prior to drug testing. In our study, differences in drug specificity were seen in both intake and preference for ethanol. DLys had a robust effect on intake compared to JMV2959, almost ablating all ethanol intake. Additionally, DLys reduced preference for ethanol without changing total fluid intake, whereas JMV2959 had no effect on preference. This suggests that DLys has more specific actions on ethanol drinking behaviors. In regards to food consumption, there was an overall reduction in food intake for all groups across test days, but there was no difference in food intake between groups. This is an important observation, indicating that although JMV2959 did not affect preference for ethanol over water, it did not have non-specific effects on overall consummatory behaviors. A potential confound in this study is that we did not see an increase in ethanol intake across all testing days following exposure to ethanol vapors, as others have reported (Becker and Lopez, 2004; Finn et al, 2007; Griffin et al, 2009). However, we did see an increase in intake on the first day in the Veh group exposed to ethanol vapor compared to Veh air vapor group, but not in the JMV2959 or DLys groups. Additionally, we did find an increase in preference for ethanol in mice exposed to ethanol vapors, an effect that was blocked by DLys but not JMV2959. This suggests that ethanol vapor exposed Veh mice were in a greater state of dependence on the first day of testing, and that the ghrelin antagonists were capable of attenuating the dependence-induced increase in drinking.

Experiment 2

Repeated passive infusions of ethanol produced an increase in ethanol intake in dependent mice compared to non-dependent (water-exposed) controls. HIC scores were increased in mice passively infused with ethanol. Although, HIC scores were not assessed in experiment 1, our previous work showed that exposure to intermittent ethanol vapor and multiple withdrawals increase withdrawal severity, measured by an increase in hourly HIC scores across 12 h of withdrawal (Finn et al, 2007). In experiment 2, DLys was only effective at reducing ethanol intake and bout size in non-dependent water infused mice. Similar to the results found in experiment 1, when mice had a voluntary choice between ethanol and water, mice administered DLys showed a reduction in ethanol intake on the first choice day. However, the fact that DLys was only effective in non-dependent mice may be due to the difference in dependence induction.

Passive IG ethanol infusion is one of the most effective and rapid ways of producing physical dependence (Fidler et al, 2006). In comparison, chronic intermittent ethanol vapors may take a few cycles of exposure to induce dependence (Finn et al, 2007). Experiment 2 did not find DLys to be effective in dependent mice and this may be due to a difference in strength of withdrawal between models, which is difficult to compare. We suggest that withdrawal severity may reduce the effectiveness of DLys. However, this hypothesis needs

further testing. A possible experiment would be to expose animals to different levels of withdrawal and test the effectiveness of DLys to reduce ethanol intake. If efficacy of DLys depends on severity of withdrawal, it would be expected that the same dose of DLys would only reduce ethanol intake in mice experiencing a sub-threshold of withdrawal severity, a threshold which would need to be determined. A second course of action may be examining the time course of plasma ghrelin following withdrawal from several methods of dependence induction such as vapor exposure versus IG ethanol exposure used in the current experiments. The interactions between the ghrelin system and withdrawal need further investigation. Finally, another possibility is the difference in alcohol access between experiments. While experiment 1 used a limited access (2 hours) paradigm, experiment 2 allowed access to alcohol over a 24 hour period, only limiting the mice to 1.5 g/kg/30min. This difference in alcohol access may have accounted for the non-effect of DLys on intake seen in dependent mice. In a previous study using non-dependent mice, we showed that DLys reduces ethanol intake and preference 4 hours after administration, while intake/preference returns to control levels 24 hours post-injection (Gomez and Ryabinin, 2014).

The IGAC study found no differences in ethanol preference during the choice days between groups. However, preference for ethanol between experiments is difficult to compare due to the parameters of each method. The ratios we reported in experiment 2 were generally consistent with those previously reported for C57BL/6J mice using the IGAC procedure (Fidler et al., 2012). It is possible that DLys may interact with the oral taste and thus, the lower ratios seen towards the end of choice days in experiment 2, may have been due to a change in taste for the sweetened Kool-Aid. However, we do not believe that DLys has an effect on taste, although we cannot be completely sure. The IGAC model presumably reduces the direct effects of ethanol's taste on the tongue when the mouse is licking (since there's no ethanol in the tube), but taste cannot be completely ruled out since there is evidence that once ethanol is absorbed into the bloodstream (and exhaled from the lungs) taste receptors on the tongue can detect ethanol (Cunningham and Linakis, 1980). There are not many studies looking at taste and ghrelin receptor antagonists. One such study showed that ghrelin disrupts conditioned taste aversion, while co-administration of a ghrelin antagonist (YIL-781) restores this ghrelin-induced change (Song et al, 2013). We would speculate that ghrelin receptor antagonists do not alter taste, but rather, ghrelin manipulates alcohol drinking behaviors through neural mechanisms (Jerlhag et al, 2009) and as seen in human studies, perception of alcohol craving (Leggio et al, 2014). A few studies have shown that ghrelin is present in saliva (Groschl et al, 2005; Li et al, 2011) and may affect taste (Shin et al, 2010), but the effects of GHSR1a antagonists on taste need further examination. Food intake was not measured in experiment 2 due to a lack of ability and accuracy given the apparatus used. However, in experiments 1 and 2, body weights did not differ between treatment groups. Based on the body weight data we speculate that food intake did not significantly differ between mice treated with saline or DLys in experiment 2.

Experiment 3

The results found that both JMV2959 and DLys decrease operant self-administration behaviors in rats after exposure to 8 weeks of intermittent ethanol vapor. Mainly, both drugs led to dose-dependent decreases in reinforcer seeking behavior (active lever presses, head

entries, and final ratios completed) in both reinforcer groups. Also, both drugs led to dose-dependent decreases in reinforcer taking behaviors (reinforcers delivered / retrieved). Treatment with JMV2959 showed non-specific effects by reducing inactive lever pressing. This effect supports previous data showing that JMV2959 in general reduces all consummatory behaviors (Gomez and Ryabinin, 2014). These results are in line with others who have shown that ethanol vapor exposure increases self-administration (O'Dell et al, 2004) and compounds used to treat alcoholism such as acamprosate and naltrexone can block this effect (Heyser et al, 2003). It should be noted that in the current experiment, JMV2959 and DLys also reduced operant behaviors of responding for sucrose, which raises the possibility of these compounds reducing reward salience, which may prove problematic in human subjects.

The role of reinforcement schedules (fixed v progressive ratio) may have played a role in differences in the results obtained between experiments 2 and 3. However, it is difficult to determine what exact role schedule differences played. It is equally possible that species differences, response demands (licking v bar pressing), length of time since withdrawal, etc. were responsible. In the IGAC model each lick was reinforced by sweetened Kool-Aid followed by infusion for every tenth lick. In contrast, there is a clear disconnect between the operant response (bar pressing) and the consummatory response in the rat model. Progressive ratio responding is generally considered more reflective of the work an animal will perform to obtain a drug or reinforcer. However, despite extensive studies on PR schedules, there is no indication that PR responding is a better reflection of motivation (Arnold and Roberts, 1997; Richardson and Roberts, 1996). With respects to taste, Overduin and colleagues (2012) found that ghrelin infused into the 3rd ventricle does not change licking behavior for food, but does increase bar pressing for food in rats. From this finding we speculate that a ghrelin receptor antagonist would not affect what Overduin describes as palatability for food, but rather alter motivation for food reward. Of course this is purely speculative and further testing is needed, but based on what we found in experiment 3, we would hypothesize that ghrelin receptor antagonists would decrease bar pressing for alcohol due to a decrease in motivation. Despite the differences between models, the data suggest that the effects of ghrelin receptor antagonists may be due to effects on intrinsic reward mechanisms.

In conclusion, similar effects were found in all three experiments of this study. All showed that ghrelin receptor antagonists are capable of reducing ethanol intake to various degrees in both mouse and rat models. The compounds tested (JMV2959 and DLys) show promise as treatments for alcoholism, as these compounds produce effects in animal models similar to those of other compounds already approved for alcoholism treatment (acamprosate and naltrexone). Although, we only tested one dose of each drug, testing other doses, either higher or lower, would not refute the current findings that ghrelin receptor antagonists show different levels of effectiveness across methods and species. Despite the differences between the current experiments, GHSR1a antagonists lead to a reduction in voluntary ethanol intake (exp.1 and 2) and a reduction in ethanol reward (exp.3). It has recently been suggested that the association between alcohol consumption and the ghrelin system are mediated via central nervous system actions and not necessarily peripheral actions (Jerlhag et al, 2014). Future experiments are being designed to test the effects of central ghrelin disruption, via

lentiviral receptor suppression and genetic models of GHSR receptor knockout. These experiments would allow for targeting of specific brain regions we believe are associated with ghrelin and alcohol intake, such as the centrally projecting Edinger-Westphal nucleus, ventral tegmental area, central nucleus of the amygdala, and arcuate nucleus. By targeting the central actions of ghrelin, we hypothesize that effects will be longer lasting. Targeting the food reward system that ghrelin engages has become a worthwhile objective towards understanding alcoholism and a conceivable treatment goal for people suffering from this disorder.

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Highlights

- Effects of ghrelin antagonism in rodents were studied on 3 models of dependence
- Selectivity of ethanol intake inhibition was seen with DLys, not JMV2959
- Antagonists inhibit ethanol intake following dependence induced by alcohol vapor
- DLys inhibits ethanol self-infusion before, not after, dependence induced by IGAC
- Antagonists lose effectiveness during repeated administration

Figure 1.

Ethanol Intake (A and B) – No difference in baseline drinking. DLys and JMV2959 reduced intake in both air and ethanol vapor exposed mice compared to Veh, but only on the first day. Ethanol Preference (C and D) – No difference in baseline preference. Only DLys reduced preference with no change by JMV2959. Food Intake (E and F) – No difference in baseline intake. A within group effect of day was seen across days. Mice decreased food intake, but no difference between groups was found. Left Side: mice exposed to air vapor; Right Side: mice exposed to ethanol vapor. Symbols: B=Baseline, VC=Vapor Chambers, Asterisk [*] = $p < 0.05$.

Figure 2.

Ethanol Intake (A and B) – Dependent mice self-infused more ethanol than non-dependent mice. DLys reduced ethanol intake in non-dependent mice during the first choice day (C1) but had no effect on intake in dependent mice. Number of Ethanol Bouts (C and D) – Dependent mice generally had more ethanol bouts than non-dependent mice. Dependent-DLys mice had fewer ethanol bouts than non-dependent-DLys mice, but Veh groups did not differ. Ethanol Bout Size (E and F) – Dependent mice generally had larger ethanol bouts than non-dependent mice. DLys reduced bout size in non-dependent mice on the first choice day (C1), but had no effect on bout size in dependent mice. Left Side: mice passively infused with water (non-dependent); Right Side: mice passively infused with ethanol (dependent). Symbols: Asterisk [*] = $p < 0.05$.

SUCROSE REINFORCER GROUPETHANOL REINFORCER GROUP **Figure 3.**

Active Lever Presses (A and B) – Doses of 2 or 4 mg/kg of either DLys or JMV2959 reduced lever pressing behavior following dependence by ethanol vapor exposure in both the sucrose and ethanol reinforced group. Head Entries (C and D) – Only the high dose (4 mg/kg) of GHSR1a antagonists reduced head entries. The effect was seen in both reinforcer groups. Deliveries (E and F) – The middle and high dose (2 and 4 mg/kg) of JMV2959 reduced deliveries while only the high dose (4 mg/kg) of DLys reduced deliveries. Left Side: rats reinforced to respond for sucrose solution; Right Side: rats reinforced to respond for ethanol solution. Symbols: [a] = significant change ($p < 0.05$) caused by JMV2959 compared to 0 mg/kg dose; [b] = significant change ($p < 0.05$) caused by DLys compared to 0 mg/kg dose.

Table 1

Mean ($\pm$ S.E.M.) preference ratios during choice days for mice infused with passive water or passive ethanol and injected with either Veh or DLys.

Groups	<u>Passive Water non-dependent</u>		<u>Passive Ethanol dependent</u>	
Choice Days	Vehicle	DLys	Vehicle	DLys
1	0.87 (0.06)	0.72 (0.13)	0.83 (0.07)	0.88 (0.07)
2	0.75 (0.07)	0.65 (0.13)	0.77 (0.09)	0.71 (0.08)
3	0.78 (0.07)	0.60 (0.12)	0.75 (0.08)	0.71 (0.06)
4	0.64 (0.08)	0.51 (0.10)	0.58 (0.09)	0.68 (0.06)
5	0.62 (0.09)	0.50 (0.11)	0.58 (0.09)	0.65 (0.07)

Table 2

Mean ($\pm$ S.E.M.) inactive lever press responses seen after administration of DLys and JMV2959 by reinforcer group (Ethanol=rats responding for 10% ethanol; Sucrose=rats responding for 2% sucrose).

Drug/Dose (mg/kg)	DLys		JMV2959	
	Ethanol	Sucrose	Ethanol	Sucrose
0	1.0 (0.4)	1.7 (0.7)	1.0 (0.3)	2.4 (0.6)
1	--	--	1.1 (0.5)	1.1 (0.5)
2	0.3 (0.2)	1.8 (0.7)	1.3 (0.4)	2.0 (0.4)
4	0.7 (0.3)	1.2 (0.5)	0.3 (0.2)	0.9 (0.5)

Table 3

Mean ($\pm$ S.E.M.) final ratios completed after administration of DLys and JMV2959 by reinforcer group (Ethanol=rats responding for 10% ethanol; Sucrose=rats responding for 2% sucrose).

Drug/Dose (mg/kg)	DLys		JMV2959	
Group	Ethanol	Sucrose	Ethanol	Sucrose
0	6.3 (0.7)	11.3 (0.8)	5.9 (0.4)	10.4 (0.4)
1	--	--	4.8 (0.4)*	9.6 (0.6)
2	4.8 (0.5)	10.0 (0.9)	4.6 (0.2)*	9.0 (0.4)*
4	3.5 (0.3)*	5.2 (1.0)*	4.3 (0.4)*	7.9 (0.4)*

* Asterisk signifies significant difference versus respective vehicle treatment, $p < 0.05$.

Table 4

Mean ($\pm$ S.E.M.) numbers of reinforcers retrieved after administration of DLys and JMV2959 by reinforcer group (Ethanol=rats responding for 10% ethanol; Sucrose=rats responding for 2% sucrose).

Drug/Dose (mg/kg)	DLys		JMV2959	
Group	Ethanol	Sucrose	Ethanol	Sucrose
0	11.2 (0.6)	16.0 (0.9)	9.8 (0.3)	15.0 (0.3)
1	--	--	8.7 (0.6)*	14.1 (0.5)
2	9.0 (0.7)*	14.7 (1.3)	8.9 (0.5)*	13.6 (0.4)*
4	6.5 (0.8)*	8.8 (0.9)*	6.0 (1.1)*	12.1 (0.3)*

* Asterisk signifies significant difference versus respective vehicle treatment, $p < 0.05$.