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Exploring sex differences in the attenuation of ethanol drinking by naltrexone in dependent rats during early and protracted abstinence

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Abstract

Background—Despite considerable efforts, few drugs are available for the treatment of alcohol (ethanol [EtOH]) use disorder (AUD). Ethanol directly or indirectly modulates several aspects of the central nervous system, including neurotransmitter/neuromodulator systems. Relapse vulnerability is a challenge for the treatment of EtOH addiction. Ethanol withdrawal symptoms create motivational states that lead to compulsive EtOH drinking and relapse even after long periods of abstinence. Among the therapeutics to treat AUD, naltrexone (NTX) is a pharmacological treatment for relapse. The present study evaluated the effect of NTX on EtOH drinking in male and female EtOH-dependent rats during abstinence.

Methods—Wistar rats (males and females) were first trained to orally self-administer 10% EtOH. Half of the rats were then made dependent by chronic intermittent EtOH (CIE) vapor exposure, and the other half were exposed to air. Using this model, rats exhibit somatic and motivational signs of withdrawal. At the end of EtOH vapor (or air) exposure, the rats were tested for the effects of NTX (10 mg/kg, p.o.) on EtOH self-administration at three abstinence time points: acute abstinence (8 h, A-Abst), late abstinence (2 weeks, L-Abst), and protracted abstinence (6 weeks, P-Abst).

Results—Naltrexone decreased EtOH intake in nondependent rats, regardless of sex and abstinence time point. In post-dependent rats, NTX decreased EtOH intake only at a delayed abstinence time point (P-Abst) in males, whereas it similarly reduced EtOH drinking in females at all abstinence time points.

Conclusions—The therapeutic efficacy of NTX depends on the time of intervention during abstinence and is different between males and females. The data further suggest that EtOH dependence causes different neuroadaptations in male and female rats, reflected by differential effects of NTX. The results underscore the significance of considering the duration of EtOH abstinence and sex as a biological variable as important factors when developing pharmacotherapies for AUD.

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Keywords

alcohol; ethanol; intake; abstinence; naltrexone; dependence

Introduction

Ethanol (EtOH) produces its effects on the central nervous system through several mechanisms, one of which involves alterations of the endogenous opioid system. Opioid receptor subtypes (μ, δ, κ) have high affinity for three main classes of endogenous opioids (β-endorphin, enkephalins, and dynorphins, respectively). Acute EtOH stimulates the release of β-endorphin, enkephalins, and dynorphin in humans and rats (Gianoulakis et al., 1996b, Marinelli et al., 2003, Marinelli et al., 2004, Marinelli et al., 2005, Marinelli et al., 2006, Dai et al., 2005, Popp and Erickson, 1998, Rasmussen et al., 1998).

Naltrexone (NTX) is an approved medication for the treatment of alcoholism, based on its efficacy in reducing the craving for and consumption of EtOH (Volpicelli et al., 1992). It is a nonspecific opioid receptor antagonist. In nondependent subjects, the NTX-induced blockade of opioid receptors for these endogenous ligands suppresses EtOH consumption (Gonzales and Weiss, 1998, Coonfield et al., 2002, Shoemaker et al., 2002, Stromberg, 2004), and this beneficial effect is blunted in post-dependent subjects (Ji et al., 2008, Sabino et al., 2006, Sabino et al., 2013, Walker and Koob, 2008). Although clinical trials have demonstrated the efficacy of NTX in reducing the risk of relapse in heavy drinkers, many patients experience no benefit of treatment (Krystal et al., 2001). This suggests that several factors influence treatment success (e.g., the time of intervention during abstinence, gender, and changes in the endogenous opioid system). Numerous investigations have sought to characterize biochemical modifications of the endogenous opioid system that are closely associated with the incidence of alcoholism or alcohol use disorder (AUD). For example, both mice and humans with a high risk of alcoholism present greater hypothalamic β-endorphin activity (Gianoulakis et al., 1996). Repeated EtOH administration induces both short- and long-term alterations of opioid levels in brain regions that are associated with motivation and reward (e.g., nucleus accumbens; (Lindholm et al., 2000). In rodents, chronic EtOH intake and EtOH withdrawal have been shown to induce a wide range of perturbations of the opioid system, suggesting a putative role for the endogenous opioid system in the effects of EtOH, such as a decrease in μ-opioid receptor expression in the nucleus accumbens and dorsal striatum (Turchan et al., 1999), an increase in prodynorphin mRNA levels in the nucleus accumbens (Przewlocka et al., 1997), a decrease in κ-opioid receptor mRNA expression in the ventral tegmental area and nucleus accumbens (Rosin et al., 1999), an increase in preproenkephalin mRNA expression in the amygdala, and a decrease in preproenkephalin mRNA expression in the nucleus accumbens (Cowen and Lawrence, 2001).

Men have been consistently shown to drink more EtOH and have a greater propensity to develop AUD than women. Recent data, however, suggest that EtOH consumption in men and women has become more similar over the past two decades (Keyes et al., 2008, Slade et al., 2016). Several studies have reported sex differences in EtOH intake in rats, in which

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females drank more than males (Blanchard et al., 1993, Morales et al., 2015, Walker et al., 2008, Li and Lumeng, 1984, Priddy et al., 2017), whereas other studies found no sex differences (van Haaren and Anderson, 1994, Moore and Lynch, 2015, Schramm-Sapyta et al., 2014). The use of different rat strains (e.g., Wistar, Sprague-Dawley, and Long Evans) or different experimental designs (e.g., two-bottle choice, self-administration, and chronic intermittent EtOH [CIE] vapor exposure) may account for such discrepancies. To our knowledge, no study to date has compared the efficacy of NTX in preventing EtOH drinking between males and females. Such information is especially important when attempting to develop efficient pharmacotherapies for the treatment of AUD.

The resumption of excessive EtOH drinking (i.e., relapse) even long after physical signs of withdrawal have dissipated is a challenge for the successful treatment of AUD. Although NTX is an approved treatment for AUD, it is unclear why treatment efficacy varies between subjects. Therefore, to gain a better understanding of the efficacy of NTX in preventing the recurrence of EtOH drinking during abstinence, the present study systematically tested the effects of NTX at different time points during abstinence in male and female rats.

Materials and Methods

Rats

Forty-eight Wistar rats (24 males and 24 females; Charles River, Wilmington, MA, USA), weighing 150–175 g upon arrival, were housed two per cage in a temperature- and humidity-controlled vivarium on a reverse 12 h/12 h light/dark cycle with *ad libitum* access to food and water. The animals were given at least 1 week to acclimate to the housing conditions and handling before testing. All of the procedures were conducted in strict adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Ethanol self-administration training (Fig. 1)

The EtOH self-administration training procedure was a modification of early studies by Wise (Wise, 1973) and revised by Simms et al. (Simms et al., 2008). The procedure did not require any fading (saccharin or sucrose) procedure to initiate voluntary EtOH drinking. On the Monday (Day 1) following the end of the housing acclimatization period, the rats were single-housed and presented with two bottles: one bottle contained H_2O, and one bottle contained 10% (w/v) EtOH (prepared in tap water from 95% w/v EtOH) for 3 days (Monday, Tuesday, Wednesday; Days 1–3). On Thursday and Friday (Days 4 and 5), the rats were grouped-housed (two per cage) and given only H_2O in their home cage. On Saturday (Day 6), the rats were given access to EtOH self-administration for 12 h in standard operant conditioning chambers (29 cm × 24 cm × 19.5 cm; Med Associates, St. Albans, VT, USA) on a fixed-ratio 1 schedule of reinforcement, in which each response at the right active lever (i.e., the only lever available at that time) resulted in the delivery of 0.1 ml of fluid and brief illumination of a cue light (0.5 s) above the lever. Food and water were available in the operant chambers during this self-administration session to avoid food or water deprivation because of the extensive length of the operant procedure. Following the 12 h session, the rats

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were returned to their home cage. On Sunday (Day 7), they were left undisturbed. On Monday and Tuesday (Days 8 and 9), the rats were given access to EtOH self-administration for 2 h/day and on Wednesday and Thursday (Day 10 and 11) for 1 h/day. Starting on Friday (Day 12) and for the rest of the self-administration days, the rats underwent 30 min self-administration sessions with the introduction of a second (left) inactive lever at which responses were recorded but had no programmed consequences. Tail bleeds were performed after each self-administration session during the last 5 days of training, and blood alcohol levels (BALs) were measured (Fig. 1A). The rats were scored for somatic withdrawal signs (WDS) upon the completion of training (Fig. 1A).

Chronic intermittent EtOH vapor exposure (Fig. 1)

At the end of EtOH self-administration training, two rats (one male and one female) did not acquire EtOH self-administration, thus reducing the number of animals to 46. After 21 sessions of EtOH self-administration, half of the rats (n = 22; 11 males and 11 females) were then made dependent (EtOH post-dependent [$EtOH_{PD}$]) by CIE vapor exposure. The other half (n = 24; 12 males and 12 females) comprised the nondependent group ($EtOH_{ND}$) that was exposed only to air. During 6-week dependence induction, the rats underwent daily cycles of 14 h ON (BALs during vapor exposure ranged between 150 and 250 mg%, measured with a blood analyzer [GC-headspace, Agilent Technologies, Santa Clara, CA, USA]) and 10 h OFF and were left undisturbed for 3 weeks except to control their BALs (measured during the last 15 min of vapor exposure) and to score WDS (at 8 h of abstinence) once per week. Behavioral signs of withdrawal were measured by a laboratory assistant who was blind to the experimental conditions using a rating scale that was adapted from an original study by Macey et al. (Macey et al., 1996) and included ventromedial limb retraction, vocalization (i.e., irritability to touch), tail rigidity, abnormal gait, and body tremors. Each sign was given a score of 0–2, based on the following severity scale: 0 = no sign, 1 = moderate, and 2 = severe. The sum of the 5 scores (0–10) was used as a quantitative measure of withdrawal severity and to confirm dependence. In this model, rats exhibit somatic and motivational signs of withdrawal (Vendruscolo and Roberts, 2014). Starting at the beginning of the fourth week of CIE vapor exposure, the rats were subjected to 30 min FR1 EtOH self-administration sessions when acute abstinence occurred (i.e., 8 h after the vapor was turned off when brain and blood alcohol levels were negligible), three times per week (Monday, Wednesday, and Friday), in which a gradual increase in EtOH intake should be measured (Vendruscolo and Roberts, 2014). The air-exposed rats underwent the same procedure.

Effects of naltrexone on EtOH self-administration during abstinence (Fig. 1)

Following 6 weeks of CIE vapor or air exposure, the animals began the abstinence phase and were tested for the effect of NTX (naltrexone hydrochloride, Sigma, St. Louis, MO, USA) on EtOH self-administration at three abstinence points: acute abstinence (A-Abst, 8 h), late abstinence (L-Abst, 2 weeks), and protracted abstinence (P-Abst, 6 weeks). At each time point of abstinence, the rats were first scored for behavioral signs of withdrawal and then received oral NTX (10 mg/kg in a volume of 3 ml/kg; n = 6) or vehicle (water, n = 5/6 animals) administration. Sixty minutes later, they were placed in the operant chambers and allowed to self-administer 10% EtOH on an FR1 schedule for 30 min. Naltrexone (or

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vehicle) was administered according to a within-subjects design, such that the same group of rats received three NTX (or vehicle) treatments: one at A-Abst, one at L-Abst, and one at P-Abst.

It is known that the plasma concentration of NTX 60 minutes following an oral administration is ~ 10 times lower when compared to the subcutaneous route (see Hussain et al., 1987 and Ciccocioppo et al., 2003 for comparison). The rationale for using a moderate 10 mg/kg oral dose of NTX was based on the literature and specifically on a recent study that reported a significant reduction of EtOH drinking in alcohol-preferring (P) rats following oral administration of 10 mg/kg NTX (Rorick-Kehn et al., 2014).The oral bioavailability of NTX needs to be taken in account when comparing the present data with other studies that used other routes of administration (Walker and Koob, 2008; Ciccocioppo et al., 2003).

Statistical analysis

Two-way analysis of variance (ANOVA) was used to compare EtOH intake and inactive lever responses between males and females during the last 5 days of training. Two-way ANOVA was used to divide male and female rats into two subgroups to obtain a similar baseline (BSL) of EtOH intake based on the last 3 days of training pre-CIE vapor exposure. Two-way ANOVA was used to analyze differences in BALs between males and females that were caused by CIE vapor exposure. Three-way ANOVA was used to analyze differences in EtOH intake, inactive lever responses, BALs, and WDS during training and CIE vapor exposure between males and females. Three-way ANOVA was used to analyze differences in EtOH intake, inactive lever responses, BALs, and WDS related to NTX (or VEH) treatment at different time points of abstinence. Although WDS were measured prior to NTX and VEH treatment, the treatment factor (NTX *vs.* VEH) was retained as a variable to maintain the group subdivision at each abstinence point and consistency among the data presentation. When no significant three-way interaction was found, two-way ANOVA was used to analyze significant two-way interactions. Significant effects in the ANOVAs were followed by the Tukey *post hoc* test. WDS values were transformed into log10 for statistical analysis and back-transformed for graphical representation. The results are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$. The statistical analysis was performed using GraphPad Prism 7 and Statistica 7.0 software.

Results

Two animals were excluded from the study, one in the male $EtOH_{PD}$ group and one in the female $EtOH_{PD}$ group, because they did not acquire EtOH self-administration, thus reducing the number of animals to 46 ($n = 12$ $EtOH_{ND}$ males and $n = 11$ $EtOH_{PD}$ males during training and CIE exposure; $n = 6$ $EtOH_{ND}$ males treated with NTX or vehicle, $n = 6$ $EtOH_{PD}$ males treated with NTX, and $n = 5$ $EtOH_{PD}$ males treated with vehicle; $n = 12$ $EtOH_{ND}$ females and $n = 11$ $EtOH_{PD}$ females during training and CIE rocedure; $n = 6$ $EtOH_{ND}$ females treated with NTX or vehicle, $n = 6$ $EtOH_{PD}$ females treated with NTX, and $n = 5$ $EtOH_{PD}$ females treated with vehicle).

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Ethanol self-administration training

During the last 5 days of EtOH self-administration training (Fig. 2), both groups of animals (males and females) presented stable and comparable EtOH intake in a 30-min daily session (two-way ANOVA: sex [male *vs.* female], $F_{1,44} = 0.1$, $p > 0.05$; time [days of self-administration], $F_{4,176} = 1.3$, $p > 0.05$; sex × time interaction, $F_{4,176} = 1.1$, $p > 0.05$). Responses at the inactive lever were low and constant across the last five days of training (two-way ANOVA: sex [male *vs.* female], $F_{1,44} = 5.8$, $p < 0.05$; time [days of self-administration], $F_{4,176} = 0.3$, $p > 0.05$; sex × time interaction, $F_{4,176} = 0.3$, $p > 0.05$; Fig. 2, bottom panel). The rats were then divided into four groups: $EtOH_{ND}$ males, $EtOH_{PD}$ males, $EtOH_{ND}$ females, and $EtOH_{PD}$ females.

Ethanol self-administration during CIE vapor exposure

Before CIE or air exposure, male and female rats were divided into two subgroups to obtain a similar baseline (BSL) of EtOH intake based on the last 3 days of training pre-CIE vapor exposure (BSL; two-way ANOVA: sex [male *vs.* female], $F_{1,42} = 0.04$, $p > 0.05$; group [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,42} = 0.06$, $p > 0.05$; sex × group interaction, $F_{1,42} = 0.54$, $p > 0.05$; Fig. 3A). At the end of CIE exposure (W4-W6), an increase in EtOH intake (calculated by averaging the measures that were obtained Monday, Wednesday, and Friday of each week; Fig. 3A) was detected in $EtOH_{PD}$ rats, with an overall difference between males and females (three-way ANOVA: sex [male *vs.* female], $F_{1,168} = 5.4$, $p < 0.05$; group [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,168} = 98.2$, $p < 0.001$; time [BSL, week 4, week 5, and week 6], $F_{3,168} = 8.0$, $p < 0.001$; sex × group × time interaction, $F_{3,168} = 0.3$, $p > 0.05$; Fig. 3A, B). Although no significant three-way interaction was detected, a significant two-way group × time interaction was found ($F_{3,168} = 11.8$, $p < 0.001$). Separate two-way ANOVAs by sex confirmed an increase in EtOH intake at weeks 4, 5, and 6 in males and females (males: group [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,21} = 36.2$, $p < 0.001$; time [BSL, week 4, week 5, and week 6], $F_{3,63} = 4.9$, $p < 0.01$; group × time interaction, $F_{3,63} = 19.7$, $p < 0.001$; females: group [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,21} = 18.7$, $p < 0.05$; time [BSL, week 4, week 5, and week 6], $F_{3,63} = 7.0$, $p < 0.001$; group × time interaction, $F_{3,63} = 4.6$, $p < 0.01$). Tukey *post hoc* tests confirmed that $EtOH_{PD}$ rats drank significantly more EtOH from week 4 to week 6 of CIE vapor exposure ($p < 0.001$) *vs.* baseline and the $EtOH_{ND}$ group. Inactive lever responses remained low and unaffected in both males and females (three-way ANOVA: sex [male *vs.* female], $F_{1,168} = 24.7$, $p < 0.001$; group [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,168} = 2.1$, $p > 0.05$; time [BSL, week 4, week 5, and week 6], $F_{3,168} = 2.4$, $p > 0.05$; sex × group × time interaction, $F_{3,168} = 0.2$, $p > 0.05$; Fig. 3A).

Blood alcohol levels

During weeks 1, 2, and 3 of CIE exposure, the EtOH delivery was adjusted to reach and maintain an average BAL of 150–250 mg%. During weeks 4, 5, and 6 of CIE, BALs remained stable within the required range (150–250 mg%). Blood was collected once per week (on Thursdays; Fig. 1B) 15 min before the EtOH vapor was turned off: week 4 (male: 171.9 ± 14.3 mg/100 ml; female: 154.0 ± 13.9 mg/100 ml), week 5 (male: 170.4 ± 22.6 mg/100 ml; female: 154.4 ± 8.8 mg/100 ml), and week 6 (male: 184.9 ± 18.9 mg/100 ml; female: 159.3 ± 6.0 mg/100 ml). No differences in BALs were found between males and

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females over the last 3 weeks of CIE vapor exposure (two-way ANOVA: sex, $F_{1,20} = 3.4$, $p > 0.05$; time, $F_{2,40} = 0.5$, $p > 0.05$; sex × time interaction, $F_{2,40} = 0.5$, $p > 0.05$).

Blood alcohol levels that were measured after each self-administration session during the CIE vapor exposure were higher in $EtOH_{PD}$ rats (both males and females) compared with their respective pre-CIE vapor exposure (BSL). Post self-administration BALs were higher in $EtOH_{PD}$ animals *vs.* $EtOH_{ND}$ for both sexes, with $EtOH_{PD}$ females having lower BALs than $EtOH_{PD}$ males across weeks 4, 5, and 6 of CIE vapor exposure ($p < 0.05$, Tukey *post hoc* test following three-way ANOVA: sex [male *vs.* female], $F_{1,168} = 26.8$, $p < 0.001$; group [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,168} = 261.3$, $p < 0.001$; time [BSL, week 4, week 5, and week 6], $F_{3,168} = 39.8$, $p < 0.001$; sex × group × time interaction, $F_{3,168} = 7.2$, $p < 0.001$; Fig. 3B).

Withdrawal scores

In males, during weeks 5, and 6 of CIE vapor exposure, somatic withdrawal signs that were measured before the self-administration session were higher in $EtOH_{PD}$ animals compared with $EtOH_{ND}$ animals and compared with BSL. In females, somatic withdrawal signs that were measured before the self-administration session were higher in $EtOH_{PD}$ animals compared with $EtOH_{ND}$ animals during weeks 4, 5, and 6 of CIE vapor exposure and compared with BSL. Compared with males, $EtOH_{PD}$ females exhibited significantly higher WDS ($p < 0.05$, Tukey *post hoc* test following three-way ANOVA: sex, $F_{1,168} = 29.9$, $p < 0.001$; group [$EtOH_{ND}$ and $EtOH_{PD}$], $F_{1,168} = 57.6$, $p < 0.001$; time [BSL, weeks 4, 5, and 6], $F_{3,168} = 8.3$, $p < 0.001$; sex × group × time interaction, $F_{3,168} = 5.2$, $p < 0.05$; Fig. 3C).

Effects of NTX on EtOH self-administration during abstinence

Ethanol intake.—Separate analyses of the differential behavioral effects of NTX across abstinence time points were performed between males and females. In males (Fig. 4A), $EtOH_{ND}$ rats responded differently to NTX than $EtOH_{PD}$ rats over the different abstinence time points (three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 6.7$, $p < 0.01$; treatment [VEH *vs.* NTX], $F_{1,54} = 43.26$, $p < 0.001$; dependence [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,54} = 32.17$, $p < 0.001$; time × treatment × dependence interaction, $F_{2,54} = 1.4$, $p > 0.05$). No significant three-way interaction was detected, but a significant two-way time × treatment interaction was found ($F_{2,54} = 4.6$, $p < 0.05$). In $EtOH_{ND}$ males, the overall effect of NTX treatment was significant (two-way ANOVA: treatment, $F_{1,9} = 26.99$, $p < 0.001$). In $EtOH_{PD}$ males, significant effects of time and NTX treatment were found (two-way ANOVA: time, $F_{2,18} = 9.6$, $p < 0.01$; treatment, $F_{1,9} = 6.0$, $p < 0.05$). Tukey *post hoc* tests confirmed that NTX reduced EtOH intake in $EtOH_{ND}$ rats at all abstinence time points ($p < 0.05$). In $EtOH_{PD}$ rats, NTX reduced EtOH intake only at P-Abst ($p < 0.05$). Inactive lever responses remained low and unaffected by NTX treatment (three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 1.7$, $p > 0.05$; treatment [VEH *vs.* NTX], $F_{1,54} = 0.2$, $p > 0.05$; dependence [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,54} = 0.7$, $p > 0.05$; time × treatment × dependence interaction, $F_{2,54} = 0.4$, $p > 0.05$; Fig. 4A).

In females (Fig. 5A), NTX decreased EtOH intake in both the $EtOH_{ND}$ and $EtOH_{PD}$ groups at all abstinence time points (three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 0.9$, $p > 0.05$; treatment [VEH *vs.* NTX], $F_{1,54} = 38.6$, $p < 0.001$; dependence [$EtOH_{ND}$ *vs.*

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$EtOH_{PD}$], $F_{1,54} = 22.5$, $p < 0.001$; time × treatment × dependence interaction, $F_{2,54} = 0.2$, $p > 0.05$). No significant three-way interaction was detected, but a significant two-way treatment × dependence interaction was found ($F_{1,54} = 4.6$, $p < 0.05$). Naltrexone reduced EtOH intake at A-Abst (two-way ANOVA: treatment, $F_{1,18} = 9.1$, $p < 0.05$; dependence, $F_{1,18} = 4.744$, $p < 0.05$; treatment × dependence interaction, $F_{1,18} = 0.4$, $p > 0.05$), L-Abst (two-way ANOVA: treatment, $F_{1,18} = 16.3$, $p < 0.001$; dependence, $F_{1,18} = 13.1$, $p < 0.01$; treatment × dependence interaction, $F_{1,18} = 3.4$, $p > 0.05$), and P-Abst (two-way ANOVA: treatment, $F_{1,18} = 16.9$, $p < 0.001$; dependence, $F_{1,18} = 9.3$, $p < 0.01$; treatment × dependence interaction, $F_{1,18} = 1.9$, $p > 0.05$) in both $EtOH_{ND}$ and $EtOH_{PD}$ rats. Tukey *post hoc* tests confirmed that NTX reduced EtOH intake in $EtOH_{ND}$ and $EtOH_{PD}$ rats at all abstinence time points ($p < 0.05$). Inactive lever responses remained low and unaffected (three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 0.9$, $p > 0.05$; treatment [VEH *vs.* NTX], $F_{1,54} = 1.1$, $p > 0.05$; dependence [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,54} = 1.0$, $p > 0.05$; time × treatment × dependence interaction, $F_{2,54} = 0.3$, $p > 0.05$; Fig. 5A).

Blood alcohol levels

In $EtOH_{ND}$ males, BALs after self-administration following NTX treatment at A-Abst, L-Abst, and P-Abst were lower compared with VEH-treated animals ($p < 0.05$, Tukey *post hoc* test). In $EtOH_{PD}$ rats, lower BALs in NTX-treated animals *vs.* vehicle-treated animals were observed at L-Abst and P-Abst ($p < 0.05$, Tukey *post hoc* test) but not at A-Abst (three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 12.5$, $p < 0.001$; treatment [VEH *vs.* NTX], $F_{1,54} = 21.6$, $p < 0.001$; dependence [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,54} = 101.4$, $p < 0.001$; time × treatment × dependence interaction, $F_{2,54} = 8.0$, $p < 0.001$; Fig. 4B). $EtOH_{ND}$ rats exhibited lower BALs *vs.* $EtOH_{PD}$ rats at A-Abst ($p < 0.05$, Tukey *post hoc* test; Fig. 4B).

In $EtOH_{ND}$ and $EtOH_{PD}$ females, BALs after self-administration following NTX treatment at A-Abst, L-Abst, and P-Abst were lower compared with VEH-treated animals ($p < 0.05$, Tukey *post hoc* test following three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 3.23$, $p < 0.05$; treatment [VEH *vs.* NTX], $F_{1,54} = 38.9$, $p < 0.001$; dependence [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,54} = 19.9$, $p < 0.001$; time × treatment × dependence interaction, $F_{2,54} = 6.5$, $p < 0.01$; Fig. 5B).

Somatic withdrawal signs

In males (Fig. 4C), overall effects of time of abstinence and dependence were found when scoring WDS (three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 9.4$, $p < 0.001$; treatment [VEH *vs.* NTX], $F_{1,54} = 0.2$, $p > 0.05$; dependence [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,54} = 26.0$, $p < 0.001$; time × treatment × dependence interaction, $F_{2,54} = 0.1$, $p > 0.05$). No significant three-way interaction was detected, but a significant two-way time × dependence interaction was found ($F_{2,54} = 15.7$, $p < 0.001$). $EtOH_{PD}$ rats had higher WDS scores compared with $EtOH_{ND}$ rats only at A-Abst in both VEH-treated animals ($p < 0.01$, Tukey *post hoc* test following two-way ANOVA: time, $F_{2,18} = 7.7$, $p < 0.01$; dependence, $F_{1,9} = 20.2$, $p < 0.001$; time × dependence interaction, $F_{2,18} = 6.4$, $p < 0.05$) and NTX-treated animals ($p < 0.01$, Tukey *post hoc* test following two-way ANOVA: time, $F_{2,18} = 9.1$, $p < 0.01$; dependence, $F_{1,9} = 11.7$, $p < 0.01$; time × dependence interaction, $F_{2,18} = 6.2$, $p < 0.05$). Further scrutiny of WDS scores between A-Abst and weeks 4–6 of CIE exposure

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suggested an increase in WDS scores at A-Abst, but this was not supported by the statistical analysis (one-way ANOVA: $F_{4,39} = 1.6$, $p > 0.05$; Fig. 3C, 4C).

In females (Fig. 5C), similar to males, overall effects of time of abstinence and dependence were found when scoring WDS (three-way ANOVA: time [A-Abst, L-Abst, P-Abst], $F_{2,54} = 19.5$, $p < 0.001$; treatment [VEH *vs.* NTX], $F_{1,54} = 0.05$, $p > 0.05$; dependence [$EtOH_{ND}$ *vs.* $EtOH_{PD}$], $F_{1,54} = 28.5$, $p < 0.001$; time × treatment × dependence interaction, $F_{2,54} = 0.1$, $p > 0.05$). No significant three-way interaction was detected, but a significant two-way time × dependence interaction was found ($F_{2,54} = 6.9$, $p < 0.01$). $EtOH_{PD}$ rats had higher WDS scores compared with $EtOH_{ND}$ rats at A-Abst and L-Abst, whereas no difference in WDS scores was observed at P-Abst between $ETOH_{ND}$ and $EtOH_{PD}$ rats in both VEH-treated animals ($p < 0.05$, Tukey *post hoc* test following two-way ANOVA: time, $F_{2,18} = 11.5$, $p < 0.01$; dependence, $F_{1,9} = 18.2$, $p < 0.01$; time × dependence interaction, $F_{2,18} = 4.8$, $p < 0.05$) and NTX-treated animals ($p < 0.05$, Tukey *post hoc* test following two-way ANOVA: time, $F_{2,18} = 14.6$, $p < 0.001$; dependence, $F_{1,9} = 5.7$, $p < 0.05$; time × dependence interaction, $F_{2,18} = 3.9$, $p < 0.05$). Further scrutiny of WDS scores between A-Abst and weeks 4–6 of CIE exposure suggested an increase in WDS scores at A-Abst, but this was not support by the statistical analysis (one-way ANOVA: $F_{4,39} = 1.6$, $p > 0.05$; Fig. 3C, 5C).

Discussion

One characteristic of AUD in humans is that dependent subjects will consume EtOH to relieve or avoid withdrawal symptoms (Peer et al., 2013). Similarly, in preclinical studies, EtOH post-dependent rats exhibit an EtOH-dependence syndrome that is characterized by both somatic and motivational withdrawal symptoms that usually begin after 6–8 h of abstinence and engage in excessive drinking when EtOH is made available again (Roberts et al., 1996, Vendruscolo and Roberts, 2014). The present study strongly supported the validity of the CIE vapor exposure model as a valid procedure to induce and study EtOH dependence in both male and female rats. Indeed, $EtOH_{PD}$ rats exhibited an enhancement (i.e., escalation) of EtOH drinking during dependence (week 4 to week 6), suggesting a transition from controlled to excessive EtOH intake, most likely in an effort to alleviate negative withdrawal states (for review, see (Koob, 2014). These behavioral changes are characteristic of dependence and reflect neuroadaptive changes that are induced by chronic intermittent EtOH that in turn disrupt brain function (e.g., reward), physiological processes, and cognitive processes (Koob, 2008, Becker and Mulholland, 2014) and could account for the changes in the efficacy of NTX in reducing EtOH drinking in the present study.

Although an overall significant effect of sex on EtOH drinking was observed during CIE vapor exposure (Fig. 3A), the possible increase in EtOH intake in females from week 4 to week 6 (suggested by further scrutiny of Fig. 3A) was not confirmed statistically. However, compared with females, males had higher BALs (Fig. 3B) and significantly lower WDS scores at weeks 4, 5, and 6 of CIE vapor exposure (Fig. 3C). The EtOH intake that was observed in males and females is difficult to reconcile with previous studies that reported a range of contradictory results. Several studies reported sex differences in EtOH intake, in which female rats drank more than males (Blanchard et al., 1993, Morales et al., 2015, Walker et al., 2008, Li and Lumeng, 1984), whereas other studies found no differences (van

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Haaren and Anderson, 1994, Moore and Lynch, 2015, Schramm-Sapyta et al., 2014). The use of different strains (e.g., Wistar, Sprague-Dawley, and Long Evans) or different experimental designs (e.g., two-bottle choice, self-administration, and CIE vapor exposure) may account for such discrepancies. For example, female Long Evans and Wistar rats consumed more EtOH than their male counterparts when given either continuous or intermittent access to EtOH in their home cages (Priddy et al., 2017). Under operant conditions, no sex or strain differences were found in drinking prior to the development of EtOH dependence (Priddy et al., 2017). Consistent with the present results, upon dependence induction by CIE exposure, Wistar rats of both sexes substantially escalated their EtOH intake compared with their nondependent drinking levels, whereas Long Evans rats only exhibited a moderate escalation of drinking, without showing any sex difference (Priddy et al., 2017). Thus, strain, sex, and drinking condition may interact to modulate EtOH drinking and are important factors to consider when exploring individual differences in EtOH drinking and dependence.

Blood alcohol levels following EtOH self-administration at weeks 4 to 6 of CIE vapor exposure were higher in males than in females (Fig. 3B). Biological (sex-related) factors, including differences in EtOH metabolism (for review, see (Cederbaum, 2012) and its effect on brain function and the levels of sex hormones, may contribute to some of these differences. For example, male and female mice differed in their sensitivity to changes in γ-aminobutyric acid-ergic neurosteroid levels with regard to EtOH exposure (Finn et al., 2010) and an interactive effect of sex and chronic EtOH consumption on blood glucose homeostasis was observed in Wistar rats (Sumida et al., 2004). Another explanation for such differences is that females may have failed to drink the EtOH that was dispensed compared with males. Dependent females exhibited the same EtOH intake at week 6 of CIE and at A-Abst after vehicle treatment, but they had different BALs after the self-administration sessions. This discrepancy is difficult to explain. One possibility could be that females metabolize EtOH differently across the estrous cycle. For example, mesocortical dopaminergic pathways are differentially sensitive to acute EtOH administration across the estrous cycle (Dazzi et al., 2007). Additionally, as mentioned above, an alternative explanation could be that females simply did not actually drink the EtOH that was delivered at A-Abst. Females not only had lower BALs than males; they also had higher WDS scores than males (Fig. 3C). These results are difficult to reconcile with previous studies that usually reported an increase in WDS in males but not in females (for review, see (Becker and Koob, 2016). However, several studies showed that females may be more sensitive to the degenerative effects of EtOH dependence (i.e., brain area volume reductions), and women seek treatment earlier in their drinking history than males (for review, see (Sharrett-Field et al., 2013), strongly suggesting a differential effect of chronic EtOH exposure in females vs. males. One explanation for the greater severity of WDS in females in the present study could be the experimental design (i.e., the duration of CIE vapor exposure to which females may be more sensitive). The observation that $EtOH_{ND}$ females had WDS scores that were comparable to $EtOH_{PD}$ males was also unexpected but may have resulted from increases in stress or anxiety responses in females (for review, see (Bangasser and Valentino, 2014). For example, vocalization, one of the WDS measures that was scored, is a known stress response

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and could have accounted for an overall higher WDS score in $EtOH_{ND}$ females (Meyer, 2015). Such a possibility, however, requires further investigation.

The neurobiological basis for sex differences in AUD is largely unknown, partially because most studies of EtOH drinking are conducted only in males. Gender differences in responses to AUD treatments are currently considered clinically, but the number of female subjects is usually limited and not always sufficient to establish the efficacy of treatment (Agabio et al., 2016). The present results showed that both male and female Wistar rats acquired EtOH self-administration and consumed the same amount of EtOH during the training phase. During the final 3 weeks of the CIE procedure (week 4 to week 6), both males and females exhibited significant escalation of EtOH intake compared with their respective air-exposed control groups. However, males and females responded differently to NTX. In $EtOH_{ND}$ animals (both males and females), NTX significantly reduced EtOH intake at all abstinence time points (A-Abst, L-Abst, and P-Abst), and a significant change in the efficacy of NTX was observed in $EtOH_{PD}$. Naltrexone was effective in $EtOH_{PD}$ males only at P-Abst but reduced EtOH drinking in $EtOH_{PD}$ females at all three abstinence time points.

Nondependent males and nondependent and post-dependent females presented a NTX-induced decrease in EtOH drinking, regardless of the duration of abstinence. However, post-dependent males were responsive to NTX after a longer period of abstinence. This variability in the efficacy of NTX is consistent with earlier studies that showed that NTX suppressed EtOH consumption in nondependent subjects (Gonzales and Weiss, 1998, Coonfield et al., 2002, Shoemaker et al., 2002, Stromberg, 2004), and this effect was blunted in post-dependent subjects (Ji et al., 2008, Sabino et al., 2006, Sabino et al., 2013, Walker and Koob, 2008). The efficacy of NTX in reducing EtOH intake may be affected by several factors, such as the model that is used (e.g., two-bottle choice *vs.* self-administration), the duration of EtOH self-administration, the severity of dependence, timing of the pharmacological intervention, sex, and changes in the endogenous opioid system. For example, acute NTX administration suppressed binge drinking in rats (Ji et al., 2008), and NTX was also highly effective in nondependent rats that were selectively bred for high alcohol preference (Sardinian alcohol-preferring [sP] rats; (Sabino et al., 2006). The lack of an effect of NTX at A-Abst in males was surprising when considering an earlier study by Walker and Koob (2008) that showed that a subcutaneous injection of NTX at lower doses than the one that was used in the present study reduced EtOH self-administration in nondependent and post-dependent animals during acute abstinence (Walker and Koob, 2008). One tentative explanation for this discrepant finding could be the lower bioavailability of NTX following oral administration (e.g., (Hussain et al., 1987). Chronic NTX administration blocked the increase in EtOH consumption after a 5-day period of forced abstinence (Heyser et al., 2003). Changes in the endogenous opioid system are observed in response to chronic exposure to EtOH and after its removal, and these changes may be responsible for the effect of NTX or lack thereof. For example, chronic EtOH administration has been reported to increase activity of the hypothalamic β-endorphin system (Schulz et al., 1980, Angelogianni and Gianoulakis, 1993), cause no change (Seizinger et al., 1983), or even decrease activity (Scanlon et al., 1992). One hypothesis could be that the greater β-endorphin release that is induced by acute exposure to EtOH induces drinking, whereas a decrease in β-endorphin activity that is induced by chronic

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EtOH intake may promote and maintain EtOH consumption because of negative reinforcement rather than positive reinforcement. However, the reason why NTX was ineffective in post-dependent males at A-Abst and L-Abst in the present study is unclear and will need further exploration.

One tentative explanation for the differential effect of NTX between males and females may be differential changes in the endogenous opioid system in response to chronic EtOH. Overall, acute EtOH has been shown to stimulate the release of β-endorphin, enkephalins, and dynorphin in humans and rats (Gianoulakis et al., 1996a, Marinelli et al., 2003, Marinelli et al., 2005, Marinelli et al., 2006, Dai et al., 2005). Chronic EtOH exposure induced changes in opioid peptide systems that involve alterations at the levels of the peptides themselves (Gianoulakis et al., 1996b, Lindholm et al., 2000), changes in receptor densities and effector systems (Turchan et al., 1999, Chen and Lawrence, 2000), and modifications of mRNA that encode both opioid peptides and receptors (Przewlocka et al., 1997, Rosin et al., 1999). Therefore, the differential effects of NTX in males and females may reflect sexual dimorphism of the endogenous opioid system and different effects of chronic EtOH use in males and females. Sexual dimorphism in the endogenous opioid system has been described in the control of pain. For example, variable responses to the analgesic effects of opioids have been attributable to sex (Dahan et al., 2008), but the mechanisms that underlie sex differences in opioid analgesia remain elusive. Sex differences in opioid analgesia are also not likely related to differences in opioid receptor density because no differences were found between male and female rats in brain μ- or δ-opioid receptor populations (Kepler et al., 1991). In humans, nonselective κ and μ ligands have been reported to produce stronger analgesic effects in women than in men (Rasakham and Liu-Chen, 2011). In animals, selective κ receptor agonists have been found to produce greater antinociceptive effects in males than in females (Rasakham and Liu-Chen, 2011). These observations suggest that the extent of sex differences in κ-, μ-, and δ-mediated analgesia is related to species, strain, ligand, and pain model. For example, the selective κ-opioid receptor agonist spiradoline was more potent in female than in male Wistar rats in the tail-withdrawal test (Terner et al., 2003). The exact nature of the changes in the endogenous opioid system during EtOH dependence and withdrawal that may explain the higher sensitivity to NTX in $EtOH_{PD}$ females remains to be established.

Clinical studies have shown that NTX treatment is more effective in patients who continue to drink EtOH during the course of treatment (Heinala et al., 2001, Killeen et al., 2004). One could argue that the efficacy of NTX treatment at P-Abst in $EtOH_{PD}$ males was the result of prior exposure to NTX with EtOH during the earlier tests (A-Abst and L-Abst). Therefore, in contrast to females, repeated NTX dosing in $EtOH_{PD}$ males may be necessary to elicit efficacy and thus be independent of the time of abstinence. Another explanation for the NTX-induced decrease in EtOH drinking could be related to an interaction between NTX and the palatability (taste) of EtOH. Supporting this hypothesis, previous studies found that NTX induced a conditioned taste aversion to EtOH in rats, monkeys, and humans and palatable food and sweet solutions (Ferraro et al., 2002, Coonfield et al., 2002, Williams and Woods, 1999b, Williams and Woods, 1999a, Mitchell et al., 2009, Stromberg et al., 2002, Beczkowska et al., 1992), consequently reducing EtOH intake. Sex differences in the palatability of EtOH could also contribute to the differential effect of NTX in reducing

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EtOH intake in $EtOH_{PD}$ rats. For example, women have been found to be more sensitive to the aversive effects of NTX (O'Malley et al., 2007, Garbutt et al., 2005, Agabio et al., 2016). Therefore, one can argue that the treatment efficacy of NTX in the present study might be correlated with the level of NTX-induced aversive side effects (Mitchell et al., 2009).

In conclusion, the present results further support targeting the endogenous opioid system to prevent excessive drinking that is characteristic of AUD, even after long periods of abstinence. The present study extends our knowledge of the efficacy of NTX treatment by showing that it varies between sexes and different time points of abstinence. This could explain some of the contradictory findings that described various effects or lack of effects of NTX in some cases (Kakidani et al., 1982, Bell and Reisine, 1993). In males, NTX was ineffective in reducing EtOH drinking during A-Abst, whereas NTX decreased EtOH drinking at A-Abst, L-Abst, and P-Abst in females. These findings suggest that NTX might modulate long-lasting craving-induced EtOH drinking in males and not acute EtOH withdrawal-induced (e.g., corticotropin-releasing factor-induced) EtOH drinking (Koob, 2015). This hypothesis will need further investigation. Overall, the present study has important implications because it points toward individual differences in the endogenous opioid system that should be considered when developing new pharmacotherapies to treat AUD.

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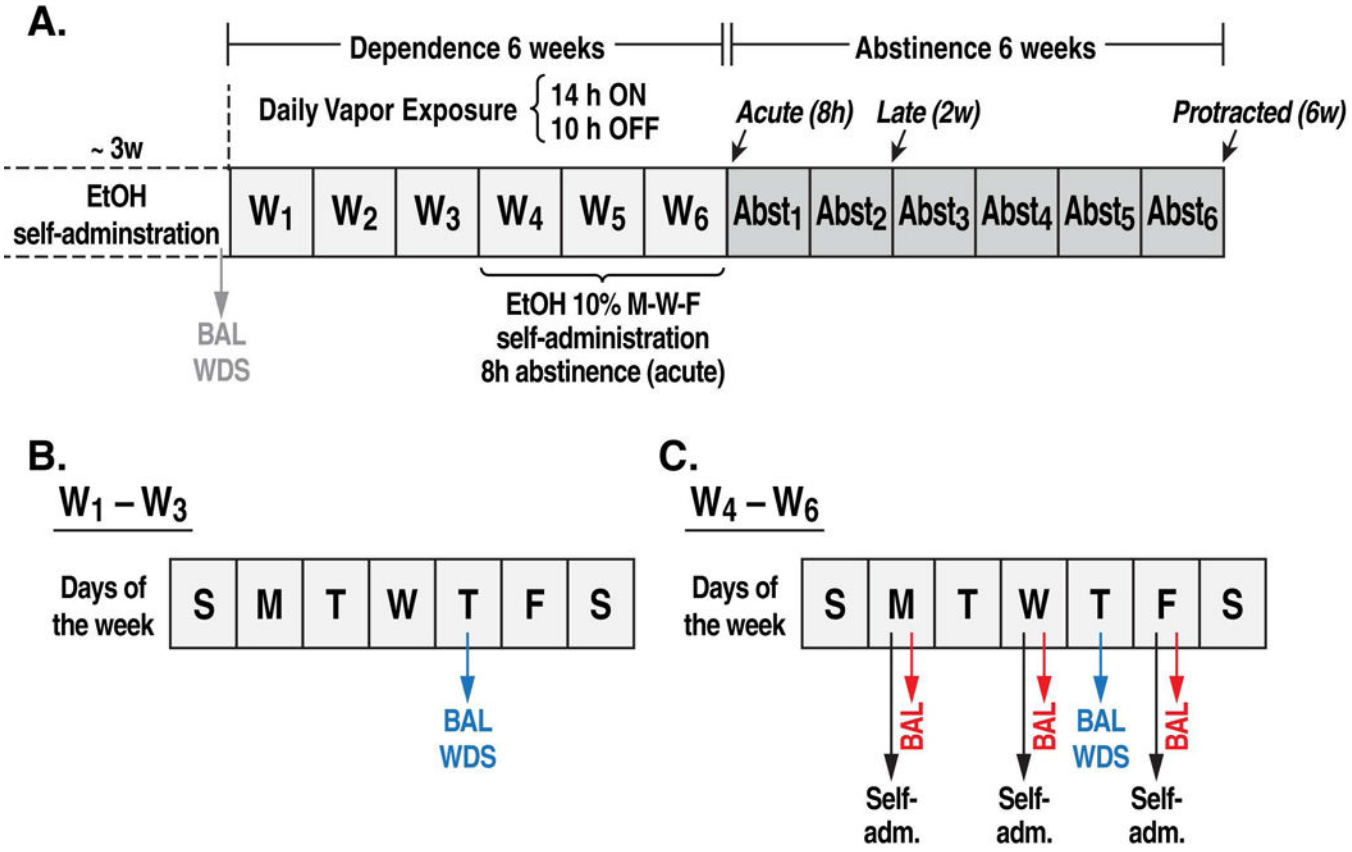

Figure 1.

Experimental procedure. At the end of EtOH self-administration training **(A)**, BALs were measured after the self-administration session during the last week of training. WDS scores were recorded upon the completion of training. **(B)** During weeks 1–3 of CIE vapor exposure, BALs were measured 15 min before the EtOH vapors were turned off, and the rats were scored for WDS 8 h after the EtOH vapor was turned off (on Thursdays). **(C)** During weeks 4–6 of CIE vapor exposure, BALs were measured an additional three times during the week immediately after the self-administration sessions (Monday, Wednesday, and Friday). BAL, blood alcohol level; WDS, somatic withdrawal signs; W, week.

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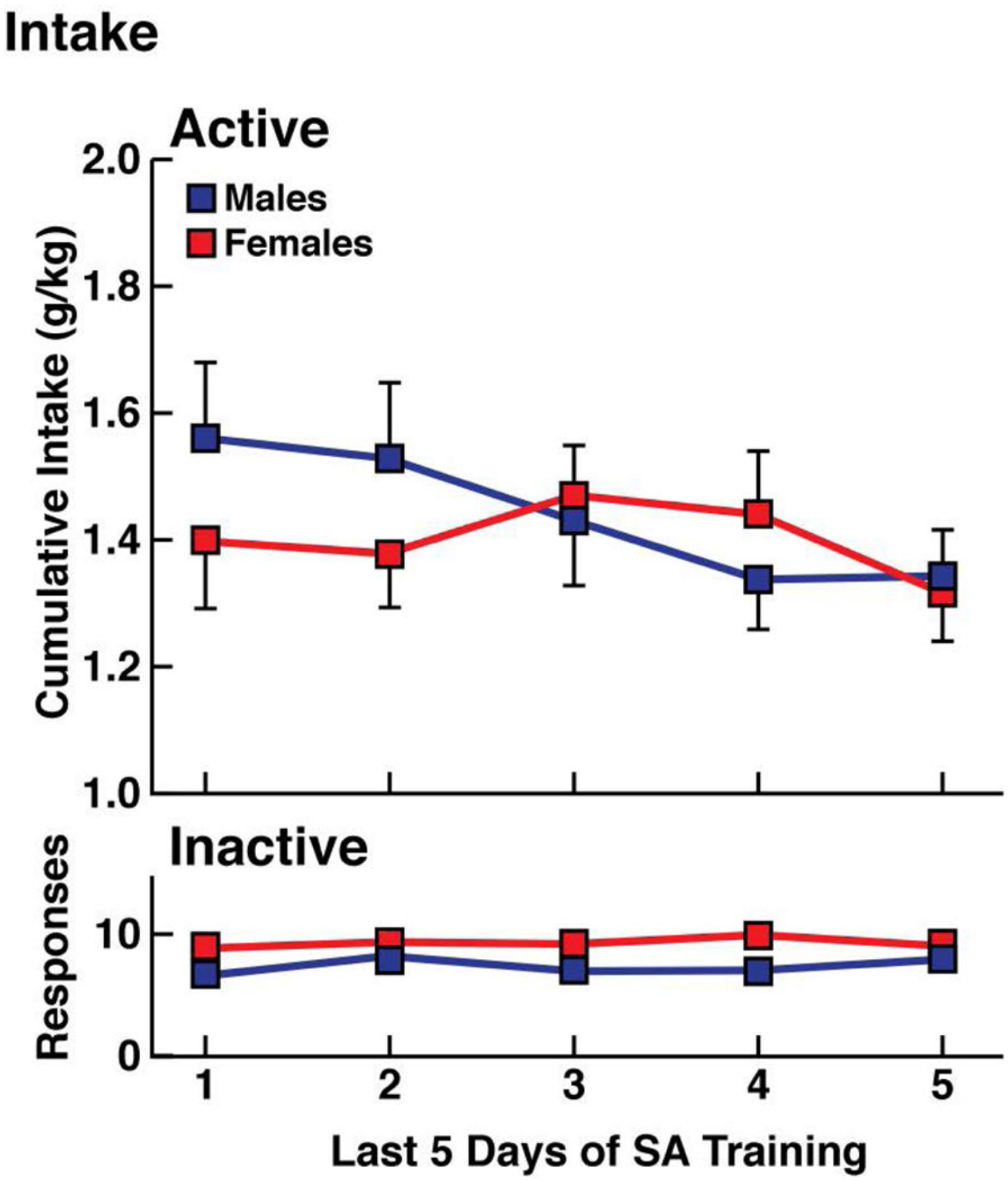

Figure 2.

Ethanol intake (**upper panel**) over the last 5 days of self-administration in daily 30 min sessions in males and females. Responses at the inactive lever are represented in the **lower panel**. *n* = 23 rats/group.

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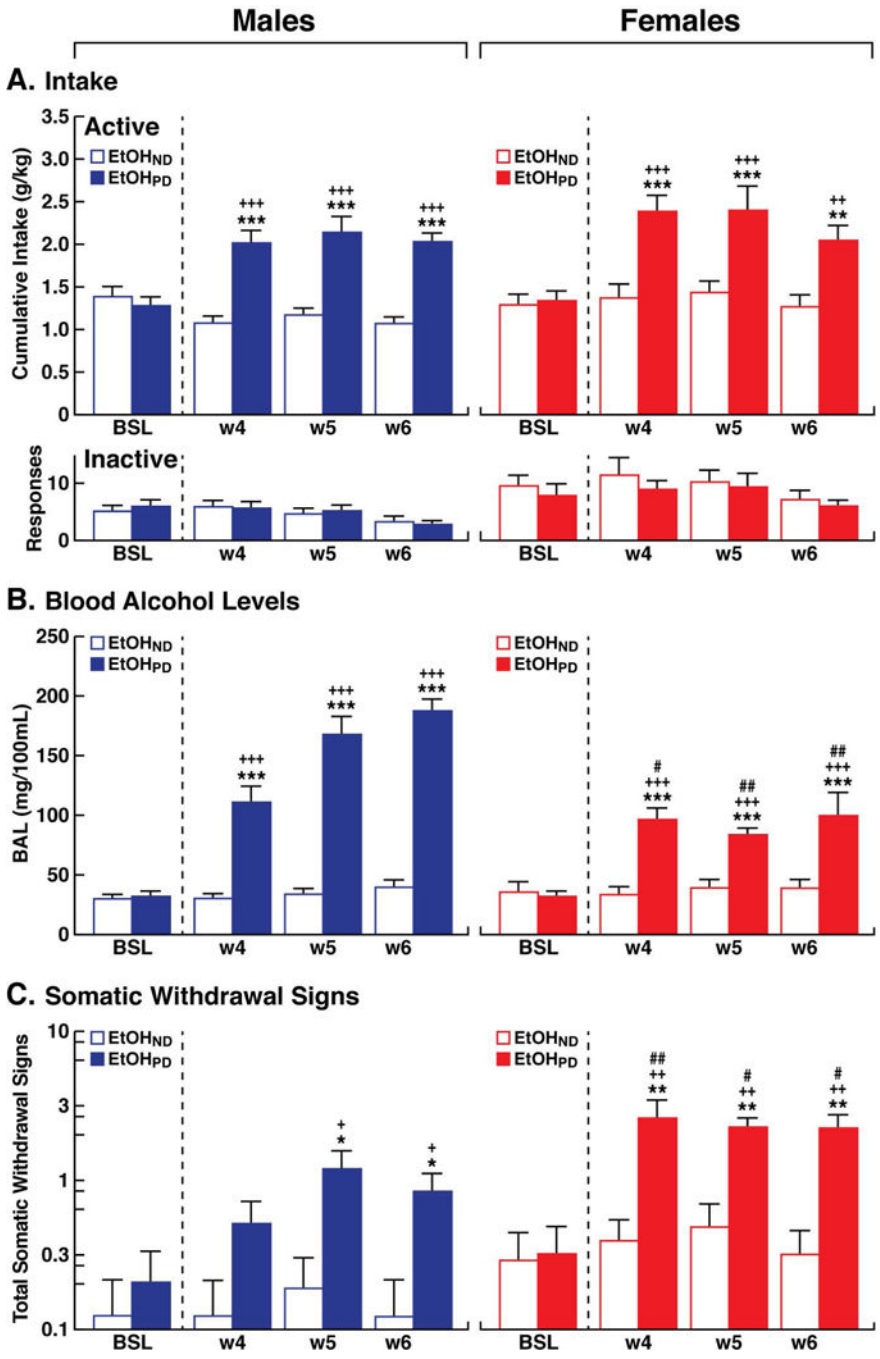

Figure 3.

(A) Upper panel. Cumulative EtOH intake was higher in $EtOH_{PD}$ animals compared with $EtOH_{ND}$ animals and compared with the training condition in both males and females. **Lower panel**. Responses at the inactive lever remained low and stable in both sexes. **(B)** Blood alcohol levels (BALs) that were measured immediately after the self-administration session during CIE vapor exposure were higher in $EtOH_{PD}$ animals compared with $EtOH_{ND}$ animals in males and females, with females having lower BALs compared with males. **(C)** Somatic withdrawal signs (WDS) that were recorded prior to the EtOH self-administration

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sessions during CIE vapor exposure at 8 h of abstinence were higher in $EtOH_{PD}$ animals than in $EtOH_{ND}$ animals in males and females, with females exhibiting higher WDS compared than males at weeks 4, 5, and 6 of CIE exposure. The Y-axis for WDS is expressed as log10. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$, *vs.* respective BSL (Tukey *post hoc* test); $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, *vs.* $EtOH_{ND}$ (Tukey *post hoc* test); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, *vs.* respective time point in males (Tukey *post hoc* test). $n = 11/12$ animals/group. BSL, baseline during training.

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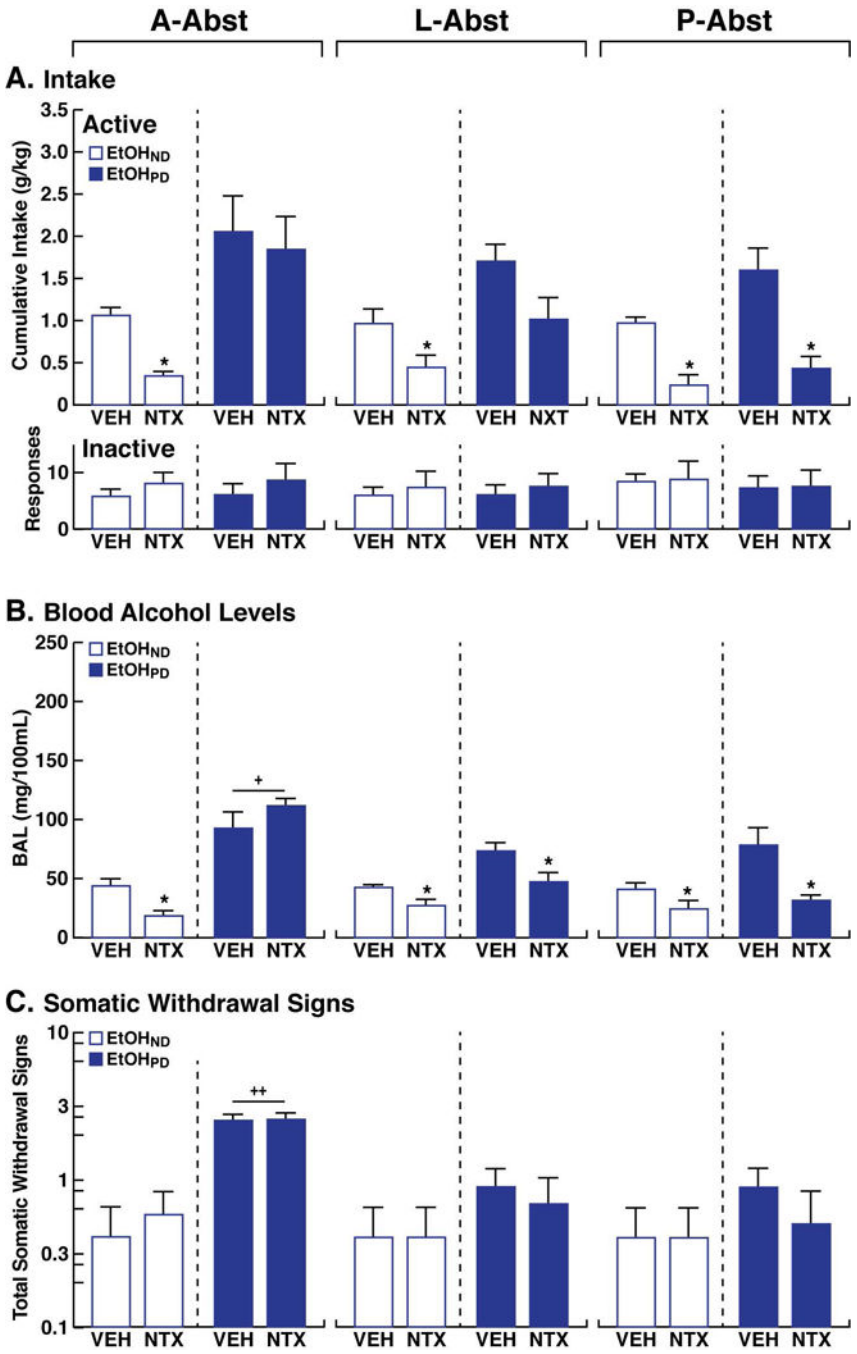

Figure 4.

Effects of NTX in males. **(A) Upper panel**. Cumulative EtOH intake after NTX or VEH administration in $EtOH_{PD}$ animals and $EtOH_{ND}$ animals at A-Abst, L-Abst, and P-Abst. **Lower panel**. Responses at the inactive lever. **(B)** Blood alcohol levels (BAL) were measured immediately after self-administration following NTX or vehicle (VEH) administration in $EtOH_{PD}$ and $EtOH_{ND}$ animals at A-Abst, L-Abst, and P-Abst. **(C)** Somatic withdrawal signs (WDS) were recorded prior to EtOH self-administration before NTX or VEH administration in $EtOH_{PD}$ and $EtOH_{ND}$ animals at A-Abst, L-Abst, and P-Abst. The

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Y-axis for WDS is expressed as log10. *$p < 0.05$, *vs.* respective VEH (Tukey *post hoc* test); $^{+}p < 0.05$, $^{++}p < 0.001$, *vs.* $EtOH_{ND}$ (Tukey *post hoc* test). $n = 5/6$ animals/group.

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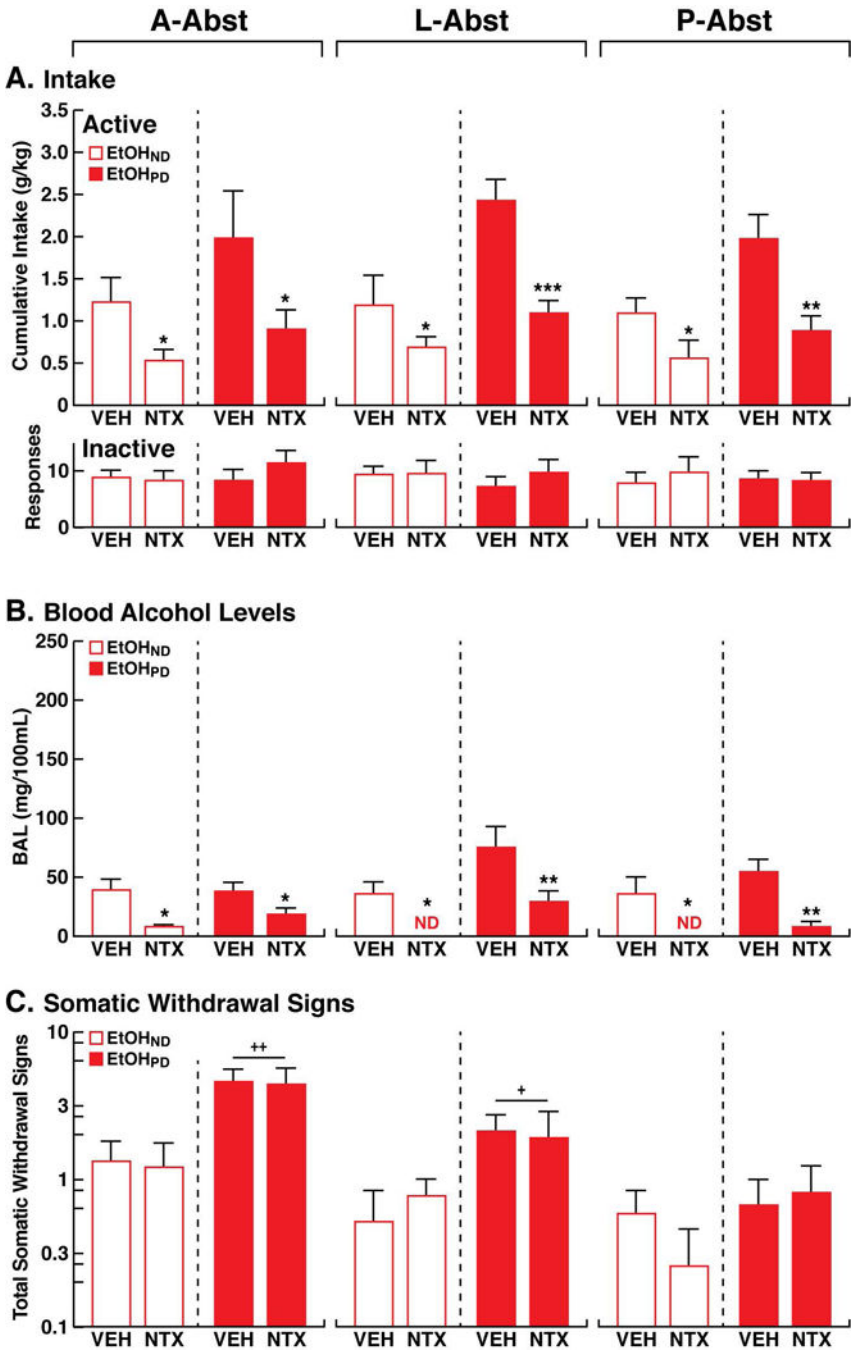

Figure 5.

(A) Upper panel. Effects of NTX in females. Cumulative EtOH intake following NTX or vehicle (VEH) administration in $EtOH_{PD}$ animals and $EtOH_{ND}$ animals at A-Abst, L-Abst, and P-Abst. **Lower panel**. Responses at the inactive lever. **(B)** Blood alcohol levels (BALs) were measured immediately after self-administration following NTX or VEH administration in $EtOH_{PD}$ and $EtOH_{ND}$ animals at A-Abst, L-Abst, and P-Abst. ND, not detectable. **(C)** Somatic withdrawal signs (WDS) were recorded immediately prior to self-administration before NTX or VEH administration in $EtOH_{PD}$ and $EtOH_{ND}$ animals at A-Abst, L-Abst, and

P-Abst. The Y-axis for WDS is expressed as log10. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, *vs.* respective VEH (Tukey *post hoc* test); $^{+}p < 0.05$, $^{++}p < 0.01$, *vs.* $EtOH_{ND}$ (Tukey *post hoc* test). $n = 5/6$ animals/group.

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