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V1b receptor antagonist SSR149415 and naltrexone synergistically decrease excessive alcohol drinking in male and female mice

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Abstract

Background: A recent clinical trial found that pharmacological blockade of V1b receptors reduces alcohol relapse in alcohol-dependent patients. SSR149415 is a selective V1b receptor antagonist that has potential for development as an alcohol dependency treatment. In this study, we investigated whether SSR149415 alone or in combination with the mu-opioid receptor [MOP-r] antagonist naltrexone (NTN) would alter excessive alcohol drinking in mice. **Methods**: Both sexes of C57BL/6J (B6) mice were subjected to a chronic intermittent access (IA) drinking paradigm (two-bottle choice, 24-h access every other day) for 3 weeks. Sucrose and saccharin drinking were used as controls for alcohol-specific drug effects. Neuronal proopiomelanocortin (POMC) enhancer *(*nPE) knockout mice with hypothalamic-specific loss of POMC (including beta-endorphin, the main endogenous ligand of MOP-r) were used as a genetic control for the effects of NTN. **Results**: Acute administration of SSR149415 (1-30 mg/kg) reduced alcohol intake and preference in a dose-dependent manner in both male and female B6 mice after IA. To investigate potential synergistic effects between NTN and SSR149415, we tested six different combination doses of SSR149415 and NTN, and found that a combination of SSR149415 (3 mg/kg) and NTN (1 mg/kg) reduced alcohol intake profoundly at doses lower than the individual effective doses in both sexes of B6 mice. We confirmed the effect of SSR149415 on reducing alcohol intake in nPE-/- male mice, consistent with independent mechanisms by which SSR149415 and NTN decrease alcohol drinking. **Conclusion**: The combination of V1b antagonist SSR149415 with NTN at individual subthreshold doses shows potential in alcoholism treatment, possibly with less adverse effects.

Keywords: SSR149415, V1b receptor, excessive alcohol drinking, naltrexone, combined therapy, nPE knockout mice.

Running title: V1b and naltrexone synergistically reduce alcohol drinking**INTRODUCTION**

There is consistent evidence suggesting that increased arginine vasopressin (AVP) neuronal activity represents an important step in the neurobiology of stress-related behaviors in several rodent models [Griebel et al, 2002; Salome et al, 2006; Roper et al, 2011] and in humans [Katz et al, 2016; Ryan et al, 2017]. Chronic high levels of alcohol consumption activate endogenous AVP systems in neuronal structures related to alcohol dependence or compulsivity. Several studies have found that chronic alcohol exposure interfered with AVP gene expression or peptide levels in several brain stress responsive regions, like the bed nucleus of the stria terminalis, medial amygdala and hypothalamic paraventricular nucleus (PVN) in mice and rats [Ishizawa et al, 1990; Silva et al, 2002; Zhou et al, 2011]. In line with these findings, reduction of

the number of AVP-immunoreactivity neurons and the AVP mRNA levels in the hypothalamus after chronic alcohol consumption has also been found in human brains [Harding et al, 1996]. These findings provide support for the importance of the AVP systems in the processes of alcohol consumption and addiction.

Central AVP binds to two different G protein-coupled receptor subtypes: V1a and V1b, and both are highly expressed in the rat extended amygdala [Veinante and Freund-Mercier, 1997]. Recently, activation of the V1b receptor system has been implicated in the negative reinforcing aspects of alcohol addiction. V1b protein levels are increased by alcohol withdrawal in the basolateral amygdala of alcohol-dependent rats [Edwards et al, 2012]. Pharmacological studies also support this notion: the systemically active, selective V1b antagonist SSR149415 [Griebel et al, 2002] reduces voluntary alcohol consumption in alcohol “dependent” rats [Edwards et al, 2012] and Sardinian alcohol preferring rats with high anxiety-like behaviors [Colombo et al, 2006; Zhou et al, 2011]. Therefore, this enhanced AVP/V1b expression and/or activity may be involved in the homeostatic adaptations of the extended amygdala after chronic drug exposure and in the negative affective state during withdrawal. In a recent phase two, double-blind, placebo-controlled randomized trial, pharmacological blockade of V1b receptor reduces alcohol consumption and relapse in alcohol-dependent patients, especially those with high stress [Ryan et al, 2017].

By targeting multiple neurotransmitter pathways implicated in different components of alcohol addiction, combination medications may have enhanced efficacy over the traditional single-medication approach. Given that naltrexone (NTN, mu-opioid receptor [MOP-r] antagonist) therapies have been used extensively in the treatment of alcoholism and V1b antagonists are in clinical trials, NTN and SSR149415 (MOP-r and V1b antagonisms, respectively) are ideal candidates for investigating the potential benefit of combined treatments. Therefore, we hypothesized that SSR149415 combined with NTN could synergistically decrease alcohol consumption in mice, and our study may provide new information about the medical potential of SSR149415 in the treatment of alcoholism. For this purpose, we first evaluated the pharmacological effect of SSR149415 alone in both male and female mice using both chronic intermittent access (IA) drinking and the drinking-in-the-dark (DID) models. In the IA model, which constitutes an appropriate animal model for studying excessive alcohol drinking [Hwa et al, 2011; Zhou et al, 2017a], the mice exposed to alcohol for 3 weeks developed high alcohol consumption. The sub-effective doses of NTN have been determined in our recent studies using the same IA paradigm [Zhou et al, 2017a, b]. Finally, we specifically tested the combinations of

SSR149415 and NTN using doses of each compound low enough that no effect on alcohol intake was found with either drug alone.

Our mechanistic hypothesis is that the MOP-r activation by endogenous ligand beta-endorphin has a different pathway driving excessive alcohol drinking from the AVP/V1b in the amygdala. In this study, we further investigated whether SSR149415 alters voluntary alcohol drinking in nPE knockout mice (targeted deletion of neuronal *Pomc* enhancers leading to the loss of central beta-endorphin and melanocortin peptide expression) [Lam et al, 2015], to explore potential neuronal mechanisms for synergistic effects of SSR149415 and NTN.

METHODS AND MATERIALS

1. Animals.

1.1. Male and female adult C57BL/6J (B6) mice. Mice (8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in a temperature-controlled room (21 ºC), with a 12-hour reverse light-dark cycle (lights off at 7:00 am) for a week prior to testing. During this week, mice were individually housed in ventilated cages fitted with steel lids and filter tops and given *ad libitum* access to food and water. Animal care and experimental procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources Commission on Life Sciences 1996), and were approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

1.2. Pomc neuronal enhancer (nPE1 and nPE2) knockout mice. The present study used intact, male and female, single-housed mice with targeted deletion of the POMC neuronal enhancers nPE1 and nPE2 and insertion of a transcriptional blocking *neo* cassette in the enhancer locus (nPE-/-) [Bumaschny et al, 2012; Lam et al, 2015]. The gene mutations were generated by homologous recombination in 129S6/SvEvTac Taffy ES cells to produce the chimeric founder mice, followed by 7-10 generations of backcrossing onto the C57BL/6J strain for the mice used in these studies. Specifically, in these transgenic mice, simultaneous deletion of nPE1 and nPE2 and insertion of a neomycin selection cassette in the enhancer vicinity in the context of the intact *Pomc* pituitary enhancer region and proximal promoter abolishes *Pomc* expression in the arcuate nucleus, without altering *Pomc* expression in pituitary cells. nPE-/- mice had greater daily food intake at 8-9 weeks of age (5.2-5.3g in both males and females) than nPE+/+ mice (3.2-3.3g in both males and females). At the time the experiments started (age 8-10 weeks), nPE-/- mice had greater body weight (~ 40g and 35g in males and females, respectively) than nPE+/+ mice (~ 27g and 23g in males and females, respectively).

2. Materials. SSR149415 (a gift from Dr. G. Griebel, Sanofi Aventis, Montpellier, France) was suspended in 5% DMSO, 5% Cremophor and saline [Griebel et al, 2002]. Ethanol solutions (7.5%, 15% and 30% v/v) were prepared from 190 proof absolute ethyl alcohol (Pharmco-AAPER, Brookfield, CT, USA) and dissolved in tap water. Sucrose and saccharin were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and diluted in tap water. Naltrexone was purchased from Sigma-Aldrich Inc. and dissolved in physiological saline.

3. Procedures.

3.1. Chronic intermittent access (IA) excessive drinking. This model in B6 mice has been widely used by many laboratories [e.g., Hwa et al, 2011; Zhou et al, 2017b, c].

3.1A. The 3-week IA model. Mice had access to alcohol drinking in their home cages for 3 weeks. Food and water were available at all times in this two-bottle choice paradigm with chronic alcohol exposure every other day. This IA protocol was described in detail in our earlier reports [Zhou et al, 2017b, c]. Briefly, starting at 10:00 am (3 hours after lights off), both water and alcohol (7.5%, 15% or 30%) solution sipper tubes were placed on the home cages. The tubes' positions (left and right) on the cage were randomly set to avoid the development of side preference. The alcohol tubes were filled with fresh alcohol solution, placed on the cage for 24 hours, and then replaced with the water tubes. After 4, 8 and 24 hours of alcohol access, alcohol and water intake values were recorded. These data were used to calculate the consumed alcohol intake (i.e., g/kg) and preference ratio for alcohol (i.e., alcohol intake/total fluid intake).

After 3 weeks of IA, male and female mice of vehicle and drug -treated groups had matched body weight and similar alcohol intake 24 hours before the test day. The compounds dissolved in vehicle were administered by an experimenter, blinded to the treatments assigned to the experimental groups.

3.1B. Acute administration in the 3-week IA model in B6 mice. On the test day, alcohol (7.5%, 15% or 30% concentrations) was presented 30 min after an injection of SSR149415 (1, 3, 10, or 30 mg/kg, i.p.) or vehicle (5% DMSO and 5% Cremophor in saline), and then alcohol and water intake values were recorded. The SSR149415 doses chosen in the present experiments were based on our previous studies in rats [Zhou et al, 2011]. Similarly, the combined effects of SSR149415 (1 or 3 mg/kg) with sub-effective doses of NTN (0.5 or 1 mg/kg) [Zhou et al, 2017b] were evaluated on alcohol drinking after the 3-week IA. On the test day, the mice received an i.p. injection of SSR149415 or vehicle followed by the second i.p. injection of NTN or saline 20 min later. Then alcohol was presented 10 min after NTN or vehicle and then alcohol and water intake values were recorded.

3.1C. Acute administration in the 3-week IA model in nPE mice. The effects of SSR149415 were measured on alcohol drinking in two genotypes (nPE+/+ and nPE-/-) of each sex, and the sub-effective dose of SSR149415 chosen (3 mg/kg) was based on the data in B6 mice in the above IA experiments. On the test day, 15% alcohol was presented 30 min after an injection of SSR149415 (i.p.) or vehicle (5% DMSO and 5% Cremophor in saline), and then alcohol and water intake values were recorded as described above.

3.2. Chronic drinking-in-the-dark (DID). This model in B6 mice has been widely used by many laboratories [e.g., Rhodes et al, 2005; Sprow et al, 2016; Zhou et al 2017b, c]. Unlike the IA model above, mice were exposed to 15% alcohol every day in this one-bottle paradigm, with one recording per day (after 4 hours of alcohol access in the dark cycle).

3.2A. The 3-week DID model. This DID protocol was described in detail in our earlier reports [Zhou et al, 2017 b, c]. Briefly, at 3 hours after lights turned off (10:00 am), the water bottle was replaced with one alcohol (15%) tube, and left for 4 hours until the original water bottle was returned. After 4 hours of alcohol access, alcohol intake values were recorded every day.

These data were used to calculate alcohol intake (i.e., g/kg). After 3 weeks of DID, male and female mice of vehicle and drug -treated groups had matched body weight and similar alcohol intake 1 day before the test day.

3.2B. Acute administration in the 3-week DID model in B6 mice. The SSR149415 doses (10 and 30 mg/kg) were based on the results of the above IA alcohol study. On the test day, 15% alcohol was presented 30 min after an i.p. injection of SSR149415 or vehicle (5% DMSO and 5% Cremophor in saline).

3.3. Sucrose (caloric reinforcer) and saccharin (non-caloric reinforcer) drinking. As the sucrose and saccharin drinking tests are sensitive to the function of brain reward systems, they are used to measure the expression of anhedonia after chronic alcohol drinking [e.g., Zhou et al 2017c]. Using the same doses, the specificity of the action of SSR149415 alone or combined with NTN on alcohol intake was further tested using sucrose or saccharin drinking behavior after acute administration of the combination following the 3-week IA. In the following experiments, 15% alcohol IA exposure was identical to those in the above experiment as described in section 3.1. After 3 weeks of IA, the alcohol tube was switched to sucrose or saccharin for 3 sessions, during which stable intake was observed after 6 days. The mice assigned to the vehicle or SSR149415 -treated groups in each sex had similar sucrose or saccharin intake 24 hours

before the test day. On the test day, sucrose (4%, 8% or 16%) and water intake values were recorded after 4, 8 and 24 hours of sucrose access. In parallel separate experiments, saccharin drinking (0.1%, 0.2% or 0.4%) was tested after 3 weeks of IA with an identical procedure.

3.3A. Acute administration of SSR149415 (10 mg/kg) on sucrose or saccharin drinking after 3-week IA in B6 mice. An i.p. injection of SSR149415 (10 mg/kg) or vehicle (5% DMSO and 5% Cremophor in saline) was given 30 min before the sucrose or saccharin solutions were presented. Male and female mice were assigned to one of two treatment groups: vehicle or SSR149415.

3.3B. Acute administration of SSR149415 (3 mg/kg) combined with NTN (1 mg/kg) on sucrose or saccharin drinking after 3-week IA in B6 mice. On the test day, the mice received the first i.p. injection of SSR149415 or vehicle followed by the second i.p. injection of NTN or saline 20 min later. Male and female mice were assigned to one of two treatment groups: vehicle or SSR149415 + NTN.

3.3C. Acute administration of SSR149415 (10 mg/kg) on sucrose or saccharin drinking in alcohol-naïve B6 mice. The procedures were identical to the above 3.3A and 3.3B experiments, except the mice were exposed to 4% sucrose or 0.1% saccharin only.

4. Data analysis. Based on the between-groups approach (i.e. separate groups of mice for each dose) and levels of differences seen previously [Zhou et al, 2017a, b, c], we performed power analyses to determine the number of animals required to provide statistically significant results and predicted that these studies require 6-8 animals per group.

There were 207 male and 211 female B6 mice analyzed in the present experiments. In the experiments with SSR149415, NTN or their combinations, alcohol (or sucrose, saccharin) intake, water intake, total fluid and preference ratio differences in each sex across the different groups were analyzed using 2-way ANOVA with repeated measures for treatment (vehicle vs drug) and for time interval (0-4, 5-8 vs. 9-24h). For dose response analysis on SSR149415 alone and SSR149415 + NTN combinations, group differences for alcohol intake and preference ratios at the 4-hour recording time were analyzed using 2-way ANOVA for treatments with different doses and for sex (male vs. female).

There were 28 male and 24 female nPE mice (divided equally between nPE+/+ and nPE-/- genotypes) analyzed in the present experiments In the nPE mouse experiment, group differences in alcohol intake, water intake and preference ratios in each sex were analyzed using 2-way ANOVA for genotype (nPE+/+ vs. nPE-/-) and treatments (vehicle vs. drug).

The 2-way ANOVAs were followed by Newman-Keuls *post-hoc* tests. The accepted level of significance for all tests was $p < 0.05$. All statistical analyses were performed using *Statistica* (version 5.5, StatSoft Inc, Tulsa, OK).

RESULTS

1. Acute administration of SSR149415 alone reduced alcohol, but not sucrose or saccharin, intake and preference after IA in both male and female B6 mice.

1.1. Dose responses of SSR149415 on 15% alcohol intake and preference. The full-dose response of acute SSR149415 administration (0, 1, 3, 10 and 30 mg/kg) in terms of 15% alcohol intake and preference at the 4-hour time point is presented in **Fig 1**. For alcohol intake (**Fig 1A**), there was a main effect of SSR149415 [2-way ANOVA, $F(10,136) = 30$, $p < 0.0000001$], and *post hoc* analysis showed that (1) in comparison with the vehicle group, the SSR149415-treated mice had less intake than the vehicle-treated mice at both 10 and 30 mg/kg doses in both males and females [*post-hoc* test $p < 0.01$ for all]; and (2) the reductions at 10 mg/kg were greater than those at 3 mg/kg [$p < 0.05$ for both sexes]. For preference ratio (**Fig 1B**), there was a main effect of SSR149415 [2-way ANOVA, $F(10,136) = 22$, $p < 0.0000001$], and *post hoc* analysis showed that (1) in comparison with the vehicle group, the SSR149415-treated mice had less preference than the vehicle-treated mice at both 10 and 30 mg/kg doses in both males and females [*post-hoc* test $p < 0.01$ for all]; and (2) the reductions at 30 mg/kg were greater than those at 3 mg/kg [$p < 0.05$ for both sexes].

1.2. Acute SSR149415 at 10 mg/kg reduced 15% alcohol intake and preference. Alcohol intake and preference ratio are presented in **Fig 2** (male) and **Fig 3** (female) after 4, 8 and 24 hours of acute SSR149415 at 10 mg/kg. SSR149415 significantly reduced alcohol intake in males [2-way ANOVA, $F(1, 13) = 6.7$, $p < 0.05$] at 4 hours [*post-hoc* test $p<0.005$] (**Fig 2A**) and in females [2-way ANOVA, $F(1, 12) = 6.2$, $p < 0.05$] at 4 hours [*post-hoc* test $p<0.001$] (**Fig 3A**). This was associated with a compensatory increase in water intake in males and in females, resulting in virtually unchanged total fluid intake in both sexes (**Table 1**). At this dose, SSR149415 also significantly reduced preference ratio in males [2-way ANOVA, $F(1, 13) = 5.4$, $p < 0.05$] at 4 hours [*post-hoc* test $p < 0.01$] (**Fig 2B**) and in females [2-way ANOVA, $F(1, 12) = 15$, $p < 0.01$] at 4 hours [*post-hoc* test $p < 0.005$] (**Fig 3B**). We did not observe any sex differences in the dose-response effects of SSR149415 alone in the above experiments, suggesting that the estrous cycle and associated hormones might not be important factors in the response to these treatments in females.

1.3. Effects of acute SSR149415 on 7.5% or 30% alcohol intake and preference. After 10 mg/kg SSR149415, there was an apparently slight decrease on either 7.5% (**Table 2A**) or

30% (**Table 3A**) alcohol intake in both male and female mice, although these could not research statistical significance. No effects of 10 mg/kg SSR149415 on alcohol preference ratio were found in either males or females with either alcohol concentration (**Tables 2A, 3A**).

1.4. No effects of acute SSR149415 on sucrose (caloric reinforcer) or saccharin (non-caloric reinforcer) intake and preference. The specificity of the effect of SSR149415 on alcohol drinking was verified by testing the effects of 10 mg/kg SSR149415 on sucrose or saccharin intake. In these experiments, the chronic 15% alcohol IA procedures were identical to those in the above experiments. The mice assigned to the vehicle or SSR149415 -treated groups had similar sucrose or saccharin intake 24 hours before the test day. On the test day, no significant effect of 10 mg/kg SSR149415 on 4% sucrose (**Table 4A**) or 0.1% saccharin (**Table 4C**) drinking was found after 4 hours in either males (left) or females (right). There was no effect on sucrose or saccharin drinking observed after 8 or 24 hours (data not shown).

Effects of SSR149415 on consumption of other concentrations of sucrose (8% or 16%) or saccharin (0.2% or 0.4%) were also tested in males and females (n = 4-5) and no significant differences were found. Similarly, there was no effect of acute SSR149415 at 10 mg/kg on sucrose or saccharin drinking in alcohol-naïve males and females (data not shown).

2. Acute administration of SSR149415 combined with naltrexone (NTN) reduced alcohol, but not sucrose or saccharin, consumption after IA in both male and female B6 mice.

2.1. Effect of acute administration of SSR149415 combined with NTN on 15% alcohol drinking. In both males and females, acute administration of SSR149415 (1 or 3 mg/kg) combined with NTN (1 mg/kg) reduced 15% alcohol intake and preference in a dose-dependent manner (data at the 4-hour time point are analyzed together and presented in **Fig 1**. Combined with NTN at 0.5 mg/kg, acute administration of SSR149415 at two doses (1 or 3 mg/kg) did not reduce alcohol intake or preference in either males or females (**Fig 1A, 1B**).

Combined with a higher dose of 1 mg/kg NTN, however, the SSR149415 at 1 mg/kg and 3 mg/kg significantly reduced alcohol intake in male [*post-hoc* test $p < 0.001$ for both] and in female mice [*post-hoc* test $p < 0.05$ and $p < 0.001$, respectively] (**Fig 1A**), when compared with the vehicle group. The reductions at 3 mg/kg SSR149415 + 1 mg/kg NTN combination were greater than those at 1 mg/kg SSR149415 + 1 mg/kg NTN combination [$p < 0.05$ for both sexes]. Furthermore, this 3 mg/kg SSR149415 + 1 mg/kg NTN combination showed greater reductions of alcohol intake than 3 mg/kg SSR149415 alone in both males and females [*post-hoc* test $p < 0.001$ for both] (**Fig 1A**). For preference ratio, only 3 mg/kg SSR149415 combined with 1 mg/kg NTN had significant reductions in both males and females [*post-hoc* test $p < 0.01$

for both] (**Fig 1B**). Similarly, the combination showed greater reductions of alcohol preference than 3 mg/kg SSR149415 alone in both males and females [*post-hoc* test $p < 0.05$] (**Fig 1B**).

Figures 2C and 2D present 15% alcohol intake and preference ratio at all three time points (4, 8 and 24 hours) following one combination dose in males. Combined with 1 mg/kg NTN, 3 mg/kg SSR149415 significantly reduced alcohol intake [2-way ANOVA, $F(1,13) = 30$, $p < 0.0001$] between 0-4 and 9-24 hour intervals [*post-hoc* test $p < 0.0001$ and $p < 0.05$, respectively] (**Fig 2C**). This combination also significantly reduced preference ratio [2-way ANOVA, $F(1,13) = 11$, $p < 0.01$] between 0-4 and 9-24 hour intervals [*post-hoc* test $p < 0.005$ and $p < 0.05$, respectively] (**Fig 2D**). There was no difference 24 hours after the test day (data not shown). **Figures 3C and 3D** present all three time points (4, 8 and 24 hours) in females. Combined with 1 mg/kg NTN, 3 mg/kg SSR149415 significantly reduced alcohol intake [2-way ANOVA, $F(1,10) = 74$, $p<0.00001$] between 0-4, 5-8 and 9-24 hour intervals [*post-hoc* test $p < 0.001$, $p < 0.001$, and $p < 0.05$, respectively] (**Fig 3C**). This combination also significantly reduced preference ratio [2-way ANOVA, $F(1,10) = 36$, $p < 0.0005$] between 0-4, 5-8 and 9-24 hour intervals [*post-hoc* test $p < 0.005$, $p < 0.005$ and $p < 0.05$, respectively] (**Fig 3D**). There was no difference 24 hours after the test day (data not shown). The combination dose had no effect on total fluid intake in either sex (**Table 1**).

2.2. Effects of acute SSR149415 + NTN on 7.5% or 30% alcohol intake and preference. There was an apparently slight decrease on either 7.5% (**Table 2B**) or 30% (**Table 3B**) alcohol intake in both males and females after 3 mg/kg SSR149415 + 1 mg/kg NTN, although these could not research statistical significance. No effects on alcohol preference ratio were found in either males or females with either concentration.

2.3. No effect of acute administration of SSR149415 combined with NTN on sucrose or saccharin drinking. The specificity of the effect of the SSR149415 + NTN combination on alcohol intake was ascertained by testing its effect on sucrose and saccharin drinking after IA. After 4 hours, no significant effect of 3 mg/kg SSR149415 + 1 mg/kg NTN (the most effective combination for reducing alcohol) on 4% sucrose or 0.1% saccharin drinking was found in either males (**Table 4B, 4D**) or females (**Table 4B, 4D**). Similarly, no significant effects of SSR149415 + NTN on other concentrations of sucrose (8% or 16%) or saccharin (0.2% or 0.4%) intake were observed in either males or females (n=4-5). There was no effect of SSR149415 + NTN on sucrose or saccharin drinking in alcohol-naïve males or females (data not shown).

3. Acute administration of SSR149415 at a sub-effective dose reduced alcohol intake after 3-week IA in male, but not female, nPE-/- mice.

3.1. Acute administration of SSR149415 (3 mg/kg) reduced alcohol intake in nPE-/- males (**Table 5A**). For alcohol intake, 2-way ANOVA revealed significant effects of genotype [$F(1, 24) = 80$, $p < 0.001$] and SSR treatment [$F(1, 24) = 5.4$, $p<0.05$]. *Post hoc* analysis showed that: (1) between genotypes, nPE-/- males had less intake than nPE+/+ males [$p < 0.01$]; and (2) SSR treatment at 3 mg/kg further reduced intake in nPE-/- males [$p < 0.05$], but not nPE+/+ males. For water intake, 2-way ANOVA revealed a significant effect of genotype [$F(1, 24) = 5.0$, $p < 0.05$]. For alcohol preference, 2-way ANOVA revealed a significant effect of genotype [$F(1, 24) = 44$, $p < 0.001$], and *post hoc* analysis showed that nPE-/- males had less preference than nPE+/+ males [$p < 0.05$].

3.2. Acute administration of SSR149415 (3 mg/kg) had no effect in nPE-/- females (**Table 5B**). For alcohol intake, 2-way ANOVA revealed significant effects of genotype [$F(1, 20) = 38$, $p < 0.001$], and *post hoc* analysis showed that nPE-/- females had less intake than nPE+/+ females [$p < 0.01$]. For water intake, 2-way ANOVA revealed a significant effect of genotype [$F(1, 20) = 5.8$, $p < 0.05$]. For alcohol preference, 2-way ANOVA revealed a significant effect of genotype [$F(1, 20) = 59$, $p < 0.001$], and *post hoc* analysis showed that nPE-/- females had less preference than nPE+/+ females [$p < 0.05$].

DISCUSSION

Our first objective of the present study was to determine the dose responses of acute administration of SSR149415 in reducing alcohol consumption in mice after chronic IA excessive alcohol drinking. At 10-30 mg/kg doses (but not 1-3 mg/kg), acute administration of SSR149415 significantly reduced alcohol intake in both males and females in the IA (**Fig 1A**), but not the DID (**Table S1**), model. Consistently, SSR149415 produced a reduction in alcohol preference in a dose-dependent (1-30 mg/kg) manner in both sexes (**Fig 1B**). It is unlikely that the effect of SSR149415 in reducing alcohol intake was secondary to a general suppression of consumption or appetitive (anhedonic effect) behaviors, since no tested doses of SSR149415 affected sucrose or saccharin consumption or preference (**Table 4**). Of note, though there are sex differences in both AVP/V1b systems [Stewart et al, 2008] and alcohol drinking behavior [Becker and Koob 2016], it is intriguing that both male and female mice had similar dose responses to SSR149415 (**Fig 1**). The new finding is in line with results showing the reducing effect of SSR149415 on alcohol consumption in alcohol-dependent male rats [Edwards et al, 2012] and alcohol-preferring male rats [Zhou et al, 2011]. To our knowledge, this is the first description of V1b antagonist SSR149415 on excessive alcohol drinking in both sexes compared side by side, suggesting that the blockade of V1b with SSR149415 may play a role in reducing alcohol drinking with no sex difference.

In a recent human study, acute administration of the selective V1b antagonist ABT-436 (3 hours before alcohol drinking) did not have any effect on mean blood alcohol levels [Katz et al 2016]. Based on this pharmacokinetic result in humans, we do not expect any significant changes of alcohol blood levels after acute administration of SSR149415 in mice.

To investigate whether alcohol drinking would alter endogenous AVP levels, many groups have measured AVP mRNA levels in both the PVN and amygdala of rats or mice after chronic alcohol exposure, and found that chronic alcohol drinking is associated with decreases in AVP mRNA levels in the PVN and the extend amygdala in rodents [e.g., Gulya et al, 1991; Silva et al, 2002; Zhou et al, 2011]. In parallel with mRNA changes, prolonged alcohol consumption is also associated with decreases in the levels of AVP-immunoreactivity in the mouse PVN and bed nucleus of the stria terminalis [Gulya et al, 1991]. In the hypothalamus of human post-mortem brains of alcoholic subjects, reduction of the number of AVP-immunoreactivity neurons and the AVP mRNA levels is also reported [Harding et al, 1996]. As AVP neurons in the PVN are distributed in both parvocellular and magnocellular divisions, and have potentially differential responses to stress, it is not clear that the altered plasma AVP levels in human studies are correlated to the AVP in parvocellular or magnocellular cells in response to alcohol withdrawal stress [Eisenhofer et al, 1985; Trabert et al, 1992]. However, recent studies suggest that activation of the V1b receptor could play important roles in acute drug withdrawal from long-term drug exposure [Zhou et al, 2008; Edwards et al, 2012; Qi et al, 2015]. Importantly, the blockade of V1b activity in the amygdala reduces the negative reinforcing action of alcohol consumption [Edwards et al, 2012]. Alternatively, the above new data suggest that the V1b expression, binding and function, as well as the downstream effects of V1b receptor signaling (rather than the AVP itself) can be more involved in the behavioral effects of alcohol drinking. Unfortunately, there is very limited research on V1b with alcohol or other drugs of abuse.

Our main objective was to investigate potential synergistic effects between NTN and SSR149415. In both preclinical and clinical studies, numerous pharmacological experiments provide consistent evidence that NTN, as a selective MOP-r antagonist, decreases alcohol consumption, reward, craving and relapse in many rodent models and human studies. SSR149415 is highly selective for the V1b receptor (60- to 800-fold more than for the V1a receptor), and displays anxiolytic, antidepressant and anti-alcohol properties in rodents [Griebel et al, 2002; Salome et al, 2006; Zhou et al, 2011; Edwards et al, 2012]. An investigation into the combination of these two compounds (NTN and SSR149415) could be particularly intriguing, given that these drugs have distinctly different mechanisms of actions. Due to its high selectivity

for the MOP-r and the low doses used in our studies, we predicted that NTN's activity at the MOP-r would not interfere with SSR149415's effect on the V1b. In fact, our results demonstrated that the combination of NTN and SSR149415 could have a synergistic, rather than additive, effect of the individual drugs on reducing alcohol intake and preference. Indications that the SSR149415 + NTN combination is more effective and potentially more beneficial in reducing alcohol intake than either drug alone include: (a) the effects of these combined, low-dose administrations of SSR149415 + NTN on alcohol drinking were 3 times greater than those of either drug alone (**Fig 1**); and (b) the combination showed a long-lasting effect after acute administration in both male (**Fig 2**) and female (**Fig 3**) mice. Finally, the specific effect of the combination on alcohol consumption was supported by the lack of any effect on sucrose or saccharin consumption (**Table 4**).

As the effectiveness of this SSR149415 + NTN combination could involve multiple neuro-pharmacological mechanisms (at least V1b and MOP-r), we hypothesized that this combination would be synergistic in reducing alcohol drinking. Indeed, neurobiological studies have found supportive observations, given the multiple actions of alcohol in the CNS and that both the MOP-r and V1b systems are profoundly altered by chronic alcohol exposure [Koob and Kreek 2007; Koob 2008]. NTN's actions are mediated through the blockade of MOP-r in the mesolimbic circuitries that may reduce the alcohol rewarding effect (positive reinforcement). In contrast, neuroanatomical distribution of V1b receptor is prominent in the stress responsive regions, like the amygdala and hypothalamus [Hernando et al, 2001; Koob 2008], and the AVP/V1b system in the extended amygdala could be a critical component contributing to the negative reinforcing effects during alcohol withdrawal. Indeed, activation of the AVP/V1b system in the amygdala has been found in drug withdrawal from several weeks of drug exposure, and the blockade of V1b activity in the amygdala reduces the negative reinforcing action of alcohol consumption [Edwards et al, 2012]. Systemic administration of V1b antagonists blocks the stress- and drug priming- triggered heroin seeking [Zhou et al, 2008] and prevents the dysphoria induced by nicotine withdrawal [Qi et al, 2015], as well as nicotine-induced locomotor sensitization [Goutier et al, 2016]. In humans, there were abnormal levels of serum and urine AVP during alcohol withdrawal, particularly when symptoms are severe [Eisenhofer et al, 1985; Trabert et al, 1992]. Therefore, by targeting MOP-r and V1b pathways implicated in both "positive" and "negative" components of alcohol addiction, the combination of NTN and SSR149415 is likely to have enhanced efficacy over the single-pathway approaches. In humans, NTN activates the hypothalamic-pituitary-adrenal (HPA) axis, and reduces alcohol drinking and craving [O'Malley et al, 2002]. As V1b activation in the anterior pituitary is also

involved in HPA regulation in humans [e.g., Katz et al, 2016], the combination of SSR149415 and NTN could synergistically modulate the HPA activity. Also, the corticotropin-releasing factor (CRF) and noradrenergic systems, the two known key stress mediators that are involved in stress and anxiety responses, probably interact with AVP systems to regulate alcohol drinking as demonstrated before [e.g., Simms et al, 2014; Tunstall et al, 2017].

Of note, the present study showed a relatively long duration (at least 24 hours) of the effect of SSR149415 + NTN combination on alcohol drinking behavior (especially the females) (**Figs 2 and 3**), which is unlikely due to SSR149415 metabolic stability and bioavailability in vivo (half-life is <1 h) [Oost et al, 2011]. In contrast, SSR149415 alone significantly reduced alcohol drinking at 4 hours with a similar profile to NTN alone (half-life is approximately 4 h), a reference compound in reducing alcohol drinking in our mouse model. Though the potential mechanisms are unknown, the development of new SSR149415 + NTN combination with improved pharmacokinetics may have the potential to yield a useful therapy for the treatment of alcoholism.

In contrast to mice in the intermittent 24-hour access IA paradigm [Hwa et al, 2011], which had excessive daily alcohol intake with 15% alcohol (~18 g/kg in males and ~23 g/kg in females, respectively), mice in the limited-access DID paradigm [Rhodes et al, 2005] had modest daily intake with 15% alcohol (~5 g/kg in males and females). For this reason, we purposely compared the effects of SSR149415 on the IA with DID, and found that single acute SSR149415 administration at 10 mg/kg reduced alcohol drinking at 4 hours after IA (**Fig 2, 3**), with no effect after DID (**Table S1**). When tested in other alcohol concentrations in the IA model, we observed that SSR149415 alone or combined with NTN had effect on high levels of daily alcohol intake with 15% alcohol, without a significant reduction on relatively low levels of daily alcohol intake with 7.5% alcohol (~10 g/kg in males and ~20 g/kg in females, respectively) [Zhou et al, 2017a, b] (**Table 2**), consistent with the above notion. However, the results (**Table 3**) that SSR149415 alone or combined with NTN showed a “selective” effect on 15% alcohol, but not on 30% alcohol with high daily alcohol intake (~23 g/kg in males and ~34 g/kg in females, respectively) [Zhou et al, 2017a, b] was unexpected. Further study to elucidate these findings is warranted.

Using the IA model, our recent study found that nPE-/- mice of both sexes had lowered intake and preference for alcohol, suggesting a reduced rewarding effect of alcohol when central beta-endorphin, the main peptide ligand of MOP-r, is reduced [Zhou et al, 2017a]. Therefore, we purposely investigated whether the blockade of V1b receptors could further affect alcohol drinking in nPE knockout mice lacking central beta-endorphin. We found that nPE-/-

males displayed a significant reduction in alcohol intake after acute administration of 3 mg/kg SSR149415 (**Table 5**), indicating a sensitized effect of V1b antagonist SSR149415 after the central POMC/beta-endorphin deletion. This also suggests that the presence of SSR149415 effects in nPE-/- males was due to an independent and different mechanism from that of NTN, as discussed above. In contrast, we observed no effect of acute treatment with 3 mg/kg SSR149415 on alcohol drinking in the IA paradigm in nPE-/- females, though the same SSR149415 treatment significantly reduced alcohol drinking in nPE-/- males. As the decreased alcohol intake in nPE-/- mice was more notable in females, the lack of significant effect of SSR149415 in nPE-/- females may be a floor effect due to their much lowered basal alcohol intake.

Together, the present study in a mouse excessive alcohol drinking model suggests that the combination of SSR149415 with NTN may be more efficacious in treating alcoholism than NTN alone. There are several precedents to test the combinations of NTN with other compounds, like acamprosate [Heyser et al, 2003], prazosin [Froehlich et al, 2013] and Mesyl Sal B [Zhou et al, 2017b]. In comparison with those combinations, this new combination with SSR149415 showed a long-lasting synergistic effect on reducing alcohol consumption, although this could be attributed to different animal models used in different laboratories. Excessive alcohol drinking is widely considered a hallmark of the transition from alcohol abuse to addiction in humans [Koob 2008]. Together, consistent with several recent studies on alcohol drinking in rats and in humans, our findings have shown further promising *in vivo* data indicating that subthreshold doses of a V1b antagonist in combination with NTN, may offer novel strategies to treat alcoholism, and possibly with less adverse effects.

Conflict of interest: All authors declare that they have no conflicts of interest.

Contributors: YZ designed the study, conducted behavioral studies, wrote the protocol, managed the literature searches and analyses, undertook the statistical analysis, and wrote the manuscript; MJL, MR, MJK contributed to the final versions of manuscript writing; and all have approved the final manuscript. Special thanks to Dr. R. Schaefer and Michelle Morochnik for providing their editing corrections on the manuscript.

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Figure Legends

Figure 1 Dose responses of acute administration of SSR149415 (SSR, 0, 1, 3, 10 or 30 mg/kg) alone or combined with naltrexone (NTN, 0, 0.5 or 1 mg/kg) on reducing 15% alcohol intake (**A**) and alcohol preference (**B**) after 3-week intermittent access drinking in both male and female B6 mice. Data were collected at the 4-hour time point on the baseline and testing day (24 hours later) and are expressed as a percentage of baseline alcohol intake to account for the differences in baseline that contribute to variation between experiments. n=6-8. *p<0.05 or **p<0.01 vs. control (both SSR and NTN at 0 mg/kg); #p<0.05 or ## p<0.01 between treatment groups.

Figure 2 Effects of acute administration of SSR149415 (SSR, 10 mg/kg) alone or 3 mg/kg SSR combined with naltrexone (NTN, 1 mg/kg) on 15% alcohol intake and preference ratio after 3-week intermittent access drinking in male B6 mice. **[A, B] SSR alone**: (1) Control group (n=7): males received one vehicle injection (5% DMSO and 5% Cremophor in saline for SSR149415 control, i.p.) before the drinking test; and (2) SSR149415 group (n=8): males received one SSR149415 injection (10 mg/kg, i.p.) before the drinking test; **[C, D] SSR + NTN**: (1) Control group (n=8): males received one vehicle (5% DMSO and 5% Cremophor in saline for SSR149415 control, i.p.) followed by saline (for NTN control, i.p.) before the drinking test; and (2) SSR149415 + NTN group (n=7): males received one SSR149415 injection (3 mg/kg, i.p.) followed by one NTN injection (1 mg/kg, i.p.) before the drinking test. On the test day, alcohol (15%) intake values were recorded after 4, 8 and 24 hours of alcohol access. * p<0.05, **p<0.01, ***p<0.005 and ****p<0.001 vs. control at the same time point.

Figure 3 Effects of acute administration of SSR149415 (SSR, 10 mg/kg) alone or 3 mg/kg SSR combined with naltrexone (NTN, 1 mg/kg) on 15% alcohol intake and preference ratio after 3-week intermittent access drinking in female B6 mice. **[A, B] SSR alone**: (1) Control group (n=7): females received one vehicle injection (5% DMSO and 5% Cremophor in saline for SSR149415 control, i.p.) before the drinking test; and (2) SSR149415 group: females (n=7) received one SSR149415 injection (10 mg/kg, i.p.) before the drinking test. **[C, D] SSR + NTN**: (1) Control group (n=6): females received one vehicle (5% DMSO and 5% Cremophor in saline for SSR149415 control, i.p.) followed by saline (for NTN control, i.p.) before the drinking test; and (2) SSR149415 + NTN group (n=6): females received one SSR149415 injection (3 mg/kg, i.p.) followed by one NTN injection (1 mg/kg, i.p.) before the drinking test. On the test day, alcohol (15%) intake values were recorded after 4, 8 and 24 hours of alcohol access. * p<0.05, **p<0.01, ***p<0.005 and ****p<0.001 vs. control at the same time point.

Table 1 Effects of acute administration of SSR149415 (SSR, 10 mg/kg) (**A**) and its combination with naltrexone (NTN) (3 mg/kg SSR + 1 mg/kg NTN) (**B**) on total fluid intake in male and female B6 mice after 3-week intermittent access drinking.

A.

Total fluid intake, ml	male (n=7-8)		female (n=7)	
	Vehicle	10 mg/kg SSR	Vehicle	10 mg/kg SSR
0-4h	1.60 ± 0.09	1.58 ± 0.20	1.88 ± 0.11	1.69 ± 0.21
5-8h	1.04 ± 0.10	1.18 ± 0.28	1.72 ± 0.22	1.65 ± 0.30
9-24h	2.84 ± 0.32	2.78 ± 0.37	3.20 ± 0.44	3.11 ± 0.55

B.

Total fluid intake, ml	male (n=7-8)		female (n=6)	
	Vehicle	3 mg/kg SSR + 1mg/kg NTN	Vehicle	3 mg/kg SSR + 1mg/kg NTN
0-4h	1.55 ± 0.11	1.49 ± 0.29	1.68 ± 0.23	1.79 ± 0.29
5-8h	1.10 ± 0.30	1.19 ± 0.45	1.59 ± 0.20	1.81 ± 0.53
9-24h	3.05 ± 0.51	2.94 ± 0.35	3.17 ± 0.61	3.23 ± 0.33

Table 2 Effects of acute administration of SSR149415 (SSR, 10 mg/kg) (**A**) and its combination with naltrexone (NTN) (3 mg/kg SSR + 1 mg/kg NTN) (**B**) on 7.5% alcohol intake and preference ratio after 3-week intermittent access drinking at 4 hours in male and female B6 mice.

A	male (n=6)		female (n=6)	
Treatment	Vehicle	10 mg/kg SSR	Vehicle	10 mg/kg SSR
Alcohol intake (g/kg/4h)	4.12 ± 0.30	3.52 ± 0.33	5.51 ± 0.51	3.76 ± 0.39
Preference ratio	0.90 ± 0.02	0.89 ± 0.01	0.91 ± 0.02	0.85 ± 0.05

B	male (n=6)		female (n=7)	
Treatment	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN
Alcohol intake (g/kg/4h)	3.75 ± 0.31	3.27 ± 0.20	5.14 ± 0.52	3.88 ± 1.02
Preference ratio	0.94 ± 0.01	0.88 ± 0.07	0.92 ± 0.01	0.86 ± 0.10

Table 3 Effects of acute administration of SSR149415 (SSR, 10 mg/kg) (**A**) and its combination with naltrexone (NTN) (3 mg/kg SSR + 1 mg/kg NTN) (**B**) on 30% alcohol intake and preference ratio after 3-week intermittent access drinking at 4 hours in male and female B6 mice.

A	male (n=7)		female (n=7-8)	
Treatment	Vehicle	10 mg/kg SSR	Vehicle	10 mg/kg SSR
Alcohol intake (g/kg/4h)	5.04 ± 0.32	4.73 ± 0.39	7.51 ± 0.82	6.57 ± 0.91
Preference ratio	0.50 ± 0.05	0.49 ± 0.04	0.64 ± 0.04	0.67 ± 0.05

B	male (n=6)		female (n=7-8)	
Treatment	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN
Alcohol intake (g/kg/4h)	5.38 ± 0.97	5.07 ± 0.50	5.91 ± 1.01	4.89 ± 0.81
Preference ratio	0.53 ± 0.04	0.55 ± 0.04	0.51 ± 0.06	0.53 ± 0.07

Table 4 Effects of acute administration of SSR149415 (SSR, 10 mg/kg) (**A**) and its combination with naltrexone (NTN) (3 mg/kg SSR + 1 mg/kg NTN) (**B**) on 4% sucrose (**A** and **B**) or 0.1% saccharin (**C** and **D**) intake and their preference ratio at 4 hours in male and female B6 mice.

A	male (n=6)		female (n=6)	
Treatment	Vehicle	10 mg/kg SSR	Vehicle	10 mg/kg SSR
4% Sucrose (g/kg/4h)	8.01 ± 0.82	9.15 ± 1.08	8.51 ± 1.15	10.7 ± 1.05
Preference ratio	0.95 ± 0.02	0.97 ± 0.03	0.96 ± 0.01	0.97 ± 0.02

B	male (n=6)		female (n=8-10)	
Treatment	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN
4% Sucrose (g/kg/4h)	7.59 ± 1.37	8.17 ± 1.01	8.14 ± 0.72	8.88 ± 1.02
Preference ratio	0.91 ± 0.05	0.97 ± 0.01	0.96 ± 0.06	0.95 ± 0.10

C	male (n=6-7)		female (n=6)	
Treatment	Vehicle	10 mg/kg SSR	Vehicle	10 mg/kg SSR
0.1% Saccharin (g/kg/4h)	0.12 ± 0.03	0.13 ± 0.01	0.15 ± 0.02	0.16 ± 0.03
Preference ratio	0.94 ± 0.03	0.98 ± 0.02	0.96 ± 0.02	0.98 ± 0.02

D	male (n=6)		female (n=6)	
Treatment	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN	Vehicle + Saline	3 mg/kg SSR + 1 mg/kg NTN
0.1% Saccharin (g/kg/4h)	0.18 ± 0.01	0.20 ± 0.02	0.19 ± 0.07	0.18 ± 0.02
Preference ratio	0.96 ± 0.01	0.97 ± 0.01	0.96 ± 0.01	0.96 ± 0.01

Table 5 Genotype differences in the effects of acute SSR149415 (SSR, 3 mg/kg) on 15% alcohol intake and preference ratio after 3-week intermittent access drinking at 4 hours in male (**A**) and female (**B**) nPE mice. Mice of each sex were assigned to one of four treatment groups: (1) nPE+/+ with vehicle (5% DMSO and 5% Cremophor in saline) as control; (2) nPE+/+ with 3 mg/kg SSR in vehicle; (3) nPE-/- with vehicle as control; and (4) nPE-/- with 3 mg/kg SSR. On the test day, 15% alcohol was presented 30 min after a single i.p. injection of SSR or vehicle, and then alcohol and water intake values were recorded after 4, 8 and 24 hours of alcohol access. Data are presented after 4 hours of alcohol access. Genotype difference: *p<0.05 or ** p<0.01 vs. nPE+/+ mice after the same treatment; SSR treatment difference: #p<0.05 vs. vehicle control in the same genotype.

A. Males (n=7)

Genotype	nPE +/+		nPE -/-	
Treatment	vehicle	3 mg/kg SSR	vehicle	3 mg/kg SSR
Intake, g/kg	5.4 ± 0.50	5.1 ± 0.47	2.8 ± 0.27 **	1.5 ± 0.28 #
Water, ml	0.32 ± 0.11	0.30 ± 0.10	0.51 ± 0.14	0.66 ± 0.16
Preference	0.77 ± 0.06	0.70 ± 0.05	0.54 ± 0.07 *	0.46 ± 0.03

B. Females (n=6)

Genotype	nPE +/+		nPE -/-	
Treatment	vehicle	3 mg/kg SSR	vehicle	3 mg/kg SSR
Intake	6.6 ± 0.49	6.1 ± 0.38	1.5 ± 0.39 **	1.4 ± 0.42
Water, ml	0.25 ± 0.09	0.24 ± 0.10	0.51 ± 0.14	0.50 ± 0.11
Preference	0.85 ± 0.06	0.82 ± 0.08	0.44 ± 0.10 **	0.40 ± 0.08

Figure 1

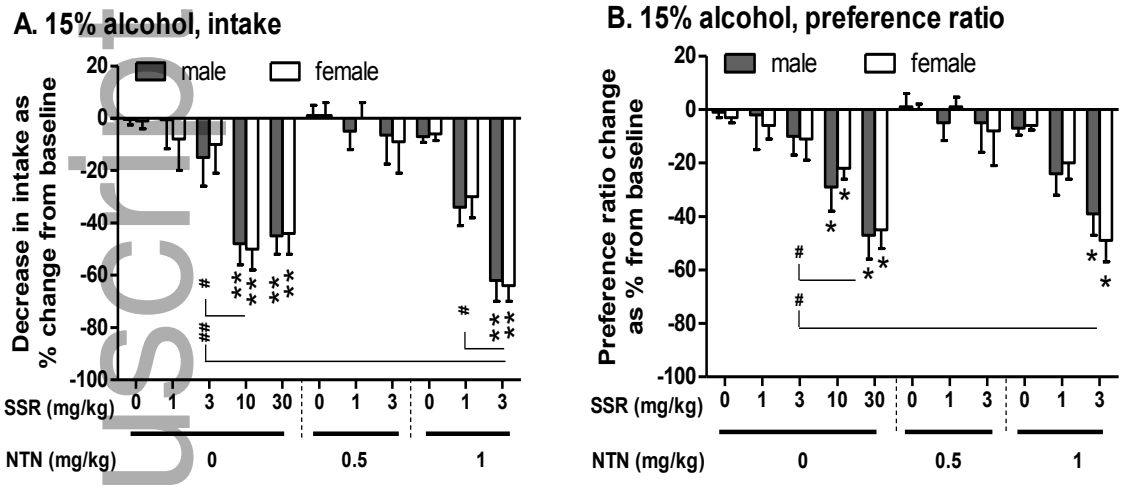

Figure 2

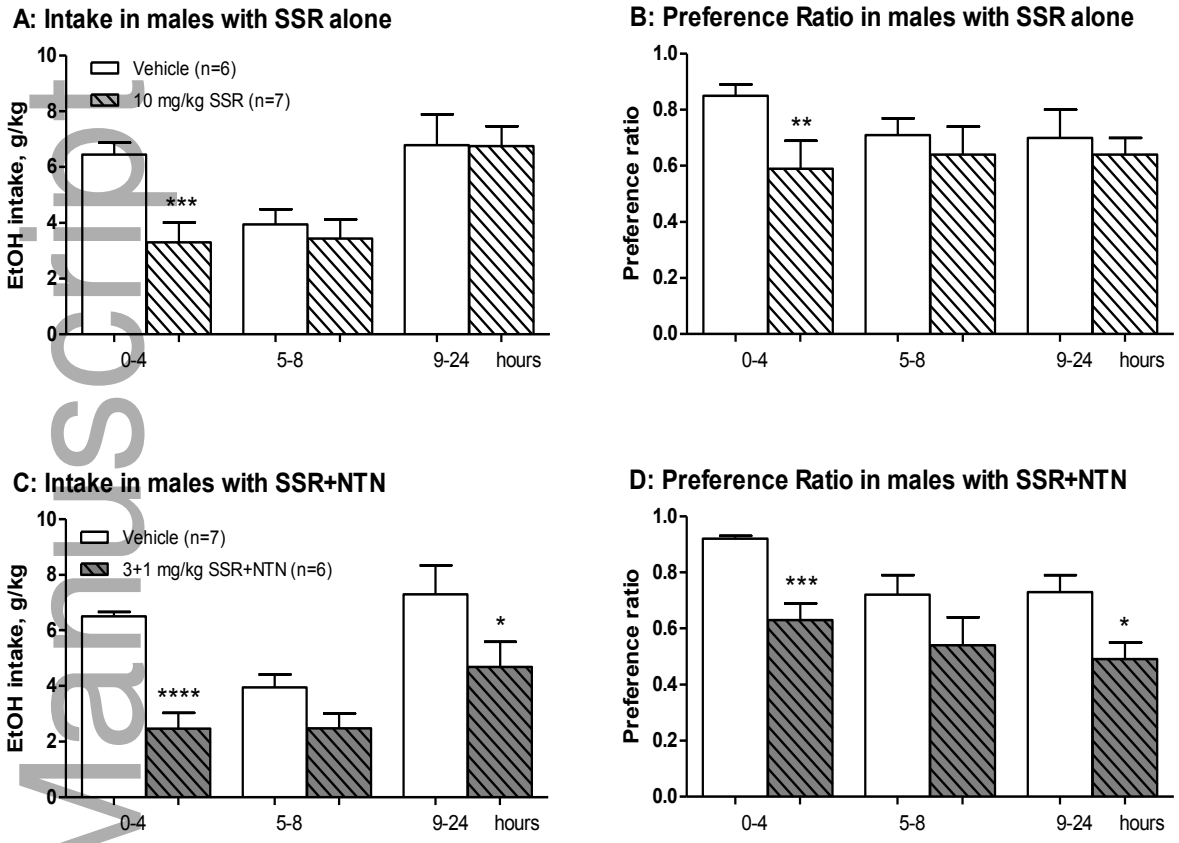

Figure 3