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Mifepristone decreases chronic voluntary ethanol consumption in rhesus macaques.

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D: ACTH: adrenocorticotropin hormone; AUD: alcohol use disorder; BEC: blood ethanol concentration; CeA: central nucleus of the amygdala; FDA: federal drug administration; GR: glucocorticoid receptor; HPA: hypothalamic pituitary adrenal axis; MIFE: Mifepristone; MR: mineralocorticoid receptor; PR: progesterone receptor; PVN: paraventricular nucleus of the hypothalamus; SIP: schedule-induced polydipsia

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ABSTRACT

The efficacy of short-term treatment with mifepristone (MIFE), a high-affinity, non-selective glucocorticoid receptor (GR) antagonist, to reduce ethanol drinking was tested in a rhesus macaque model. Stable individual daily ethanol intakes were established, ranging from 1.6 g/kg/day to 4.0 g/kg/day (n=9 monkeys). Following establishment of chronic ethanol intake, a MIFE dosing regimen that modeled a study of rodent drinking and human alcohol craving was evaluated. Three doses of MIFE (17, 30, and 56 mg/kg/day) were each administered for four consecutive days. Both 30 and 56 mg/kg decreased ethanol intake compared to baseline drinking levels without a change in water intake. 56 mg/kg/day of MIFE produced the largest reduction in ethanol self-administration with the average intake at 57% of baseline intakes. Cortisol was elevated during MIFE dosing and a mediation analysis revealed that the effect on ethanol drinking was fully mediated through cortisol. During a forced abstinence phase, access to 1.5 g/kg ethanol resulted in relapse in all drinkers and was not altered by 56 mg/kg MIFE treatment. Overall, these results show that during active drinking MIFE is efficacious in reducing heavy alcohol intakes in a monkey model, an effect that was related to MIFE-induced increase in cortisol. However, MIFE treatment did not eliminate ethanol drinking. Further, cessation of MIFE treatment resulted in a rapid return to baseline intakes and MIFE was not effective in preventing a relapse during early abstinence.

Significance: Mifepristone reliably decreases average daily ethanol self-administration in a non-human primate model. This effect was mediated by cortisol, was most effective during open-access conditions, and did not prevent or reduce relapse drinking.

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INTRODUCTION

Approximately 14.8 million people in the United States, or 1 in 19, had an alcohol use disorder (AUD) in 2018 (Substance Abuse and Mental Health Services Administration, 2019). The prevalence of a lifetime diagnosis of AUD between 2012-2013 was 29.1%, a substantial increase from 2001-2002, with fewer than 20% seeking treatment (Grant et al., 2015). The Food and Drug Administration (FDA) has approved three medications for the treatment of AUD between 1951 and 2006: Disulfiram, oral and extended release Naltrexone and Acamprosate. Two additional drugs, Topiramate and Gabapentin are recommended for the off-label treatment of AUD by the American Psychiatric Association (Reus et al., 2018) and a number of other treatments are under investigation (Litton et al., 2015; Witkiewitz et al., 2019; Swift and Aston, 2015). A major challenge in the development of successful pharmacologic intervention is that AUD is a highly heterogeneous disorder with multiple biological and environmental factors (Litten et al., 2015). In fact, the efficacy of currently approved medications can be related to neurobiological features present in subsets of individuals diagnosed with AUD (Litten et al., 2015; Witkiewitz et al., 2019). The heterogeneity of AUD and the interaction with specific pharmacotherapies highlight both the need and challenge of developing treatment options that target subsets of individuals.

Pharmacotherapies that target the stress system are a promising avenue for novel interventions. The hypothalamic pituitary adrenal (HPA) axis is a fundamental system in maintaining homeostasis and is disrupted by long-term ethanol consumption and abstinence in humans and animals (Blaine et al., 2017; Becker, 2012; Jimenez and Grant, 2017). Cortisol, the primary glucocorticoid secreted by the adrenal cortex, is a

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primary endpoint of HPA axis activation. The HPA axis response to stress (psychological and pharmacological) is blunted in alcohol-dependent subjects relative to non-alcoholic controls (Adinoff et al., 2005a; Adinoff et al., 2005b; Lovallo et al., 2000), although cortisol is elevated during ethanol withdrawal (Adinoff et al., 2003; Iranmanesh et al., 1989). Furthermore, stress is a salient risk factor for relapse (Blaine and Sinha, 2017), suggesting treatments that target the HPA response may be efficacious in treating AUD. An antagonist at the glucocorticoid receptor, Mifepristone (MIFE) has been tested in a variety of preclinical models of AUD. In rodents, MIFE shows efficacy on a large number of alcohol phenotypes including: blocking ethanol-induced place preference (Rotter et al., 2012), reducing ethanol intake (Koenig and Olive, 2004; Vendruscolo et al., 2012; Vendruscolo et al., 2015), reducing cognitive deficits in ethanol withdrawal (Jacquot et al., 2008), protecting hippocampal neurons from injury due to binge-like ethanol consumption (Cippitelli et al., 2014), reducing ethanol withdrawal severity (Sharrett-Field et al., 2013), reducing stress-induced reinstatement of ethanol-seeking (Simms et al., 2012) and decreasing the escalation of alcohol self-administration following protracted abstinence (Repunte-Canonigo et al., 2015).

Non-human Primates (NHPs) are less represented in preclinical studies of ethanol pharmacotherapy evaluation (Weerts et al., 2007). Rhesus monkeys are an excellent model for studies of individual differences in propensity to drink large amounts of alcohol associated with an AUD diagnosis and biomedical consequences (>8-12 drink equivalent/day) (Baker et al., 2014) and stress-related interventions due to their similar endocrine physiology with humans, particularly adrenal physiology (Conley et al., 2004; Jimenez and Grant, 2017). In this study, we tested the effect of a four-day oral

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MIFE administration on the ability to reduce ethanol intake and blood ethanol concentrations (BEC) in rhesus monkeys with a history of daily open-access to ethanol self-administration (22 hrs/day) and to determine if drinking would return to baseline intakes following the cessation of MIFE treatment. We further investigated if a three-day MIFE treatment could prevent relapse drinking during forced abstinence.

METHODS

Animals

Twelve adult male rhesus macaques (*Macaca mulatta*) were assigned as ethanol drinkers (n=9) or ethanol-naïve controls (n=3). Animals were housed in quadrant cages (0.8 x 0.8 x 0.9 m) with constant temperature (20-22 C), humidity (65%), and an 11-hour light cycle (lights on at 07:00). Animals had visual, auditory, and olfactory contact with other animals in the protocol. All animals were maintained on a positive caloric and fluid balance throughout the experiment, and body weights were recorded weekly. Monkeys were 5.6 – 5.8 years of age at the start of open-access conditions. Other data that have been collected and/or published on this cohort of animals (Rhesus 14) can be found through the Monkey Alcohol and Tissue Research Resource (www.MATRR.com) (Daunais et al., 2014). All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the NIH guidelines for the care and use of laboratory animal resources and approved by the Oregon National Primate Research Center IACUC.

Operant panel

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Operant panels dispense food and fluids, as previously described (Grant et al., 2008; Schnitko et al., 2019; Schnitko et al., 2020). Briefly, each panel has two spouts, each below a set of three stimulus lights (white, red, and green) that indicate an active session, food, or fluid availability, respectively. A centrally located recessed dowel activates the fluid spouts, and an infrared finger-poke activates the pellet dispenser. Dowel pulls, finger pokes and fluid consumption are recorded in real time (approximately every 500 ms) using custom hardware and programing using National Instruments interface and Labview Software. Operant panels run daily from 11:00 – 09:00 (the next morning). Between 09:00-11:00 each day, operant panels were turned off while data was downloaded, husbandry tasks performed, food and fluids were replenished, and enrichment was provided.

Ethanol Induction

A schedule-induced polydipsia (SIP) procedure was used to induce ethanol self-administration in daily 16 hour sessions, as previously described (Grant et al., 2008; Vivian et al., 2001). Briefly, a 1 g banana food pellet was delivered every 300 seconds until a water volume equivalent to 1.5 g/kg of 4% (w/v) ethanol was consistently consumed in the inter-pellet interval. Following water induction, 4% ethanol replaced water. In approximately 30-day increments, each animal consumed increasing daily doses of 4% ethanol: 0.5 g/kg/day, 1.0 g/kg/day then 1.5 g/kg/day. Following consumption of the ethanol dose, water was immediately available and any remaining pellets were available on a fixed-ratio-1 schedule following a two-hour delay.

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Ethanol self-administration and forced abstinence

After 1.5 g/kg/day ethanol induction, open-access ethanol self-administration began where water and ethanol were concurrently available in daily 22 hour sessions. Starting at the session onset, food pellets (up to 1/3 of the daily ration) were available on a fixed-ratio-1 schedule in at least three daily meals with 2 hour intervals between meals. A meal ended when one-third of the daily food allotment was obtained, or, if the monkey took longer than 2-minutes to obtain a pellet. Between meals, red stimulus lights above the spouts signaled a 2 hr time out where food pellets were not available.

After 467 consecutive daily open-access alcohol sessions, the animals entered the first forced abstinence phase. During abstinence, the stimuli on the operant panel were identical to open-access conditions with the only change being that the ethanol reservoir was replaced with water. The abstinence phases lasted 34, 41 and 39-46 days (variable due to the timing of necropsy), respectively. Following the first and second abstinence phases were two open-access phases lasting 76 and 104 days, respectively. The experimental timeline is shown in **Figure 1A**.

Control subjects

Ethanol-naïve control subjects were housed in the same room as the ethanol drinking subjects and participated in all experimental manipulations (blood collections, MIFE, etc). SIP and self-administration conditions were identical, with the exception that both spouts dispensed water. A maltose-dextran solution (10% in water) was given to the controls to calorically match the drinkers and controls. Each control subject was yoked to an ethanol drinker of similar body weight. Each week the average daily

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calories consumed from ethanol was calculated to make an isocaloric maltose-dextrin solution for the yoked control animal. Maltose-dextrin was given at the beginning of each daily session by attaching a bottle to the front of the housing cage beginning in 0.5 g/kg/day induction. Maltose-dextrin was not available during abstinence.

Blood Samples

Femoral blood samples were obtained with a 22-g × 1-inch Vacutainer needle and a 3 ml Vacutainer hematology tube (Becton Dickinson, Franklin Lakes, NJ, USA). All blood samples were stored on ice (~ 15 min) until centrifuged (3,000 rpm, 15 min at 4°C, Model Allegra 21R, Beckman Coulter, Fullerton, CA, USA). Plasma samples (300 µl aliquots) were frozen at −80°C until processing.

Blood ethanol concentration (BEC) was measured by collecting blood (20 µl) 7 hours into the drinking session, approximately once per week. Whole blood was placed into airtight containers and stored at -4°C until assayed using headspace gas chromatography (Agilent Technologies, Santa Clara, CA) and analyzed using linear regression and a standard curve ranging from 25-400 mg/dl.

Mifepristone administration

Mifepristone (Corcept Therapeutics, Menlo Park, CA) powder was prepared for oral consumption in fruit tape wrapping, a peanut butter ball, or an apple with honey and peanut butter. Three doses (17 mg/kg/day, 30 mg/kg/day, and 56 mg/kg/day) were tested during open-access (22hr/day) to ethanol, each for four consecutive days. MIFE was given at approximately 10:00 each morning, during the two-hour window when

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ethanol was not available. The 56 mg/kg dose was administered as two 28 mg/kg doses, with the second daily dose given at 18:00, right before the lights went off. A detailed timeline of the 56 mg/kg MIFE administration is provided in **Figure 1B**. MIFE dosing during open-access was evaluated across a 16 day dosing regimen, including 4 days of baseline (days 1-4; blue), 4 days of MIFE (days 5-8; red), first 4 days of post-MIFE (days 9-12; Post-1; green), and second 4 days of post-MIFE (days 13-16; Post-2; purple) (see **Figure 1B**). These bins were used for statistical analyses. The 17 mg/kg/day and 30 mg/kg/day timelines varied from the 56 mg/kg timeline in that MIFE was given once each day (10:00 AM). There was no afternoon blood draw on Day 7 following 17 mg/kg MIFE and the 30 mg/kg had an afternoon blood draw at 16:00 rather than 18:00 on Day 7.

Both controls and drinkers were given MIFE. The amount of time to consume a particular dose varied by animal. Overall, MIFE was reliably consumed. Among individuals, there were no more than 3 occasions per dose where MIFE was only partially consumed (for example, a small amount observed on the floor of the cage). In these instances, the delivery method was changed (for example, from a peanut butter ball to a fruit tape pouch) for the following dose to increase compliance. During abstinence, non-compliance remained low with no more than 2 suspected partial doses per animal. One animal was excluded from analysis due to having partially consumed or refused more than half the doses (animal 10243). There was a washout period of at least 10 days between doses during open-access and there were 222 days between the last MIFE dose during open-access and the abstinence.

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Ethanol relapse and mifepristone during abstinence

To test the effect of MIFE on reducing individual differences in the vulnerability to relapse, one week after the beginning of the second abstinence phase (abstinent Day 8), a single dose of 1.5 g/kg ethanol was made available two hours after the session began (**Figure 1C**). On abstinent Days 10-12, 56 mg/kg MIFE was administered, as previously described. On abstinent Day 13, the monkeys were again given access to 1.5 g/kg ethanol. We evaluated the rate of intake and preference before and after the MIFE dosing regimen. The rate of drinking was defined as the time elapsed between the first drink and the completion of 1.5 g/kg. Preference was defined as the volume (ml) of ethanol consumed divided by the total volume (ethanol + water) consumed during the time to reach the 1.5 g/kg cutoff.

Mifepristone Assay

Monkey plasma samples containing mifepristone and D4-mifepristone (internal standard) were extracted using a mixture of hexane and MTBE. The organic layer was evaporated to dryness under nitrogen and the residue was reconstituted in water/acetonitrile/formic acid (75:25:0.1, v/v/v). the sample extracts were analyzed by reversed phase chromatography using a Zorbax SB-phenyl column maintained at 50°C. The mobile phase was nebulized using a heated nitrogen in a Z-spray source/interface and the ionized compounds were detected using a tandem quadrupole mass spectrometer.

Hormone Assays

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Plasma aliquots were assayed by the Endocrine Technology Core at Oregon National Primate Research Center (Beaverton, OR). A Roche Cobas e411 automatic clinical platform was used to assay cortisol (0.036–63.4 µg/dl sensitivity).

Statistical Methods

A two-way repeated measures ANOVA was used to detect differences in the concentration of MIFE using group (control vs drinker) and time (Day 7 vs Day 9) as factors. The effect of MIFE on ethanol and water intake were analyzed using a repeated measures mixed-effects model with time (two levels: baseline, MIFE), dose (three levels: 17 mg/kg, 30 mg/kg, 56 mg/kg), and group (for water analysis) as independent variables. Repeated measures ANOVAs were used to determine whether the effects of 30 mg/kg and 56 mg/kg MIFE persisted after dosing ended and their effects on circulating cortisol concentration using phase (four levels: baseline, MIFE, Post-1 and Post-2) as the independent variable. The effects of MIFE during abstinence were evaluated by comparing the rate of ethanol intake and preference between the two ethanol challenges (as described above) using repeated measures ANOVA. Significant results are reported based on post hoc comparisons using Bonferroni corrected t-tests. A mediation analysis of MIFE-induced cortisol on ethanol intake was performed using the ‘mediate’ package in R (Tingley et al., 2014, R Core Team, 2020). Cortisol and MIFE concentrations and the percent change in ethanol intake were log transformed prior to the mediation analysis. All animals were included in analysis (n = 9 drinkers, n = 3 controls), unless otherwise stated. Data are presented as mean ± SD, with 95%

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confidence intervals (CI). All analyses were conducted in Prism, (version 8) or RStudio (version 1.2), $\alpha < 0.05$.

RESULTS

Mifepristone plasma concentrations

There were wide individual differences in circulating MIFE concentrations that led to large variance in average data. For the 17 mg/kg dose, the average concentration of MIFE the morning after the final dose (Day 9) was 6.0 ± 4.1 ng/ml (95% CI [3.4, 8.6]). For 30 mg/kg/day MIFE, the average evening concentration after the third dose (Day 7), was 39.3 ± 36.1 ng/ml (95% CI [16.4, 62.3]), and had decreased to 14.7 ± 13.6 ng/ml (95% CI [6.1, 23.3]) the morning after the final dose (Day 9; **Figure 2A**). A RM-ANOVA revealed a main effect of time ($F_{(1,10)} = 8.5$, $p = 0.016$), but not group (control vs drinker: $F_{(1,10)} = 0.5$, $p > 0.05$) and no interaction ($F_{(1,10)} = 0.6$, $p > 0.05$). With 56 mg/kg/day MIFE, the average evening concentration on Day 7 was 259.1 ± 203.6 ng/ml (95% CI [129.7, 388.4]) and decreased to 176.1 ± 124.7 ng/ml (95% CI [96.9, 255.3]) the morning after the final dose (Day 9; **Figure 2B)**. A RM-ANOVA revealed a main effect of time ($F_{(1,10)} = 5.2$, $p = 0.046$), but not group ($F_{(1,10)} = 0.03$, $p > 0.05$) and no interaction ($F_{(1,10)} = 0.8$, $p > 0.05$).

Mifepristone effect on ethanol and water intake in open-access availability

By six months of daily ethanol self-administration (151 to 153 consecutive open-access sessions), average daily ethanol intake ranged from 1.64 to 4.02 g/kg/day. The average BEC across 30-31 samples ranged from 23 to 135 mg/dl. These intakes and

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BECs represent a stage of ethanol intake when between-subject daily drinking patterns are predictable and demonstrate this cohort had two light drinkers, one binge drinker, four heavy drinkers and two very heavy drinkers, as previously defined (Baker et al., 2014). MIFE testing began on the 223rd day of open-access and continued until day 331st day of open-access (see **Figure 1A**).

Average daily ethanol and water intake was calculated during the four days prior to each MIFE dose (baseline) and compared to the four-day average intake during each dose of MIFE (see **Figure 1B**). The average daily intake between the three baseline phases had a coefficient of variation of less than 15% for each subject. A mixed effects model yielded a main effect of dose ($F_{(2,16)} = 5.9$, $p = 0.012$) and phase (two levels: baseline and MIFE; $F_{(1,8)} = 45.2$, $p < 0.001$), with lower ethanol intake during MIFE administration (**Figure 3A**). The interaction was also significant ($F_{(2,16)} = 17.9$, $p < 0.001$). Post hoc comparisons revealed an effect of baseline (mean: 3.2 g/kg/day, 95% CI [2.5, 3.9]) vs. 30 mg/kg/day MIFE (mean: 2.6 g/kg/day, 95% CI[1.9, 3.4]; $p_{adj} = 0.0018$) and baseline (mean: 3.2 g/kg/day; 95% CI [2.3, 4.1]) vs 56 mg/kg/day MIFE (mean: 1.8 g/kg/day, 95% CI [1.2, 2.5]; $p_{adj} < 0.0001$), but no effect of the 17 mg/kg/day dose and no differences among the three baseline phases. There were differences in intake between 30 and 56 mg/kg/day ($p_{adj} <0.001$) and between 17 and 56 mg/kg/day ($p_{adj} <0.001$). These data demonstrate a MIFE dose response where 30 and 56 mg/kg/day were effective in decreasing ethanol consumption. During the four days of MIFE dosing, the subjects drank an average of 81% and 57% of their baseline intake levels for 30 and 56 mg/kg/day, respectively.

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The effect of MIFE on water consumption was evaluated with a mixed effects model and yielded no main effect of phase ($F_{(5,50)}$ = 0.5, p > 0.05) or dose ($F_{(2,50)}$ = 2.3, p > 0.05), but a main effect of group, where controls consumed more water (controls: 151.9 ± 9.2 g/kg/day, 95% CI [143.9, 159.8]; drinkers: 92.8 ± 14.4 g/kg/day, 95% CI [81.1, 104.6]; $F_{(1,10)}$ = 5.5, p = 0.041). Therefore, while MIFE effectively reduced ethanol intake at both 30 and 56 mg/kg/day, water intake was not affected (**Figure 3B**). No food remained at the end of the sessions and body weights remained unchanged.

For the two effective doses of MIFE (30 and 56 mg/kg/day), the post-MIFE ethanol intakes were evaluated over the baseline, MIFE dosing and 8 sessions following the last MIFE administration, (post-1, and post-2) in 4 session "bins" (see **Figure 1B**). Using a RM-ANOVA, the 30 mg/kg/day dose had an effect on ethanol intake over time ($F_{(1.9, 15.2)}$ = 11.0, p = 0.0012). Post-hoc analyses compared each 4-day bin to baseline. There was a decrease in ethanol intake from baseline to MIFE dosing (Baseline: 3.2 ± 0.3 g/kg/day, (95% CI [2.5, 3.9]); 30 mg/kg/day MIFE: 2.6 ± 0.3 g/kg/day, (95% CI [1.9, 3.4]; p_{adj} = 0.0003) and from baseline to post-2 (2.8 ± 0.3 g/kg/day, 95% CI [2.0, 3.5]; p_{adj} = 0.0028) (**Figure 4A**). The 56 mg/kg/day dose also had decreased ethanol intake over time ($F_{(2, 16)}$ = 12.4, p = 0.0006). Post-hoc analyses indicate a decrease from baseline ethanol intake only during the four days of MIFE administration (Baseline: 3.2 ± 0.4 g/kg/day, 95% CI [2.3, 4.1]; 56 mg/kg/day MIFE: 1.8 ± 0.3 g/kg/day, 95% CI [1.2, 2.5]; p_{adj} = 0.0014) (**Figure 4B**).

During the 30 mg/kg MIFE dosing, blood ethanol concentration (BEC) was measured at baseline (Day 1; 103 ± 56 mg/dl, 95% CI [59, 146), on the third MIFE dosing day (Day 7; 59 ± 49 mg/dl, 95% CI [21, 96]), and four days following the end of

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the MIFE dosing during Post-1 (Day 12; 90 ± 65 mg/dl, 95% CI [40, 140]) (**Figure 4C**). A RM-ANOVA indicated a main effect of phase ($F_{(1.9, 15.2)}$ = 8.2, p = 0.0043). Correcting for multiple comparisons, BECs decreased during dosing (Day 7) when compared to baseline (p_{adj} = 0.0082). To evaluate the effect of 56 mg/kg/day MIFE on BEC, samples were measured at baseline (Day -2; 106 ± 80 mg/dl, 95% CI [45, 168]), the first day of MIFE dosing (Day 5; 83 ± 73 mg/dl, 95% CI [27, 139]), the last day of MIFE dosing (Day 8; 27 ± 47 mg/dl, 95% CI [-9, 64]), and six days following the last dose (Day 14; 91 ± 40 mg/dl, 95% CI [60, 121]) (**Figure 3D**). A RM-ANOVA indicated a main effect of phase ($F_{(1.8, 14.6)}$ = 6.2, p = 0.0124). Correcting for multiple comparisons, BECs had decreased on the last day of MIFE dosing (Day 8) compared to baseline (p_{adj} = 0.04), and between the last day of MIFE dosing (Day 8) and BECs 6 days later (Day 14), with BECs increasing when MIFE is no longer being administered (p = 0.0122). No differences were found between baseline and the first day of MIFE dosing (Day 5).

Cortisol response to MIFE treatment

Cortisol increased during MIFE administration, similar to previous reports (Yuen et al., 2017; Pal'chikova et al., 2016; Bertagna et al., 1984, Bertagna et al., 1984). For 30 mg/kg/day MIFE, a mixed effects model revealed a main effect of time ($F_{(3.30)}$ = 9.81, p = 0.0001), but not group. The interaction between time and group was significant ($F_{(3,30)}$ = 5.04, p = 0.006; **Figure 5A**). Post-hoc comparisons revealed that cortisol increased following the final dose of 30 mg/kg/day MIFE (Day 9: 16.9 ± 8.1 ug/dl, 95% CI [11.8, 22.0]) compared to pre-MIFE (Day 5, immediately before the first daily dose: 12.1 ± 4.9 ug/dl, 95% CI [9.0, 15.2]; p = 0.0003), but that this effect was driven by the

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ethanol-naïve controls (p = 0.0003). For 56 mg/kg/day MIFE, a mixed effects model revealed a main effect of time ($F_{(3.30)}$ = 41.68, p < 0.0001), but not group or an interaction (**Figure 5B**). Post-hoc comparisons revealed that cortisol following the final dose of 56 mg/kg/day MIFE (Day 9: 44.7 ± 14.2 ug/dl, 95% CI [35.7, 53.7]) increased when compared to pre-MIFE (Day 5, immediately before the first dose: 11.9 ± 3.5 ug/dl, 95% CI [9.7, 14.2]; p < 0.0001). The concentration of cortisol measured from plasma collected at 09:00 the morning after the final MIFE dose (Day 9) positively correlated with the concentration of MIFE in the same sample (r = 0.86, p < 0.0001; **Figure 5C**). Additionally, there was a negative correlation between MIFE concentration measured on Day 9 and the average percent change in ethanol intake during dosing (r = -0.63, p = 0.005; **Figure 5D**). With increasing MIFE concentrations there was a greater decrease in average ethanol intake.

Because MIFE concentration in plasma was highly correlated with both plasma cortisol concentration and ethanol intake, we evaluated whether cortisol was mediating the effect of MIFE on ethanol intake (**Figure 6**). Unlike covariates or moderators, mediators have a causal (in the current case, biological) rationale between the independent and dependent variables. Specifically, MIFE increases cortisol. This is the indirect effect. Regression analysis indicated that MIFE concentration the morning after the final dose (Day 9) predicted the change in ethanol intake during MIFE administration, β = -0.132, SE = 0.057, p = 0.0.035 and cortisol concentration on Day 9, β = 0.446, SE = 0.056, p < 0.0001. Cortisol was a predictor of the percent change in ethanol intake during MIFE administration, β = -0.311, SE = 0.107, p = 0.010. MIFE was no longer a predictor of percent change in ethanol intake after controlling for cortisol, β =

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0.034, SE = 0.122, p = 0.782, consistent with full mediation. The mediation effect was tested using nonparametric bootstrap confidence intervals with the percentile method using 500 simulations. These average causal mediation effect (ACME) shows that the indirect coefficient was significant, β = -0.166, 95% CI [-0.430, -0.02], p = 0.036. The average direct effect (ADE) when controlling for the mediator also supported mediation, β = 0.034, 95% CI [-0.17, 0.32], p = 0.74. The bootstrap analysis revealed that the proportion mediated to be 126%, again supporting that the effect of MIFE on ethanol consumption was mediated by the increase in circulating cortisol.

Effect of MIFE on relapse drinking

Similar to previous cohorts, cortisol was elevated during forced abstinence and all animals resumed drinking when ethanol in reintroduced (Cuzon Carlson et al., 2011; Allen et al., 2018). In these subjects, cortisol concentration was compared between open-access, collected one week prior to the second abstinence phase, and abstinence, 24 hours and 23 days into the second abstinence phase. A mixed effects model revealed a main effect of phase ($F_{(1.9, 18.7)}$ = 3.95, p = 0.040) where compared to open-access access (relapse 1: 11.0 $\pm$ 4.1 ug/dl, 95% CI [8.4, 13.6]) cortisol was higher during early (24 hour: 13.9 $\pm$ 5.1 ug/dl, 95% CI [10.7, 17.2], p = 0.017) and protracted abstinence (23 days: 14.6 $\pm$ 4.0 ug/dl, 95% CI [12.0, 17.1], p = 0.033, **Figure 7a**).

We compared the time to consume 1.5 g/kg ethanol and preference for ethanol on the first day of ethanol open-access following the first and second abstinence phases to the limited relapses during early abstinence presented above. A mixed effects model revealed a main effect of phase ($F_{(1.9, 17.2)}$ = 16.5, p = 0.0001) where posthoc analysis

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revealed that the time to consume 1.5 g/kg ethanol was higher following the first abstinence period (267.3 ± 92.7 minutes, 95% CI [181.6, 353.0]) compared to the pre-MIFE relapse (82.8 ± 57.9 minutes, 95% CI [34.3, 131.2]; p_{adj} = 0.0464), the post-MIFE relapse (20.2 ± 6.8 minutes, 95% CI [14.5, 25.9]; p_{adj} = 0.0022) and following the second abstinence phase (76.2 ± 94.6 minutes, 95% CI [-2.8, 155.3]; p_{adj} = 0.0449; **Figure 7b**). There was also a main effect of phase for preference ($F_{(3,28)}$ = 4.6, p = 0.0099). Posthoc analysis revealed that preference for ethanol following the first abstinence phase was lower (34.5 ± 12.0%, 95% CI [24.5, 44,5]) when compared to both post-MIFE during abstinence (61.0 ± 17.5%, 95% CI [46.4, 75.6; p_{adj} = 0.0219) and the second abstinence phase (61.4 ± 18.9%, 95% CI [45.6, 77.2]; p_{adj} = 0.0196; **Figure 7c**).

DISCUSSION

MIFE, an antagonist at both the glucocorticoid (GR) and progesterone receptors (PR), reduced ethanol consumption in this rhesus macaque model of long-term ethanol self-administration in a selective and dose-dependent manner. Extended use of MIFE is currently approved for cases of Cushing's disease, but at a maximum of 20 mg/kg/day and is not recommended to exceed 600 mg/day (about 8-10 mg/kg) in patients with hepatic impairment. MIFE has been suspected of causing drug induced liver impairment (Funke and Rockey, 2018; Shah et al., 2019), In this study, the threshold effective dose regimen was 30 mg/kg/day for 4 days. This dose decreased the average daily ethanol intake of 9 monkeys from 3.2 to 2.3 g/kg/day. In contrast, a study in baboons with limited daily access to ethanol and consuming about 1 g/kg/day found 30 mg/kg MIFE

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was not effective in reducing ethanol self-administration (Holtyn and Weerts, 2019). A greater decrease in average daily intake was observed during 56 mg/kg of MIFE for four days with average daily ethanol intake decreasing from 3.2 to 1.8 g/kg/day, or 57% of baseline. This is approximately 12-13 drink-equivalents cut to 7-8 drink-equivalents. Although consumption remained high, the health benefits of reducing ethanol intake by almost half are clinically relevant (Witkiewitz et al., 2018; Knox et al., 2018; Pearson et al., 2017; Charlet and Heinz, 2017). One important caveat in the current study is the rapid return to baseline drinking when MIFE treatment ended. It remains to be determined if longer-term administration of MIFE would have been more effective in reducing ethanol intake. However, long-term MIFE administration at these doses may increase the risk of adrenal insufficiency seen in clinical populations, such as Cushing's syndrome, although we did not find evidence of this in our study. In this study there were no observed changes in food or fluid intake that would indicate illness. Hypokalemia was not detected and, in fact, potassium concentration increased following MIFE administration (**Supplementary Figure 1**). Hypoglycemia resulting from MIFE administration has been reported (Humayun and Masding, 2016). Blood glucose decreased following 56 mg/kg MIFE when administered during open-access, but not during abstinence (Supplementary Figure 2) Thus, the doses used here are a concern, particularly for extended treatment, and may be a barrier for use in a clinical setting. As stated above, the FDA has capped daily MIFE administration at 20 mg/kg/day (Sai et al., 2019; Castinetti et al., 2010). However, 600 mg/day for seven days was effective in reducing craving and the number of drinks per week in a small study of non-treatment seeking alcohol-dependent subjects (Vendruscolo et al., 2015).

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One implication of the NHP studies is that the ability of MIFE to reduce ethanol drinking may be restricted to chronic heavy intakes greater than 8 drink equivalents (>2 g/kg) per day. That MIFE efficacy be restricted to heavy drinking is supported by rodent studies where MIFE was effective only in dependent animals whose intakes were higher than non-dependent animals (Repunte-Canonigo et al., 2015; Simms et al., 2012; Somkuwar et al., 2017; Vendruscolo et al., 2012, 2015). In addition, the two lightest drinkers in the current study increased their average daily ethanol intake following the 56 mg/kg MIFE treatment, indicating the possibility that some subjects may be vulnerable to a rebound effect. Together, these data suggest that there may be a minimum threshold of daily ethanol consumption for MIFE to be therapeutic, and that below this threshold MIFE may be ineffective or contraindicated.

In terms of receptor specificity related to the decrease in alcohol intake, both GR and PR act as ligand-dependent transcription factors and also have non-genomic actions mediated by second-messenger signaling pathways (Leonhardt et al., 2003; Lösel and Wehling, 2003; Rainville et al., 2020). Although MIFE is not a selective antagonist, the effects on ethanol drinking in rodents appear to be through GRs. Vendruscolo and colleagues (2015) demonstrated that MIFE reduced ethanol intake in dependent rats and the effect was similar when CORT113176, a GR-specific antagonist, was administered. More recently, selective breeding for high binge-like ethanol intake drinking found in ‘High Drinking in the Dark’ (HDID-1) mice were more sensitive to GR antagonism, as demonstrated by both MIFE and CORT113176 dosing (Savarese et al., 2020). Further, central GR expression is influenced by ethanol exposure and withdrawal in several limbic and reward regions (Vendruscolo et al., 2012;

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Roy et al., 2002; Repunte-Canonigo et al., 2015). However, there are mixed outcomes with site-specific MIFE administration, with both positive (Simms et al., 2012; Vendruscolo et al., 2012, 2015), and negative (Repunte-Canonigo et al., 2015) outcomes when MIFE is delivered to the central nucleus of the amygdala (CeA) and one report that MIFE administration to the ventral tegmental area and the nucleus accumbens decreased ethanol intake (Repunte-Canonigo et al., 2015). There are no studies in macaques directly addressing site-specific effects of MIFE, but the positive correlation between cortisol and circulating MIFE strongly suggests an effect through blocking GRs and inhibiting negative feedback to regulate cortisol levels in macaques. A further finding was that the increase in cortisol following MIFE administration was a mediating factor in decreasing ethanol drinking. Cortisol was not solely responsible for the change in intake, but the explained variance between MIFE and intake is greater when considering the effect of MIFE on cortisol. The analysis reported here supports a full mediation, however additional resesarch should be done to validate these findings. Of particular interest is whether cortisol mediates the effect of MIFE on ethanol intake in human alcoholics at doses that have been shown previously to reduce intake (Vendruscolo et al., 2015). Plasma cortisol also mediates the effect of MIFE on psychotic symptoms in a recent study (Block et al., 2018). Alternatively, MIFE-induced increases in cortisol may only be a biomarker of MIFE action at the GR and not directly related to reducing alcohol intake. This interpretation is not supported by studies of naloxone, a mu-opioid receptor antagonist that decreases craving and drinks consumed and also increases cortisol (Hendershot et al., 2017; O'Malley et al., 2002; Wand et al., 2001). Together, MIFE and naloxone outcomes on ethanol drinking suggesting multiple

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mechanisms that increase cortisol may underlie positive outcomes in alcohol pharmacotherapy. However, this may be too simplistic of an explanation, as increased cortisol due to forced abstinence did not prevent relapse in this monkey model and stressful events, that presumably increase cortisol, increase probability of a relapse in humans (Keyes et al., 2012; Wemm et al., 2019). Further, the relationship between cortisol and MIFE's effects on intake is counter-intuitive as they each have opposite effects on the GR.

An alternative explanation of MIFE induced increases in cortisol mediating a decrease in alcohol intake is that cortisol has approximately 10-fold higher affinity for the mineralocorticoid receptor (MR). Under healthy non-stressful basal conditions most cortisol is bound to MRs. The role of MR in alcohol dependence and withdrawal has not been studied as extensively as GRs, but recent data suggest that MR may also be a promising pharmacological target. In humans, the principal ligand for the MR, aldosterone, is positively correlated with craving and the number of drinks consumed (Leggio et al., 2008; Aoun et al., 2017). In this rhesus macaque model during long-term daily self-administration, circulating aldosterone was increased and expression of the MR gene (NR3C2) in the CeA was negatively correlated with average daily intake (Aoun et al., 2017). In rodents, ethanol exposure did not alter MR expression (Vendruscolo et al., 2012) however MR-antagonism by spirolactone was effective in reducing ethanol seeking (Makjijani et al., 2018). Importantly, the balance between MR and GR is critical for maintaining homeostasis (de Kloet and Joëls, 2020). The indication that both long-term ethanol and repeated MIFE administration impact HPA axis activation and the balance of MR and GR warrants further investigation.

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Repeated periods of forced abstinence (up to 34 days) following 12 months of daily access to alcohol in macaques reliably results in elevated cortisol and an immediate relapse to drinking once ethanol is reintroduced (Cuzon Carlson et al., 2011; Allen et al., 2018). Synaptic recordings from abstinent monkeys revealed increased excitatory activity onto parvocellular neurons in the hypothalamic paraventricular nucleus (PVN), the apex of the HPA axis, compared to ethanol-naïve controls (Jimenez et al., 2019). The frequency of excitatory events onto parvocellular neurons correlated with circulating cortisol and was normalized by applying 20 mM (approximately 92 mg/dl) ethanol. Thus, forced abstinence revealed an allostatic shift in glutamatergic activity within the PVN that was related to cortisol and influenced by ethanol. These synaptic studies demonstrate an important allostatic shift in activity at the apex of the stress response that may be driven by changes in GR expression or activity in the PVN and other limbic regions, such as the central nucleus of the amygdala (Simms et al., 2012; Vendruscolo et al., 2015). Furthermore, the rodent studies discussed previously and the data presented here demonstrate that ethanol dependence is necessary for MIFE to reduce ethanol consumption, suggesting that cycling between drinking and abstaining may expose vulnerability in the GR system that is targetable using GR-antagonists.

To test if MIFE treatment could block relapse to alcohol drinking, the highest effective dose of MIFE (56 mg/kg/day) was tested in a modified dosing of 3 daily doses during early abstinence. Under these conditions, MIFE did not block relapse. In fact, the time to consume 1.5 g/kg decreased and preference for ethanol increased following MIFE administration in relapse. In rodents MIFE blocks the post-abstinence escalation

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of ethanol intake but also did not eliminate intake (Somkuwar et al., 2017; Vendruscolo et al., 2012; Vendruscolo et al., 2015). Given the decline in MIFE concentration the morning after the final dose (Figure 2B) and the rapid return to drinking after dosing ended during open-access conditions (Figure 4B), MIFE may have been more effective if administration had continued during the ethanol slip, rather than ending the evening prior. However, these data do not suggest that tolerance to MIFE is responsible for the lack of effect in abstinence as there were over 7 months (222 days) between the last MIFE dose during open-access and abstinence.

In conclusion, epidemiological data suggest that there have been similar rates of treatment seeking over the past several decades (Hasin et al., 2007; Substance Abuse and Mental Health Services Administration, 2019). Pharmacological treatment options that are not tied to abstinence may help encourage a greater number of people to seek help (McGinty et al., 2015). The data presented here, and a previous report on non-treatment seeking alcohol-dependent subjects (Vendruscolo et al., 2015) suggest MIFE may be an effective pharmacological option for harm reduction in individuals who are not able, or interested, in abstinence. However, additional research is needed to determine whether MIFE can be an effective treatment in acute situations, similar to Disulfiram, or would be safe for long-term administration.

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AUTHORSHIP CONTRIBUTIONS

- Participated in research design: KA Grant, H Hunt
- Conducted experiments: N Newman, K Diem, H Vanderhooft, TA Shnitko
- Performed data analysis: NAR Walter, VA Jimenez
- Wrote or contributed to the writing of the manuscript: VA Jimenez, NAR Walter, KA Grant

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FOOTNOTES

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FIGURE LEGENDS

Figure 1 | Experimental timeline and mifepristone (MIFE) administration. A) Complete experimental timeline. B) The dosing regimen occurred in a 16 day cycle, divided into four experimental phases which are referred to by dosing day throughout the results. Detailed hour-by-hour timeline during 56 mg/kg/day MIFE dosing in open-access. C) A 1.5 g/kg ethanol dose was available on the 8th day of the second abstinence phase two hours into the daily session and again on the 13th day of abstinence, after three days of 56 mg/kg/day MIFE. AB1: first forced abstinence, AB2: second forced abstinence, Nec: necropsy, BEC: blood ethanol concentration. Note the phases, days and colors shown in panels B and C are used in the following figures.

Figure 2 | MIFE concentration in plasma measured in the evening of the third daily dose (Day 7) and the morning after the final dose (Day 9) for 30 mg/kg (A) and 56 mg/kg (B). Data represents individual (n=9 drinkers, filled circles; n=3 controls, open circles) and average (bars) MIFE concentration. Note the y-axis scales are not identical. *$p < 0.05$.

Figure 3 | Effect of MIFE on average ethanol (A) and water (B) intake during baseline (blue bars, four days immediately prior to each MIFE dose) and during MIFE administration (red bars, four days). Data represents individual (n=9 drinkers, filled circles; n=3 controls, open circles) and average (bars). * $p < 0.05$, *** $p < 0.001$.

Figure 4 | The effect of MIFE on average daily ethanol intake and blood ethanol concentration (BEC) across the phases shown in Figure 1B. A and B: Individual (circles)

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and average (bars) ethanol intake during experimental phases (4 days consecutive days) with 30 mg/kg and 56 mg/kg MIFE. C and D: Effect of 30 mg/kg and 56 mg/kg on BEC. Bars represent the group average, individuals represented by circles, experimental day shown in parenthesis below. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5 | Effect of MIFE on cortisol concentration measured in the AM during 30 mg/kg (A) and 56 mg/kg (B) dosing. Cortisol on Day 5 was collected prior to the first MIFE dose and represents a baseline. (C) Relationship between cortisol and MIFE concentrations measured the morning after the final MIFE dose (Day 9). (D) Relationship between MIFE concentration measured the morning after the final dose (Day 9) and the average individual percent change in daily ethanol intake between baseline (Days 1-4) and MIFE dosing (Days 5-8). Data represent individuals (n = 9 drinkers, n = 3 controls). * $p < 0.05$, *** $p < 0.001$.

Figure 6 | Summary of mediation analysis. (A) The direct model of MIFE concentration the morning after the final dose (Day 9) and the percent change in ethanol intake during MIFE (Days 5-8) relative to baseline (Days 1-4). (B) The mediation model with cortisol measured the morning after the final dose (Day 9) as a mediator between MIFE concentration and percent change in ethanol self-administration. Path values correspond to unstandardized coefficients and significance. ACME: average causal mediation effect. * $p < 0.05$, *** $p < 0.0001$, ns: $p > 0.05$.

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Figure 7 | Cortisol concentration in open-access (Relapse 1) and during early (24 hour) and late (23 days) abstinence 2 (A). The time to consume a limited 1.5 g/kg ethanol dose (B) and preference for ethanol (C) were evaluated during the first day of Relapse-1 (post-ABS1), during abstinence 2 before and after 56 mg/kg MIFE administration and the first day of Relapse-2 (post-ABS2). Grey bars represent abstinence 2 (AB2). Data represent average (bars) and individuals (closed circles: n = 8 drinkers, open-circles: n = 3 controls). * $p < 0.05$, ** $p < 0.01$.