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The mineralocorticoid receptor antagonist spironolactone reduces alcohol self-administration in female and male rats

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Abstract

Cortisol/corticosterone and the hypothalamic-pituitary-adrenal (HPA) axis serve an important role in modulating alcohol drinking behaviors. To date most alcohol research has focused on the functional involvement of corticosterone and the glucocorticoid receptor (GR), the primary receptor for corticosterone. Recent studies have indicated that the related mineralocorticoid receptor (MR), which binds both corticosterone and aldosterone, may also play a role in alcohol drinking. Therefore, the purpose of the present study was to test the functional role of MR signaling in alcohol self-administration via pharmacological antagonism of the MR with spironolactone. Male and female Long-Evans rats were trained to self-administer a sweetened alcohol solution (15% (v/v) alcohol + 2% (w/v) sucrose). The effects of spironolactone (0, 10, 25, 50 mg/kg; IP) were tested on alcohol self-administration and under “probe extinction” conditions to measure the persistence of responding in the absence of the alcohol reinforcer. Parallel experiments in sucrose self-administration trained rats were used to confirm the specificity of spironolactone effects to an alcohol reinforcer. In female rats spironolactone (50 mg/kg) reduced alcohol self-administration and persistence of alcohol responding. In male rats spironolactone (25 and 50 mg/kg) reduced alcohol self-administration, but not persistence of alcohol responding. Spironolactone reduced sucrose intake in female rats only, and locomotion in male and female rats during sucrose self-administration. There was no effect of spironolactone on persistence of sucrose responding. These studies add to growing evidence that the MR is involved in alcohol drinking, while underscoring the importance of studying both male and female animals.

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Keywords

aldosterone; alcohol; drinking; glucocorticoids; relapse; spironolactone

Introduction

There is a wealth of literature examining the role of the hypothalamic-pituitary- adrenal (HPA) axis and corticosteroids in alcohol consumption, seeking, and dependence (Koenig and Olive, 2004, Vendruscolo et al., 2012, Vendruscolo et al., 2015). Within the HPA axis, cortisol (corticosterone in rodents) is one of the primary hormonal stress signals, and several studies in male adrenalectomized rats have shown that corticosterone moderates alcohol drinking (Fahlke et al., 1994a, Fahlke et al., 1995). To date, most alcohol studies have focused on the role of glucocorticoid receptors (GRs), which primarily bind corticosterone, in alcohol-related behaviors. For example, in preclinical studies of male rats, the GR and progesterone receptor antagonist mifepristone has been shown to block escalation of alcohol drinking following induction of dependence by chronic alcohol vapor exposure (Vendruscolo et al., 2012), and reduce alcohol consumption in a homecage limited-access two-bottle choice study (Koenig and Olive, 2004). Additionally, in alcohol-dependent male rats, GRs are downregulated in the prefrontal cortex (PFC), nucleus accumbens (NAc), and bed nucleus of the stria terminalis (BNST) during acute alcohol withdrawal, and upregulated in the NAc core, ventral BNST, and central amygdala (CeA) 3 weeks into abstinence (Vendruscolo et al., 2012). Overall these studies suggest that glucocorticoid signaling via the GR plays a dynamic role in both acute alcohol consumption and alcohol dependence, though it is important to note the bias towards utilizing male subjects in the literature as the HPA axis is known to be sexually dimorphic, both in normal and diseased states (Bangasser and Valentino, 2014). For example, in female mice with a history of predator stress and alcohol drinking GR is upregulated in the PFC during acute withdrawal, while males show no change (Finn et al., 2018).

Surprisingly, few alcohol-related studies have focused on the functional role of the mineralocorticoid receptor (MR). The MR has mainly been studied for its peripheral effects such as modulating fluid balance and blood pressure via its endogenous ligand, aldosterone, but is also known to modulate memory formation, fear extinction, and recall in male rats (Zhou et al., 2010, Dorey et al., 2011, Zhou et al., 2011, Ter Horst et al., 2012, Gomez-Sanchez and Gomez-Sanchez, 2014). Furthermore, there are sizeable sex differences in the role of MR in fear extinction, with female mice showing greater extinction deficits following MR deletion in the forebrain than male mice (Ter Horst et al., 2012). While the MR is traditionally thought of as a cytosolic ligand-dependent transcription factor that effects genomic changes on the time-scale of hours, recent studies have identified a membrane bound variant of the MR that can act on the time-scale of minutes (Karst et al., 2005, Khaksari et al., 2007, Dorey et al., 2011, Gomez-Sanchez and Gomez-Sanchez, 2014). In fact, the MR also binds corticosterone, and is expressed in brain regions generally associated with addiction such as the prefrontal cortex, hippocampus, and amygdala (Reul and de Kloet, 1986, Fuller et al., 2000). The MR has also been shown to mediate some responses to corticosterone that GR does not, such as modulating hippocampal glutamate signaling (Karst

et al., 2005) and corticosterone-induced impairment of memory retrieval (Khaksari et al., 2007). Earlier studies reported the lack of modulatory effect of MR antagonism on alcohol drinking (Koenig and Olive, 2004, O'Callaghan et al., 2005) (see later discussion), and MR mRNA levels are not changed during acute withdrawal in alcohol dependent male rats (Vendruscolo et al., 2012). However, a recent multi-species study linked lower MR gene expression levels in the central amygdala (CeA) to higher alcohol drinking behavior in male primates with a history of alcohol consumption and more compulsive-like alcohol drinking in alcohol dependent male rats (Aoun et al., 2017). In male and female humans it was confirmed that higher levels of the MR ligand aldosterone correlated with higher alcohol craving in recovering alcohol use disorder (AUD) patients, and that non-abstinent patients had higher levels of aldosterone than abstinent patients (Leggio et al., 2008, Aoun et al., 2017). As such, there is growing evidence that MR signaling may play an important role in alcohol drinking behavior, and that there may be sex differences in this role, but it is unclear if this receptor may prove a potential therapeutic target.

One of the goals of the present study was to assess the role of MR signaling in the maintenance of alcohol self-administration using the MR antagonist spironolactone. Another goal of this study was to examine the effects of spironolactone on behavior under extinction conditions. To do this, probe extinction tests were used in which the cues associated with the reinforcer were presented, but alcohol delivery was withheld. This test allows for the examination of the persistence of responding in the presence of drug-associated cues, but absence of the primary reinforcer, which is an important feature of drug seeking behavior. Based on the literature, we hypothesized that MR antagonism would reduce alcohol self-administration, the persistence of non-reinforced alcohol responding. We also hypothesized that females would be more sensitive to this MR antagonism given greater behavioral response to MR knockout as well as documented HPA axis dimorphism (Ter Horst et al., 2012, Bangasser and Valentino, 2014). To test this hypothesis male and female Long-Evans rats were trained to self-administer a sweetened alcohol solution (15% (v/v) alcohol + 2% (w/v) sucrose) and administered spironolactone prior to alcohol self-administration and probe extinction sessions. Furthermore, to explore the specificity of this effect to an alcohol reinforcer, spironolactone was tested in a separate group of male and female Long-Evans rats trained on sucrose self-administration. Together with recent studies implicating MR signaling in alcohol drinking behavior, these findings suggest that the MR may be an important avenue for research in the alcohol field.

Materials and Methods

Animals

38 adult Long-Evans rats (19 male/19 female) were single housed under a 12h light/dark cycle (7am/pm). All experiments were conducted during the light cycle. Animals were continuously monitored and cared for by the veterinary staff of the UNC-Chapel Hill Division of Comparative Medicine. All procedures were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals and institutional guidelines. All protocols were approved by the UNC Institutional Animal Care and Use Committee

(IACUC). UNC-Chapel Hill is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Apparatus

Self-administration was conducted in operant chambers (Med Associates, Georgia, VT) located within sound-attenuating cabinets equipped with an exhaust fan to provide ventilation and mask outside noise. Chambers were equipped with two retractable levers on opposite sides of the chamber (left and right), and a cue light was located above each lever. When the response requirement was met on the left (active) lever, a cue light (directly above the lever) and a stimulus tone were presented for the duration of the alcohol reinforcer delivery (0.1mL of solution into a well on the left side of the chamber across 1.66s). Responding during reinforcer delivery and on the right (inactive) lever was recorded, but had no programmed consequences. Chambers were also equipped with 4 parallel infrared beams across the bar floor to measure general locomotor activity throughout the session. The number of beam breaks for the entire session was collected and this total was divided by the session length (30 min) to represent the locomotor rate (beam breaks/min).

Alcohol self-administration training

Rats were trained to self-administer a 15% (v/v) alcohol + 2% (w/v) sucrose solution (15A/2S) on a fixed ratio 2 (FR2) schedule of reinforcement in 30 minute sessions, five days a week (M-F) via sucrose fading as described in (Randall et al., 2017). Sucrose fading began with self-administration of 10% sucrose (10S), then 2A/10S, 5A/10S, 10A/10S, 10A/5S, 15A/5S, 15A/2S on subsequent sessions, 5 sessions of 15A, and then remained at 15A/2S for the duration of training. A sweetened alcohol reinforcer was used as we find this results in stable alcohol self-administration in these long-term studies (Randall et al., 2015, Randall et al., 2017, Jaramillo et al., 2018). Rats that did not consistently self-administer at least 0.4 g/kg alcohol were excluded, there were two male rats that did not meet this criterion and were not included in this study. Rats had approximately 4 months of self-administration training and were used in an unrelated non- drug study (i.e., involved exposure to a single stressor and self-administration was unaltered (unpublished)) a month prior to the initiation of this study.

Sucrose self-administration training

Rats were trained to self-administer a 0.8% (w/v) sucrose solution (0.8S) on an FR2 schedule over 30 minute sessions, five days a week (M-F) via sucrose fading as described above. Sucrose fading began with self-administration of 10S, then 5S, 2S, 1S, then 0.5S for one week before returning to 0.8S for the remainder of the study. This dose of sucrose was selected as it resulted in similar levels of lever responding as the alcohol self-administration group (Jaramillo et al., 2018).

Experiment 1: Effect of spironolactone on maintenance of alcohol self-administration—To measure the effect of spironolactone on maintenance of alcohol self-administration, rats received spironolactone (0, 10, 25, 50 mg/kg, IP) 30 minutes prior to a self-administration session. A within-subject design was used such that each rat (n=12 females; n=10 males) received each treatment in a random order and doses were equally

represented for each sex on each test day. Test days were on Tuesdays and Thursdays with standard self-administration sessions on Monday, Wednesday, and Friday. Alcohol lever responses had to be at least 80% of baseline (average responding in the 2 sessions preceding the study) self-administration in order for a rat to be tested, all rats met this criterion. One week after the conclusion of testing, rats began Experiment 2.

Experiment 2: Effect of spironolactone on alcohol response persistence—To assess the effects of spironolactone on alcohol lever responding in the absence of the alcohol reinforcer, but presence of the cues associated with alcohol, probe extinction tests were used as we previously describe in (Randall et al., 2015). During these tests, responses on the alcohol lever were not reinforced with alcohol (i.e., extinction). That is, for each completed FR2 the cue light and tone were activated but no alcohol was delivered. Rats received spironolactone (0, 25, 50 mg/kg, IP) 30 minutes prior to test session. A within subject design was used and test days and intervening standard self-administration days were scheduled as described in Experiment 1.

Experiment 3: Effect of spironolactone on maintenance of sucrose self-administration—To measure the effect of spironolactone on maintenance of sucrose self-administration the same testing procedure described in Experiment 1 was used (n=7 females; n=7 males). At the conclusion of testing the spironolactone dose range (0, 10, 25, 50 mg/kg, IP), rats underwent a follow-up test in which all rats received a higher spironolactone dose (100 mg/kg, IP). One week after this follow-up test, rats began Experiment 4.

Experiment 4: Effect of spironolactone on sucrose response persistence—To assess the effects of spironolactone on persistent responding in the absence of sucrose reinforcement, the same probe extinction testing was used as described in Experiment 2. Rats received spironolactone (0, 25, 50 mg/kg, IP) 30 minutes prior to these tests.

Drugs

Alcohol (95% (v/v), Pharmaco-AAPER, Shelbyville, KY) and sucrose were diluted with tap water for all self-administration sessions. Spironolactone (Sigma-Aldrich, St. Louis, MO; Lot # MKBV2039V, MKBZ9531V, MKCD7812) was suspended in 45% 2- hydroxypropyl- β -cyclodextrin (Sigma-Aldrich, St. Louis, MO) and injected at a volume of 1 mL/kg.

Data Analysis

Alcohol or sucrose lever responses, inactive lever responses, alcohol or sucrose intake (g/kg or mL/kg, respectively; estimated from the number of reinforcers received) and locomotor rate were analyzed with two-way repeated measures analysis of variance (RM- ANOVA) with sex and spironolactone dose as factors. Based on our hypothesis that females and males would respond differently to MR antagonism, a significant main effect of sex or spironolactone dose was followed up with one-way ANOVAs to examine the effects of spironolactone within each sex separately. Cumulative alcohol lever responses across the session were analyzed by two-way RM-ANOVA with spironolactone dose and session time as factors for each sex separately. In all cases, post-hoc analysis (Tukey) was used to

determine specific differences from the vehicle condition. For all analyses, significance was set at $p = 0.05$.

Results

Experiment 1: Effect of spironolactone on maintenance of alcohol self-administration

The baseline alcohol lever responses (mean and S.E.M. of the two days prior to the initiation of spironolactone testing) was as follows: females 42.1 ± 3.51 and males 88.0 ± 9.53 . Males had significantly higher responses ($t(20) = 4.84$, $p < 0.001$), but there was no significant difference in alcohol intake (females: 0.80 ± 0.06 g/kg; males: 0.93 ± 0.10 g/kg). Female rats had a higher locomotor rate (27.1 ± 1.40 beam breaks/min) than male rats (21.9 ± 0.10 beam breaks/min; ($t(20) = 2.92$, $p = 0.008$)).

Alcohol lever responses following spironolactone treatment in males and females are shown in Figure 1A. The two-way ANOVA showed a significant main effect of spironolactone dose ($F(3,60)=8.52$, $p<0.001$), and a significant main effect of sex ($F(1,60)=7.47$, $p=0.01$), with overall higher alcohol lever responses in the males ($p<0.05$), consistent with our previous work (Randall et al., 2017). In the females, there was a significant reduction in alcohol lever responses following the 50 mg/kg dose relative to vehicle ($F(3,33) = 6.03$, $p = 0.002$). In the males, there was a significant reduction in alcohol lever responses following the 25 mg/kg dose relative to vehicle ($F(3,27) = 5.07$, $p = 0.006$). There was no significant sex by spironolactone dose interaction. The corresponding alcohol intake is shown in Table 2. There was a significant main effect of spironolactone dose ($F(3,60) = 8.49$, $p < 0.001$), with a significant reduction in alcohol intake following 50 mg/kg spironolactone. There was no main effect of sex on alcohol intake, indicating that while alcohol lever responses differed between sexes, alcohol intake was the same, consistent with our previous work (Randall et al., 2017). In the females, alcohol intake was reduced at the 50 mg/kg spironolactone dose relative to vehicle ($F(3,33) = 6.23$, $p = 0.002$; Table 2). In the males, alcohol intake was reduced at the 25 mg/kg spironolactone dose relative to vehicle ($F(3,27) = 4.56$, $p = 0.01$). There was no significant sex by dose interaction. There was no significant effect of spironolactone on inactive lever responses (Table 1).

Given the lack of interaction between sex and spironolactone dose on total session alcohol lever responses, the pattern of alcohol lever responses across the session were examined in females and males separately. In the females (Fig 1B), there was a significant main effect of dose ($F(3,165) = 5.56$, $p = 0.003$), time ($F(5,165) = 52.40$, $p < 0.001$), and a significant dose x time interaction ($F(15,165) = 4.71$, $p < 0.001$). Treatment with 50 mg/kg spironolactone reduced alcohol lever responses at 10 min and throughout the remainder of the session relative to vehicle ($p<0.05$). In the males (Fig 1C), there was a significant main effect of dose ($F(3,135) = 5.63$, $p = 0.004$), time ($F(5,135) = 60.08$, $p < 0.001$), and a significant dose x time interaction ($F(15,135) = 3.68$, $p < 0.001$). Relative to vehicle, treatment with 25 mg/kg spironolactone reduced alcohol lever responses at 20 min and throughout the remainder of the session ($p<0.05$), and following 50 mg/kg spironolactone responses were reduced at 25 min and throughout the remainder of the session ($p<0.05$).

Analysis of spironolactone effects on locomotor rate (Fig 1D) showed a significant main effect of spironolactone dose ($F(3,60)=8.00$, $p<0.001$), and a significant main effect of sex ($F(1,60)=7.01$, $p=0.02$). In the females, spironolactone treatment did not affect locomotor rate. In the males, there was a significant reduction in locomotor rate at both the 25 mg/kg and 50 mg/kg spironolactone doses relative to vehicle ($F(3,27) = 11.94$, $p < 0.001$). There was no spironolactone dose by sex interaction. These results suggest that the decreases in alcohol self-administration in the males may be related to a general suppression of motor activity.

Experiment 2: Effect of spironolactone on alcohol response persistence

Examination of spironolactone on alcohol lever responding during the probe extinction sessions (Fig 2A), showed a significant main effect of spironolactone dose ($F(2,40)=11.84$, $p<0.001$), with a significant reduction at the 50 mg/kg spironolactone dose relative to vehicle ($p<0.05$). In female rats, there was a significant reduction in alcohol lever responses at 50 mg/kg spironolactone relative to vehicle ($F(2,22) = 8.89$, $p = 0.001$). In the males, there was a significant reduction in alcohol lever responses, but no spironolactone dose was significantly different from vehicle ($F(2,18) = 3.84$, $p = 0.04$). There was no main effect of sex and no spironolactone dose by sex interaction. The lack of a main effect of sex suggests that the females and males show similar persistence of lever responding under extinction conditions, which is interesting given that males have higher rates of alcohol lever responses under alcohol reinforced conditions (see Discussion). Spironolactone treatment did not affect inactive lever responses (Table 1). In the females, analysis of cumulative alcohol lever responses across the probe extinction session (Fig 2B) showed a significant main effect of dose ($F(2,110) = 9.68$, $p < 0.001$), time ($F(5,110) = 53.36$, $p < 0.001$), and a significant dose x time interaction ($F(10,110) = 3.23$, $p = 0.001$). Alcohol lever responding was reduced following treatment with 50 mg/kg spironolactone at 10 min and continuing throughout the session relative to vehicle ($p<0.05$). In the males (Fig 2C), there was a significant main effect of dose ($F(2,90) = 3.61$, $p = 0.048$), time ($F(5,90) = 43.54$, $p < 0.001$), with a trend for a significant dose by time interaction ($F(10,90)=1.85$, $p=0.06$).

Analysis of locomotor activity (Fig 2D) showed a significant main effect of spironolactone dose ($F(2,40)=16.42$, $p<0.001$), and a significant main effect of sex ($F(1,40)=8.93$, $p=0.007$), with significantly greater locomotor activity in the females. In both the females and males, there was a significant reduction in locomotor activity at the 50 mg/kg spironolactone dose relative to vehicle ($F(2,22) = 8.06$, $p = 0.002$; $F(2,18) = 12.15$, $p < 0.001$, respectively). This pattern of results suggests that the reductions in alcohol lever responses during these extinction sessions following treatment with the 50 mg/kg dose may be related to a general inhibition of locomotor behavior.

Experiment 3: Effect of spironolactone on maintenance of sucrose self- administration

The baseline sucrose lever responses (mean and S.E.M. of the two days prior to the initiation of spironolactone testing) was as follows: females 36.4 ± 8.02 and males 80.4 ± 14.3 . Males had significantly higher responses ($t(12) = 2.69$, $p = 0.02$), but there was no significant difference in sucrose intake (females: 6.70 ± 1.39 mL/kg; males: 9.13 ± 1.57 mL/kg). There

was no difference in locomotor rate (females: 27.5 ± 1.16 beam breaks/min; males: 26.3 ± 1.65 beam breaks/min).

Sucrose self-administration in males and females is shown in Figure 3A. The two-way RM-ANOVA showed a significant main effect of sex ($F(1,48) = 6.98$, $p=0.02$), with overall higher sucrose lever responses in the males ($p<0.05$). In the females, sucrose lever responses were significantly reduced following spironolactone ($F(4,24)=5.43$, $p=0.003$), but no dose was significantly different from vehicle - there was a trend for a reduction at the 100 mg/kg dose ($p=0.068$). Spironolactone did not affect sucrose self-administration in the males. There was no main effect of spironolactone dose and no sex by spironolactone dose interaction. The corresponding sucrose intake is shown in Table 2. There was a significant main effect of spironolactone dose on sucrose intake ($F(4,48) = 4.62$, $p = 0.003$) with 100 mg/kg spironolactone significantly reducing sucrose intake. The significant main effect of spironolactone dose in sucrose intake by the two-way ANOVA, but not in sucrose lever responding may be a contribution of the overall reduced variability given that there is no sex difference in sucrose intake. In the females, sucrose intake was significantly reduced following the 100 mg/kg spironolactone dose ($F(4,24)=7.46$, $p<0.001$). Spironolactone did not affect sucrose intake in the males. There was no main effect of sex, and no spironolactone dose by sex interactions on sucrose intake. There was no significant effect of spironolactone on inactive lever responses (Table 1).

Given the lack of interaction between sex and spironolactone dose on total session sucrose lever responses, the pattern of alcohol lever responses across the session were examined in females and males separately. In the females (Fig 3B), there was a significant main effect of spironolactone dose ($F(4,120) = 4.78$, $p = 0.006$), time ($F(5,120) = 41.03$, $p < 0.001$), and a significant dose by time interaction ($F(20,120) = 4.60$, $p < 0.001$). Treatment with 100 mg/kg spironolactone reduced sucrose lever responses at 25 min and 30 min relative to vehicle ($p<0.05$). In the males (Fig 3C), there was a significant main effect of time ($F(5,120) = 53.79$, $p < 0.001$), and no effect of dose or dose by time interaction.

Analysis of spironolactone on locomotor rate showed a significant main effect of spironolactone dose ($F(4,48)=7.05$, $p<0.001$; Fig D), with 100 mg/kg spironolactone significantly reducing locomotor rate. In the females, there was a significant reduction in locomotor rate at the 100 mg/kg spironolactone dose relative to vehicle ($F(4,24)=3.88$, $p=0.01$). In the males, there was also a significant reduction in locomotor rate, but no spironolactone dose was significantly different from vehicle ($F(4,24)=3.38$, $p=0.03$). There was no effect of sex, and no sex by dose interaction. These data suggest an overall suppression of motor activity at the highest spironolactone dose (100 mg/kg).

Experiment 4: Effect of spironolactone on sucrose response persistence

Analysis of sucrose lever responding during the probe extinction session is shown in Figure 4A. Two way RM-ANOVA revealed no main effect of sex or spironolactone dose, and no sex by spironolactone dose interaction. Spironolactone treatment did not affect inactive lever responses (Table 1). In the females (Fig 4B) and males (Fig 4C), cumulative sucrose lever responses across the probe extinction session showed a significant main effect of time (females: $F(5,60) = 25.63$, $p < 0.001$; males: $F(5,60) = 81.98$, $p < 0.001$). There were no

main effects of spironolactone dose, and no time by spironolactone dose interactions. Spironolactone treatment did not affect locomotor rate (Fig 4D). As there was no main effect of spironolactone dose, no main effect of sex, and no sex by dose interaction. Overall, spironolactone did not appear to affect the persistence of sucrose lever responding under these conditions.

Discussion

This study demonstrates that pharmacological antagonism of the MR with spironolactone reduces alcohol self-administration in both male and female rats, albeit at a higher dose in female rats. In male, but not female rats this reduction in self-administration was accompanied by a reduction in locomotor activity during the self-administration session. Spironolactone also reduced the persistence of alcohol responding in the presence of alcohol-associated cues in female rats, but not male rats; however, at these spironolactone doses locomotor activity during the session was reduced in both sexes. Interestingly, the same spironolactone doses that were found to reduce alcohol self-administration did not affect sucrose self-administration or persistence of sucrose responding under probe extinction conditions, suggesting specificity to the alcohol reinforcer at those doses. Together these findings add to the growing body of literature suggesting that MR signaling may play a role in ongoing alcohol drinking.

The results of Experiment 1 suggest that MR signaling is involved in self-administration of a sweetened alcohol reinforcer in female rats, while in male rats this relationship is less clear due to the concomitant reduction in locomotor activity during the session. Additionally, females were less sensitive to the effects of spironolactone than males, showing a reduction in alcohol self-administration at 50 mg/kg as compared to 25 mg/kg in males. This contradicts our original hypothesis that female rats would be more sensitive to MR antagonism and could be due to the influence of gonadal hormones such as estrogen which play a complex modulatory role in both aldosterone and corticosterone signaling, both acutely and as part of the estrous cycle (Xue et al., 2013, Bangasser and Valentino, 2014, Minni et al., 2014). In humans, spironolactone can cause hypotension when combined with alcohol (Pfizer, 2008), and this may contribute to the reduction in locomotor activity observed in the male rats. This possible interaction is supported by the finding that the spironolactone-induced reduction in alcohol self-administration emerges late in the self-administration session after alcohol has been consumed. Interestingly, female rats show reduced alcohol self-administration without concomitant reduction in locomotor activity, which may be due to reduced effects of spironolactone on blood pressure in females, as suggested by one study (Michaelis et al., 2012). Together, this pattern of results could suggest potentiated sensitivity to the motor suppressant effects of spironolactone in the presence of alcohol and that females may be less sensitive to this interaction. While we did not directly assess any interactions between spironolactone and alcohol, it is also possible that the reduction in locomotor activity seen in male rats is due to spironolactone potentiating alcohol-induced ataxia.

In the sucrose self-administration group, the increased spironolactone dose (100 mg/kg) resulted in a significant decrease in sucrose intake (ml/kg) in the females only, while there

was an overall reduction in sucrose lever responses and a trend for a reduction at 100 mg/kg. This could be related to the increased variance in lever responses at this dose, difference between lever responses and reinforcers delivered (i.e. lever responses during reinforcer delivery), or variance in animal weight that was corrected by examining intake rather than responding. These differences may also arise, in part, due to the low sample size (n=7) which is a limitation of this experiment.

In the present work, probe extinction sessions were used to examine the persistence of responding in the absence of the primary reinforcer. Interestingly, in both the alcohol self-administration trained and sucrose self-administration trained rats, no sex differences were observed under these conditions. This is important given that males in both self-administration groups had greater lever responses than the females during self-administration. Therefore, the males had a greater conditioning history than females (i.e., greater number of pairings with the cues associated with alcohol/sucrose delivery), which would be predicted to result in greater persistence of responding or greater resistance to extinction in the presence of the cues than the females (Jimenez-Gomez and Shahan, 2007, Nevin, 2012). As such, the lack of a sex difference in responding under these extinction conditions suggests that for the males this richer conditioning history did not result in greater response persistence or greater resistance to extinction than the females, which is interesting. This result could suggest that the males were more sensitive to the change in the reinforcement conditions. Consistent with this suggestion, we have previously shown that male and female Long Evans rats trained under self-administration conditions similar to those used here have similar breakpoints under progressive ratio conditions, even though males have greater alcohol lever response during self-administration training (Randall et al., 2017).

The results of Experiment 2 show that spironolactone (50 mg/kg) reduced the persistence of responding in female, but not male rats. However, this dose resulted in reduced locomotor activity in both sexes, making it difficult to definitively conclude whether MR is implicated in persistence of alcohol responding as measured under these probe extinction tests. Additionally, the lack of effect of spironolactone on alcohol lever responding in the males is an example where the reduction in locomotor activity was not accompanied by a reduction in alcohol lever responses, and where this reduction in locomotor activity occurs in the absence of alcohol consumption. Interestingly, Experiment 4 showed that spironolactone had no effect on the persistence of sucrose responding under the same extinction conditions in male or female rats trained to self-administer sucrose. One interpretation of the locomotor data is that having a history of alcohol self-administration promoted a neuroadaptation that increased sensitivity to the motor suppressant effects of spironolactone. While rats with a history of alcohol self-administration as in the present work are generally not considered alcohol dependent and do not exhibit somatic signs of withdrawal, it has been shown that daily intake of at least 1.2 g/kg alcohol over six months is sufficient to increase serum aldosterone in male rhesus macaques (Aoun et al., 2017). This suggests potential for an interaction between MR and alcohol drinking that long-term alcohol self-administration history may be sufficient to induce. As such, future studies should be conducted to examine the importance of alcohol history in the effects of spironolactone on alcohol self-administration and persistence of alcohol responding.

The present findings are in contrast to two previous studies which reported no effect of spironolactone on alcohol consumption or preference in rats and mice under home cage drinking conditions (Koenig and Olive, 2004, O'Callaghan et al., 2005). However, there are some key methodological differences that may have contributed to the different outcomes. Koenig et al. (2004) tested MR antagonism in male Long-Evans rats in a limited-access two-bottle choice model of homecage drinking that incorporated 23 hours of water deprivation in between 1 hour sessions of drinking 10% (v/v) alcohol and water. Given that MR and its ligand aldosterone are important in regulating fluid balance, it is possible that the stress of water deprivation dysregulated the MR system, and thus masked the effects of spironolactone on alcohol drinking (Gomori et al., 1960, Tang et al., 2011, Ali et al., 2012). The present studies were conducted under ad-libitum fluid access. Additionally, in that study spironolactone was administered immediately prior to the 1 hour drinking session, whereas in the present study a 30 minute pretreatment time was used. This may be important because while spironolactone itself is an MR antagonist with a half-life under 2 hours, it also produces two long-acting MR antagonist active metabolites, canrenone and 7-thiomethyl-spironolactone with half-lives of 13.8 and 16.5 hours respectively (Kolkhof and Barfacker, 2017), which may be important for the reductions in alcohol self-administration observed in the present work. The O'Callaghan et al. (2005) study was conducted in male and female C57/BL10 mice with 24 hour free-access to 8% (v/v) alcohol and water. Spironolactone (50 mg/kg) was administered daily over 3 weeks and alcohol consumption was measured 3 times a week. One major difference between this study and the present work was the timescale over which alcohol drinking was measured, as shown here, spironolactone can reduce alcohol self-administration in a 30 minute session. An additional consideration of both studies is the length of alcohol history that the animals have prior to spironolactone testing (15 weeks in the present study versus 3 and 4 weeks, respectively in these two cited studies), as this long-term alcohol history may be important for the effects of spironolactone.

An important consideration for the present findings is the potential off-target effects of spironolactone. While spironolactone is the MR antagonist most frequently used in the literature (Zhou et al., 2010, Zhou et al., 2011, Nasca et al., 2015), in addition to the MR ($K_i = 2.32$ nM) spironolactone is active at the glucocorticoid receptor ($K_i = 32.6$ nM), androgen receptor ($K_i = 39.4$ nM), progesterone receptor ($K_i = 400$ nM), and estrogen receptor ($K_i > 1100$ nM) (Bell et al., 2007). Therefore, while the spironolactone doses used in this study are within the range used in the literature (with the exception of 100 mg/kg), we cannot definitively conclude the contribution of these off target effects to the observed reductions in alcohol self-administration and locomotor behavior. Future studies may benefit from testing newer MR antagonists such as eplerenone, that exhibit greater selectivity for the MR and greater safety profile (spironolactone carries an FDA black box warning for tumorigenicity) (de Gasparo et al., 1987, Struthers et al., 2008), to clarify the role of MR in alcohol self-administration. Another interesting consideration is whether aldosterone and/or corticosterone mediates MR's role in alcohol self-administration, as MR can bind both (Fuller et al., 2000). Metyrapone, which blocks synthesis of both corticosterone and aldosterone (Tucci et al., 1967, Gomez-Sanchez et al., 1997), can reduce alcohol preference and intake in high alcohol preferring male Wistar rats, and this effect is reversed by exogenous administration of corticosterone (Fahlke et al., 1994b). However corticosterone is

a precursor for aldosterone (Ikeda et al., 2012), so an additional study using an aldosterone synthesis inhibitor such as FAD286 could clarify this point.

The results from the present work show a functional role for the MR in alcohol self-administration. Future experiments will be important to clarify the role of MR signaling in alcohol drinking-related behaviors (i.e. examining the effects of MR antagonism on alcohol-seeking and self-administration following abstinence or extinction, under progressive ratio schedules of reinforcement, or compulsive drinking of quinine adulterated alcohol). Additionally, interesting differences observed in response to MR antagonism in males and females merit further investigation and highlight the importance of studying both male and female subjects. Altogether, these findings suggest that the MR-aldosterone system poses an exciting and novel avenue to study in relation to alcohol reinforcement processes contributing to alcohol consumption and neuroadaptations that may accompany alcohol drinking and dependence as a potential target for novel therapeutics to treat AUDs.

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Highlights

- MR antagonism reduces alcohol self-administration in male and female rats
- In female rats, MR antagonism reduces response persistence in absence of alcohol
- In female rats, higher dose of MR antagonist reduced sucrose self-administration
- Response persistence in absence of sucrose is unchanged following MR antagonism
- MR signaling may play a role in regulating ongoing alcohol drinking

Figure 1.

(A) Effects of spironolactone on alcohol self-administration. Mean ($\pm$ S.E.M.) alcohol lever responses for female ($n = 12$) and male ($n = 10$) rats treated with spironolactone. (B, C) Cumulative alcohol lever responses across the session in females and males, respectively. (D) Locomotor activity (beam breaks/min) during the self-administration session in males and females. Spironolactone reduced alcohol lever responses in male and female rats, and decreased locomotor rate in male, but not female, rats. * - $p < 0.05$ versus female vehicle, † - $p < 0.05$ versus male vehicle.

Figure 2.

(A) Effects of spironolactone on alcohol response persistence (under “probe extinction” conditions). Mean ($\pm$ S.E.M.) alcohol lever responses for male and female rats treated with spironolactone. (B, C) Cumulative alcohol lever responses across the session in females and males, respectively. (D) Locomotor activity (beam breaks/min) during the probe-extinction session in males and females. Spironolactone reduced alcohol response persistence in female, but not male rats, and decreased locomotor rate in both male and female rats. * - $p < 0.05$ versus female vehicle, † - $p < 0.05$ versus male vehicle.

Figure 3.

(A) Effects of spironolactone on sucrose self-administration. Mean ($\pm$ S.E.M.) sucrose lever responses for female ($n = 7$) and male ($n = 7$) rats treated with spironolactone. (B, C) Cumulative sucrose lever responses across the session in females and males, respectively. (D) Locomotor activity (beam breaks/min) during the self-administration session in males and females. Spironolactone reduced sucrose self-administration across the session in female, but not male rats, and decreased locomotor rate in both male and female rats. * - $p < 0.05$ versus female vehicle, † - $p < 0.05$ versus male vehicle.

Figure 4.

(A) Effects of spironolactone on sucrose response persistence (under “probe extinction” conditions). Mean ($\pm$ S.E.M.) sucrose lever responses for male and female rats treated with spironolactone. (B, C) Cumulative sucrose lever responses across the session in females and males, respectively. (D) Locomotor activity (beam breaks/min) during the probe extinction session in males and females. Spironolactone had no effect on sucrose response persistence or locomotor activity.

Table 1.

Inactive lever responses for all experiments.

Spironolactone Dose (mg/kg)					
Males	0	10	25	50	100
Alcohol Self-Admin.	1.00 ± 0.36	0.25 ± 0.25	0.50 ± 0.29	0.58 ± 0.50	--
Alcohol Probe Ext.	1.20 ± 0.59	--	0.60 ± 0.34	0.30 ± 0.30	--
Sucrose Self-Admin.	0.00 ± 0.00	1.00 ± 0.38	1.71 ± 0.89	2.57 ± 2.57	0.00 ± 0.0
Sucrose Probe Ext.	1.57 ± 0.92	--	0.43 ± 0.20	0.57 ± 0.30	--
Females	0	10	25	50	100
Alcohol Self-Admin.	1.42 ± 0.43	1.58 ± 0.45	1.00 ± 0.35	0.67 ± 0.19	--
Alcohol Probe Ext.	1.00 ± 0.34	--	1.00 ± 0.39	0.42 ± 0.15	--
Sucrose Self-Admin.	1.43 ± 0.61	0.86 ± 0.55	1.29 ± 0.42	0.71 ± 0.42	0.00 ± 0.0
Sucrose Probe Ext.	1.71 ± 0.84	--	0.57 ± 0.30	2.14 ± 1.58	--

Table 2.

Alcohol and sucrose intake for Experiments 1 and 3.

Spironolactone Dose (mg/kg)					
Males	0	10	25	50	100
Alcohol SA (g/kg)	0.84 ± 0.09	0.84 ± 0.09	0.50 ± 0.08 [*]	0.59 ± 0.09	--
Sucrose SA (mL/kg)	7.00 ± 1.56	6.98 ± 0.88	6.70 ± 1.81	7.93 ± 1.53	5.72 ± 1.07
Females	0	10	25	50	100
Alcohol SA (g/kg)	0.95 ± 0.12	1.04 ± 0.11	0.70 ± 0.13	0.54 ± 0.09 [*]	--
Sucrose SA (mL/kg)	7.58 ± 1.57	7.83 ± 1.25	10.40 ± 1.82	7.65 ± 1.22	1.70 ± 1.34 [*]

^{*}
p<0.05 vs. vehicle (0)