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Mifepristone in the Central Nucleus of the Amygdala Reduces Yohimbine Stress-Induced Reinstatement of Ethanol-Seeking

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Chronic ethanol exposure leads to dysregulation of the hypothalamic-pituitary-adrenal axis, leading to changes in glucocorticoid release and function that have been proposed to maintain pathological alcohol consumption and increase vulnerability to relapse during abstinence. The objective of this study was to determine whether mifepristone, a glucocorticoid receptor antagonist, plays a role in ethanol self-administration and reinstatement. Male, Long–Evans rats were trained to self-administer either ethanol or sucrose in daily 30 min operant self-administration sessions using a fixed ratio 3 schedule of reinforcement. Following establishment of stable baseline responding, we examined the effects of mifepristone on maintained responding and yohimbine-induced increases in responding for ethanol and sucrose. Lever responding was extinguished in separate groups of rats and animals were tested for yohimbine-induced reinstatement and corticosterone release. We also investigated the effects of local mifepristone infusions into the central amygdala (CeA) on yohimbine-induced reinstatement of ethanol- and sucrose-seeking. In addition, we infused mifepristone into the basolateral amygdala (BLA) in ethanol-seeking animals as an anatomical control. We show that both systemic and intra-CeA (but not BLA) mifepristone administration suppressed yohimbine-induced reinstatement of ethanol-seeking, while only systemic injections attenuated sucrose-seeking. In contrast, baseline consumption, yohimbine-induced increases in responding, and circulating CORT levels were unaffected. The data indicate that the CeA plays an important role in the effects of mifepristone on yohimbine-induced reinstatement of ethanol-seeking. Mifepristone may be a valuable pharmacotherapeutic strategy for preventing relapse to alcohol use disorders and, as it is FDA approved, may be a candidate for clinical trials in the near future.
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INTRODUCTION

The link between stress and chronic relapsing alcohol-use disorders (AUDs) has long been established in the alcohol research field (Brown *et al*, 1995; Cooper *et al*, 1992; Koob and Le Moal, 1997; Russell and Mehrabian, 1975; Sinha, 2001). Stress and re-exposure to cues or to the context previously associated with drug availability are common reasons for relapse to drug-seeking in humans and induce reinstatement of drug-seeking in rodents (Brown *et al*, 1995; Liu and Weiss, 2003; Shaham *et al*, 2000; Zironi *et al*, 2006). Response to stress begins with the activation of the hypothalamic-pituitary-adrenal (HPA) axis, leading to increases in glucocorticoid (GC) release. GCs, which bind to GC receptors (GRs), mediate adaptation to stress and regulate termination of the stress response through negative feedback at the level of the HPA axis (De Kloet and Reul, 1987; Diorio *et al*, 1993; Magarinos *et al*, 1987). Chronic stress and impaired GR feedback have been proposed to lead to the dysregulation of HPA axis activity. The feedback response in the extrinsic HPA structures such as the amygdala, hippocampus, and prefrontal cortex is regulated by the GR, which, in the absence of hormone, resides in the cytoplasmic compartment (Adzic *et al*, 2009). Upon ligand binding, GR translocates to the nucleus, and regulates neuronal target gene expression (Beato and Sanchez-Pacheco, 1996), including downregulation of the GR itself. However, under chronic stress this feedback becomes deregulated, leading to the variety of maladaptive syndromes such as anxiety and various forms of depressive disorders (Sapolsky, 2000) and addiction, including alcohol dependence (Koob, 2008). Indeed, chronic ethanol intake leads to alterations in the homeostatic functioning of GCs

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(cortisol in humans, corticosterone (CORT) in rodents), which can lead to neuroadaptations that increase susceptibility to AUDs (Koob, 2008; Shaham and Hope, 2005).

Mifepristone, also known as RU486, is a derivative of the 19-norprogestin norethindrone and potently competes with type II GRs and progesterone receptors (PRs). Antagonism of GRs has been shown to modulate several physiological systems, including the central nervous system, immune response, metabolism, digestive, renal, and reproductive systems (Lu *et al*, 2006). The combination of mifepristone (Mifeprex) with misoprostol (Cytotec), a prostaglandin analog, is currently FDA approved for the termination of early pregnancy (Lu *et al*, 2006; Mahajan and London, 1997). The anti-GC activity of mifepristone has made it a potential treatment for Cushing's syndrome (Johanssen and Allolio, 2007) and neurological and psychological disorders (DeBattista and Belanoff, 2006; Gallagher and Young, 2006; Gallagher *et al*, 2005, 2008; Wulsin *et al*, 2010; Young, 2006). The drug has also been examined in the self-administration of amphetamine (De Vries *et al*, 1996), cocaine (Deroche-Gamonet *et al*, 2003; Fiancette *et al*, 2010), morphine (Mesripour *et al*, 2008), and ethanol, where it has been shown to have either no effect or decrease baseline ethanol consumption (Fahlke *et al*, 1995; Jacquot *et al*, 2008; Koenig and Olive, 2004; Lowery *et al*, 2010; O'Callaghan *et al*, 2005; Roberts *et al*, 1995; Yang *et al*, 2008). However, the role of mifepristone in stress-induced reinstatement of ethanol-seeking is not known.

Intermittent footshock is often used to study stress-induced reinstatement in rodents and effectively reinstates heroin (Shaham *et al*, 1997), cocaine (Erb *et al*, 1998), nicotine (Buczek *et al*, 1998), and ethanol-seeking (Le *et al*, 1998). Recently, the pharmacological stressor yohimbine has been shown to be an alternative, not only in its ability to reinstate drug-seeking, but also in its effects on corticotrophin-releasing factor (CRF) production and activation of the same circuitry as footshock (Funk *et al*, 2006). Yohimbine is an alkaloid that acts as an α-2 adrenoceptor antagonist, leading to the release of noradrenaline, which stimulates the sympathetic nervous system and CORT release. Yohimbine induces reinstatement of methamphetamine (Shepard *et al*, 2004), cocaine (Lee *et al*, 2004), heroin (Banna *et al*, 2010), palatable food (Ghitza *et al*, 2006), and ethanol-seeking (Le *et al*, 2005; Simms *et al*, 2011). Furthermore, in humans recovering from AUDs, yohimbine stress increases anxiety and cortisol levels and the cortisol response is elevated in subjects with AUDs compared with controls (Krystal *et al*, 1994, 1996). The main aim of this study was to determine the role of the GR antagonist, mifepristone, in yohimbine-induced reinstatement of ethanol-seeking. We present evidence which suggests that GRs (and potentially PRs) in the central nucleus of the amygdala (CeA) play a role in yohimbine-induced reinstatement of ethanol-seeking.

MATERIALS AND METHODS

Subjects

Male, Long–Evans rats weighing 150–180 g upon arrival (Harlan Indianapolis, IN) were individually housed in ventilated Plexiglas cages. Rats were housed in a climate-controlled room on a 12 h light–dark cycle (lights on at 0700 h). Food and water were available *ad libitum*, except for short periods during initial training, as outlined below. All procedures were pre-approved by the Gallo Center Institutional Animal Care and Use Committee and were in accordance with NIH guidelines for the Humane Care and Use of Laboratory Animals.

Drugs

Yohimbine (Sigma-Aldrich, St Louis, MO) was dissolved in distilled water and administered at a dose of 2 mg/kg in a volume of 0.5 ml/kg intraperitoneally (i.p.). Mifepristone (Sigma-Aldrich) was suspended in 1% Tween-80 and 25% β-cyclodextrin in saline and stirred for 2 h before systemic injections (i.p.). For intra-amygdala infusions, mifepristone was dissolved in 100% DMSO. Owing to the small size of the central amygdala (CeA) and basolateral amygdala (BLA) and to limit diffusion, the drug was infused in a volume of 0.3 µl over 2 min, after which the injectors were left in position for an additional minute. To examine brain tissue for signs of cell death following drug or vehicle delivery, a group of rats was cannulated in the CeA ($n = 3$) and administered six infusions of either vehicle (100% DMSO), mifepristone (10 µg) dissolved in DMSO, or phosphate-buffered saline (PBS). For analysis, the tissue was then stained with Hoechst 33342 for labeling viable nuclei and quantified using the Imaris Neuroscience software pack (v.7.1.1; Andor Technology, Belfast, UK) (for details, see Supplementary materials and Lin *et al* (2006)). Sucrose solutions, 5 or 10% (w/v) (Fisher Scientific, Fairlawn, NJ), were prepared using filtered water. The 10 and 20% ethanol (v/v) solutions were prepared using 95% ethyl alcohol and filtered water. In the sucrose-fade experiments, 10, 5, 3, and 1.5% sucrose, respectively, were dissolved in 10% ethanol (w/v).

Self-Administration Apparatus and Training

Self-administration testing was conducted in standard operant conditioning chambers (Coulbourn Instruments, Allentown, PA). Details regarding this apparatus have been described elsewhere (Richards *et al*, 2008). Long–Evans rats ($n = 12$–14 per group) were trained to self-administer 10% ethanol using a modified sucrose-fading procedure, as described previously (Simms *et al*, 2010). Separate groups of Long–Evans rats ($n = 12$–15 per group) were trained to self-administer 5% sucrose, using a protocol similar to that used for 10% ethanol, except for the substitution of 5% sucrose as the reinforcer throughout the experiment. Two additional groups of Long–Evans rats ($n = 7$–11 per group) were trained to self-administer 20% ethanol without the use of a sucrose-fading procedure, as described previously (Simms *et al*, 2010). All animals were trained to self-administer their respective solution in daily 30-min fixed ratio 3 (FR3) sessions (0.1 ml per reinforcer) for a minimum of 20 sessions. For ethanol-trained groups, animals not reaching 0.25 g/kg intake per session were excluded from further study. For sucrose-trained groups, animals not reaching 40 active lever presses per session were excluded from further study (for detailed methods, see Supplementary materials).

Extinction

To extinguish lever pressing, rats continued with daily operant sessions under FR3 conditions; however, active lever pressing did not result in reinforcer delivery despite light and tone cues being presented. Extinction training continued until the rats responded with $<10\%$ of their baseline pressing on the active lever for two consecutive sessions. After this time, yohimbine-induced reinstatement tests were performed with regular extinction sessions on non-reinstatement days.

Effect of Mifepristone on Yohimbine-Induced Reinstatement of 10% Ethanol- and 5% Sucrose-Seeking

Animals trained to self-administer 10% ethanol ($n=26$) and 5% sucrose ($n=12$) were extinguished as described above and assigned to groups matched for their previous operant self-administration and extinction responding. We used the between-subjects factor of mifepristone dose (0, 5, or 30 mg/kg, i.p.) and the within-subjects factor of yohimbine dose (0 and 2 mg/kg, i.p.) to assess the effect of mifepristone on yohimbine-induced reinstatement of ethanol-seeking. Animals received two injections on test days. The rats were first injected with either mifepristone or its vehicle and 30 min later with either yohimbine or vehicle. Rats were placed into the operant self-administration chambers 30 min after the second injection. Reinstatement sessions were conducted under the same conditions as the extinction sessions; successful FR3 responses at the previously active lever resulted in light and tone cue presentation with no reinforcer delivery. Inactive lever responding had no programmed consequences as a measure of nonspecific behavioral activation. Yohimbine and vehicle were administered over two test sessions, 7 days apart for animals trained to self-administer 10% ethanol with regular extinction sessions on the days between tests. Ethanol-trained rats were tested for reinstatement only following the first yohimbine exposure as we have recently shown that this behavior decays with subsequent yohimbine challenges in animals trained with a sucrose-fading procedure (Simms *et al*, 2011). Animals trained to self-administer 5% sucrose were tested as described above for 10% ethanol, with the exception being that mifepristone was administered in a Latin-square design (ie, each animal was assigned to one mifepristone dose per week, with a vehicle challenge on Wednesday and a yohimbine challenge on Friday). Each sucrose animal received all the doses of mifepristone across a 3-week test period.

CORT Measurements following Mifepristone and Yohimbine Administration

To examine the effects of mifepristone and yohimbine on circulating CORT levels, blood was collected from extinguished rats trained to respond for 10% ethanol ($n=12$). Tests were conducted across 2 weeks. Each animal received either a mifepristone (30 mg/kg, i.p.) or vehicle injection, followed 30 min later by a vehicle injection in week 1 and a yohimbine (2 mg/kg) injection in week 2. At 30 min after the yohimbine or vehicle treatment, the animals were anesthetized with isoflurane and blood was collected from the lateral tail vein. Samples were centrifuged at 4 °C for 13 min at 8000 r.p.m., and then stored at −80 °C. Serum CORT concentrations were determined by ELISA (Assay Designs, Ann Arbor, MI).

Effect of Microinfusions of Mifepristone into the Amygdala on Yohimbine-Induced Reinstatement of 20% Ethanol- and 5% Sucrose-Seeking

We have recently shown that yohimbine-induced reinstatement of 20% ethanol-seeking is stable across multiple yohimbine test sessions, thereby making within-subject experimental designs possible (Simms *et al*, 2011). Therefore, we utilized this model to determine the role of the amygdala in mifepristone's effect on yohimbine-induced reinstatement. Animals trained to self-administer 20% ethanol were bilaterally implanted with guide cannulae (C315G, 26 G; Plastics One) aimed dorsal to the CeA ($n=18$; AP −2.1, ML ±4.1, DV −6.0) and the BLA ($n=10$; AP −2.12, ML ±5.0, DV −6.5) according to Paxinos and Watson (1997). In addition, one group of animals trained to self-administer 5% sucrose ($n=12$) was cannulated in the CeA as a control for the ethanol experiments. For detailed methods on the surgical and histological procedures, see Supplementary materials. Following surgery, the animals were given two additional weeks of reinforced self-administration to insure that the surgery did not disrupt responding. Lever responding was then extinguished over 12–18 sessions. The first week of reinstatement testing was carried out without any mifepristone manipulation to determine baseline reinstatement levels. Yohimbine vehicle was administered on Wednesday and yohimbine (2 mg/kg) was administered on Friday with regular extinction sessions run on non-test days. The rats were then assigned to groups matched for their previous operant self-administration, extinction, and week 1 reinstatement responding. Starting with the second reinstatement week, we used the within-subjects factors of mifepristone dose (0, 5, or 10 μg per side for CeA animals; 0 or 10 μg per side and PBS for BLA animals) and yohimbine dose (0 or 2 mg/kg, i.p.) to assess the effect of mifepristone in the CeA and BLA on yohimbine-induced reinstatement. Animals received an intra-amygdala infusion and an i.p. injection on test days. The rats were first infused with either mifepristone or its vehicle and 10 min later they were injected with either yohimbine or yohimbine vehicle. Rats were placed into the operant self-administration chambers 30 min after the yohimbine or vehicle injection for the reinstatement test session. Each animal received all the doses of mifepristone across a 3-week test period. Reinstatement sessions were conducted under the same conditions as described above.

Effect of Mifepristone on 10% Ethanol and 5% Sucrose Self-Administration and Yohimbine-Induced Increases in 10% Ethanol and 5% Sucrose Self-Administration

The effects of mifepristone were also assessed on maintained responding for 10% ethanol ($n=12$) and 5% sucrose ($n=15$), and yohimbine-induced increases in responding for 10% ethanol ($n=11$) and 5% sucrose ($n=14$), following a minimum of 20 FR3 operant sessions. For detailed methods, see Supplementary material.

Data Analysis

Statistical analyses were performed using SigmaStat version 3.5 (Systat Software, San Jose, CA). Lever presses for the yohimbine reinstatement groups were analyzed by one- and two-way analysis of variance (ANOVA) and paired *t*-test for baseline reinstatement in the amygdala groups. CORT was analyzed using a repeated-measures two-way ANOVA. Data for the mifepristone self-administration studies in the maintenance phase were analyzed by repeated-measures one-way ANOVA. All ANOVA tests were followed by Newman–Keuls *post hoc* test, where statistical significance was $p<0.05$. All data are presented as mean ± SEM.

RESULTS

Effect of Mifepristone on Yohimbine-Induced Reinstatement of 10% Ethanol-Seeking

Pretreatment with mifepristone significantly attenuated yohimbine-induced reinstatement of 10% ethanol-seeking. Statistical analysis of active lever pressing using a two-way ANOVA revealed an overall effect of pretreatment dose of mifepristone (0, 5, or 30 mg/kg) ($F(2,47)=3.67$, $p<0.05$) and yohimbine dose (0 or 2 mg/kg) ($F(1,47)=24.40$, $p<0.001$), but no interaction between mifepristone dose × yohimbine dose (although there was a trend, $p=0.09$). To further explore the effect of mifepristone on yohimbine-induced reinstatement, we performed a one-way ANOVA focusing just on the effect of mifepristone following yohimbine treatment. This analysis revealed a significant effect of mifepristone (0, 5, or 30 mg/kg) ($F(2,23)=3.85$, $p<0.05$), and *post hoc* analysis revealed that there was a significant difference between active lever presses for the vehicle and the 30 mg/kg doses ($p<0.05$; Figure 1a). Mifepristone pretreatment had no effect on responding on the active lever following yohimbine vehicle delivery. Statistical analysis of inactive lever pressing using a two-way ANOVA revealed an overall effect of yohimbine dose (0 or 2 mg/kg) ($F(1,47)=9.06$, $p<0.01$; Table 1), but no effect of pretreatment dose of mifepristone or interaction between mifepristone dose × yohimbine dose. Yohimbine-induced increases in inactive lever presses have been reported in the literature (Le *et al*, 2011; Marinelli *et al*, 2007); however, these effects are inconsistent as our group and others have failed to find such an effect (Le *et al*, 2005; Nielsen *et al*, 2011; Richards *et al*, 2008). More evidence of this inconsistency is offered in this study, where we show that inactive lever responding is increased only in the animals trained to respond for 10% ethanol, and not 20% ethanol or 5% sucrose. Two ethanol-trained animals were excluded from statistical analysis: one failed to extinguish and the other failed to consume 0.25 g/kg per session in the maintenance phase.

Effect of Mifepristone on Yohimbine-Induced Reinstatement of 5% Sucrose-Seeking

Pretreatment with mifepristone attenuated yohimbine-induced reinstatement of sucrose-seeking. Statistical analysis of active lever pressing using repeated-measures two-way ANOVA revealed an overall effect of yohimbine dose (0 or 2 mg/kg) ($F(1,53)=14.18$, $p<0.01$), but no effect of mifepristone pretreatment and no interaction between mifepristone dose × yohimbine dose (although there was a strong trend, $p=0.07$). To further explore the effect of

Table 1 Inactive Lever Presses in Groups of Ethanol-Extinguished Rats given Mifepristone prior to Yohimbine (or Vehicle)

Mifepristone (mg/kg)	Inactive lever presses	
	Vehicle group	Yohimbine group
Vehicle	0.63 ± 0.28	2.13 ± 0.68
5	0.38 ± 0.28	1.25 ± 0.44
30	0.25 ± 0.17	0.63 ± 0.28

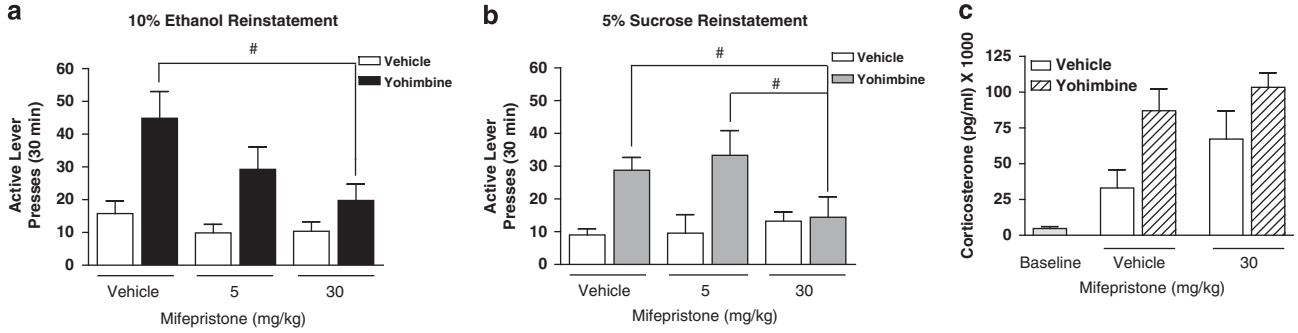

Figure 1 Systemic administration of the glucocorticoid receptor (GR) antagonist mifepristone significantly attenuates yohimbine-induced reinstatement of ethanol- and sucrose-seeking, but does not affect sera corticosterone (CORT) levels. Animals were pretreated with mifepristone (0, 5, or 30 mg/kg) to examine its effects on yohimbine- (2 mg/kg, intraperitoneally (i.p.)) induced reinstatement of ethanol- and sucrose-seeking. (a) Yohimbine significantly reinstates active lever responding in ethanol-trained rats, an effect that is attenuated by mifepristone ($^{\#}p<0.05$ comparing yohimbine reinstatement following mifepristone vehicle pretreatment to reinstatement following 30 mg/kg mifepristone pretreatment). (b) Yohimbine significantly reinstates active lever responding in sucrose-trained rats, an effect that is attenuated by mifepristone ($^{\#}p<0.05$ comparing yohimbine reinstatement following mifepristone vehicle and 5 mg/kg pretreatment to reinstatement following 30 mg/kg mifepristone pretreatment). (c) Mifepristone pretreatment (30 mg/kg) has no effect on yohimbine-induced increases in CORT. Data are presented as mean ± SEM ($n=24$ for ethanol reinstatement study, $n=9$ for sucrose reinstatement study, and $n=12$ for CORT study). Statistical analysis was performed by analysis of variance (ANOVA) with Newman–Keuls *post hoc* testing.

mifepristone on yohimbine-induced reinstatement, we performed a repeated-measures one-way ANOVA focusing just on the effect of mifepristone following yohimbine treatment. This analysis revealed a significant effect of mifepristone (0, 5, or 30 mg/kg) ($F(2,26) = 4.14$, $p < 0.05$), and *post hoc* analysis revealed that there were significant differences between active lever presses for both vehicle and the 5 mg/kg doses compared to the 30 mg/kg dose ($p < 0.05$; Figure 1b). Mifepristone pretreatment had no effect on responding on the active lever following yohimbine vehicle delivery. Statistical analysis of inactive lever pressing using a repeated-measures two-way ANOVA for the sucrose-trained rats revealed no overall effect of yohimbine or mifepristone treatment (data not shown). Five sucrose-trained animals were excluded from statistical analysis: three failed to reinstate in any of the test weeks and two others failed to meet the acquisition criteria (<40 presses) in the maintenance phase.

The Effect of Mifepristone on Blood CORT Levels

Mifepristone had no effect on yohimbine-induced increases in CORT. Statistical analysis of sera CORT, using a repeated-measures two-way ANOVA, revealed an overall effect of yohimbine treatment ($F(1,23) = 14.22$, $p < 0.01$), but no overall effect of mifepristone pretreatment or yohimbine × mifepristone interaction (Figure 1c).

Effect of Microinfusions of Mifepristone into the Amygdala on Yohimbine-Induced Reinstatement of 20% Ethanol- and 5% Sucrose-Seeking

Microinfusions of mifepristone into the CeA significantly attenuated yohimbine-induced reinstatement of 20% ethanol-seeking. A paired *t*-test comparing the baseline vehicle and baseline yohimbine reinstatement levels revealed that yohimbine treatment significantly increased responding on the active lever ($p < 0.01$; Figure 2a). Statistical analysis using a repeated-measures two-way ANOVA of active lever pressing for the ethanol-trained animals pretreated with mifepristone revealed an overall effect of pretreatment dose of mifepristone (0, 5, or 10 μg) ($F(2,65) = 4.09$, $p < 0.05$), yohimbine dose (0 or 2 mg/kg) ($F(1,65) = 10.50$, $p < 0.01$), and a significant interaction between mifepristone dose × yohimbine dose ($F(2,65) = 4.02$, $p < 0.05$). *Post hoc* analysis revealed that yohimbine significantly reinstated

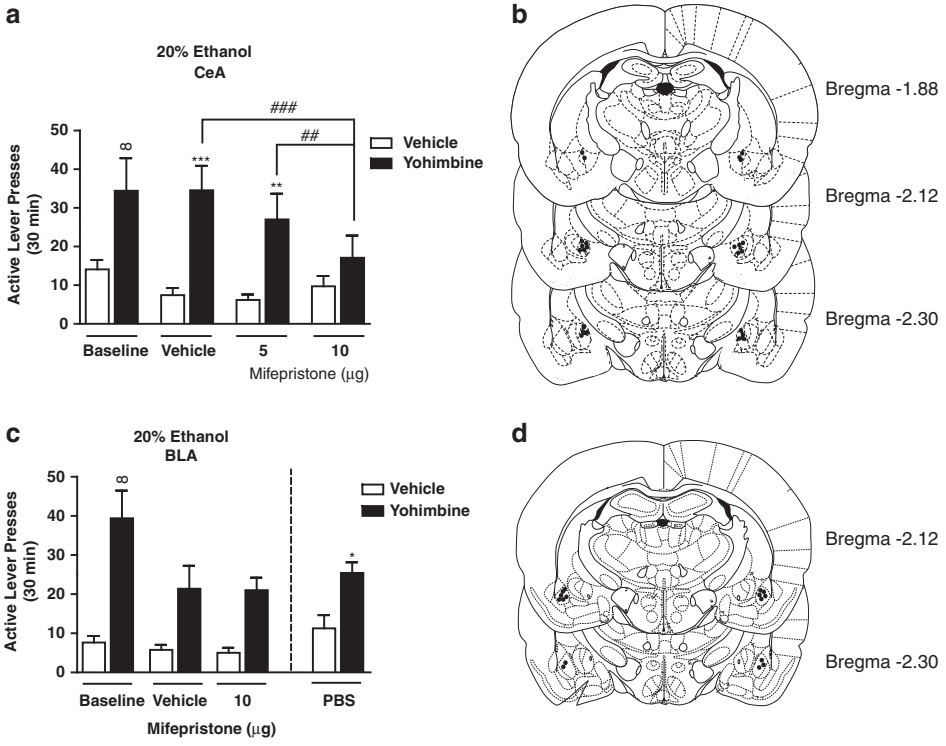

Figure 2 Intra-central amygdala (CeA), but not basolateral amygdala (BLA), administration of mifepristone significantly attenuates yohimbine-induced reinstatement of ethanol-seeking. Animals were pretreated with mifepristone (0, 5, or 10 μg) into the CeA and BLA to examine its effects on yohimbine-(2 mg/kg, intraperitoneally (i.p.)) induced reinstatement of ethanol-seeking. (a) Yohimbine significantly reinstates active lever responding (∞, $p < 0.01$ comparing baseline vehicle to baseline yohimbine: ***$p < 0.001$; **$p < 0.01$ compared with yohimbine vehicle treatment for animals pretreated with mifepristone vehicle and 5 μg, respectively), an effect that is dose-dependently attenuated by mifepristone ($^{###}p < 0.001$ and $^{##}p < 0.01$ comparing yohimbine reinstatement following mifepristone vehicle and 5 μg pretreatment, respectively, to reinstatement following 10 μg mifepristone pretreatment). (b) Schematic representations adapted from Paxinos and Watson of the injection cannulae placements in coronal sections of the CeA of rats included in the data analysis. (c) Intra-BLA mifepristone has no effect on yohimbine-induced reinstatement of active lever responding ($p < 0.01$ comparing baseline vehicle to baseline yohimbine; *$p < 0.05$ comparing phosphate-buffered saline (PBS) vehicle to PBS yohimbine). (d) Schematic representations adapted from Paxinos and Watson of the injection cannulae placements in coronal sections of the BLA of rats included in the data analysis. Data are presented as mean active lever presses ± SEM ($n = 11$ for CeA and $n = 8$ for BLA). Statistical analysis was performed by paired *t*-test for baseline and PBS data and repeated-measures two-way analysis of variance (ANOVA) with Newman–Keuls *post hoc* testing for animals pretreated with mifepristone.

ethanol-seeking in the groups that were pretreated with 0 and 5 μg of mifepristone ($p<0.001$ and <0.01, respectively; Figure 2a), but not in those that received 10 μg. *Post hoc* analysis also revealed there was a significant difference in the active lever pressing following yohimbine challenge between the groups pretreated with mifepristone vehicle and mifepristone (10 μg, $p<0.001$; Figure 2a). Mifepristone pretreatment had no effect on responding on the active lever following yohimbine vehicle administration. Statistical analysis of inactive lever pressing using repeated-measures two-way ANOVA revealed no significant differences (data not shown). Histological placements are shown in Figure 2b. Seven animals were excluded from the CeA studies: one failed to consume 0.25 g/kg per session in the maintenance phase, one failed to extinguish, one failed to reinstate in any of the test weeks, and four others were excluded owing to cannula placements outside the CeA.

Microinfusions of mifepristone into the BLA had no effect on yohimbine-induced reinstatement of 20% ethanol-seeking. A paired *t*-test comparing the baseline vehicle and baseline yohimbine reinstatement levels revealed that yohimbine treatment significantly increased responding on the active lever ($p<0.01$; Figure 2c). Statistical analysis using a repeated-measures two-way ANOVA of active lever pressing for the ethanol-trained animals pretreated with mifepristone revealed an overall effect of yohimbine dose (0 or 2 mg/kg) ($F(1,31)=19.04$, $p<0.01$; Figure 2c), but no effect of pretreatment dose of mifepristone (0 or 10 μg) or interaction between mifepristone dose × yohimbine dose. Mifepristone pretreatment had no effect on responding on the active lever following yohimbine vehicle administration. Mifepristone and vehicle infusions into the BLA caused a dramatic decrease in reinstatement when compared with the baseline reinstatement level. Therefore, to verify that the drop in responding was not caused by the DMSO vehicle, in the third week of reinstatement testing, PBS was infused before yohimbine vehicle and yohimbine administration. A paired *t*-test of the active lever responding revealed that yohimbine-induced a significant reinstatement ($p<0.05$; Figure 2c) following the PBS infusion. The reinstatement was similar in magnitude to the reinstatement following DMSO or mifepristone pretreatment, suggesting that the infusion of any solution into the BLA can decrease the magnitude of the reinstatement. Statistical analysis of inactive lever pressing using repeated-measures two-way ANOVA revealed no significant differences (data not shown). Histological placements are shown in Figure 2d. Two animals were excluded from the BLA studies owing to failure to complete the dose–response due to health issues perhaps caused by BLA infusions.

Six microinfusions of 100% DMSO and mifepristone (10 μg) into the CeA did not result in increased cell death when compared with PBS controls. Statistical analysis using a one-way ANOVA of viable nuclei in the three treatment groups revealed no significant effects (Figure 3e). This extends previous findings in which a 50% DMSO solution infused into the amygdala failed to produce signs of cell death (Lin *et al*, 2006).

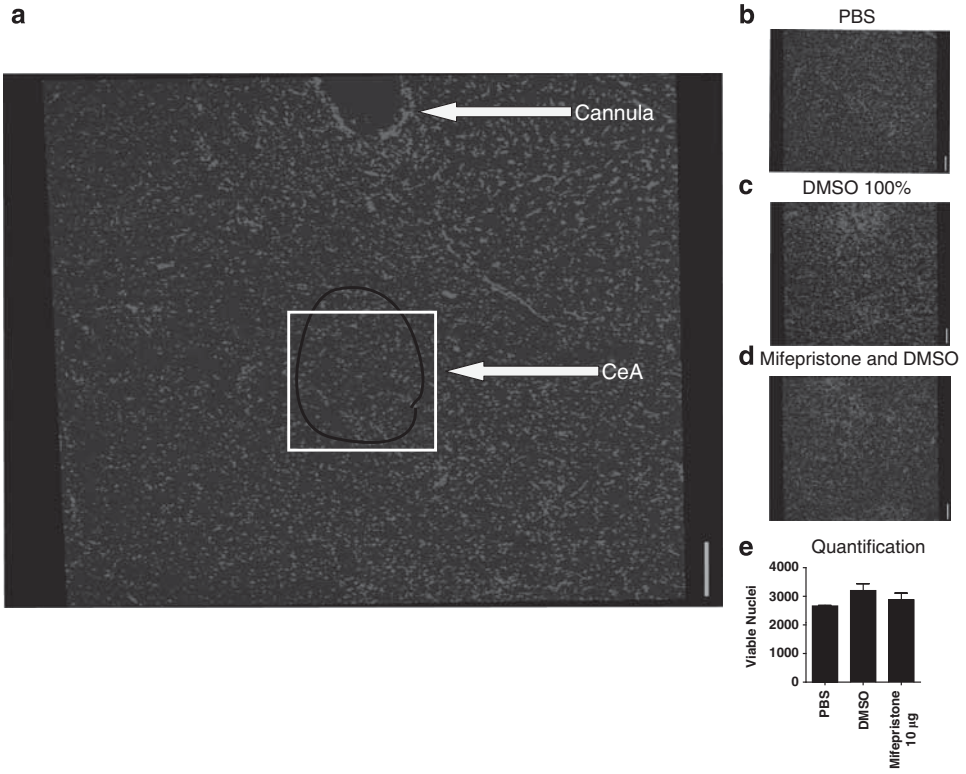

Figure 3 Intra-central amygdala (CeA) infusions of dimethylsulfoxide (DMSO) or mifepristone do not cause cell death in the amygdala. (a) Representative slide for amygdala slices with cannula and CeA highlighted. Morphological studies were conducted in CeA sections of rats pretreated with six microinfusions of (b) phosphate-buffered saline (PBS), (c) 100% DMSO, and (d) mifepristone dissolved in 100% DMSO using Hoechst 33342 staining 24 h after the last infusion. (e) Quantification of viable cells was performed by the Imaris Neuroscience software pack. Data are presented as mean ± SEM, ($n=3$). Statistical analysis was performed by one-way analysis of variance (ANOVA).

Microinfusions of mifepristone into the CeA had no effect on yohimbine-induced reinstatement of 5% sucrose-seeking. A paired *t*-test comparing the baseline vehicle and baseline yohimbine reinstatement levels revealed that yohimbine treatment significantly increased responding on the active lever ($p<0.01$; Figure 4). Statistical analysis of active lever pressing for the sucrose-trained animals pretreated with mifepristone using a repeated-measures two-way ANOVA revealed an overall effect of yohimbine dose (0 or 2 mg/kg) ($F(1,59)=10.60$, $p<0.05$; Figure 4), but no effect of mifepristone pretreatment or interaction between mifepristone dose × yohimbine dose. Mifepristone pretreatment had no effect on responding on the active lever following yohimbine vehicle administration. Statistical analysis of inactive lever pressing using repeated-measures two-way ANOVA revealed no significant differences (data not shown). Two animals were excluded from the sucrose CeA studies: one failed to complete the dose–response due to problems with cannula patency and one failed to reinstate in any of the test weeks.

Effect of Mifepristone on 10% Ethanol and 5% Sucrose Self-Administration

To examine the effects of mifepristone on maintained responding for both ethanol and sucrose, we administered mifepristone (5, 10, or 30 mg/kg, i.p.) or vehicle 30 min before the onset of regular, reinforced self-administration sessions. We found that mifepristone had no effect on the responding for either solution. Repeated-measures one-way ANOVA for ethanol-trained animals revealed no significant effects of treatment on active lever presses (Figure 5a), inactive lever presses (data not shown), or ethanol intake (g/kg) (Figure 5b). Repeated measures one-way ANOVA for sucrose-responding animals revealed no significant effects of treatment on active lever presses (Figure 5c), inactive lever presses (data not shown), or sucrose intake (g/kg) (Figure 5d). One animal was excluded from the ethanol group owing to failure to consume 0.25 g/kg per session.

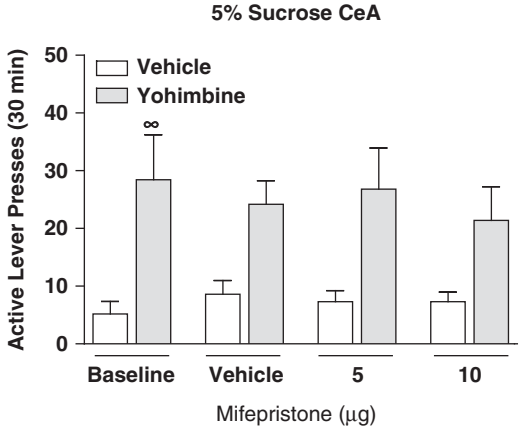

Figure 4 Intra-central amygdala (CeA) administration of mifepristone has no effect on yohimbine-induced reinstatement of sucrose-seeking. Animals were pretreated with mifepristone (0, 5, or 10 μg) into the CeA to examine its effects on yohimbine- (2 mg/kg, intraperitoneally (i.p.)) induced reinstatement of sucrose-seeking. Mifepristone had no effect on yohimbine-induced reinstatement of active lever responding (∞, $p<0.01$ comparing baseline vehicle to baseline yohimbine). Data are presented as mean active lever presses ± SEM ($n=10$). Statistical analysis was performed by paired *t*-test for baseline data and repeated-measures two-way analysis of variance (ANOVA) for animals pretreated with mifepristone.

Effect of Mifepristone on Yohimbine-Induced Increases in 10% Ethanol and 5% Sucrose Intake

To examine the effects of mifepristone on yohimbine-induced increases in responding for ethanol and sucrose, mifepristone (5 or 30 mg/kg, i.p.) or vehicle was administered 30 min before yohimbine administration, which was delivered 30 min before the onset of regular, reinforced self-administration sessions. We found that mifepristone had no effect on the increases in responding following yohimbine challenge for either solution. Repeated-measures one-way ANOVA for the ethanol-trained animals revealed a significant effect of yohimbine treatment on active lever responding ($F(3,39)=9.12$, $p<0.001$) and ethanol intake (g/kg) ($F(3,39)=13.59$, $p<0.001$), but no effect on inactive lever responding (data not shown). *Post hoc* analysis revealed that animals responded on the active lever and consumed significantly more ethanol following the yohimbine challenge when compared with baseline, regardless of mifepristone pretreatment dose (Figure 6a and b). Repeated-measures one-way ANOVA for the sucrose-trained animals revealed a significant effect of yohimbine treatment on active lever responding ($F(3,43)=9.58$, $p<0.001$) and sucrose intake (g/kg) ($F(3,43)=11.58$, $p<0.001$), but no effect on inactive lever responding (data not shown). *Post hoc* analysis revealed that animals responded on the active lever and consumed significantly more sucrose following the yohimbine challenge when compared with baseline, regardless of mifepristone pretreatment dose (Figure 6c and d). One animal was excluded from each group owing to failure to meet the acquisition criteria.

DISCUSSION

The major finding of this study is that mifepristone, delivered both systemically and directly into the CeA, but not into the BLA, attenuates yohimbine-induced reinstatement of ethanol-seeking, while failing to decrease circulating CORT levels. We also found that the high dose of mifepristone can block yohimbine-induced reinstatement of sucrose-seeking when delivered systemically, but not into the CeA. It has previously been shown that yohimbine-induced reinstatement of ethanol-seeking can be modulated by many receptors, including: CRF (Marinelli *et al*, 2007), orexin (Richards *et al*, 2008), serotonin (Le *et al*, 2009), neuropeptide Y (Cippitelli *et al*, 2010), peroxisome proliferator-activated receptor-γ (Stopponi *et al*, 2011), adrenoreceptors (Le *et al*, 2011), and delta-opioid receptors (Nielsen *et al*, 2011). This study is the first, to our knowledge, to indicate that GRs play a role in the reinstatement behavior elicited by yohimbine. Mifepristone is a potent antagonist at both GR and PR, with binding affinity far greater than the endogenous steroids, CORT and progesterone (Sitruk-Ware and Spitz, 2003). It seems likely that the effects of mifepristone on yohimbine-induced reinstatement of ethanol- and sucrose-seeking are mediated

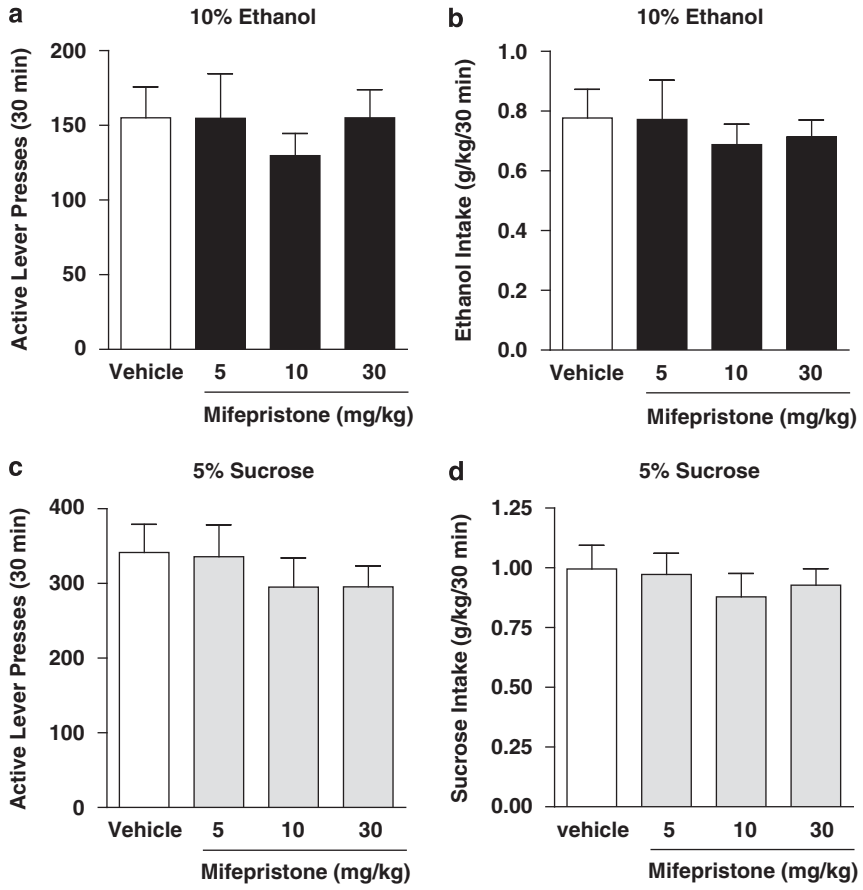

Figure 5 Mifepristone has no effect on baseline operant responding for ethanol or sucrose. Mifepristone (0, 5, 10, and 30 mg/kg) treatment has no effect on (a) active lever pressing and (b) consumption (g/kg per 30 min) in animals trained to respond for 10% ethanol. Similarly, mifepristone treatment has no effect on (c) active lever pressing and (d) consumption (g/kg per 30 min) in animals trained to respond for 5% sucrose. Data are presented as mean ± SEM ($n = 11$ for ethanol-trained rats and $n = 15$ for sucrose-trained rats). Statistical analysis was performed by repeated-measures one-way analysis of variance (ANOVA).

by antagonism of the stress hormone, CORT, and less likely by progesterone. To date, the role of progesterone in drug-seeking behaviors has been mixed. Progesterone has been shown to be negatively correlated with drug-seeking behavior in cocaine-trained, female rats (Anker *et al*, 2007; Feltenstein *et al*, 2009; Larson *et al*, 2007). However, it has been shown that the progesterone metabolite, allopregnanolone, can have both a stimulatory or inhibitory effect on ethanol intake depending on the dose administered and the drinking paradigm used (Ford *et al*, 2005; Janak *et al*, 1998; Morrow *et al*, 2001). Our group (Simms *et al*, 2011) and others (Marinelli *et al*, 2007) have previously shown that yohimbine produces significant increases in circulating CORT levels and, in the present study, we show that yohimbine administration in ethanol-extinguished rats causes a nonsignificant increase in progesterone levels (Supplementary Figure 1). However, the levels in the male rats described here remain very low and are quite close to basal levels reported in the literature (Andersen *et al*, 2004, 2005; Auger *et al*, 2006). Additional experiments with more selective ligands for GR and PR are necessary to fully determine the mechanism of action for mifepristone's effect on yohimbine-induced reinstatement of ethanol-seeking.

The two stressors known to elicit reinstatement of ethanol-seeking behavior, intermittent footshock and yohimbine, have been shown to activate the BLA, CeA, and nucleus accumbens (NAc), and also induce CRF mRNA in the dorsal region of the bed nucleus of the stria terminalis (BNST), as well as in the CeA (Funk *et al*, 2006). Of these sites, it has been demonstrated that mifepristone decreases neuronal activity in the CeA (but not the BLA), where it acts to suppress the stress response elicited by the forced swim test (Wulsin *et al*, 2010). Importantly, Wulsin and co-workers also found no effect of mifepristone on the stress-induced response in the paraventricular nucleus of the hypothalamus, which points to extrahypothalamic site of action for its anti-stress and anti-depressant effects. CORT binding has been shown to negatively regulate HPA axis function in the hypothalamus, while it has stimulatory effects in extrahypothalamic regions, including the CeA (Makino *et al*, 1994; Pastor *et al*, 2008). Furthermore, inactivation of the CeA, but not the BLA, has been shown to block footshock-induced reinstatement of cocaine-seeking (McFarland *et al*, 2004). In agreement with this literature, the data presented here demonstrates that mifepristone infused directly into the CeA, but not the BLA, abolishes yohimbine-induced reinstatement of ethanol-seeking. Large

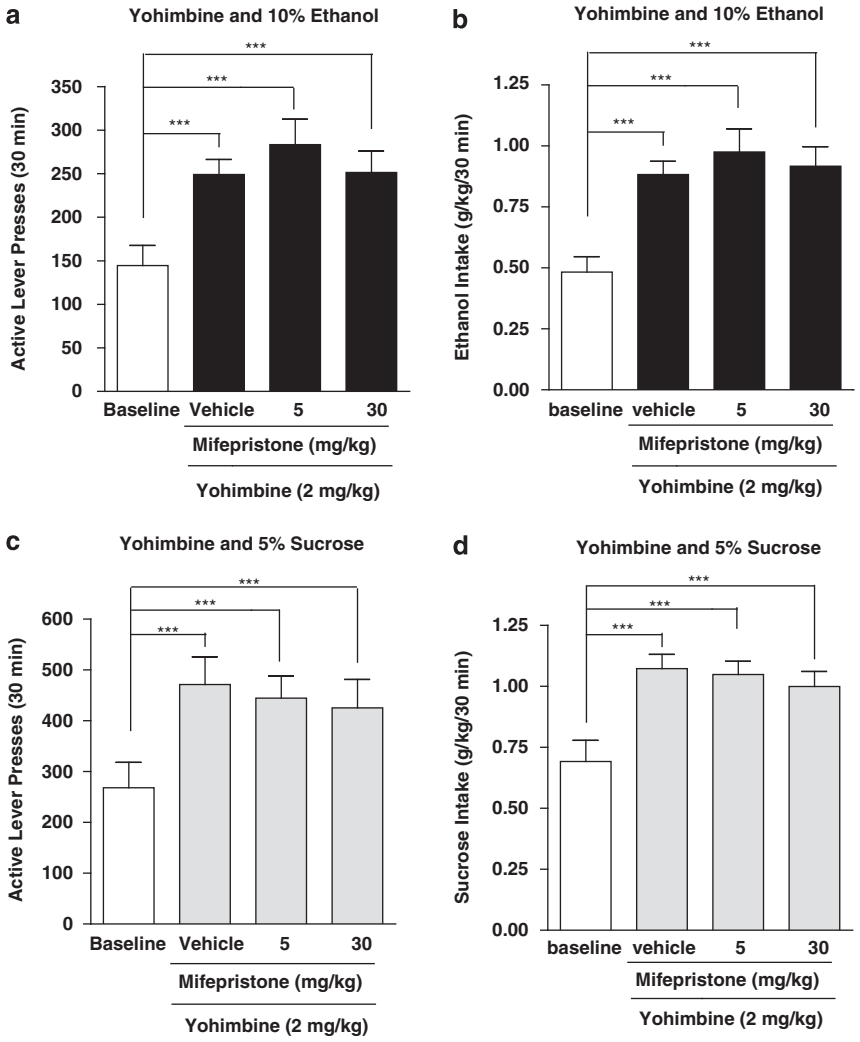

Figure 6 Mifepristone does not attenuate yohimbine-induced increases in ethanol and sucrose responding. Yohimbine (2 mg/kg) significantly increases operant responding for and consumption (g/kg per 30 min) of (a, b) ethanol and (c, d) sucrose (***$p<0.001$ compared with baseline responding), while mifepristone pretreatment has no effect on yohimbine-induced increases in ethanol and sucrose responding or consumption. Data are presented as mean ± SEM ($n=10$ for ethanol-trained rats and $n=11$ for sucrose-trained rats). Statistical analysis was performed by repeated-measures one-way analysis of variance (ANOVA).

numbers of GRs are expressed in the CeA (Reul and de Kloet, 1985; Sapolsky *et al*, 1983) and these receptors have been shown to directly mediate anxiety-like behaviors in rodents (Myers and Greenwood-Van Meerveld, 2007, 2010). The current explanation is that the effects of GRs in the CeA are mediated by CRF, as intracerebral ventricular administration of a selective CRF-R1 antagonist blocks the long-term anxiogenic effects of CORT exposure in the CeA (Myers and Greenwood-Van Meerveld, 2010). Although GRs are primarily described as nuclear receptors, which require time to translocate from the cytoplasmic compartment to the nucleus to alter gene expression (Beato and Sanchez-Pacheco, 1996), there are several recent studies which indicate that there may be fast-acting non-genomic effects of GRs, as well as for mineralocorticoid receptors (MRs) (for a review, see Groeneweg *et al* (2011)). Evidence for the existence of GR and MR near the membrane has been verified using synaptosome extracts (Komatsuzaki *et al*, 2005; Qiu *et al*, 2010; Wang and Wang, 2009) and using electron microscopy of the neuronal membrane (Johnson *et al*, 2005; Prager *et al*, 2010). The presence of GRs near the membrane may account for the rapid behavioral effects seen following both the systemic and intra-CeA administration (30 and 10 min pretreatment, respectively) of mifepristone in this study. We hypothesize that membrane-associated GRs, on CRF-R1-containing neurons of the CeA, modulate yohimbine-induced reinstatement of ethanol-seeking. In addition, future studies exploring the effects of the MR in models of stress-induced ethanol-seeking could potentially yield interesting data.

Chronic exposure to high levels of GCs in the brain, similar to that caused by long-term ethanol exposure, has been shown to upregulate CRF expression in the amygdala (Koob, 2008; Makino *et al*, 1994, 2002; Shepard *et al*, 2000; Swanson and Simmons, 1989). Blocking GRs in the amygdala prevents the excitation of CRF-containing neurons (Gray and Bingaman, 1996), which may decrease the CRF activation necessary for reinstatement. Drugs of abuse (including alcohol) and stress have been shown to enhance excitatory synaptic strength, long-term potentiation (LTP),

at ventral tegmental area (VTA) dopamine neurons (Saal *et al*, 2003) while inhibiting LTP in inhibitory γ-aminobutyric acid cells in the same brain region (Niehaus *et al*, 2010). Taken together, these studies demonstrate how a state of increased excitation and decreased inhibition can lead to a hyperactivation of dopamine neurons that may facilitate later drug self-administration and reinstatement. Interestingly, both Saal and Niehaus showed that the VTA LTP effects of stress are blocked by mifepristone treatment before stress exposure. In this study, a similar phenomenon could be occurring in the CeA, as mifepristone blocks the neuronal activation elicited by yohimbine in ethanol-trained animals and prevents further activation of the HPA axis, although further studies are needed to establish this effect. Although it has been shown that CRF, but not CORT, modulates footshock-induced reinstatement of ethanol-seeking (Le *et al*, 2000), the effects of mifepristone on footshock-induced reinstatement have yet to be examined. However, it is possible that GR blockade may also decrease or abolish footshock-induced reinstatement by decreasing activation in the amygdala. Work from several other laboratories has shown that reinstatement of drug-seeking is dependent on the extended amygdala. The extended amygdala consists of the BNST, CeA, and shell of the NAc (Alheid *et al*, 1998). This system is important in several stress-related components of drug withdrawal (Smith and Aston-Jones, 2008), and inactivation of CeA, BNST, shell of the NAc, or VTA has been shown to block footshock-induced reinstatement of cocaine-seeking (McFarland *et al*, 2004).

An unexpected finding in this study is that while systemic administration of mifepristone attenuated yohimbine-induced reinstatement of sucrose-seeking, microinfusions directly into the CeA had no effect. These data indicate that while GRs are important for the expression of this behavior in both ethanol- and sucrose-trained animals, different mechanisms and brain pathways govern the reinstatement behavior in the two groups. We have previously shown that animals trained to self-administer both 10 and 20% ethanol have an exaggerated CORT response to yohimbine when compared with sucrose controls (Simms *et al*, 2011). We have also shown that yohimbine treatment alters receptor signaling in the pooled midbrain (including the amygdala) of ethanol-experienced animals: changes that are not present in ethanol-naïve animals (Nielsen *et al*, 2011). Moreover, clinical evidence suggests that the cortisol response to yohimbine challenge in patients with a history of ethanol dependence is elevated compared with controls (Krystal *et al*, 1994, 1996). Funk *et al* (2006) have elegantly demonstrated the brain activation patterns of intermittent footshock and yohimbine administration in ethanol-naïve animals. Perhaps, an extension of these studies examining the brain activation patterns in animals trained to self-administer ethanol and sucrose would provide additional information about the differential effects observed here.

Although systemic mifepristone was effective in decreasing yohimbine-induced reinstatement, it had no effect on the self-administration of ethanol or sucrose, even when yohimbine was administered to increase ethanol consumption. The literature is somewhat mixed when examining the effects of mifepristone on ethanol consumption as some authors have reported decreases in consumption following drug treatment (Koenig and Olive, 2004; O'Callaghan *et al*, 2005), while others have reported no change (Fahlke *et al*, 1994, 1995, 1996; Lowery *et al*, 2010; Yang *et al*, 2008). Koenig and Olive (2004) demonstrated significant decreases in consumption using a limited-access, two-bottle-choice paradigm; however, it is important to note that the animals were fluid restricted for 23 h a day (O'Callaghan *et al*, 2005). Water restriction has been shown to activate the HPA axis and elevate CORT in rats (Aguilera *et al*, 1993; Kiss *et al*, 1994). Therefore, the decreases in consumption may have been due to artificially elevated GR function in this chronic stress state. In agreement with this analysis, it has been shown that while mifepristone has no effect on baseline drinking in mice, it blocks stress-induced increases in drinking caused by daily vehicle injections (O'Callaghan *et al*, 2005). In conditions where stress is limited, mifepristone has no effect on ethanol intake (Fahlke *et al*, 1994, 1995, 1996; Lowery *et al*, 2010; Yang *et al*, 2008).

Somewhat surprisingly, mifepristone had no effect on yohimbine-induced increases in consumption. It has been previously shown that these increases are modulated by extrahypothalamic mechanisms, specifically CRF (Marinelli *et al*, 2007) and serotonin systems (Le *et al*, 2009). These investigators have demonstrated that serotonin and CRF systems interact within the MRN to modulate reinstatement to ethanol-seeking (Le *et al*, 2002). Interestingly, yohimbine has actions as a serotonin, 5-hydroxytryptamine 1A (5-HT1A), receptor agonist where it decreases cell firing and release in a manner similar to intra-MRN infusions of CRF and the selective 5-HT1A receptor agonist 8-OH-DPAT, which also reinstate ethanol-seeking (Le *et al*, 2002). Finally, yohimbine-induced increases in drinking and reinstatement have been shown to be attenuated with the 5-HT1A antagonist WAY 100635 (Le *et al*, 2009). We have demonstrated that mifepristone attenuates yohimbine-induced reinstatement without affecting yohimbine-induced increases in responding, which indicates that the drug may be modulating ethanol reinstatement without effecting serotonin systems.

It is essential that pharmacotherapies be developed for AUDs with the primary aim of reducing relapse rates. Anti-GC agents, such as mifepristone, may have value in the treatment of AUDs because they may have the potential to reset a dysregulated HPA axis following treatment. Animal and human studies have shown that brief treatment with mifepristone upregulates GR function and restores normal HPA feedback (Belanoff *et al*, 2001; Young, 2006), decreases depression-like behavior as measured by the forced swim test (Wulsin *et al*, 2010), and attenuates the cognitive deficits caused by ethanol withdrawal (Jacquot *et al*, 2008). Mifepristone may be a strong candidate for clinical trials because it is FDA approved and has exhibited a limited toxicity profile following brief treatment (Sitruk-Ware and Spitz, 2003).

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1RO1AA017924-01 to SEB. The experiments contained herein comply with the current laws of the United States of America. All procedures were pre-approved by the Gallo Center Institutional Animal Care and Use Committee and were in accordance with NIH guidelines for the Humane Care and Use of Laboratory Animals.

DISCLOSURE

The authors declare that, except for income received from my primary employer, SEB has received financial support for research for an unrelated clinical study, but has not received compensation from any individual or corporate entity over the past 3 years for research or professional service and there are no personal financial holding that could be perceived as constituting a potential conflict of interest.

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