Behavioral/Systems/Cognitive

Corticosteroid-Dependent Plasticity Mediates Compulsive Alcohol Drinking in Rats

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Alcoholism is characterized by a compulsion to seek and ingest alcohol, loss of control over intake, and the emergence of a negative emotional state during abstinence. We hypothesized that sustained activation of neuroendocrine stress systems (e.g., corticosteroid release via the hypothalamic-pituitary-adrenal axis) by alcohol intoxication and withdrawal and consequent alterations in glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) activation drive compulsive alcohol drinking. Our results showed that rats exposed to alcohol vapor to the point of dependence displayed increased alcohol intake, compulsive drinking measured by progressive-ratio responding, and persistent alcohol consumption despite punishment, assessed by adding quinine to the alcohol solution, compared with control rats that were not exposed to alcohol vapor. No group differences were observed in the self-administration of saccharin-sweetened water. Acute alcohol withdrawal was accompanied by downregulated GR mRNA in various stress/reward-related brain regions [i.e., prefrontal cortex, nucleus accumbens (NAc), and bed nucleus of the stria terminalis (BNST)], whereas protracted alcohol abstinence was accompanied by upregulated GR mRNA in the NAc core, ventral BNST, and central nucleus of the amygdala. No significant alterations in MR mRNA levels were found. Chronic GR antagonism with mifepristone (RU38486) prevented the escalation of alcohol intake and compulsive responding induced by chronic, intermittent alcohol vapor exposure. Chronic treatment with mifepristone also blocked escalated alcohol drinking and compulsive responding during protracted abstinence. Thus, the GR system appears to be involved in the development of alcohol dependence and may represent a potential pharmacological target for the treatment of alcoholism.

Introduction

Alcoholism is a complex psychiatric condition characterized by compulsive alcohol seeking and ingestion, loss of control over intake, and the emergence of a negative emotional state during withdrawal (Koob and Volkow, 2010). Similar to stress, alcohol intake activates the hypothalamic-pituitary-adrenal (HPA) axis to release cortisol/corticosterone (CORT; cortisol in humans and corticosterone in rodents) from the adrenal gland (Ellis, 1966; Lee and Rivier, 1997; Richardson et al., 2008). CORT contributes to the reinforcing effects of drugs (Fahlke et al., 1995, 1996; Goeders, 1997; Piazza and Le Moal, 1998; Mantsch et al., 1998; Uhart and Wand, 2009). Upon release, CORT binds to two types of brain receptors: mineralocorticoid receptors (MRs or type I, which have high affinity for CORT) and glucocorticoid receptors (GRs or type II, which have a lower affinity for CORT and are activated predominantly at high circulating CORT levels; McEwen et al., 1968, McEwen, 2007). A consequence of high CORT levels is the dysregulation of gene transcription, including corticotropin-releasing factor (CRF), a critical factor in alcohol dependence-related neuroadaptations (Heilig and Koob, 2007).

In alcohol dependence, the neuroendocrine stress system is dysregulated in both humans (Adinoff et al., 1990; 2003; Lovallo et al., 2000; Uhart and Wand, 2009; Sinha et al., 2011) and rodents (Rasmussen et al., 2000; Zorrilla et al., 2001; Richardson et al., 2008), but the consequences of this dysregulation for the escalation of alcohol intake, compulsive use, and relapse remain unclear. Following a prolonged history of alcohol dependence, negative reinforcement becomes a dominant motivational factor for continued alcohol use (i.e., alcohol is used to alleviate or prevent negative emotional symptoms, such as anxiety, dysphoria, and hypohedonia that emerge in the absence of the drug; Edwards and Koob, 2010). The transition from positive reinforcement in nondependent individuals to negative reinforce-

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ment in alcohol dependence may therefore be driven by dysregulated HPA axis function, yet the role of MRs and GRs in the transition to alcohol dependence remains to be investigated.

We tested the hypothesis that CORT receptors are involved in the escalated and compulsive alcohol intake that results when alcohol dependence is induced by alcohol vapor exposure. Compulsive drinking was assessed by a progressive-ratio (PR) test, in which the "price" (number of lever presses) required for the next reinforcement increases progressively (Hodos, 1961), and by a quinine adulteration test that measures persistent alcohol consumption despite the aversive bitter taste of quinine (added to the alcohol solution; Wolffgramm and Heyne, 1995). We found that acute alcohol withdrawal and protracted alcohol abstinence were associated with changes in glucocorticoid receptor (GR) expression levels in several stress/reward-related brain areas. Chronic GR antagonism prevented the development of escalated and compulsive alcohol drinking produced by alcohol vapor exposure during acute withdrawal and blocked the escalated and compulsive alcohol drinking during protracted abstinence in animals with a history of alcohol dependence. Altogether, the results suggest a critical role for GRs in the development and maintenance of escalated drinking in alcohol dependence.

Materials and Methods

Subjects. Adult male Wistar rats (Charles River), weighing 225–275 g at the beginning of the experiments, were housed in groups of 2–3 per cage in a temperature-controlled (22°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 8:00 P.M.) with *ad libitum* access to food and water. All behavioral tests were conducted during the dark phase of the light/dark cycle. All procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute.

Operant self-administration. Self-administration sessions were conducted in standard operant conditioning chambers (Med Associates). The rats were first trained to self-administer alcohol using a modified (Walker and Koob, 2007) sucrose-fading procedure (Samson, 1986), in which 10% (w/v) alcohol was added to a sweet solution and then sweeteners were gradually removed from the experimental solution. Upon completion of this procedure, the animals were allowed to self-administer a 10% (w/v) alcohol solution and water on a fixed-ratio 1 (FR1) schedule of reinforcement (i.e., each operant response was reinforced with 0.1 ml of the solution). For the pharmacological tests with mifepristone, rats were trained to self-administer alcohol according to the following protocol that generated the same amount of baseline drinking but with less testing in preliminary studies. First, the rats were given free-choice access to alcohol (10% w/v) and water for 1 d in their home cages to habituate them to the taste of alcohol. Second, the rats were subjected to an overnight session in the operant chambers with access to one lever (right lever) that delivered water (FR1). Food was available *ad libitum* during this training. Third, after 1 d off, the rats were subjected to a 2 h session (FR1) for 1 d and a 1 h session (FR1) the next day, with one lever delivering alcohol (right lever). All of the subsequent sessions lasted 30 min, and two levers were available (left lever: water; right lever: alcohol) until stable levels of intake were reached. Responding obtained with this procedure is equivalent to the sucrose-fading procedure (Samson, 1986; Walker and Koob, 2007). Upon completion of this procedure, the animals were allowed to self-administer a 10% (w/v) alcohol solution and water on an FR1 schedule of reinforcement.

Alcohol vapor chambers. The rats were made dependent by chronic, intermittent exposure to alcohol vapors as previously described (O'Dell et al., 2004; Gilpin et al., 2008). They underwent cycles of 14 h on (blood alcohol levels during vapor exposure ranged between 150 and 250 mg%) and 10 h off, during which behavioral testing for acute withdrawal occurred (i.e., 6–8 h after vapor was turned off when brain and blood alcohol levels are negligible; Gilpin et al., 2009). In this model, rats exhibit somatic withdrawal signs and negative emotional symptoms reflected by anxiety-like responses and elevated brain reward thresholds (Schulteis et al., 1995; Roberts et al., 2000; Valdez et al., 2002; Rimondini et al., 2003; O'Dell et al., 2004; Zhao et al., 2007; Sommer et al., 2008; Edwards et al., 2012). Nondependent rats were not exposed to alcohol vapor. For protracted abstinence, the animals were tested at the same time of the day as for acute withdrawal but 3–6 weeks after the vapor was turned off.

Operant self-administration during alcohol vapor exposure. Behavioral testing occurred 2–4 times per week. The rats were tested for alcohol (and water) self-administration on an FR1 schedule of reinforcement for 13 30-min sessions. Operant self-administration on an FR1 schedule requires minimal effort by the animal to obtain the reinforcer and herein was considered a measure of intake. For five sessions, the rats were tested on a PR schedule, under which the number of lever presses necessary to obtain the next reinforcer progressively increased according to the following progression: 1, 1, 2, 2, 3, 3, 4, 4, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, etc. The PR session stopped after 90 min or when 15 min had elapsed without the rat obtaining a reinforcer. In this test, the workload ("price") for the next alcohol reinforcer increases progressively until the rat reaches a "breakpoint" (i.e., a measure of motivation/compulsivity) beyond which it no longer responds for alcohol.

The rats were then maintained on an FR1 schedule until stable levels of alcohol self-administration were reattained, and the alcohol solution was adulterated with increasing concentrations of quinine (0.0005, 0.001, 0.0025, and 0.005 g/L) presented between-sessions (one concentration per session). This test measures the persistence of animals to consume alcohol despite the aversive bitter taste of quinine that was added to the alcohol solution and was considered herein a measure of compulsive intake.

Finally, the rats were tested for saccharin (0.004%, w/v) self-administration under an FR1 schedule for five 30 min sessions to determine whether the effects were specific for alcohol or generalized to other types of reward. A submaximal rewarding saccharin concentration was chosen based on previous studies (Vendruscolo et al., 2010) to prevent reaching a "ceiling effect" in any group and maintain similar response rates as alcohol.

Brain collection. Brains from dependent and nondependent rats were collected and snap-frozen with isopentane for measurements of GR and MR mRNA levels during acute alcohol withdrawal (24 h after the vapor was turned off). This time point was chosen because we were interested in more stable dysregulations of gene expression and to avoid any transient effects caused by earlier withdrawal. Importantly, the escalation of alcohol consumption has been demonstrated in rats at 2–8 h and 24 h of withdrawal and 2–7 weeks post-vapor (Valdez et al., 2002; Rimondini et al., 2003; O'Dell et al., 2004; Gilpin et al., 2008; Sommer et al., 2008). Brains from a separate cohort of animals were dissected 3 weeks after the vapor was turned off to investigate whether changes in GR mRNA levels could be detected during protracted alcohol abstinence. The brains were sliced on a cryostat, and bilateral punches (300 μm thickness, 2 mm diameter) were collected from the prefrontal cortex (PFC), nucleus accumbens (NAc), bed nucleus of the stria terminalis (BNST), amygdala, and hippocampus. Because we expected that smaller gene expression changes might occur during protracted alcohol abstinence, we dissected subregions of some of the regions used above for GR quantification from 300 μm cryostat-cut slices (Cuello and Carson, 1983).

Quantitative nuclease protection array. The expression of GR and MR mRNA during acute alcohol withdrawal was assessed using quantitative nuclease protection assays (qNPA, High Throughput Genomics). This assay was used because it allowed us to quantitatively measure several stress/reward-related genes at the same time. The primer sequences were the following: GR (position 85, GACTTTTATAAAAGCCTGAGGGGAGGAGCTACA GTCAAGGTTTCTGCATC; position 318, GGGGCTGTATATGGGAGAGACAGAAACAAAAGTGATGGGGAATGACTTGG; position 1474, CCAGCATGCCGCTATCGGAAATGTCTTCAGGCTGGAATGAACCTTGAAGC; position 1734, CACACTCAACA TGTTAGGTGGGCGTCAAGTGATTGCAGCAGTGAAATGGC), MR (position 346, CCTCTCCATCCTCATTGCCGATCAGCCAGTATTGGACTTGCTGGTAGCGG; position 1764, GTCAAGCAAGCACTCATGTTCAGGCGCCTCTTTTAAAGGGAACC CCACGG; position 3380, CTGGGAATGCCAAACCCCTTTACTTTCACAGAAAGTG ACGGGAGATACCG; position 4508, CCCGCGTGGGA

AGTGTTCGTGAGACTCTAGTGCGCAGCTGTGGCTTCTGC). RNA was extracted and purified from brain tissue using the PureLinkTM RNA Mini Kit (Ambion) according to the manufacturer's instructions. Two hundred nanograms of total RNA were diluted in lysis buffer (up to 25 μl) that contained the cDNA riboprobes complementary to targeted RNA and added to a 96-well plate. The plate was then heated at 95°C for 15 min (denaturation) and incubated for 16 h at 60°C for hybridization of the probes to RNA. The single-strand nucleic acids (unbound RNA and probes) were eliminated by S1-nuclease digestion for 90 min at 50°C. The enzymatic reaction was terminated by adding 10 μl of S1 stop solution, followed by incubation at 95°C for 15 min to inactivate the S1 nuclease and hydrolyze bound RNA. Ten microliters of neutralizing solution were added to hydrolyze the DNA:RNA heteroduplexes and degrade the RNA, leaving the sample with the selected probe only. The samples were then transferred for RNA quantification to a plate, in which 16 spots, each containing a linker to specifically capture each probe, had been printed into each well, and incubated at 50°C for 24 h to allow for probe hybridization to the plate. After several washes, 40 μl of detection linker solution was added to each well, and the ArrayPlate was incubated at 60°C for 90 min to allow the detection linker to hybridize to the ArrayPlate. The plate was washed for another cycle and incubated at 37°C for 30 min after the addition of the detection enzyme (40 μl). This step was followed by another wash and the addition of horseradish peroxidase chemiluminescent substrate. The chemiluminescent signal from each well of the ArrayPlate was quantified and reported by SuperCapella Imager.

Reverse transcription and quantitative PCR. Given that GR mRNA levels and not MR mRNA levels were differentially expressed in dependent and nondependent rats during acute alcohol withdrawal, we only measured GR mRNA levels during protracted alcohol abstinence. Total RNA was extracted using the PicoPure RNA Isolation kit (Applied Biosystems) and treated with DNase I (Qiagen). Concentrations were determined using the Quant-iT RiboGreen RNA Assay Kit (Invitrogen). cDNA was reverse-transcribed from total RNA using iScript cDNA (Bio-Rad) in the presence of Oligo (dT) and random primers according to the manufacturer's instructions. Gene expression levels were determined by quantitative PCR (qPCR) using a SYBR Green-based detection system (iQ SYBR Green Supermix, Bio-Rad Laboratories). Reactions were performed on laser-equipped thermal cyclers to detect changes in fluorescence in real time, and cDNA concentrations of GR were calculated according to the relative quantification (ddCt) method, corrected for differences in PCR efficiency, and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), cyclophilin A (*Ppia*), or β-actin (*Actb*). The following primers were used: GR primer pair 1 (forward, 5′ TACAAAGATTGCAGGTATCCTATGA 3′; reverse, 5′ ACTCTT GGCTCTTCAGACCTTC 3′), primer pair 2 (forward, 5′ GCACCAGCTA TCAGAAGACC 3′; reverse, 5′ GCTCTACACCAGTTAGGACG 3′), *Ppia* (forward, 5′ TATCTGCACTGCCAAGACTGAGTG 3′; reverse, 5′ CTTCTT GCTGGTCTTGCCATTCC 3′), *Actb* (forward, 5′ AGATTACTGCCCT GGCTCCT 3′; reverse, 5′ CAGTGAGGCCAGGATAGAGC 3′).

Mifepristone (RU38486) treatment. To investigate the functional role of GRs in the escalation of alcohol self-administration during alcohol vapor exposure, the rats were trained to self-administer alcohol as described above and subcutaneously implanted with mifepristone pellets (a GR/progesterone receptor antagonist; 150 mg; Innovative Research of America) or placebo pellets for chronic release (21 d). The mifepristone dose was chosen based on previous studies (Schneider et al., 2003; Nephew et al., 2008) and adjusted for bodyweight. Twenty-four hours later, alcohol vapor exposure and behavioral testing began. To investigate the functional role of GRs in escalated alcohol self-administration during protracted alcohol abstinence, additional groups of dependent and nondependent rats were subcutaneously implanted with mifepristone or placebo pellets 1 week after the vapor was turned off. Behavioral testing began 1 week after pellet implantation. Three animals from this experiment were excluded: one mifepristone-treated dependent rat and one placebo-treated nondependent rat that showed rejection of the pellet and one placebo-treated dependent rat outlier.

Statistical analysis. The data are expressed as mean and SEM. The data were analyzed using ANOVA with or without repeated measures, with session as the within-subjects factor and group (dependent vs nondependent) and treatment (mifepristone vs placebo pellet) as between-subjects factors. When appropriate, *post hoc* comparisons were performed using Fisher's Least Significant Difference (LSD) test. Glucocorticoid receptor and MR mRNA levels were compared using Student's *t* test. The accepted level of significance for all tests was $p \leq 0.05$.

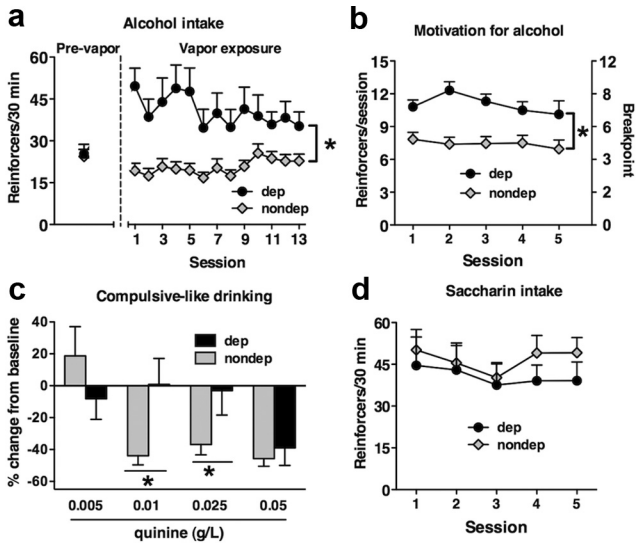

Figure 1. Specific increase in alcohol intake and compulsive drinking in alcohol vapor-exposed rats during acute alcohol withdrawal. ***a***, Number of lever presses for alcohol before (pre-vapor) and during alcohol vapor exposure on a fixed-ratio 1 (FR1) schedule of reinforcement (i.e., every active lever press was reinforced with 0.1 ml of 10% alcohol, w/v). ***b***, Number of alcohol reinforcers earned and last ratio achieved in a progressive-ratio test. ***c***, Compulsive-like drinking (i.e., persistent alcohol drinking despite the aversive bitter taste of quinine added the alcohol solution). The data represent the percentage change from baseline (i.e., lever presses for alcohol alone before adulteration with quinine). ***d***, Number of lever presses for a saccharin (0.004%, w/v) solution (FR1). The data represent mean and SE. *$p < 0.05$, significant difference between dependent and nondependent. $n = 16–18$ per group.

Results

Alcohol vapor-exposed animals show increased alcohol intake, motivation for alcohol, and compulsive drinking

Pre-vapor operant responding for alcohol is shown in Figure 1*a*. Subsequent testing began after 1 month of intermittent alcohol vapor exposure ("dependence induction" for the dependent group) and was performed during acute withdrawal (6–8 h after the vapor was turned off). Vapor-exposed dependent rats showed increased lever press responding for alcohol (group effect: $F_{(1,32)} = 7.6, p < 0.01$) compared with nondependent rats (Fig. 1*a*). Similar results were obtained for alcohol intake (in g/kg). With the exception of session 10, dependent rats (0.68 ± 0.08 g/kg/30 min) showed higher intake than nondependent rats (0.3 ± 0.02 g/kg/30 min) in all self-administration sessions (group × session interaction: $F_{(12,384)} = 2.8, p < 0.001$; LSD *post hoc* test: $p < 0.05$). Dependent rats also showed increased responding compared with nondependent rats (group effect: $F_{(1,32)} = 17.1, p < 0.0005$) in the PR test (Fig. 1*b*).

For the quinine-adulteration test (Fig. 1*c*), the analyses revealed a significant group × concentration interaction ($F_{(3,96)} = 5.6, p < 0.001$) for the percentage change from baseline consumption. Dependent rats showed more resistance to the aversive taste of quinine, with significantly greater intake by dependent rats than nondependent rats of solutions adulterated with 0.01 g/L quinine ($p < 0.01$) and 0.025 g/L quinine ($p < 0.05$). Because quinine may be appetitive at low concentrations (Da Silva et al., 2005), and a trend toward increased drinking was observed in nondependent rats at the lowest quinine concentration, we additionally performed a statistical analysis that excluded the lowest

quinine concentration. The results again revealed a significant group × concentration interaction ($F_{(2,64)} = 5.0$, $p < 0.01$), thus confirming the robustness of the observed effect.

To control for taste differences between groups, another set of rats was given quinine solution (0.025 g/L) without alcohol. A 36.6% versus 32.9% reduction in responding was observed in vapor-exposed versus control rats ($p = 0.8$), respectively, compared with baseline responding for alcohol. When 10% alcohol was added to the quinine solution, vapor-exposed animals increased their intake to the previous levels of alcohol self-administration, whereas control animals showed even lower levels of alcohol/quinine intake (group effect: $p < 0.01$). Thus, dependent and nondependent rats were equally sensitive to the aversive bitter taste of quinine, but only dependent rats were "motivated" to overcome the aversiveness of quinine to obtain alcohol.

The increased alcohol intake, motivation for alcohol consumption, and compulsive drinking exhibited by dependent rats was specific for alcohol because dependent and nondependent rats did not differ in the self-administration of saccharin-sweetened water (Fig. 1*d*).

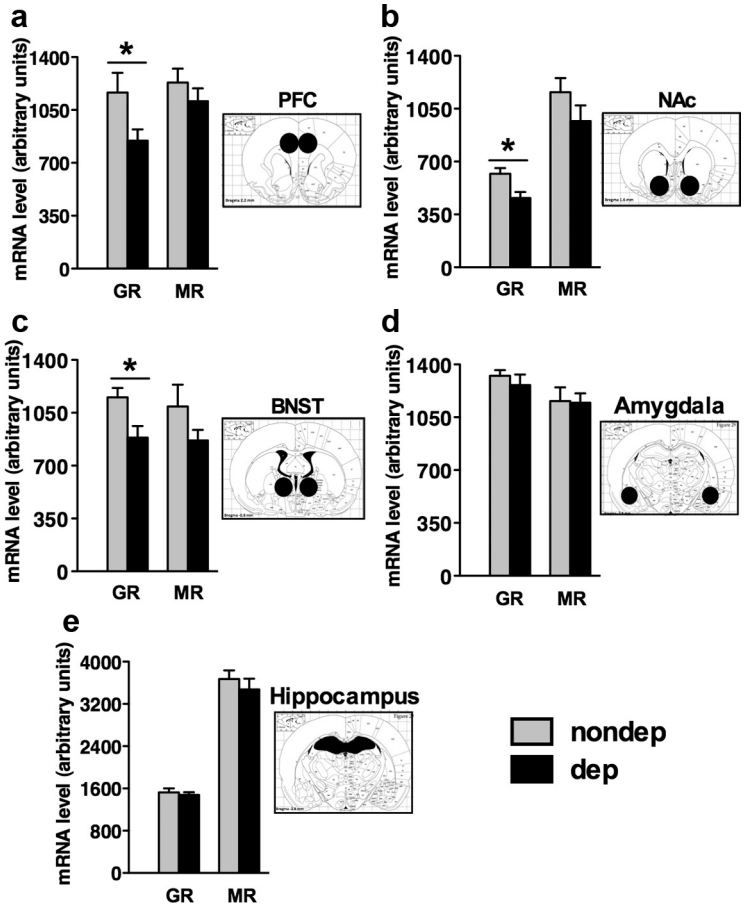

Figure 2. Acute alcohol withdrawal (24 h after the vapor was turned off) was accompanied by GR downregulation in stress/reward-related brain areas. Dependent rats showed lower GR mRNA levels in the (***a***) PFC, (***b***) NAc, and (***c***) BNST but not (***d***) amygdala or (***e***) hippocampus compared with nondependent rats. Insets illustrate the approximate location of brain punches. Data represent mean and SE. *$p < 0.05$, significant difference between dependent and nondependent. $n = 7$–17 per group.

Alcohol withdrawal is associated with GR mRNA expression changes in stress/reward-related brain regions

We next determined GR and MR mRNA expression levels in several stress/reward-related brain regions. Acute alcohol withdrawal (24 h after the alcohol vapor was turned off) was associated with GR mRNA downregulation in the PFC ($t_{20} = 2.1$, $p < 0.05$; Fig. 2*a*), NAc ($t_{18} = 2.7$, $p < 0.05$; Fig. 2*b*), and BNST ($t_{24} = 2.7$, $p < 0.05$; Fig. 2*c*) but not amygdala (Fig. 2*d*) or hippocampus (Fig. 2*e*). No group differences were found for MR mRNA levels in any brain areas.

For protracted alcohol abstinence (3 weeks after the alcohol vapor was turned off), we found that GR mRNA was significantly higher in dependent rats than in nondependent rats in the NAc core ($t_{12} = 3.6$, $p < 0.005$), ventral BNST ($t_{10} = 2.6$, $p < 0.05$), and central nucleus of the amygdala (CeA; $t_{11} = 4.4$, $p < 0.005$) but not PFC, NAc shell, dorsolateral BNST, or basolateral nucleus of the amygdala (BLA; Fig. 3).

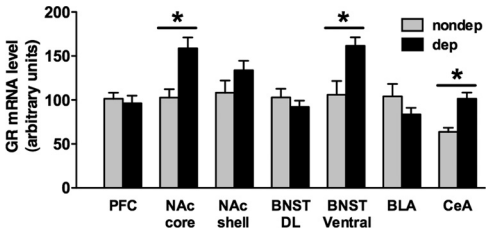

Figure 3. Protracted alcohol abstinence (3 weeks after the vapor was turned off) was accompanied by GR upregulation in stress/reward-related brain areas. Dependent rats showed higher GR mRNA levels in the NAc shell, ventral BNST, and central nucleus of the amygdala (CeA) but not PFC, NAc core, dorsolateral (DL) BNST, or basolateral amygdala (BLA) compared with nondependent rats. The data represent mean and SE. *$p < 0.05$, significant difference between dependent and nondependent. $n = 5$–8 per group.

Antagonism of GRs prevents the development of alcohol dependence and blocks escalated alcohol intake during protracted abstinence

Before vapor exposure, the groups selected as dependent and nondependent did not differ significantly with regard to baseline alcohol intake. Chronic mifepristone treatment selectively blocked the escalation of alcohol intake in vapor-exposed rats (group × treatment × day interaction: $F_{(4,136)} = 2.8$, $p < 0.05$). Placebo-treated, vapor-exposed rats exhibited a significant increase in lever pressing for alcohol compared with placebo-treated nondependent rats on day 10 ($p < 0.05$), day 13 ($p < 0.005$), and day 17 ($p < 0.05$) of vapor exposure and compared with mifepristone-treated vapor-exposed rats on day 6 ($p < 0.05$), day 10 ($p < 0.005$), day 13 ($p < 0.001$), and day 17 ($p < 0.001$) of vapor exposure. Mifepristone-treated, vapor-exposed rats did not differ from nondependent rats, and mifepristone did not affect lever pressing for alcohol in nondependent rats (Fig. 4*b*). Although a marginal, nonsignificant effect was detected for alcohol intake (in g/kg; treatment × group × session interaction: $F_{(4,136)} = 2.2$, $p = 0.07$), additional analyses performed separately by group revealed an overall treatment effect in dependent rats only ($F_{(5,85)} = 3.3$, $p < 0.01$), with mifepristone-treated dependent rats displaying lower alcohol intake compared with placebo-

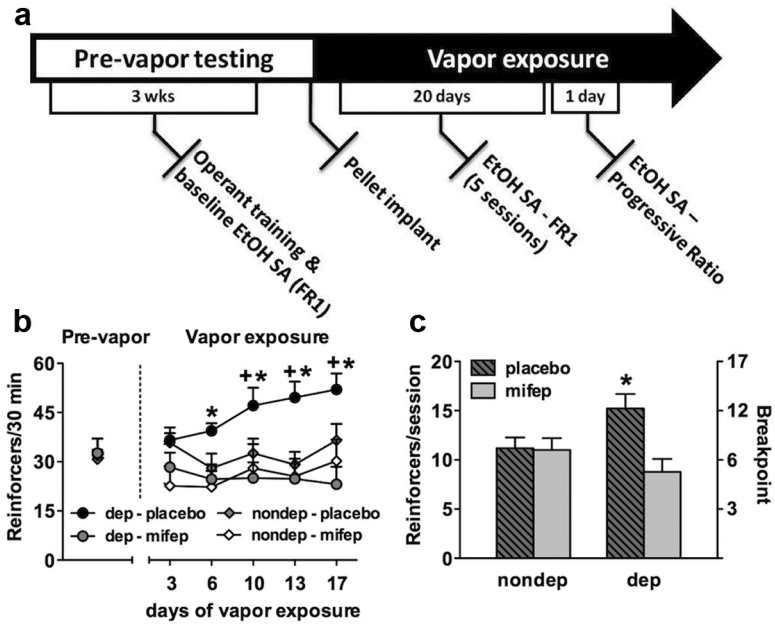

Figure 4. Chronic GR blockade by mifepristone prevented the escalation of alcohol intake and motivation for alcohol in vapor-exposed animals during acute alcohol withdrawal. ***a***, Timeline of the experiment. Dependent and nondependent rats were implanted with pellets for the chronic release of the GR antagonist mifepristone (150 mg for 21 d) or placebo before exposure to alcohol vapor. Mifepristone-treated vapor-exposed rats did not exhibit an escalation of alcohol intake (***b***) or increased PR responding (***c***) compared with placebo-treated vapor-exposed rats. Mifepristone did not influence alcohol intake in nondependent rats. The data represent mean and SE. $^{*}p < 0.05$, significant difference from mifepristone-treated vapor exposed rats; $^{+}p < 0.05$, significant difference from placebo-treated nondependent rats. $n = 9–10$ per group.

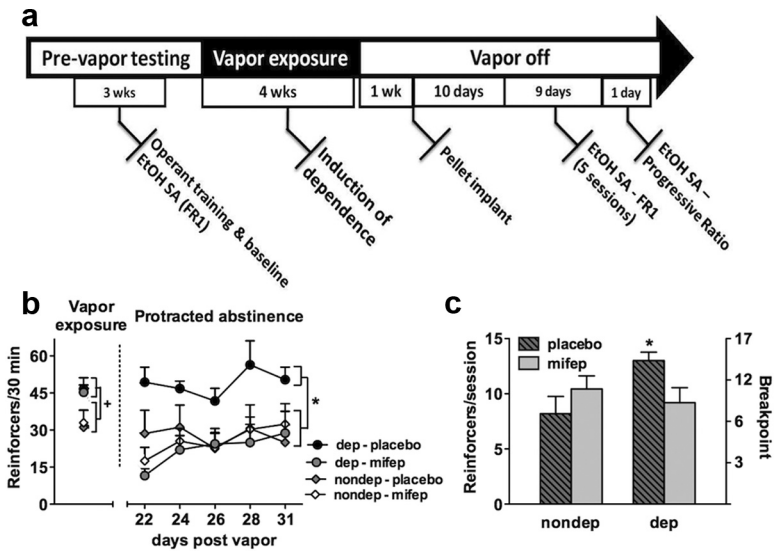

Figure 5. Chronic GR blockade by mifepristone decreased escalated alcohol intake in vapor-exposed animals during protracted alcohol abstinence. ***a***, Timeline of the experiment. The rats were made dependent on alcohol by exposure to chronic, intermittent vapor exposure and then removed from the vapor chambers. One week later, dependent and nondependent rats were implanted with pellets for the chronic release of the GR antagonist mifepristone (150 mg for 21 d) or placebo. Behavioral testing began 1 week after pellet implantation (3 weeks of protracted withdrawal). Mifepristone-treated vapor-exposed rats did not exhibit escalated alcohol intake (***b***) or increased PR responding (***c***) compared with placebo-treated vapor-exposed rats. Mifepristone did not influence alcohol intake in nondependent rats. The data represent mean and SE. $^{*}p < 0.05$, significant different from mifepristone-treated dependent rats and placebo-treated nondependent rats. $n = 5–7$ per group.

treated dependent rats. The average intake was 0.51 ± 0.12 versus 0.98 ± 0.08 g/kg/30 min for mifepristone- and placebo-treated dependent rats, respectively.

For the PR test, a group × treatment interaction ($F_{(1,34)} = 6.1$, $p < 0.05$) was detected. Mifepristone blocked the increased responding for alcohol produced by alcohol vapor exposure ($p < 0.005$), without altering responding to obtain alcohol in nondependent controls (Fig. 4*c*).

The results for the effect of mifepristone on alcohol self-administration during protracted alcohol abstinence are shown in Figure 5. During alcohol vapor exposure, dependent rats displayed higher alcohol self-administration compared with nondependent rats ($F_{(1,18)} = 6.6$, $p < 0.05$). Placebo-treated rats with a history of alcohol dependence displayed escalated alcohol intake during protracted abstinence, and mifepristone treatment blocked this effect. The treatment did not affect alcohol intake in nondependent rats. Indeed, placebo-treated dependent rats displayed higher alcohol intake compared with all of the other groups (group vs treatment interaction: $F_{(1,18)} = 5.1$, $p < 0.05$; LSD *post hoc* test: $p < 0.05$).

For the PR test, a group × treatment interaction ($F_{(1,18)} = 5.7$, $p < 0.05$) was detected. Placebo-treated dependent rats displayed increased responding for alcohol compared with mifepristone-treated dependent rats and placebo-treated nondependent rats ($p < 0.05$; Fig. 5*c*).

Discussion

We report here that rats made dependent on alcohol by chronic, intermittent alcohol vapor exposure displayed a specific increase in alcohol intake and compulsive alcohol drinking compared with nondependent rats, traits that are thought to be hallmarks of alcohol dependence. Compared with nondependent rats, dependent rats showed GR mRNA downregulation in several stress/reward-related brain areas during acute withdrawal and GR upregulation during protracted alcohol abstinence. A functional role for GRs in alcohol dependence was demonstrated by showing that chronic GR blockade during the course of alcohol vapor exposure prevented the escalation of alcohol intake and blocked the increase in PR responding. Chronic GR antagonism also blocked escalated and compulsive alcohol drinking during protracted abstinence in rats with a history of alcohol dependence. These results suggest a critical role for GR in the development and maintenance of alcohol dependence.

Alcohol withdrawal-associated GR mRNA changes in stress/reward-related brain regions

Glucocorticoid receptor mRNA was downregulated in the PFC, NAc, and BNST during acute alcohol withdrawal, an effect also observed with chronic stress (de Kloet et al., 2005; Noguchi et al., 2010). The lack of effect in the amygdala and hippocampus does not exclude the possibility of

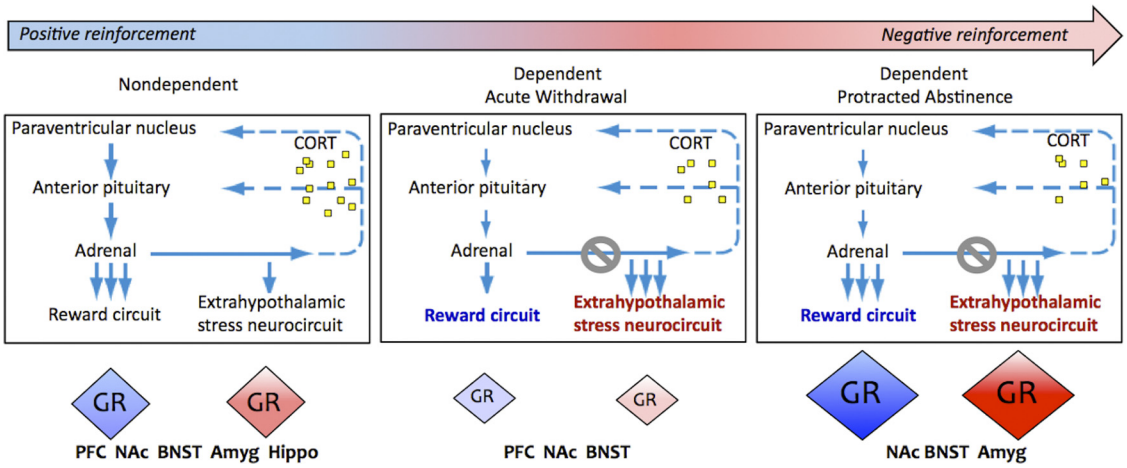

Figure 6. Hypothalamic-pituitary-adrenal axis, extrahypothalamic GR levels, and brain stress/reward function hypothesized to be recruited at different stages of the addiction cycle as addiction moves from positive reinforcement to negative reinforcement. Left, In nondependent subjects, alcohol activates the HPA axis to release CORT from the adrenal gland (Ellis, 1966; Lee and Rivier, 1997; Richardson et al., 2008), and CORT facilitates the reinforcing effects of alcohol (Fahlke et al., 1995, 1996) via positive reinforcement. High CORT levels decrease HPA axis activity via negative feedback. Middle, Repeated cycles of alcohol intoxication/withdrawal induce overactivation of the HPA axis that disrupt HPA axis function (i.e., blunted activity; Richardson et al., 2008) and downregulate GRs levels in stress/reward-related brain regions. These long-lasting changes can "sensitize" extrahypothalamic stress systems (e.g., CRF) involved in the behavioral response to stressors and further drive escalated and compulsive drug intake (Makino et al., 2002) via negative reinforcement (Edwards and Koob, 2010). Right, During protracted abstinence, GR levels are upregulated in stress/reward-related brain regions, suggesting receptor adaptation when alcohol vapor exposure ceases. Although peak HPA axis activation is blunted during protracted abstinence in alcohol dependence (Adinoff et al., 1990; Zorrilla et al., 2001), alcohol dependence-mediated dysregulation of GRs remains. A sensitized GR system would be expected to increase the "gain" for a neuronal response to CORT, sustaining the escalation of alcohol intake even in the absence of peak levels of released CORT. There are several steps between GR binding, mRNA expression, and function. Pinpointing the single molecular mechanism that underlies escalated alcohol intake is difficult at this time. The bidirectional regulation of GRs at different withdrawal time-points suggests that GR expression is dynamically regulated in the alcohol-dependent and postdependent brain and mediates escalated alcohol drinking. Amyg, amygdala; Hippo, hippocampus.

alterations in GR levels in these brain regions because the measurements included the whole structures rather than discrete subregions. In contrast, GR levels were increased in the NAc core, CeA, and ventral BNST during protracted alcohol abstinence, suggesting receptor adaptation when alcohol exposure ceased. This bidirectional regulation of GRs at different withdrawal time-points suggests that GR expression is dynamically regulated in the alcohol-dependent and postdependent brain. Evidence shows that both increased GR activity and decreased GR activity are associated with increased anxiety (Wei et al., 2004; Ridder et al., 2005; Jakovcevski et al., 2008), but escalated alcohol intake during protracted abstinence may also involve GR and reward system sensitization (Piazza and Le Moal, 1998). Thus, opposite changes in GR levels during acute alcohol withdrawal and protracted abstinence may play a role in sensitivity to stress/reward and escalated alcohol intake during these distinct phases of alcohol dependence (Valdez et al., 2002; Rimondini et al., 2003; O'Dell et al., 2004; Zhao et al., 2007; Gilpin et al., 2008; Sommer et al., 2008). Corticosterone levels have been shown to be blunted during acute withdrawal and protracted abstinence in our models of alcohol dependence (Zorrilla et al., 2001; Richardson et al., 2008), but directly relating CORT levels to GR changes during these phases would be an important future study. Altogether, the present findings suggest the occurrence of allostatic-like changes in the extrahypothalamic GR system in alcohol dependence.

Chronic GR antagonism blocks the escalation of alcohol intake during acute alcohol withdrawal and reverses escalated alcohol intake during protracted alcohol abstinence

Chronic mifepristone treatment during alcohol vapor exposure completely prevented the escalation of alcohol intake in vapor-exposed rats, indicating that functional GRs are required for the escalation of alcohol intake that is associated with the development of alcohol dependence. Mifepristone did not affect alcohol consumption in nondependent rats, as previously reported (Fahlke et al., 1995; 1996; O'Callaghan et al., 2005; Yang et al., 2008; Lowery et al., 2010). This result suggests that mifepristone prevents neuroplasticity involved in escalated alcohol drinking but does not influence the positive reinforcing properties of alcohol that are hypothesized to maintain moderate alcohol drinking in nondependent rats. Mifepristone also blocked the increased responding on a PR schedule produced by dependence, suggesting a decrease in the development of compulsive drinking. Progressive-ratio responding can be linked to the construct of compulsivity, in which responding to alcohol is persistent in the face of adverse consequences (i.e., increased cost for each subsequent reward) (Koob, 2012). Mifepristone has also been shown to attenuate behavioral sensitization produced by repeated stress or alcohol injections (Roberts et al., 1995), decrease alcohol drinking in limited-access conditions (Koenig and Olive, 2004), and reduce the stress-induced reinstatement of alcohol seeking (Simms et al., 2012) in nondependent rats, suggesting that mifepristone can affect alcohol-related behaviors under stressful conditions. Additionally, placebo-treated rats with a history of alcohol dependence drank more alcohol during protracted abstinence compared with nondependent rats, as previously reported (Valdez et al., 2002; Rimondini et al., 2003; Gilpin et al., 2008; Sommer et al., 2008). In the present study, chronic mifepristone treatment also decreased alcohol intake during protracted abstinence in rats with a history of alcohol dependence to the levels of nondependent rats. This effect was selective for alcohol dependence (i.e., nondependent alcohol drinking was unaffected by mifepristone). Possibly, low doses of mifepristone block the reinforcing effects of alcohol only when the GR and reward systems are hypersensitive, similar to protracted abstinence. These results support a functional role for GRs in escalated drinking during protracted abstinence.

Although peak HPA axis activation is blunted during protracted abstinence in alcohol dependence (Adinoff et al., 1990; Zorrilla et al., 2001), alcohol dependence-mediated GR dysregu-

lation remains. A sensitized GR system would be expected to increase the "gain" for a neuronal response to CORT, sustaining the escalation of alcohol intake even in the absence of peak levels of released CORT. This sensitization of GR function may occur at several levels. One possibility is that there is a greater overall number of cytosolic GRs that facilitate ligand-dependent receptor dimerization and translocation to the nucleus. Alternatively, there may be altered phosphorylation of the GR (e.g., Sr203, Ser211, Ser226), thus modulating its transcriptional activity and altering its protein stability and subcellular location (Wallace and Cidlowski, 2001; Itoh et al., 2002; Miller et al., 2005; Chen et al., 2008; Avenant et al., 2010). Another possibility is that GR dissociation with cytosolic binding partners (HSPs, FKBPs) and nuclear translocation may be sensitized following repeated activation of these pathways. Additionally, the nuclear interactions between GR and other cofactors or transcriptional regulators (e.g., AP-1, NFKB) may remain altered during protracted abstinence and sensitize the GR system indirectly. Ligand-independent GR function (Eickelberg et al., 1999; Verhoog et al., 2011; Galliher-Beckley et al., 2011; Ritter et al., 2012) may also be sensitized. Each of these molecular processes may work to sensitize the GR system and maintain escalation despite blunted CORT release. However, given the several steps between GR binding, mRNA expression, and function (Reichardt and Schütz, 1998; Davies et al., 2008), pinpointing a single molecular mechanism that underlies escalated alcohol intake is difficult. Understanding this process will clearly require further research.

Mifepristone also inhibits the progesterone receptor (Peeters et al., 2004), which may participate in alcohol-related behaviors (Janis et al., 1998). However, the effects of mifepristone during alcohol withdrawal appear to be GR-specific (Jacquot et al., 2008). Moreover, one question is whether mifepristone can penetrate the blood–brain barrier. Mifepristone has somewhat limited blood–brain barrier permeability, but the concentrations attained in the brain are still significant (almost one-third those of serum; Heikinheimo and Kekkonen, 1993). Accordingly, systemic mifepristone administration produces behavioral changes (Roberts et al., 1995; Koenig and Olive, 2004; Simms et al., 2012), inhibits neurogenesis (Oomen et al., 2007), and, most directly, competes with dexamethasone for binding sites in the rat CNS (Allen et al., 1988). Our results likewise suggest that mifepristone reaches central GRs at concentrations sufficient for receptor occupancy.

Furthermore, CORT is released in hourly pulses with a higher amplitude of release during the active period (i.e., the dark in rodents), thus reflecting the circadian pattern of release (Young et al., 2004). Because of the relatively low affinity of CORT for GRs, these receptors are only activated during stressful conditions or during the circadian peaks of CORT release. Thus, chronic blockade of GRs by mifepristone in hypothalamic and extrahypothalamic structures may prevent GR activation and function during the high levels of CORT observed during alcohol intoxication/withdrawal, but chronic blockade may also influence the circadian rhythm of CORT release, which also can affect stress-related behaviors (Young et al., 2004; Sarabdjitsingh et al., 2010).

Conclusions

Previous work has shown that CORT facilitates the reinforcing effects of alcohol in nondependent subjects (Fahlke et al., 1995, 1996). However, high circulating levels of CORT during alcohol intoxication/withdrawal can feed back to shut off the HPA axis, "sensitize" extrahypothalamic stress systems (e.g., CRF) involved in the behavioral response to stressors, and further drive escalated and compulsive drug intake (Makino et al., 2002). Therefore, although activation of the HPA axis may contribute to the reinforcing effects of alcohol during initial drug use, its excessive activation may lead to the sensitization of brain stress systems that mediate negative reinforcement in the transition to alcohol dependence. The present study suggests that changes in GR function in extrahypothalamic stress systems, possibly via a hypothesized initial excessive activation of GRs, are involved in alcohol dependence (Fig. 6). Normalizing GR signaling early in the transition to dependence may block the sensitization of the brain stress systems, and the normalization of GR function after alcohol detoxification may reset the reward system to result in a shift from negative reinforcement to "normal" positive reinforcement or block the well documented sensitization of reward associated with protracted abstinence. Importantly, altered GR levels have been found in the superior frontal cortex of alcoholics (Liu et al., 2007; Ponomarev et al., 2012), and mifepristone has some promise for treating dysregulated mood (Flores et al., 2006; Nihalani and Schwartz, 2007; Blasey et al., 2009). The present results indicate that the GR system may be an attractive potential pharmacological target for the treatment of alcohol dependence.

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