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Alcohol self-administration acutely stimulates the hypothalamic-pituitary-adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state

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Abstract

Clinical studies link disruption of the neuroendocrine stress system with alcoholism, but remaining unknown is whether functional differences in the hypothalamic-pituitary-adrenal (HPA) axis precede alcohol abuse and dependence or result from chronic exposure to this drug. Using an operant self-administration animal model of alcohol dependence and serial blood sampling, we show that long-term exposure to alcohol causes significant impairment of HPA function in adult male Wistar rats. Acute alcohol (voluntary self-administration or experimenter-administered) stimulated the release of corticosterone and its upstream regulator, adrenocorticotrophic hormone, but chronic exposure sufficient to produce dependence led to a dampened neuroendocrine state. HPA responses to alcohol were most robust in 'low-responding' non-dependent animals (averaging < 0.2 mg/kg/session), intermediate in non-dependent animals (averaging ~0.4 mg/kg/session), and most blunted in dependent animals (averaging ~1.0 mg/kg/session) following several weeks of daily 30-min self-administration sessions, suggesting that neuroendocrine tolerance can be initiated prior to dependence and relates to the amount of alcohol consumed. Decreased expression of corticotropin-releasing factor (CRF) mRNA expression in the paraventricular nucleus of the hypothalamus and reduced sensitivity of the pituitary to CRF may contribute to, but do not completely explain, neuroendocrine tolerance. The present results, combined with previous studies, suggest that multiple adaptations to stress regulatory systems may be brought about by excessive drinking, including a compromised hormonal response and a sensitized brain stress response that together contribute to dependence.

Keywords

ACTH; alcohol; corticosterone; corticotropin-releasing factor mRNA; self-administration; withdrawal

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Introduction

Alcoholism is a chronically relapsing disorder characterized by cycles of repeated high alcohol intake and negative emotional consequences of withdrawal thought to contribute to excessive drinking and susceptibility to relapse (Koob, 2003; Breese *et al.*, 2005; Heilig & Egli, 2006). Similar to human alcoholics, animals made ‘dependent’ using chronic exposure to high alcohol exhibit uncontrolled excessive intake of alcohol during periods of withdrawal and enhanced anxiety-like behaviors (Roberts *et al.*, 1996; Overstreet *et al.*, 2002; Rimondini *et al.*, 2002; Valdez *et al.*, 2002; O’Dell *et al.*, 2004; Koob, 2006). Identifying the effect of alcohol on the nervous system and neuroadaptive changes emerging during the progression of alcohol dependence in rodent models will help elucidate the pathology of alcoholism in humans.

Stress and its related hormones of the hypothalamic-pituitary-adrenal (HPA) axis are implicated in alcohol abuse and dependence (Wand & Dobs, 1991; Froehlich *et al.*, 2003; Koob & Kreek, 2007). Clinical studies link disruption of the HPA axis with alcoholism, including a dampened ability to cope with stress, and negative correlations between cortisol and craving and relapse in alcoholics (Lovallo *et al.*, 2000; O’Malley *et al.*, 2002; Kiefer & Wiedemann, 2004; Adinoff *et al.*, 2005). In rodents, experimenter-administered alcohol stimulates the HPA axis, and the response is significantly blunted by chronic alcohol exposure (Lee & Rivier, 1997). However, investigation of the relationship between voluntary alcohol intake and HPA function is limited. Most animal studies exploring the behavioral components underlying alcohol drinking in self-administering animal models of dependence have not thoroughly and properly explored questions about the HPA axis, largely due to the technical challenges of combining stress-sensitive neuroendocrine and behavioral studies.

To test the hypothesis that the effects of voluntary alcohol administration on the HPA axis vary with alcohol dependence, neuroendocrine measures of HPA activity were determined using serial blood sampling in a well-established operant self-administration animal model of alcohol dependence (O’Dell *et al.*, 2004; Funk *et al.*, 2006; Walker & Koob, 2007; Gilpin *et al.*, 2008b; Richardson *et al.*, 2008; for related models, see Roberts *et al.*, 1996; Overstreet *et al.*, 2002; Rimondini *et al.*, 2002; Valdez *et al.*, 2002). In this model, animals trained to self-administer alcohol and exposed for several weeks to bouts of high alcohol (e.g. vapors) exhibit an alcohol-dependence syndrome characterized by somatic and motivational withdrawal symptoms and engage in excessive drinking when alcohol is made available again (‘dependent animals’). Animals trained to self-administer alcohol but exposed for several weeks to control air do not exhibit the dependence syndrome and continue to respond for alcohol at baseline levels when alcohol is made available again (‘non-dependent’ animals). The present study focused on a particular period of acute withdrawal (6–8 h after removal from chronic alcohol). This is a well-studied period of withdrawal when dependent animals display mild signs of physical withdrawal (Roberts *et al.*, 1996) but robust signs of motivational withdrawal: (i) increased expression of anxiety-like behavior (Baldwin *et al.*, 1991; Rassnick *et al.*, 1993); (ii) elevations in brain reward thresholds (‘reward deficits’; Schulteis *et al.*, 1995); (iii) increased extracellular levels of the stress peptide corticotropin-releasing factor (CRF) in the central nucleus of the amygdala (CeA; Merlo Pich *et al.*, 1995) and lateral bed nucleus of the stria terminalis (BNST; Olive *et al.*, 2002); and (iv) excessive drinking that is sensitive to blockade by CRF₁ receptor antagonists (Gilpin *et al.*, 2008a; Richardson *et al.*, 2008). Accordingly, the goal of the present work was to ascertain the neuroendocrine profile of dependent animals at this time to establish the role the HPA axis may play in dependence. We show evidence for acute activation of the HPA axis by alcohol and demonstrate that functional changes in the HPA axis and neuroadaptive changes in CRF gene expression within the hypothalamus can be brought on with chronic exposure to alcohol sufficient to produce dependence.

Materials and methods

Animals

Adult male Wistar rats weighing 150–225 g at the start of the experiment were obtained from Charles River Laboratory (Kingston, NY, USA). Animals weighed ~550–650 g by the end of each experiment (4–6 months later), and body weight between treatment groups was not significantly different (data not shown). Rats were housed two or three per cage with food and water available *ad libitum*. Lights were on a 12 h light: dark cycle, with lights on at 06.00 h (Experiments 1, 3 and 4) or 20.00 h (Experiment 2). Because of the extensive length and technical challenges associated with these studies, animals were occasionally excluded during the course of the experiment (~25% attrition rate, which did not vary across treatment groups) due to a variety of reasons (e.g. training/baseline criteria, complications during or following surgery or during dependence induction, patency problems, collection and/or processing of plasma samples or brain tissue, etc.). Thus, for simplicity, animal numbers and descriptions below include only those animals that completed the entire experiment. All animals were allowed 4–7 days of acclimation to the laboratory and were frequently handled prior to the start of all experiments. All procedures met the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee at The Scripps Research Institute.

Experimental design and animal model of alcohol dependence

In addition to the animals described below for Experiments 1–4, a group of untrained animals was used to characterize physical signs of withdrawal and blood alcohol levels following several weeks of exposure to alcohol vapors ($n = 8$) vs. control air ($n = 8$). Unless stated otherwise, all other animals were processed through the self-administration training, maintenance, dependence induction and operant testing procedures described below in detail and in Fig. 1 before being assigned to separate groups in Experiments 1–4. All behavioral, endocrine and molecular measures in Experiments 1–4 occurred at a time when dependent animals were 6–8 h into withdrawal from alcohol vapors (during the afternoon of the light cycle for Experiments 1, 3 and 4; ~4 h into the dark cycle for Experiment 2).

Experiment 1: effect of voluntary alcohol self-administration on the HPA axis and blood alcohol levels in non-dependent and dependent animals—The goals of this experiment were to determine whether: (i) voluntary operant self-administration of alcohol stimulated the HPA axis at the level of corticosterone and its upstream regulator, adrenocorticotrophic hormone (ACTH); (ii) whether HPA hormone levels were blunted under basal conditions in dependent animals; (iii) whether self-administration of alcohol elevated ACTH and corticosterone to levels observed in non-dependent animals. Fifteen trained animals ($n = 7$ non-dependent, $n = 8$ dependent) were fitted with jugular catheters, and blood samples were measured before, during and after a 30-min operant self-administration session in which animals were allowed to respond for alcohol and water. Blood alcohol levels, ACTH and corticosterone levels were assessed at 0, 15, 30 and 45 min time-points. Details of all procedures are described below.

Experiment 2: effect of 1 g/kg alcohol on the HPA axis and blood alcohol levels in low-responding non-dependent, non-dependent and dependent animals—This experiment sought to characterize HPA responses to a controlled dose of alcohol (1 g/kg) in animals self-administering alcohol chronically but consuming different levels. Alcohol self-administration elicited a mild increase in ACTH and corticosterone (Experiment 1) compared with the robust HPA responses observed in alcohol-naïve animals following an experimenter-administered alcohol challenge (e.g. Lee & Rivier, 1997; Lee *et al.*, 2000), suggesting that some degree of HPA downregulation may occur following chronic daily use of alcohol, even

under non-dependent conditions. Thus, the primary goal of Experiment 2 was to measure ACTH and corticosterone responses to alcohol in three groups of trained animals: (i) dependent animals ($n = 5$); (ii) non-dependent animals ($n = 7$); and (iii) 'low-responding' non-dependent animals ($n = 4$). The low-responding group consisted of non-dependent animals consuming low levels of alcohol (averaging <10 responses for alcohol or 0.2 g/kg per session throughout training). Low-responding animals are normally eliminated from our studies during baseline training because of limited space and resources but nonetheless show the same relative susceptibility to dependence induction (doubling alcohol intake) as 'normal' non-dependent animals (N.W. Gilpin, A. Smith, M. Cole, F. Weiss, G.F. Koob and H.N. Richardson, unpublished results). An alcohol dose of 1 g/kg (i.v.) was chosen for the alcohol challenge because it reflected the amount voluntarily consumed by dependent animals during self-administration sessions. Animals were fitted with jugular catheters, and ACTH and corticosterone were serially measured before (0 min) and after (15 , 45 and 75 min) the alcohol injection. The timing of withdrawal (6 – 8 h) was shifted to occur during the active (dark) phase of the animals' light: dark cycle, allowing us to determine whether dampened HPA function in dependent animals persisted beyond the afternoon of the light cycle. Details of all procedures are described below.

Experiment 3: effect of CRF administration on the HPA axis in alcohol-naive, non-dependent and dependent animals—We next established whether reduced HPA axis activity associated with dependence was of peripheral or central origin by testing dependent animals for decreased responsiveness of the pituitary gland to CRF. ACTH responses were measured following administration of rat/human CRF (r/hCRF). A dose–response curve was first conducted in alcohol-naive animals to determine the dose of r/hCRF that elicits a mid-range ACTH response; this same dose was then tested in non-dependent and dependent animals. Thus, 24 alcohol-naive ($n = 6$, for each of four r/hCRF doses: 3.0 , 1.0 , 0.3 or 0.1 $\mu\text{g}/\text{kg}$) and nine trained animals (non-dependent, $n = 5$; dependent, $n = 4$) were fitted with jugular catheters, blood samples were obtained before (0 min) and after (15 , 30 and 45 min) an injection of r/hCRF, and ACTH levels were measured. Details of all procedures are described below.

Experiment 4: CRF mRNA in the paraventricular nucleus of the hypothalamus (PVN) in alcohol-naive, non-dependent and dependent animals—The final experiment determined whether decreased function of the HPA axis associated with long-term alcohol exposure and dependence was associated with alterations in CRF expression in the hypothalamic CRF system. CRF mRNA levels were measured using *in situ* hybridization histochemistry in the PVN in alcohol-naive ($n = 6$), non-dependent ($n = 10$) and dependent ($n = 13$) animals. Details of all procedures are described below.

Operant self-administration apparatus

The self-administration system consisted of operant boxes (Coulbourn Instruments, Allentown, PA, USA) contained within wooden sound-attenuated ventilated cubicles. The operant boxes were equipped with two retractable levers located 4 cm above the grid floor and 4.5 cm to either side of a small stainless steel receptacle containing two drinking cups. Two infusion pumps (Razel Scientific Instruments, Stamford, CT, USA) were connected to the system so that a lever press resulted in delivery of 0.1 mL of solution. Tap water was delivered to one dish, and the experimental solution (e.g. sweetened solution or alcohol) was delivered to the other dish. Fluid delivery and recording of operant self-administration were controlled by a microcomputer. Lever presses were not recorded during the 0.5 s inter-response time-out interval when solution was being delivered.

Operant self-administration procedure

Animals were trained to orally self-administer alcohol or water in a concurrent, two-lever, free-choice contingency. A continuous reinforcement (fixed ratio-1, FR1) schedule was used in which each lever press was reinforced. Animals acquired alcohol self-administration using a variation of the previously described saccharin fading free-choice operant conditioning protocol (Samson, 1986). The present procedure culminates in pharmacologically relevant levels of alcohol self-administration, defined by blood alcohol levels, in non-dependent animals with limited access to alcohol over a 6-week period (Roberts *et al.*, 1999). The modified procedure in the present study utilized a sweetened solution containing 3% glucose and 0.125% saccharin (Sigma, St Louis, MO, USA) instead of water restriction, and 0.2% saccharin to initiate and maintain operant responding (Funk *et al.*, 2006). Animals respond for this solution within just one–two training sessions, thereby circumventing the need for water restriction to initiate lever pressing. Operant sessions during training and post-vapor testing were conducted 5 days per week between 09.00 h and 15.00 h (lights on at 06.00 h for Experiments 1, 3 and 4, and lights off at 08.00 h for Experiment 2). Operant sessions were 30 min in duration, except during the initial days of training in which sessions lasted up to 2 h to permit acquisition of responding for the sweetened solution. Alcohol (10% w/v) then was added to the sweetened solution and, once mean responding stabilized (about 1 week), the glucose was removed from the solution, leaving only 0.125% saccharin and 10% w/v alcohol. Animals were kept at this stage until mean responding again stabilized (about 1 week), and saccharin concentrations were gradually reduced in ~50% successive steps over 2–10 days, ultimately leaving an unsweetened 10% w/v alcohol solution. Animals then were maintained on 10% w/v alcohol for at least 3 weeks, and stable responders ($\pm 25\%$ across three consecutive sessions and averaging > 10 presses for alcohol) were evenly divided into two groups matched for baseline responding and exposed to intermittent alcohol vapors (dependent) or air (non-dependent) as described below. As stated above, Experiment 2 included an additional group of trained animals normally excluded before groups are matched for baseline responding and split into non-dependent vs. dependent groups. Low-responding non-dependent animals that average ~10 or fewer alcohol responses (< 0.20 g/kg/30 min session) were included in this experiment for comparison with the non-dependent group that averages ~20–25 alcohol responses (~4.0 g/kg/30 min session) and the dependent group that averages ~55–70 alcohol responses (~1.0 g/kg/30 min session) 6–8 h into withdrawal from alcohol vapors (e.g. O'Dell *et al.*, 2004; Walker & Koob, 2007; Richardson *et al.*, 2008; see Fig. 1).

Solutions for oral self-administration

Alcohol (10% w/v) was prepared with 95% ethyl alcohol and tap water. Glucose (3%) and/or saccharin (0–0.125%; Sigma Chemical, St Louis, MO, USA) were added to water or the alcohol solutions to achieve the appropriate concentration.

Dependence induction by alcohol vapor chambers

A recent modification of the alcohol dependence model was made to reflect the natural progression of alcohol dependence in which alcohol exposure occurs in a series of extended exposures followed by periods of withdrawal (O'Dell *et al.*, 2004). Chronic exposure to intermittent vapor exposure elicits even higher alcohol self-administration than continuous vapor (O'Dell *et al.*, 2004); therefore, the procedure was used to induce dependence in trained animals in the present study. Vapors were delivered on an intermittent schedule (on at 18.00 h, off at 08.00 h) for a period of 4 weeks before post-vapor testing began. This schedule of exposure has been shown to induce dependence (O'Dell *et al.*, 2004). In the chambers, 95% alcohol flows from a large reservoir to a peristaltic pump (model QG-6, FMI Laboratory; Fluid Metering, Syosett, NY, USA). Alcohol is delivered from the pump to a sidearm flask at a flow rate that can be regulated. The flask is placed on a heater so that the drops of alcohol hitting

the bottom of the flask are vaporized. Airflow controlled by a pressure gage is delivered to the flask and carries the alcohol vapors to the vapor chamber that contains the animal cages. The flow rate was set between 22 and 27 mg/L to deliver vapors that result in blood alcohol levels between 0.125 and 0.250 g% (g/dL) or 27.2–54.4 mM, which was confirmed by weekly blood samples. Blood samples were collected by the tail-snip method (0.1–0.2 mL) from all animals (both alcohol vapor-exposed dependent and control air-exposed non-dependent groups) just after the vapors turned off (08.00 h), and 5 μ L of plasma was used for measurement of blood alcohol levels, as described below. When dependent animals had blood alcohol levels outside the target range, the evaporated alcohol values were adjusted to reestablish the correct range. As expected, blood alcohol levels were always undetectable (< 0.02 g% or 4.4 mM) in non-dependent animals, but tail bleeding was performed to control for any stress experienced during this procedure.

Measurements of physical withdrawal

Previous work using a related model (chronic continuous vapor exposure) indicated that dependent animals show mild, but not severe (e.g. spontaneous tonic-clonic convulsions; Majchrowicz, 1975) signs of physical withdrawal following removal from alcohol vapors (Roberts *et al.*, 1996). To characterize withdrawal severity in the present model, withdrawal signs were quantified in 2-h bouts over 24 h after removal from chronic intermittent alcohol vapors or control air using methodology similar to earlier reports (Macey *et al.*, 1996; Roberts *et al.*, 1996). Specifically, distal limb flexion response, tail stiffness and abnormal body posture were measured using a subjective 0–2 point scale to score withdrawal symptoms (0 = undetectable, 1 = moderate, 3 = severe). Ventromedial distal limb flexion responses were determined by lifting the rat up by the scruff of the neck and observing retraction of the lower legs toward the body. Tails were examined for rigidity and/or curvature upward toward the back. The presence of a broad-based stance and/or abnormal gait indicated abnormal body posture. Statistical analyses were conducted on overall withdrawal severity scores, that were determined for each animal by summing the scores for the three signs, yielding a range from 0 to 6.

Intravenous catheterization surgery

Animals used in blood sampling experiments were anesthetized by isoflurane inhalation, and intravenous catheters were aseptically inserted in the right jugular vein using a modified version of a procedure previously described (Caine *et al.*, 1993). The catheter assembly consisted of a 14-cm length of silastic tubing (0.025 inch inner diameter, 0.047 inch outer diameter; Dow Corning, Midland, MI, USA) that was attached to a guide cannula (Plastics One, Roanoke, VA, USA) bent at a near right angle and embedded in dental acrylic anchored with a 2 \times 2 cm square of mesh. The catheter was fed subcutaneously from where it was secured in the jugular vein to the animal's back. It exited through a small incision on the back, and the base was sealed with a small plastic cap and metal cover cap. This design helps keep the catheter base sterile and protected from cage mates (animals were housed in groups of two–three). Catheters were flushed with heparinized saline (10 U/mL of heparin sodium; American Pharmaceutical Partners, Schaumburg, IL, USA; in 0.9% bacteriostatic sodium chloride; Hospira, Lake Forest, IL, USA) containing antibiotic (20 mg/0.2 mL; Timetin, GlaxoSmithKline; once daily for 1 week, then once every 7–14 days) and heparinized saline afterward until the end of the experiment.

Blood sampling: alcohol challenge, self-administration and CRF challenge experiments

Blood sampling experiments were conducted in the following manner. In Experiments 1, 3 and 4, hormone levels were measured during the afternoon of the light phase of the light: dark cycle (lights on at 06.00 h and off at 18.00 h) when dependent animals were 6–8 h into withdrawal

(~14.00–16.00 h) and blood alcohol levels were back at baseline (preliminary work; see also Fig. 1B). In Experiment 2, the animals were housed in a reverse light cycle (lights on at 20.00 h and off at 08.00 h) that allowed for analysis of HPA hormones during the dark phase while maintaining the same withdrawal period as Experiments 1–3 (6–8 h after removal from alcohol vapors for dependent animals). To minimize the effects of extraneous stress on stress-sensitive hormones, animals were allowed at least 1 week of recovery following surgery and were sampled under basal conditions (see below). In the self-administration blood sampling experiment (Experiment 1) following surgical implantation of jugular catheters and post-surgery recovery, animals were tested every 2–4 days in the operant chambers while connected to tethers and sampling tubing until baseline responding stabilized. This ensured that on the day of the experiment, animals were fully habituated to the sampling apparatus, and the experimental procedures would not interfere with operant responding, that is highly sensitive to environmental changes.

On the day of sampling, catheters were connected to tubing attached to a syringe containing heparinized saline. Rats were placed in individual buckets (alcohol and CRF challenge experiments) or operant boxes (self-administration experiment) where they remained awake and freely moving, allowing for collection of blood without disturbing the animals. Animals rested for 3 h after connection to allow stress-responsive hormones to return to basal levels (C. Rivier, unpublished results) before serial sampling began. Blood samples were taken via the catheter (0.3 mL each sample), and fluid was replaced with heparinized saline. In the self-administration behavior/sampling experiment (Experiment 1), the first sample (0 min, basal) was followed by presentation of the operant levers, two samples were taken during self-administration (15 and 30 min), and a final sample was taken 15 min after retraction of the levers (45 min). In the alcohol challenge experiment (Experiment 2), the first sample (basal) was followed by i.v. administration of alcohol (1 g/kg of 20% w/v alcohol diluted in heparinized saline), and three samples were taken thereafter (15, 45 and 75 min). During the injection, alcohol was delivered in small pulses evenly distributed over the course of 5 min to approximate the blood alcohol levels observed during self-administration of alcohol in the previous experiment. In the CRF challenge experiment (Experiment 3), the first sample (basal) was followed by i.v. administration of r/hCRF, and three subsequent blood samples were taken (15, 30 and 45 min). Blood was collected into 1.5-mL Eppendorf tubes on ice containing 10 μ L EDTA and capped immediately to avoid evaporation of alcohol in the blood. Samples were spun at 3000 rpm for 15 min, and plasma was collected and stored at -80°C until ACTH, corticosterone and blood alcohol level assays were performed (described below).

Reagent

r/hCRF was synthesized by solid phase methodology and generously provided by Dr Jean Rivier (The Salk Institute, La Jolla, CA, USA; Kornreich *et al.*, 1992). It was dissolved in 0.04 M phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and 0.01% ascorbic acid.

Measurement of ACTH levels

Plasma ACTH levels were determined using a commercially available two-site immunoradiometric assay (IRMA) kit (Allegro kit; Nichols Institute, San Juan Capistrano, CA, USA). IRMA was selected for measurement of plasma ACTH levels because it uses two monoclonal antibodies to measure ACTH_{1–39} in plasma, resulting in cross-reactivity < 0.5% with ACTH fragments (Gibson *et al.*, 1989). Data are expressed in pg/mL plasma, and the lowest limit of detectability was 5 pg/mL or 1.1 pM. This assay has been validated for rats (Rivier & Shen, 1994) and has intra- and interassay coefficients of variation of 3.2% and 6.8%, respectively.

Measurement of corticosterone levels

Total plasma corticosterone levels were determined using a commercially available radioimmunoassay kit (MP Biomedicals, Orangeburg, NY, USA). Data are expressed in ng/mL plasma, and the lowest limit of detectability was 5 ng/mL or 14.4 nM. Intra- and interassay coefficients of variation are 7.3% and 13.2%, respectively.

Measurement of blood alcohol levels

Plasma (5 μ L) was used for measurement of blood alcohol levels using an Analox AM 1 analyzer (Analox Instruments, Lunenburg, MA, USA). The reaction is based on the oxidation of alcohol by alcohol oxidase in the presence of molecular oxygen (alcohol + O₂ \rightarrow acetaldehyde + H₂O₂). The rate of oxygen consumption is directly proportional to the alcohol concentration. Single-point calibrations were done for each set of samples with reagents provided by Analox Instruments (0.025–0.400 g% or 5.4–87.0 mM).

Intracardial perfusion and brain tissue preparation for in situ hybridization histochemistry

Brain tissue collection was timed to correspond to the period when animals would normally be tested for self-administration to best represent the neural state under which animals were motivated to lever press for alcohol. Thus, 2–4 days after the last 30-min self-administration session and at a time when dependent animals were 6–8 h into withdrawal from vapors, dependent, non-dependent and alcohol-naive control rats (matched for age, body weight and handling) were deeply anesthetized with 35% chloral hydrate, a drug that does not increase immediate-early gene RNA levels or ACTH concentrations (C. L. Rivier, unpublished results). Tissue collection and processing procedures were similar to those described previously (Potter *et al.*, 1994). Briefly, animals were perfused intracardially with saline followed by cold 4% paraformaldehyde/0.1 M borate buffer, pH 9.5. The brains were removed, post-fixed in 4% paraformaldehyde for 4–5 days, and placed overnight in 10% sucrose/4% paraformaldehyde/0.1 M borate buffer. Brains were sectioned into 30- μ m coronal slices obtained at 120- μ m intervals and stored at –20°C in a cryoprotectant solution (50% 0.1 M phosphate-buffered saline, 30% ethylene glycol and 20% glycerol) until *in situ* hybridization histochemistry.

Synthesis and preparation of probe for in situ hybridization histochemistry

All solutions were treated with diethylpyrocarbonate (DepC) and autoclaved to prevent degradation of RNA. The pGEM4 plasmid containing a 1.2-kb EcoRI fragment of rat CRF cDNA (kindly provided by Dr K. Mayo) was linearized with HindIII. The antisense probe was transcribed in a reaction mixture containing 250 ng of linearized plasmid in 6 mM MgCl₂, 36 mM Tris (pH 7.5), 2 mM spermidine, 8 mM dithiothreitol, 25 mM ATP/GTP/CTP, 5 μ M of γ -³⁵S-UTP, 1 U of RNasin (Promega, Madison, WI, USA) and 10 U of SP6 for 60 min at 37°C. Unincorporated nucleotides were removed using Quick-Spin columns (Boehringer Mannheim, Indianapolis, IN, USA). A sense probe was used as a control for non-specific signals in some adjacent sections for *in situ* hybridization.

In situ hybridization histochemistry and microscopic analysis

Hybridization labeling of CRF-expressing cells was performed using ³⁵S-labeled CRF cRNA probe. Protocols for riboprobe synthesis, hybridization and autoradiographic localization of mRNA signals were adapted from Simmons *et al.* (1989) and Potter *et al.* (1994). Brain paste standards containing serial dilutions of ³⁵S-UTP, used for quantification of mRNA signal, were prepared concurrently to ensure that optical density was found within the linear range of the standard curve. Brain tissue was processed in two hybridization runs, and tissue from dependent and non-dependent rats was included in both runs for CRF mRNA levels. Transcript expression did not differ across runs, and animals were combined for data analyses. All solutions were treated with DepC and sterilized to prevent RNA degradation. Sections mounted onto gelatin-

and poly-L-lysine-coated slides were desiccated under vacuum overnight, fixed in 4% paraformaldehyde for 30 min and digested by proteinase K (10 µg/mL in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA at 37°C for 25 min). Slides were then rinsed in sterile DepC water followed by a solution of 0.1 M triethanolamine (TEA), pH 8.0, acetylated in 0.25% acetic anhydride in 0.1 M TEA and dehydrated via graded concentrations of alcohol (50, 70, 95 and 100%). After vacuum drying for a minimum of 2 h, 90 µL of hybridization mixture (1×10^7 cpm/mL) was spotted on each slide, sealed under a coverslip and incubated at 60°C overnight in a slide warmer. Coverslips were then removed, and the slides were rinsed in 4× standard sodium citrate (SSC) (1× SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0) at room temperature. Sections were digested by RNase A (20 µg/mL, 37°C, 30 min), rinsed in descending concentrations of SSC (2×, 1×, 0.5× SSC), washed in 0.1× SSC for 30 min at 65°C, and dehydrated via graded concentrations of alcohol. Slides were then vacuum-dried and exposed at 4°C to X-ray film (Eastman Kodak, Rochester, NY, USA) for 24–48 h, defatted in xylene and dipped in NTB2 nuclear emulsion (Kodak; diluted 1: 1 with distilled water). Slides were exposed for 7–10 days, developed in D19 developer (Kodak) for 3.5 min at 15°C, and fixed in rapid fixer (Kodak) for 6 min. Thereafter, slides were rinsed in running distilled water, counterstained with thionin (0.25%), dehydrated via graded concentrations of alcohol, cleared in xylenes and coverslipped with DPX.

Semi-quantitative densitometric microscopic analyses of CRF mRNA were conducted on silver grain-developed emulsion-dipped slides using darkfield microscopy. All microscopic analyses were obtained using a Zeiss Axiophot photomicroscope fitted with a Zeiss ZVS video camera in which images were captured under darkfield illumination at 100× magnification. Captured sections were analyzed for optical density (OD, arbitrary units) by measuring silver grain intensity over the area of interest and subtracting background silver grain intensity using NIH Scion Image 4.03 software. CRF mRNA signal was measured from both sides of the PVN (parvocellular and magnocellular divisions, –1.4 to –2.12 mm from bregma). Three–four matched sections were used to determine mean values for each animal.

Data presentation and statistical analyses

Operant behavior averages presented in Fig. 1 and Table 1 were obtained from three–six operant tests prior to and two–eight operant tests following dependence induction by chronic exposure to intermittent alcohol vapors (dependent animals) or air control (non-dependent animals) from Experiments 1–4. Data collected from operant sessions during the light phase (Experiments 1, 3 and 4) were analyzed separately from data collected from operant sessions during the dark phase (Experiment 2) of the light: dark cycle. All behavioral (operant responding and withdrawal signs), plasma blood alcohol, ACTH and corticosterone levels, and brain transcript data were analyzed using analyses of variance ANOVAs). Because animals were allowed 3 h of rest after hook-up before the sample was obtained, separate analyses of ACTH and corticosterone levels at the 0 min time-point were performed as an index of basal HPA activity. Simple regression analyses were used to establish the relationship between total alcohol consumption, blood alcohol levels and HPA responses. Unless stated otherwise, all analyses were followed by Bonferroni–Dunn *post hoc* tests, and $P < 0.05$ was considered statistically significant. To allow comparisons with *in vitro* studies (Little, 2003), conversion constants are provided for blood alcohol levels and ACTH and corticosterone levels in the figure legends.

Results

Animal model of alcohol dependence

Figure 1 illustrates the design of the alcohol dependence model (Fig. 1A), blood alcohol levels and physical withdrawal signs following removal from chronic intermittent exposure to alcohol

vapors or air control in untrained animals (Fig. 1B), and operant self-administration data from trained animals of Experiments 1–4, summarizing enhanced responding for alcohol in dependent animals following dependence induction by chronic intermittent exposure to alcohol vapors (Fig. 1C and D). Figure 1B (left graph) shows the drop in blood alcohol levels following removal from vapors, reaching undetectable levels by 4–6 h into withdrawal (main effect of Withdrawal Time, $F_{6,42} = 80.57$, $P < 0.0001$, Power = 1.00). Blood alcohol levels were significantly lower at all withdrawal times compared with 0 h, and blood alcohol levels at 8, 10 and 12 h were significantly lower compared with blood alcohol levels at 2 h (all $P < 0.001$; data are from alcohol vapor-exposed animals only). Figure 1B (right graph) shows that mild physical withdrawal signs were evident as early as 2 h into withdrawal, peaked from 6 to 8 h, and descended thereafter (main effect of Group, $F_{1,98} = 25.89$, $P = 0.0002$, Power = 1.00; and Withdrawal Time, $F_{1,98} = 4.75$, $P = 0.0001$, Power = 1.00; interaction between both factors, $F_{1,98} = 4.26$, $P = 0.0004$, Power = 0.99). *Post hoc* analyses indicated significant differences between air control and alcohol vapor-exposed animals at 6, 8, 10, 12 and 24 h time-points (all $P < 0.01$) and significant increases in withdrawal signs at 6 and 8 h compared with 0 h in dependent animals only ($P < 0.00$). Operant response data in Fig. 1C and D are self-administration averages (g/kg/session) occurring pre- or post-vapor (i.e. compared with dependence induction using chronic exposure to intermittent alcohol vapors in dependent animals or control air in non-dependent animals; model shown in Fig. 1A). All post-vapor testing was conducted when dependent animals were 6–8 h into withdrawal from vapors, which occurred in the light (Experiments 1, 3 and 4; Fig. 1C) or dark (Experiment 2; Fig. 1D) phase of the light: dark cycle. Main effects of Group ($F_{1,48} = 9.45$, $P = 0.003$, Power = 0.87) and Testing Period ($F_{1,48} = 22.60$, $P < 0.0001$, Power = 1.00) were observed compared with dependence induction by alcohol vapors, with an interaction between the two variables ($F_{1,48} = 22.73$, $P < 0.0001$, Power = 1.00) on alcohol self-administration (g/kg) when animals were tested during the light cycle (Fig. 1C). *Post hoc* analysis indicated that pre-vapor alcohol self-administration was not different between the groups, but post-vapor responding for alcohol was higher in dependent compared with non-dependent animals and compared with pre-vapor responding ($P < 0.0001$; Fig. 1C). Figure 1D shows the alcohol intake during the dark phase in Experiment 4. Main effects of Group ($F_{2,13} = 11.95$, $P = 0.001$, Power = 0.98) and Testing Period ($F_{2,13} = 28.56$, $P < 0.0001$, Power = 1.00) were observed compared with dependence induction by alcohol vapors, with an interaction between the two variables ($F_{2,13} = 13.45$, $P < 0.0002$, Power = 0.99) on alcohol self-administration (g/kg; Fig. 1D). *Post hoc* analysis indicated that low-responding non-dependent animals consumed less alcohol pre-vapor compared with both non-dependent and dependent animals ($P < 0.01$; Fig. 1D). Post-vapor alcohol self-administration was higher in dependent animals compared with pre-vapor responding in this group and compared with post-vapor responding in both low-responding and non-dependent animals ($P < 0.01$; Fig. 1D).

Self-administration of water (number of responses/session) is shown in Table 1. Water responding did not differ between non-dependent and dependent groups or with dependence induction [pre-vs. post-vapor testing in animals tested during the light (Experiments 1, 3 and 4) or dark (Experiment 2) cycle (all $P > 0.05$; Table 1)]. As expected, the data confirm that chronic intermittent vapor-induced increases in alcohol drinking in dependent animals were not attributable to non-specific increases in self-administration behavior.

Experiment 1: effect of voluntary alcohol self-administration on the HPA axis and blood alcohol levels in non-dependent and dependent animals

Alcohol consumption and blood alcohol levels during self-administration—

Alcohol consumption (cumulative responses and total g/kg intake) and blood alcohol levels during a 30-min operant session in non-dependent and dependent animals 6–8 h into withdrawal are shown in Fig. 2A (alcohol consumption), Fig. 2B (regression plot for alcohol

consumption and 30 min blood alcohol levels) and Fig. 2C (group average blood alcohol levels at 0, 15, 30 and 45 min). As expected, one-way ANOVAs indicated that dependent animals self-administered significantly more alcohol than non-dependent animals over the course of the operant session (total alcohol responses: $F_{1,16} = 4.29$, $P = 0.05$, Power = 0.49, Fig. 3A; total alcohol g/kg intake: $F_{1,16} = 6.09$, $P = 0.02$, Power = 0.64, Fig. 3A inset), while responding for water was not different between groups (non-dependent: 2.71 ± 0.18 ; dependent: 2.63 ± 0.31 responses; $P > 0.05$).

Self-administration of an average of 1 g/kg of alcohol in 30 min produced average blood alcohol levels above 0.150 g% (32.6 mM) in dependent animals vs. self-administration of an average of 0.5 g/kg (~0.050 g% or 10.9 mM blood alcohol levels) in non-dependent animals (Fig. 3B and C). Simple regression analysis of total alcohol consumption and blood alcohol levels at the end of the session (30 min) confirmed a strong positive correlation between the two variables ($r = 0.85$, $F_{1,16} = 44.89$, $P < 0.0001$, Power = 1.00; Fig. 3B). Significant main effects of Group ($F_{1,48} = 7.60$, $P = 0.01$, Power = 0.72) and Sample Time ($F_{3,48} = 25.20$, $P < 0.0001$, Power = 1.00), and a significant Group \times Sample Time interaction ($F_{3,48} = 6.50$, $P = 0.0009$, Power = 0.97) were observed on blood alcohol levels during the self-administration session. Specifically, blood alcohol levels were significantly higher at 15, 30 and 45 min in dependent animals (all $P < 0.001$), and at 30 and 45 min in non-dependent animals (all $P < 0.05$) compared with basal levels (0 min, Fig. 3C). Blood alcohol levels were significantly higher in dependent animals compared with non-dependent animals at all sampling time-points after the start of the self-administration session (15, 30 and 45 min, all $P < 0.05$; Fig. 3C), reflecting the higher amount of alcohol consumed in dependent animals during the test session.

Attenuated ACTH and corticosterone in dependent animals: stimulation of the HPA axis by alcohol self-administration—ACTH levels obtained before (basal, 0 min), during (15 and 30 min) and after (45 min) self-administration of alcohol are shown in Fig. 3D. Under basal conditions, ACTH levels were significantly blunted in dependent animals ($F_{1,16} = 11.60$, $P = 0.003$, Power = 0.91; Fig. 3D). Self-administration of alcohol elevated ACTH in both groups of animals ($F_{3,48} = 3.56$, $P = 0.02$, Power = 0.76; Fig. 3D) at 15 min, and the degree to which ACTH was increased from basal levels was positively correlated with blood levels of alcohol [15 min blood alcohol levels and the percent change in ACTH at 15 min compared with basal levels (~60% increase), $r = 0.52$, $F_{1,17} = 5.98$, $P = 0.02$]. Blood alcohol levels were not correlated with percent increase in ACTH at any other time-point. Two-way ANOVA indicated that, despite alcohol-elicited increases, ACTH remained lower at all time-points in dependent compared with non-dependent animals (main effect of Group, $F_{1,48} = 7.78$, $P = 0.01$, Power = 0.75).

Corticosterone levels obtained before (basal, 0 min), during (15 and 30 min) and after (45 min) self-administration of alcohol are shown in Fig. 3E. Basal corticosterone was lower in dependent compared with non-dependent animals (0 min, $F_{1,16} = 4.77$, $P = 0.04$, Power = 0.53; Fig. 3E). Self-administration of alcohol elicited a slight increase in corticosterone at 15 min, demonstrated by a significant positive correlation between 15 min blood alcohol levels and the percent change in corticosterone at 15 min (~17% increase) compared with basal levels ($r = 0.48$, $F_{1,16} = 4.79$, $P = 0.04$). Blood alcohol levels were not correlated with percent increase in corticosterone at any other time-point.

Experiment 2: effect of 1 g/kg of alcohol on the HPA axis and blood alcohol levels in low-responding, non-dependent and dependent animals

Administration of alcohol (1 g/kg, i.v.) elicited drastically different HPA responses among the three self-administration groups, with the most robust responses occurring in the low-responding non-dependent group, intermediate responses occurring in the standard non-

dependent group and significantly blunted responses occurring in the dependent group (Fig. 3). Alcohol stimulated similar elevations in blood alcohol levels in all groups equally, with peaks occurring at 15 min (main effect of Sampling Time, $F_{3,39} = 66.71$, $P < 0.0001$, Power = 1.00), with no significant main effect of Group and no interaction ($P > 0.05$; Fig. 3A). Despite similar blood alcohol level responses, main effects of Group ($F_{2,36} = 4.36$, $P = 0.03$, Power = 0.64) and Sample Time ($F_{3,36} = 23.51$, $P < 0.0001$, Power = 1.00), and a significant Group \times Sample Time interaction ($F_{6,36} = 5.13$, $P = 0.0007$, Power = 0.99) were observed on ACTH levels (Fig. 2B). *Post hoc* analyses indicated significant elevations in ACTH at 15 min in low-responding and non-dependent groups (all $P < 0.008$; Fig. 3B). Likewise, main effects of Group ($F_{2,39} = 15.98$, $P = 0.0003$, Power = 1.00) and Sample Time ($F_{3,39} = 12.41$, $P < 0.0001$, Power = 1.00), and a significant interaction between these two variables ($F_{6,39} = 2.39$, $P = 0.04$, Power = 0.75) were observed on corticosterone levels (Fig. 2C). *Post hoc* analyses indicated significant elevations in corticosterone at 15 and 45 min in low-responding animals, and at 15 min in non-dependent animals compared with 0 min (all $P < 0.008$; Fig. 2B). Low-responding animals had higher ACTH and corticosterone levels compared with dependent animals at 15 and 45 min ($P < 0.01$; Fig. 2B and C). Accordingly, trained animals that chronically self-administered low levels of alcohol (averaging less than 0.2 g/kg/session) elicited higher or 'normal' (comparable with the alcohol-naive animals in Lee & Rivier, 1997; Lee *et al.*, 2000) HPA responses to an alcohol challenge compared with animals chronically self-administering moderate (non-dependent animals, averaging ~0.3–0.4 g/kg/session) or high (dependent animals, averaging 1.0 g/kg/session) levels of alcohol. Interestingly, prior to the alcohol injection, basal levels of ACTH and corticosterone were not significantly lower in dependent animals compared with non-dependent animals or low-responding non-dependent animals (all $P > 0.05$), suggesting that dependence-induced reductions in basal HPA activity that were observed in the afternoon of the light cycle (Experiments 1, 3, 4) may reflect potential perturbation of diurnal regulation of the HPA axis.

Experiment 3: effect of a CRF challenge on the HPA axis in alcohol-naive, non-dependent and dependent animals

The next experiment explored whether ACTH and corticosterone responses to exogenously administered CRF differed in dependent and non-dependent animals because pituitary responsiveness could mediate neuroendocrine tolerance. A dose–response curve was first conducted in alcohol-naive animals (Fig. 4A). As expected, CRF induced dose-related ($F_{3,60} = 55.94$, $P < 0.0001$, Power = 1.00) and time-related ($F_{3,60} = 507.40$, $P < 0.0001$, Power = 1.00) increases in plasma ACTH levels, with peak responses measured at the 15 min time-point (Fig. 4A). The 0.3 $\mu\text{g}/\text{kg}$ dose of CRF elicited a mid-range ACTH response in alcohol-naive control animals (elevated ACTH to levels ~300 pg/mL or 66.1 pM) and was selected for administration in dependent and non-dependent rats. Similar to Experiment 1, basal ACTH was lower in dependent animals ($F_{1,7} = 32.66$, $P = 0.0007$, Power = 0.99; Fig. 4B). However, CRF elicited an increase in ACTH in dependent and non-dependent animals, eliminating the difference between the two groups ($F_{3,21} = 71.13$, $P < 0.0001$, Power = 1.00; Fig. 4B). Notably, CRF elicited ACTH responses that were about half the magnitude observed in alcohol-naive controls (0.3 $\mu\text{g}/\text{kg}$ dose in Fig. 4A and B).

Experiment 4: CRF mRNA in the PVN in alcohol-naive, non-dependent and dependent animals

Photomicrographs illustrating CRF mRNA signal in the PVN from alcohol-naive, non-dependent and dependent animals are shown in Fig. 5A. Group means of OD (arbitrary units) are shown in Fig. 5B for the parvocellular division of the PVN (pPVN). CRF mRNA levels in the pPVN were reduced in alcohol-exposed animals compared with alcohol-naive controls, but this reduction reached significance only in dependent animals (main effect of Group: $F_{2,26} = 3.20$, $P = 0.05$, Power = 0.55; naive > dependent, $P = 0.01$; Fig. 5B). Non-dependent and

dependent animals did not differ in CRF expression levels in the pPVN, and no groups differed in CRF mRNA levels in the magnocellular division of the PVN, a region that contains a small number of CRF cells (data not shown; Swanson *et al.*, 1983).

Discussion

The HPA axis has long been hypothesized to play a role in alcoholism and dependence (Wand & Dobs, 1991; Froehlich *et al.*, 2003). Combined behavioral, neuroendocrine and molecular approaches were used to investigate the relationship between alcohol drinking, chronic alcohol exposure and the HPA axis using an animal model of alcohol dependence. Operant self-administration of alcohol acutely elevated HPA hormone levels, but chronic exposure resulted in significant impairment of HPA function. Dependence was associated with reduced basal levels of corticosterone and its upstream regulator, ACTH, in the afternoon of the light cycle and robust deficits in HPA responsivity to alcohol (voluntary and involuntary) that persisted into the dark phase. An injection of CRF 'normalized' ACTH levels in dependent animals compared with non-dependent controls, implicating central rather than peripheral mechanisms in this deficit. While neuroendocrine tolerance may be accompanied by decreased CRF mRNA in the PVN in dependent animals compared with alcohol-naïve controls, alterations in hypothalamic CRF expression do not fully explain the severe impairment of HPA function observed in dependent animals compared with non-dependent animals. Overall, the findings support clinical reports showing impaired stress responsivity in alcoholics (Lovallo *et al.*, 2000; O'Malley *et al.*, 2002; Kiefer & Wiedemann, 2004; Adinoff *et al.*, 2005) and suggest a pathway by which high chronic alcohol use could lead to a dampened neuroendocrine state. The neuroendocrine changes may underlie some of the negative characteristics experienced in the acute withdrawal state that lead to excessive alcohol intake, but this remains to be determined. Nevertheless, severe alterations in HPA function associated with chronic alcohol exposure are particularly important because they have the potential to negatively impact overall health and the ability to respond appropriately to internal and environmental challenges.

Chronic exposure to high alcohol induces physical and motivational dependence, as measured by increased anxiety-like responses and elevated brain reward thresholds during acute withdrawal (Rassnick *et al.*, 1993; Schulteis *et al.*, 1995; Roberts *et al.*, 1996; Valdez *et al.*, 2002; Overstreet *et al.*, 2004; Gehlert *et al.*, 2007). Increased responding for alcohol in dependent animals was two–threefold higher than pre-vapor responding, which is consistent with previous reports that tested during acute withdrawal (2 h; Funk *et al.*, 2006; 6–8 h, O'Dell *et al.*, 2004; Walker & Koob, 2007; Richardson *et al.*, 2008) and protracted abstinence (Roberts *et al.*, 1996; Valdez *et al.*, 2002). Animals made dependent by exposure to intermittent alcohol vapors had mild physical signs of withdrawal that began to rise about 2 h following removal from alcohol vapors, peaked at 8 h and gradually descended thereafter. The physical signs of withdrawal observed here were milder and dissipated earlier compared with animals made dependent using continuous alcohol vapor exposure (Roberts *et al.*, 1996). In contrast, responding for alcohol is higher when animals are exposed to alcohol vapors on an intermittent vs. continuous schedule (O'Dell *et al.*, 2004), further confirming the dissociation between physical withdrawal and high alcohol consumption. Together these results provide support for the hypothesis that the 'emotional' aspects of withdrawal (indexed by changes in anxiety-like behavior and reward thresholds in animal studies) are motivators at least as powerful as, if not more than, physical aspects of withdrawal for driving excessive drinking in dependent animals (Rassnick *et al.*, 1993; Schulteis *et al.*, 1995; Valdez *et al.*, 2002; Overstreet *et al.*, 2004; Sabino *et al.*, 2006; Chu *et al.*, 2007; Funk *et al.*, 2007; Gehlert *et al.*, 2007; Richardson *et al.*, 2008). Consistent with this hypothesis, physical symptoms of withdrawal are not always observed in alcoholics, and the motivational signs of withdrawal, such as anxiety, dysphoria and malaise, are considered important in continued use and relapse. Alcoholics are hypothesized to drink alcohol not simply for its euphorogenic effects, but also to avoid or reduce the negative affective

states associated with withdrawal or to self-medicate existing negative affective states (Cappell & LeBlanc, 1979; Lowman *et al.*, 1996; Koob, 2003).

The present study showed that basal ACTH and corticosterone levels were attenuated in dependent compared with non-dependent animals self-administering alcohol, extending earlier studies showing blunted HPA hormones several weeks after withdrawal from chronic alcohol liquid diet compared with alcohol-naïve controls (Rasmussen *et al.*, 2000; Zorrilla *et al.*, 2001). Additionally, consistent with these findings, clinical studies link disruption of the HPA axis with alcoholism, including a dampened ability to cope with stress and negative correlations between cortisol and craving and relapse in alcoholics (Lovallo *et al.*, 2000; O'Malley *et al.*, 2002; Kiefer & Wiedemann, 2004; Adinoff *et al.*, 2005). Conversely, some clinical and rodent studies have demonstrated elevated, rather than reduced, basal glucocorticoid levels during acute withdrawal or early abstinence. Many factors may contribute to the seemingly opposite findings, including species differences in rodent models (Tabakoff *et al.*, 1978; Roberts *et al.*, 1992), co-presence of a depression syndrome (Heinz *et al.*, 1995; Sher, 2007), developmental age (Sher, 2007) and measurements of free vs. total glucocorticoid levels (Keedwell *et al.*, 2001). Future studies in which free corticosterone levels are also measured (a better index of bioavailability) in non-dependent and dependent self-administering animals could also elucidate whether the acute and chronic effects of alcohol on total corticosterone levels (measured in the present study) parallel changes in free circulating hormone levels more readily available for actions within the brain and periphery.

Another important factor that may explain some differences among studies is glucocorticoid rhythmicity (Adinoff *et al.*, 1991). In the afternoon of the light cycle, when glucocorticoid levels are normally rising to peak levels, dependent animals had lower ACTH and corticosterone compared with non-dependent animals (Experiments 1, 3 and 4). However, a few hours into the dark phase (Experiment 2), basal HPA hormone levels were elevated in dependent animals and no longer differed from non-dependent animals. Thus, detection of attenuated basal HPA activity in dependent animals depends on circadian time, suggesting possible perturbation in the diurnal regulation of ACTH and corticosterone with dependence. Nevertheless, blunted release of ACTH and corticosterone following voluntary and involuntary alcohol in dependent animals persisted across light and dark cycles, indicating dissociation in the underlying mechanisms driving the dependence-induced changes in basal HPA hormone levels vs. HPA responsiveness.

The current work indicates that a dampened neuroendocrine state did not occur prior to, but rather was an 'outcome' of, chronic exposure to alcohol vapors in dependent animals (but see Gianoulakis *et al.*, 2005). A dampened neuroendocrine state may still underlie some of the negative emotions experienced during withdrawal and motivate drinking behavior to establish normal endocrine function. Glucocorticoids are hypothesized to initially play a stimulatory role on alcohol consumption during training in non-dependent animals through positive reinforcement mechanisms (Piazza *et al.*, 1993, 1996; Fahlke *et al.*, 1995; Koenig & Olive, 2004). Subsequently in the dependent state (present report), attenuated HPA could function as a motivating factor for excessive drinking because considerably higher alcohol consumption would be needed to achieve the same level of glucocorticoids. Notably, baseline ACTH and corticosterone prior to initiation of drinking are not tightly linked to the amount of alcohol dependent animals consume (regression analyses not shown), indicating that low basal hormone levels by themselves are not enough to 'initiate' high drinking. Instead, the failure to significantly elevate ACTH and corticosterone during self-administration would be one means by which a dampened neuroendocrine state could affect drinking behavior possibly through altered reward or satiety thresholds. In support of this hypothesis, anti-craving drugs used to prevent relapse, such as opioid antagonists, activate the HPA axis, and sensitivity to this

activation is most predominant in subjects with a strong family history for alcohol dependence (Wand *et al.*, 1999; O'Malley *et al.*, 2002; Kiefer *et al.*, 2006).

Several important inferences can be made by inclusion of alcohol-naïve animals and low alcohol-consuming animals (low-responding, non-dependent group) for comparison to moderate (non-dependent groups) and high (dependent groups) alcohol-consuming animals. First, neuroendocrine tolerance appears to be first initiated prior to dependence. HPA responses to alcohol (Experiment 2) and CRF (Experiment 3) were substantially more robust ('normal') in groups that had little (low-responding, non-dependent animals) or no exposure (alcohol-naïve) to alcohol compared with non-dependent animals that consumed moderate levels of alcohol and dependent animals that consumed high levels of alcohol. CRF mRNA normally shows high constitutive expression in the PVN (Sawchenko *et al.*, 1993), and transcript levels were high in alcohol-naïve controls, slightly (albeit not significantly) reduced in non-dependent animals and significantly reduced in dependent animals. The findings altogether suggest that downregulation of the HPA axis may be initiated before dependence. The early stages of neuroendocrine tolerance could potentially be mediated at the level of the pituitary (decreased sensitivity to CRF; Rivier *et al.*, 1990) and eventually through adaptations in the hypothalamus. The data also argue against these mechanisms completely explaining the robust blunting of basal ACTH and corticosterone levels and HPA responsiveness in dependent compared with non-dependent animals. The two groups did not differ in either pituitary responsiveness to CRF or constitutive expression of CRF mRNA in the PVN, precluding peripheral and implicating (but not identifying) other central mediators of neuroendocrine tolerance in the later stages of addiction.

Although all of the exact mechanisms driving reduced HPA function in dependent animals remain unknown, the current report, in conjunction with previous findings, suggests a possible pathway by which high alcohol consumption may eventually lead to dependence. CRF neurons in limbic structures, such as the CeA and BNST are involved in mediating the negative affective state experienced during withdrawal (Alheid & Heimer, 1988; Herman & Cullinan, 1997; Wand, 2005; Koob, 2006). The present study demonstrates that oral self-administration of alcohol stimulates the HPA axis to release ACTH and corticosterone, one means by which the HPA axis can drive neuroadaptive changes in the limbic CRF system. High corticosterone increases CRF mRNA in the CeA and lateral BNST and decreases CRF mRNA in the PVN (Makino *et al.*, 1994; Albeck *et al.*, 1997; Schulkin *et al.*, 1998; Shepard *et al.*, 2000). Furthermore, the CeA has stimulatory influence and the lateral BNST has both stimulatory and inhibitory influence on the CRF system in the PVN, and this complex interplay is vulnerable to allostatic overload under chronic high stress conditions (Cullinan *et al.*, 1993; Gray *et al.*, 1993; Herman *et al.*, 1994, 1996; Herman & Cullinan, 1997; Schulkin *et al.*, 1998; Schulkin, 1999). Given that moderate to high drinking (present study) stimulates the HPA axis, the initial exposure to high corticosterone may stimulate CRF expression in the CeA and lateral BNST, eventually leading to neuroadaptive changes, including further sensitization of CRF activation in the extended amygdala and decreased HPA function. Thus, multiple mechanisms of the brain stress axis may be engaged with excessive drinking, including a compromised hormonal response (present study) and a sensitized brain stress response (previous studies), both of which contribute to dependence.

In conclusion, the present study shows that alcohol dependence is associated with dysregulation of the HPA axis. Alterations of the HPA axis brought on by heavy binge drinking may begin with a history of high drinking, whether or not the subject is physically dependent, and may ultimately lead to the dysregulation associated with the dependent state. HPA adaptations occur at various stages of the addictive cycle and appear to be mediated by multiple mechanisms. Decreased HPA function provides support for the hypothesized shift in stress regulation (decreased activation of the hypothalamic/neuroendocrine CRF system and enhanced

activation and sensitivity of the extrahypothalamic/behavioral CRF system; Koob, 2008). Changes in HPA function could directly or indirectly contribute to the negative affective state experienced during acute withdrawal to motivate excessive alcohol intake. Moreover, impaired HPA function associated with dependence could ultimately negatively impact overall health because of the decreased ability to respond appropriately to internal and environmental challenges.

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Abbreviations

ACTH	adrenocorticotrophic hormone
BNST	bed nucleus of the stria terminalis
CeA	central nucleus of the amygdala
CRF	corticotropin-releasing factor
CRF₁	CRF type I receptor
DepC	diethylpyrocarbonate
HPA	hypothalamic-pituitary-adrenal
IRMA	immunoradiometric assay
OD	optical density
pPVN	parvocellular division of the paraventricular nucleus of the hypothalamus
PVN	paraventricular nucleus of the hypothalamus
r/hCRF	rat/human CRF
SSC	standard sodium citrate
TEA	triethanolamine

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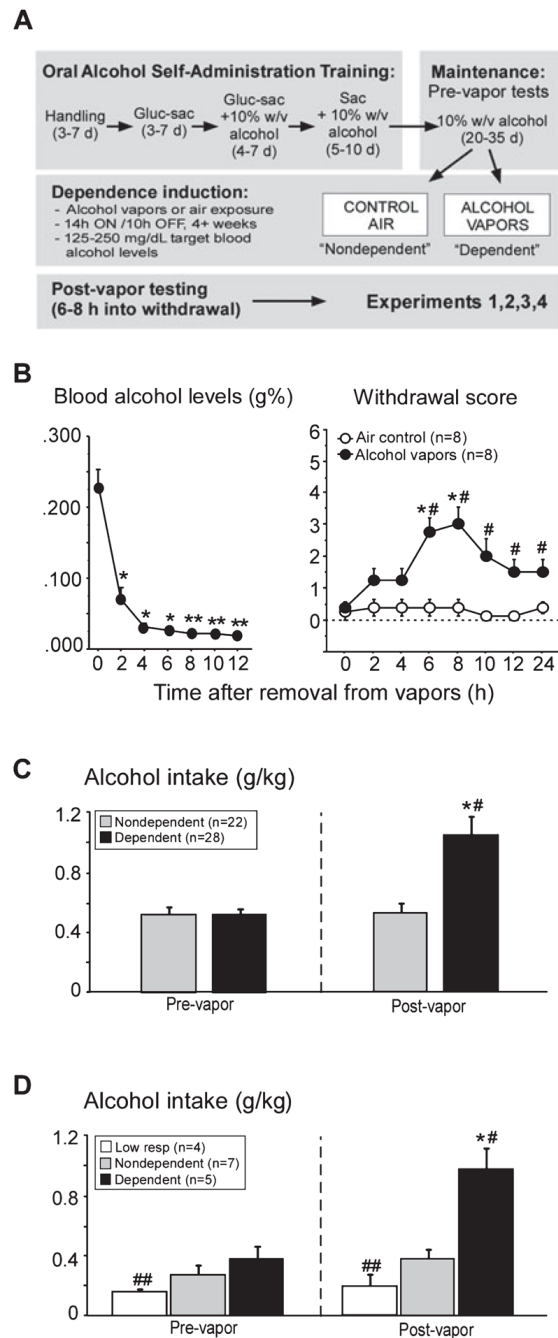


Fig. 1. Design of the alcohol dependence model (A), blood alcohol levels (left graph), and physical withdrawal signs (right graph) following removal from chronic intermittent exposure to alcohol vapors or air control (B), and operant self-administration data summarizing enhanced responding for alcohol in dependent animals following dependence induction (C). Adult male Wistar rats were trained using a sweetened-solution fading paradigm to orally self-administer alcohol (10% w/v ethanol, alcohol) or water. Following maintenance on 10% w/v alcohol for 20–30 days, stable responders were evenly divided into two groups and exposed to intermittent alcohol vapors, 14 h on/10 h off (‘dependent’) or control air (‘non-dependent’). After 4 weeks of dependence induction, animals were tested at least twice before being assigned to

Experiments 1–4. After removal from vapors, blood alcohol levels dropped rapidly, reaching undetectable levels within 4–6 h ($*P < 0.001$, compared with 0 h; $**P < 0.001$, blood alcohol levels compared with 2 h; B, left graph). Mild physical withdrawal began to rise 2 h into withdrawal, peaked from 6 to 8 h, and descended thereafter ($*P < 0.002$, compared with 0 h; $\#P < 0.01$, compared with air control group; B, right graph). Operant data from all trained animals of the present study are shown in (C) (Experiments 1, 3 and 4, light cycle) and (D) (Experiment 2, dark cycle). Post-vapor testing was done when dependent animals were 6–8 h into withdrawal. Bars in (C) represent mean alcohol intake (g/kg) in dependent animals (black bars, $n = 22$) tested before ('pre-vapor') and after ('post-vapor') dependence induction compared with non-dependent animals (gray bars, $n = 28$) tested at these same times (data from Experiments 1, 3 and 4 combined). Bars in (D) represent mean alcohol intake (g/kg) in dependent animals (black bars, $n = 5$) tested before ('pre-vapor') and after ('post-vapor') dependence induction compared with non-dependent (gray bars, $n = 7$) and low-responding non-dependent (white bars, $n = 4$) controls tested at these same times (Experiment 2). Enhanced responding for alcohol was evident in dependent animals compared with non-dependent controls following chronic intermittent alcohol vapor. *Post hoc* analysis indicated that post-vapor responding for alcohol was higher in dependent animals ($*P < 0.0001$, compared with pre-vapor responding; $\#P < 0.001$, compared with non-dependent controls during post-vapor testing; C and D). Low-responding animals had reduced alcohol intake compared with non-dependent and dependent animals pre-vapor ($^{\#\#}P < 0.01$) and compared with dependent animals post-vapor ($^{\#\#}P < 0.01$). To convert blood alcohol levels from g% (g/dL) to mM, divide values by 0.0046 (e.g. 0.200 g% is 43.5 mM). Data are expressed as mean \pm SEM.

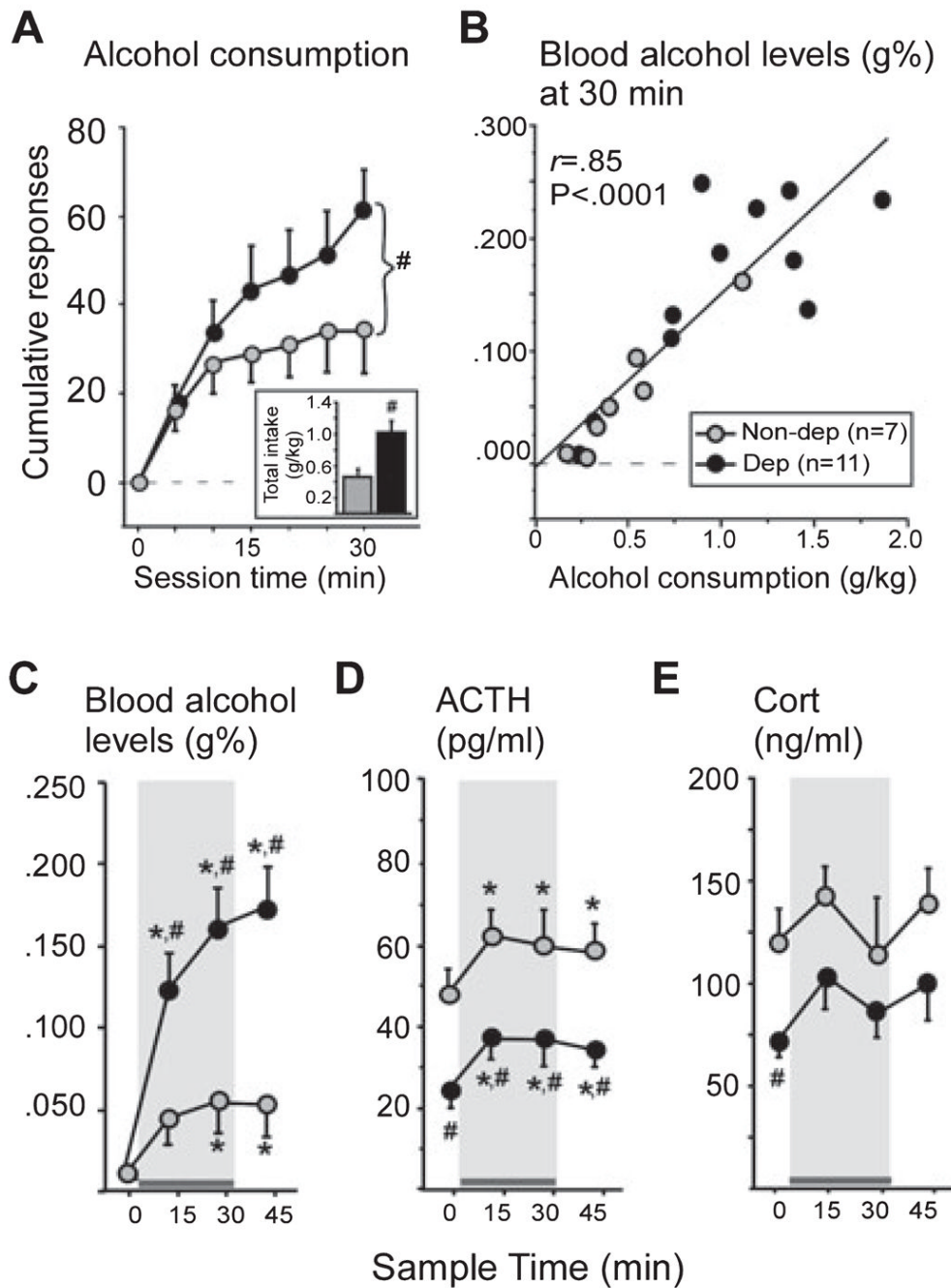


Fig. 2. Alcohol consumption (cumulative responses, A, and total g/kg intake, inset), blood alcohol levels (blood alcohol levels, g%; simple regression scatter plot of alcohol consumption and 30 min blood alcohol levels, B; blood alcohol levels over time, C), plasma adrenocorticotrophic hormone (ACTH; pg/mL, D), and plasma corticosterone (cort; ng/mL, E) responses to self-administered alcohol (10% w/v) in non-dependent (gray bar/circles, $n = 7$) and dependent (black bar/circles, $n = 11$) animals 6–8 h into withdrawal from vapors. Gray shading indicates when the levers were available in the operant chambers. Dependent animals consumed twice as much alcohol as non-dependent animals ($\#P = 0.01$, A, inset), reflected in blood alcohol levels that were > 0.150 g% (32.6 mM) in this group (C). The amount of alcohol consumed

was strongly correlated with blood alcohol levels immediately following the session (B). (C) Self-administration of alcohol significantly increased blood alcohol levels in both groups ($*P < 0.05$, compared with 0 min, C), but blood alcohol levels were significantly higher in dependent animals at all time-points except at 0 min, when blood alcohol levels were at baseline ($^{\#}P < 0.05$, compared with non-dependent animals). Self-administration of alcohol elevated ACTH in both groups of animals ($*P = 0.02$, compared with 0 min, D), and the degree to which ACTH was increased from basal levels was positively correlated with blood alcohol levels (15 min blood alcohol levels and percent increase in ACTH from basal levels, $r = 0.52$, $P = 0.02$, no indicator shown). Despite the increase, ACTH remained lower at all time-points in dependent animals ($^{\#}P = 0.01$, compared with non-dependent animals, D). Corticosterone was also lower in dependent animals at 0 min ($^{\#}P < 0.02$, compared with non-dependent animals, E). Self-administration of alcohol elicited a slight increase in corticosterone at 15 min, and the degree to which corticosterone increased was positively correlated with blood alcohol levels ($r = 0.58$, $P = 0.04$, no indicator shown). To convert blood alcohol levels from g% (g/dL) to mM, divide values by 0.0046 (e.g. 0.150 g% is 32.6 mM); to convert ACTH₁₋₃₉ (pg/mL) to pM, divide values by 4.5410 (e.g. 50 pg/mL is 11.01 pM); to convert corticosterone (ng/mL) to nM, divide values by 0.3464 (e.g. 100 ng/mL is 288.7 nM). Data are expressed as mean \pm SEM.

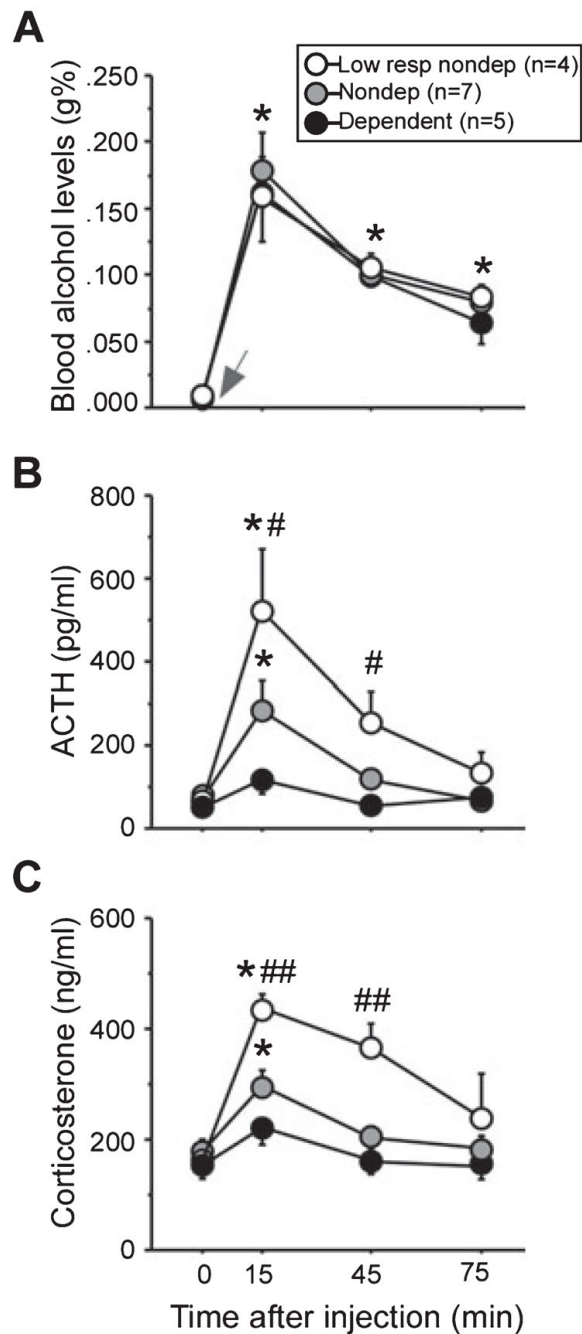


Fig. 3. Blood alcohol levels (g%, A), adrenocorticotrophic hormone (ACTH; pg/mL, B) and corticosterone levels (ng/mL, C) before and after an alcohol challenge (1 g/kg, i.v.) in low-responding (white circles, $n = 4$), non-dependent ($n = 7$) and dependent (black circles, $n = 5$) animals 6–8 h into withdrawal from vapors (dark cycle). Alcohol was administered (gray arrow) immediately after the first sample was collected (basal, 0 min). Baseline (0 min) blood alcohol levels were undetectable, and alcohol elicited equivalent increases in blood alcohol levels in all groups ($*P < 0.0001$; compared with 0 min). No differences in HPA axis activity were evident under basal conditions, but alcohol elicited vastly different ACTH and corticosterone responses in the three groups. The most robust HPA responses were observed

in low-responding animals, intermediate responses in non-dependent animals and nearly flattened responses in dependent animals. *Post hoc* analyses indicated significant peaks in ACTH and corticosterone at 15 min in low-responding and non-dependent animals, and corticosterone levels remained elevated at 45 min only in low-responding animals ($*P < 0.008$, compared with 0 min). Low-responding animals had significantly higher ACTH and corticosterone levels 15 and 30 min after the alcohol challenge ($\#P < 0.01$, compared with dependent animals; $\#\#P < 0.01$, compared with dependent and non-dependent animals). To convert blood alcohol levels from g% (g/dL) to mM, divide values by 0.0046 (e.g. 0.200 g% is 43.5 mM); to convert ACTH₁₋₃₉ (pg/mL) to pM, divide values by 4.5410 (e.g. 500 pg/mL is 110.1 pM); to convert corticosterone (ng/mL) to nM, divide values by 0.3464 (e.g. 400 ng/mL is 1154.7 nM). Data are expressed as mean \pm SEM.

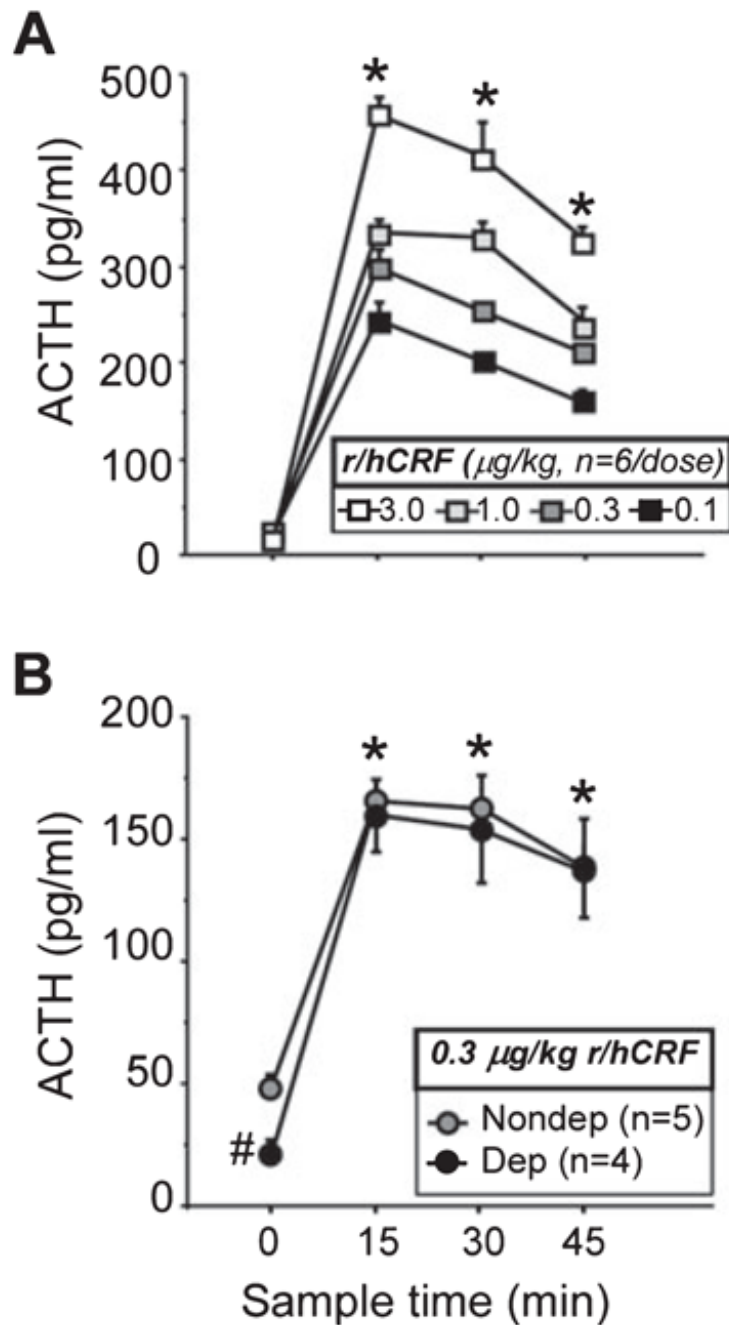


Fig. 4. Adrenocorticotrophic hormone (ACTH) responses following exogenously administered rat/human corticotropin-releasing factor (r/hCRF). A dose-response curve for r/hCRF was first conducted in alcohol-naive animals ($n = 6$ per dose: 0.1, 0.3, 1.0 and 3.0 $\mu\text{g}/\text{kg}$, i.v.), indicating that all doses increased ACTH ($*P < 0.0001$, A), with a peak at 15 min, and that the 0.3 $\mu\text{g}/\text{kg}$ dose of CRF elicited a mid-range ACTH response. The 0.3 $\mu\text{g}/\text{kg}$ dose of r/hCRF was then administered to non-dependent (gray circles, $n = 5$) and dependent (black circles, $n = 4$) rats 6–8 h into withdrawal from vapors (14.00–16.00 h), and blood samples were obtained 0 (basal, before injection), 15, 30 and 45 min later for measurement of plasma ACTH. Although basal ACTH was significantly lower in dependent animals ($\#P = 0.0007$, compared with non-

dependent animals at 0 min, B), CRF elicited a rapid rise in ACTH and eliminated the hormonal difference between groups ($*P < 0.0001$, compared with 0 min, B). To convert ACTH₁₋₃₉ (pg/mL) to pM, divide values by 4.5410 (e.g. 200 pg/mL is 44.0 pM). Data are expressed as mean \pm SEM.

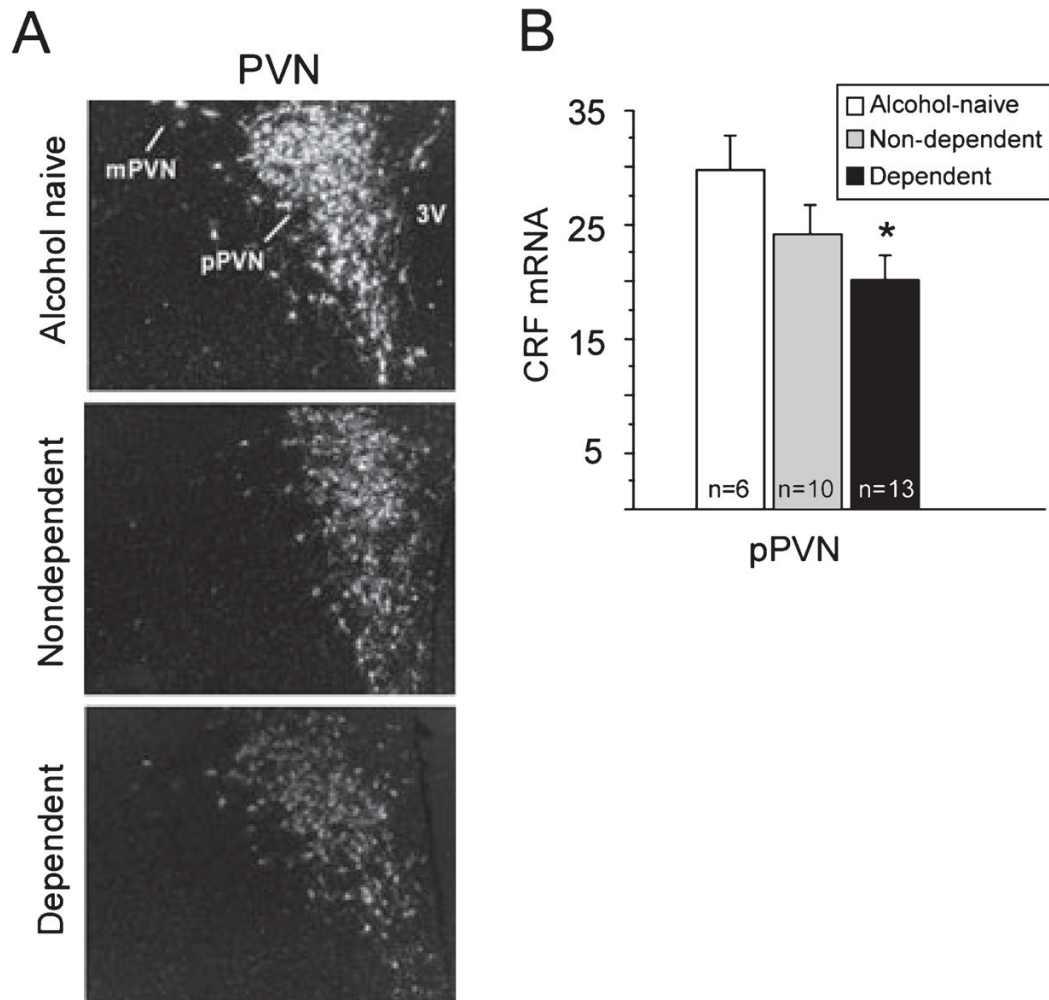


Fig. 5. Photomicrographs showing corticotropin-releasing factor (CRF) mRNA signal in the paraventricular nucleus of the hypothalamus (PVN, A) and group means of transcript optical density (OD, arbitrary units of signal intensity corrected for background) in the parvocellular portion of the PVN (pPVN) in alcohol-naive (white bar, $n = 6$), non-dependent (gray bar, $n = 10$) and dependent (black bar, $n = 13$) animals 6–8 h into withdrawal from vapors (14.00–16.00 h, B). CRF mRNA was significantly decreased in the pPVN in dependent animals compared with alcohol-naive controls ($*P = 0.01$, B), but not compared with non-dependent animals. Groups did not differ in CRF mRNA levels in the magnocellular division of the PVN (mPVN, data not shown). Data are expressed as mean \pm SEM.

Table 1

Mean operant responding for water (\pm SEM) during the light or dark phase prior to ('pre-vapor') and following ('post-vapor') dependence induction by alcohol vapors in dependent animals compared with non-dependent control animals

Operant testing	Group	Pre-vapor responses for water	Post-vapor responses for water
Experiments 1, 3 and 4 (light cycle)	Non-dependent ($n = 22$)	14.85 ± 3.52	11.59 ± 3.00
	Dependent ($n = 28$)	10.61 ± 2.02	10.73 ± 2.56
Experiment 2 (dark cycle)	Non-dependent ($n = 7$)	26.81 ± 8.18	28.43 ± 8.33
	Dependent ($n = 5$)	18.13 ± 4.76	22.40 ± 6.96
	Low-responding non-dependent ($n = 4$)	32.67 ± 11.48	27.50 ± 12.43

In all experiments, operant responses for water were not different between treatment groups before or after dependence induction by alcohol vapors in dependent animals (all $P > 0.05$). Animals were tested when dependent animals were 6–8 h into withdrawal from alcohol vapors.