Corticotropin Releasing Factor–Induced Amygdala Gamma-Aminobutyric Acid Release Plays a Key Role in Alcohol Dependence

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Background: Corticotropin-releasing factor (CRF) and gamma-aminobutyric acid (GABA)ergic systems in the central amygdala (CeA) are implicated in the high-anxiety, high-drinking profile associated with ethanol dependence. Ethanol augments CeA GABA release in ethanol-naive rats and mice.

Methods: Using naive and ethanol-dependent rats, we compared electrophysiologic effects and interactions of CRF and ethanol on CeA GABAergic transmission, and we measured GABA dialyzate in CeA after injection of CRF1 antagonists and ethanol. We also compared mRNA expression in CeA for CRF and CRF1 using real-time polymerase chain reaction. We assessed effects of chronic treatment with a CRF1 antagonist on withdrawal-induced increases in alcohol consumption in dependent rats.

Results: CRF and ethanol augmented CeA GABAergic transmission in naive rats via increased GABA release. Three CRF1 receptor (CRF1) antagonists decreased basal GABAergic responses and abolished ethanol effects. Ethanol-dependent rats exhibited heightened sensitivity to CRF and CRF1 antagonists on CeA GABA release. Intra-CeA CRF1 antagonist administration reversed dependence–related elevations in GABA dialysate and blocked ethanol-induced increases in GABA dialyzate in both dependent and naive rats. Polymerase chain reaction studies indicate increased expression of CRF and CRF1 in CeA of dependent rats. Chronic CRF1 antagonist treatment blocked withdrawal-induced increases in alcohol drinking by dependent rats and tempered moderate increases in alcohol consumption by nondependent rats in intermittent testing.

Conclusions: These combined findings suggest a key role for specific presynaptic CRF-GABA interactions in CeA in the development and maintenance of ethanol dependence.

Key Words: Alcohol, dependence, electrophysiology, microdialysis, mRNA

Alcohol addiction is a chronic relapsing disorder characterized by compulsive alcohol use, loss of control in limiting intake, and a withdrawal syndrome characterized by a negative emotional state during abstinence. Several brain neurochemical systems and brain regions are engaged in the development of alcohol dependence. The gamma-aminobutyric acid (GABA)ergic and corticotropin releasing factor (CRF) systems in the central amygdala (CeA) are thought to play an important role in anxiety associated with ethanol dependence and in mediating the increased ethanol intake during withdrawal in dependent animals (1–6).

CRF release in the CeA is increased during withdrawal in alcohol-dependent animals (5,6) and appears to contribute to withdrawal-related anxiety, which is reduced by injection of CRF receptor antagonists into the CeA (7). CRF contributes to increased alcohol consumption in dependent animals, because CRF1 receptor (CRF1) antagonists (8,9) and CRF1 deletion (10) both reduced the increased ethanol self-administration in dependent but not nondependent animals. These results lead to the hypothesis that negative emotional states like anxiety contribute to the compulsive alcohol intake associated with dependence via negative reinforcement (1).

At the cellular level, acute ethanol augments evoked GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs) by increasing GABA release in both rat (11,12) and mouse CeA neurons (13,14). CRF1 mediates this effect in CeA of naive mice (15,16) via protein kinase C epsilon (PKCε) signaling (14). However, there is no published electrophysiologic evidence on the role of CRF in ethanol effects in rat CeA. Therefore, in this study, we validated involvement of the CRF-CRF1 system in ethanol effects on GABAergic transmission in rat CeA slices: CRF increased GABAergic transmission in rat CeA neurons as in mice, and CRF1 antagonists blocked ethanol-induced GABA release in rat CeA.

We recently demonstrated increased basal GABA release and no tolerance to the acute ethanol-induced presynaptic release of GABA in CeA from ethanol-dependent rats compared with naive rats (12). To our knowledge, the effects of CRF on GABAergic responses in CeA neurons from ethanol-dependent rats have not been investigated. Therefore, in this study, we tested the hypothesis that neuroadaptations in the CeA CRF-GABA systems play a role in the development of ethanol dependence. We find increased sensitivity to CRF and CRF1 antagonists on GABA release in CeA slices of ethanol-dependent rats, and we demonstrate that in vivo intra-CeA administration of a CRF1 antagonist via retromicrodialysis reverses dependence-related elevations in extracel-
lular GABA and blocks ethanol-induced increases in GABA in both dependent and nondependent rats. We also find increased mRNA levels for CRF and CRF₁ in CeA of dependent rats.

Given the prominent action of acute administration of CRF₁ antagonists in reducing ethanol-self-administration in dependent rats (9), here we tested chronic treatment of dependent rats with a CRF₁ antagonist. This antagonist administered for 25 days blocked withdrawal-induced increases in alcohol consumption in dependent rats and also moderate drinking produced by intermittent testing in nondependent rats.

These combined findings suggest a specific CRF-GABA interaction in CeA that undergoes neuroadaptative changes during ethanol dependence and that may underlie the development and maintenance of ethanol dependence.

Methods and Materials

Electrophysiologic Studies

Slice Preparation. We prepared transverse CeA slices (400 μm thick) as previously described (11,12), from male Sprague–Dawley rats (120–300 g, 4–7 weeks old) anesthetized with halothane (3%) and decapitated. We continuously superfused (at 2–4 mL/min) slices with warm (32°C), gassed artificial cerebrospinal fluid (ACSF) of the following composition in mmol/L: NaCl, 130; KCl, 3.5; NaH₂PO₄, 1.25; MgSO₄, 0.97; CaCl₂, 2.0; NaHCO₃, 24; glucose, 10. We added drugs to the ACSF from stock solutions to obtain known concentrations in the superfusate.

Chronic Ethanol Treatment. We used the standard ethanol inhalation method of the Scripps Research Institute Alcohol Research Center to induce ethanol dependence in rats continuously exposed to ethanol vapors for 2 to 4 weeks (12,16) (Supplement 1). We made recordings in ethanol-free ACSF from slices of ethanol-dependent rats 2 to 8 hours after cutting the slices, as previously described (12).

Blood Alcohol Level (BAL) and Body Weight. Three times per week we determined BALs of the ethanol-dependent animals from tail-blood samples. The mean BAL of all ethanol-dependent animals was 171.8 ± 14 mg/dL (n = 40), and their body weight was 255 ± 14 g (n = 27), compared with 235 ± 18 g (n = 36) for controls.

Electrophysiology. We recorded from CeA neurons predominantly with sharp micropipettes (3 mol/L KCl) using discontinuous voltage- or current-clamp mode (12). We evoked pharmacologically isolated GABAergic receptor-mediated IPSCs by stimulating locally within the CeA through a bipolar electrode (11,12) (Supplement 1). We examined paired-pulse facilitation (PPF) in each neuron using paired stimuli at 50-msec interstimulus intervals (12). We express all values as mean ± SEM, analyzed via between-subjects or within-subject analysis of variance (ANOVA) with repeated measures and, when appropriate, the Newman-Keuls post hoc test, with p < .05 considered statistically significant. In some cases, we used a Student’s paired or unpaired t test for individual means comparisons.

Miniature IPSCs. We recorded from a subset of CeA neurons using the “blind” whole-cell patch-clamp method, in the presence of 10 μmol/L CNQX, 30 μmol/L APV, 1 μmol/L CGP 55845A and 1 μmol/L tetrodotoxin to isolate spontaneous, action potential-independent GABAergic miniature IPSCs (mIPSCs; for details, see Supplement 1).

Microdialysis

Sprague-Dawley rats (250–370 g; Charles River, Hollister, California) were anesthetized with isoflurane (1%–2%) and stereotaxically implanted with a stainless steel microdialysis guide cannula that terminated at the dorsal surface of the medial CeA (Supplement 1) (12,17,18).

We continuously exposed rats to ethanol vapors for at least 2 weeks before microdialysis was initiated. The mean BAL of these ethanol-dependent animals was 165.8 mg/dL (n = 7). We treated sham, naive rats similarly but without ethanol vapors. On experiment days, animals were maintained in the vapor chambers until the microdialysis probes were inserted and secured to the guide-cannulae. We began collection of the dialysate samples 3 hours after implantation. Dialysate samples were frozen at −70°C until they were derivatized and analyzed for amino acid content using capillary electrophoresis with laser-induced fluorescence detection (12) (Supplement 1).

Molecular Biology

Quantitative Polymerase Chain Reaction (qPCR). For quantification of mRNA levels of CRF in CeA, we used ethanol-naive/sham (n = 11) and acutely withdrawn ethanol-dependent Wistar (n = 8) rats. Rats in the qPCR experiment were rapidly anesthetized by isoflurane inhalation and sacrificed 24 hours after air/ethanol vapor offset. We sectioned brains coronally (2-mm slices) in a rat brain matrix. Separate punches containing the CeA, basolateral amygdala, or nucleus accumbens were obtained using a 14-gauge needle, guided by atlas (19), and stored at −80°C until processing. The real-time qPCR was performed as described previously (20) (see also Supplement 1). Results were analyzed by second derivative methods and expressed in arbitrary units, normalized to Cyp expression levels. We ran standards and samples in duplicate and determined gene-specific amplification by melting curve analysis as one peak at expected melting temperatures and by agarose gel electrophoresis. Quantitative PCR data were analyzed by Student’s t test.

Behavioral Studies

Adult male Wistar rats obtained from Charles River (n = 29; Kingston, New York) weighed 336.4 ± 9.8 g (~8 weeks of age) at the start of operant training (Supplement 1). We trained rats to orally self-administer 10% (w/v) ethanol or water in a concurrent, two-lever, free-choice contingency in operant chambers (Coulbourn Instruments, Allentown, Pennsylvania), using a supersaccharin fading procedure (21). Upon stabilization of operant responding (~15 sessions), we divided rats into two groups counterbalanced for baseline alcohol intake: one group made dependent on alcohol and the other nondependent. To induce ethanol dependence, rats were exposed to intermittent (14-hour vapor ON/10-hour vapor OFF per day) ethanol vapor that produces BALs between 150 and 200 mg/100 ml (22,23) for 23 consecutive days. The mean BAL for dependent rats was 163 ± 11 mg/100 mL across 23 days of exposure. We systemically injected rats with R121919 (10 mg/2 mL/kg subcutaneously) or vehicle (20% wt/vol hydroxypropyl-β-cyclodextrin) on even-numbered days at 6- to 8-hour withdrawal from vapor exposure. We tested rats for operant alcohol responding on Days 3, 7, 11, 15, 19, and 23 at 6- to 8-hour withdrawal from vapor exposure to monitor development of excessive ethanol drinking during the transition to ethanol dependence. Ethanol intake was normalized for body weight (i.e., g ethanol/kg body weight) and is expressed as mean ± SEM. We analyzed self-administration data using three-way mixed-design ANOVA, with ethanol dependence history and R121919 dose as between-subjects factors and day as the within-subjects factor. We conducted post hoc com-
parisons using Student-Newman-Keuls test and set statistical significance at $p < .05$.

**Results**

**Electrophysiology**

**Sensitization to CRF Effects in CeA of Dependent Rats.** We performed electrophysiologic experiments 2 to 8 hours after preparation of CeA slices from ethanol-dependent or naive control rats. We recorded from 178 CeA neurons with mean resting membrane potential of $-75 \pm 2$ mV and mean input resistance of $105 \pm 4$ MΩ. We evoked isolated GABAA-IPSCs by stimulating locally within the rat CeA.

Acute superfusion of 25 to 400 nmol/L CRF for 10 to 20 min dose-dependently increased the amplitude of evoked IPSCs (Figure 1A; black logistic line, filled squares) in CeA neurons of naive rats, with an apparent EC$_{50}$ of 77 nmol/L. The highest CRF concentration tested, 400 nmol/L, enhanced IPSCs to the same extent as 200 nmol/L; therefore, we regarded the latter concentration as maximal. Superfusion of 200 nmol/L CRF significantly ($p < .05$) increased (by 45%) the mean amplitude of evoked IPSCs measured over all stimulus strengths (Figure 1C; $n = 9$).

Figure 1B plots an averaged time course for 200 nmol/L CRF applied for 20 min at a moderate stimulus intensity (adjusted for an IPSC amplitude 50% of maximal, determined from I/O

![Figure 1](https://www.sobp.org/journal)

**Figure 1.** Corticotropin releasing factor (CRF) dose-dependently increases gamma-aminobutyric acid (GABA)ergic transmission in rat central amygdala (CeA) neurons, and the maximal CRF effect is increased in CeA neurons from dependent rats. (A) Black line, filled squares: concentration–response data from 4 to 7 CeA neurons from naive rats for each point, representing percent increase in mean ($\pm$ SEM) inhibitory postsynaptic currents (IPSC) peak amplitudes plotted against a log scale of CRF concentration. The black solid curve is a logistic fit of the data plotted by origin software (OriginLab Corporation, Northampton, Massachusetts), using $y = (A1 - A2)/[1 + (x/xo)^p] + A2$, where A1 is the initial value of IPSC increase (5%), A2 the estimated final maximum value (49%), xo is the center value (77 nmol/L; unfixed), and $p$ is the rate or power (2.3). All values were fixed except center. Dashed lines: apparent EC$_{50}$ for CRF. Gray line, open squares: concentration–response data from 3 to 9 CeA neurons from ethanol-dependent rats; each point represents percent change in mean ($\pm$ SEM) IPSC peak amplitude pooled and plotted versus log of CRF concentration. The gray solid curve is a logistic fit, where A1 is the initial value of IPSC change ($-3$%), A2 the estimated final maximum value (66%), xo is the center value (82 nmol/L; unfixed), and $p$ is the rate or power (3.7). All values fixed except center. Dashed lines: apparent EC$_{50}$ for CRF. * Indicates significance ($p < .05$) between the two groups. (B) Time course of CRF on IPSPCs. Top panel: representative IPSCs in a CeA neuron from a naive rat recorded before and during 200 nmol/L CRF (20 min) and washout (30 min). Bottom panel: averaged responses obtained from 7 AA neurons. CRF significantly increased mean IPSC amplitudes with partial recover on washout. (C) IPSC input-output (I-O) curves: CRF (200 nmol/L) superfusion (at 10–15 min) significantly ($p < .05$) increased the mean evoked IPSCs over three stimulus intensities in CeA from naive rats. Top insert: representative recordings of evoked IPSCs. (D) I-O curves: CRF (200 nmol/L, 10- to 15-min application) superfusion significantly ($p < .05$) increased the mean evoked IPSCs over all three stimulus strengths, with recovery on washout in CeA from ethanol-dependent rats. Top insert: representative recordings of evoked IPSCs.
relationships in Figure 1C). The CRF effect began after approximately 5 min of CRF superfusion; we saw no desensitization with prolonged (20 min) superfusion. Subsequent washout of CRF with ACSF alone led to recovery of IPSCs to near control levels (Figure 1B).

We then made recordings in ethanol-free ACSF from slices of ethanol-dependent rats 2 to 8 hours after cutting the slices, as previously described (12). As in CeA of naive rats, acute CRF (25–400 nmol/L) dose-dependently increased evoked IPSC amplitudes (Figure 1A; gray logistic line, open squares) in CeA from ethanol-dependent rats, with an apparent EC50 of about 82 nmol/L, equivalent to that for naive rats. However, in CeA of dependent rats, the ability of maximal (200 nmol/L) and a submaximal (100 nmol/L) concentration of CRF to augment evoked IPSCs was significantly (p < .05) enhanced (by 65% and 38%, respectively; n = 9; Figure 1A, open squares) compared with naive CeA (by 40% and 28%, n = 9, Figure 1A, black logistic line, filled squares). Superfusion of 200 nmol/L CRF increased (by 65%; p < .05) the mean amplitude of evoked IPSCs measured over all stimulus strengths (Figure 1D; n = 9).

To determine whether CRF alters presynaptic transmitter release, we examined PPF of evoked IPSCs, thought to be inversely related to transmitter release (11,24). CRF significantly decreased PPF of IPSCs (n = 9; Figure 2A) in CeA from naive rats, suggesting increased GABA release. Next, we recorded spontaneous mIPSCs (see Methods and Materials). CRF superfusion increased the mean mIPSC frequency (to 148.5 ± 8% of control) but not the mean amplitude of mIPSCs, in CeA from naive rats (Figure 2C; n = 4), further indicating increased presynaptic release of GABA. CRF also decreased PPF of IPSCs in CeA of ethanol-dependent rats (Figure 2B; n = 9), suggesting increased GABA release. Notably, in ethanol-dependent rats, PPF was lower than in naive rats (Figure 2A and 2B), suggesting increased basal GABA release. In CeA neurons of dependent rats, CRF also significantly (p < .05) increased mean mIPSC frequencies to 148.5 ± 9%; Figure 2C) in CeA from naive rats, p < .05; for NIH-3, 25% ± 8% of control (n = 9). This CRF-induced frequency increase (200.9%) was significantly (p < .05) greater than that (148.5%; Figure 2C) of CeA of naive rats.

We previously reported that ethanol augments IPSCs in both mouse and rat CeA neurons (11,13–15). This effect in mouse CeA is mediated by presynaptic CRF1 (13,15). Here, to determine whether CRF, also mediates ethanol effects in rat CeA, we tested three selective CRF1 antagonists: antalarmin, LWH-63 (also known as NIH-3) and R121919. As previously demonstrated (11), 44 nmol/L ethanol alone significantly (p < .05) increased evoked IPSC amplitudes to 145 ± 6% (n = 6) in CeA neurons from naive rats (Figure 3A). Antalarmin (10 μmol/L; n = 7), NIH-3 (10 μmol/L; n = 5), and R121919 (1 μmol/L; n = 7) directly increased evoked IPSC amplitudes to 91 ± 6% (p > .05), 85 ± 7% (p < .05), 91 ± 5% (p < .05) and 85 ± 7% (p < .05) of control, respectively, and abolished acute ethanol-induced increases of IPSCs (Figure 3B and 3C). The three antagonists applied alone also increased the mean PPF of IPSCs by 10% to 15% (for antalarmin, 10% ± 5%, n = 7, p > .05; for NIH-3, 15% ± 3%, n = 5, p < .05; for R121919 13% ± 4%, n = 7, p < .05; not shown) and blocked the acute ethanol-induced decrease in PPF, thus opposing the presynaptic effect of ethanol. NIH-3 alone also significantly (p < .05) decreased the frequency of mIPSCs by 30% ± 5%, n = 4) and completely prevented the increase of mIPSC frequency induced by ethanol (n = 3; not shown).

As in our previous studies (12), acute ethanol superfusion significantly (p < .5; n = 6) increased IPSC amplitudes to 147% ± 9% (n = 7) of baseline in CeA neurons of ethanol-dependent rats (Figure 3D), suggesting lack of tolerance to acute ethanol effects on GABA release. In CeA slices from dependent rats, inhibition of basal IPSCs by antalarmin (n = 10), NIH-3 (n = 5), or R121919 (n = 8) alone was significantly (p < .05) greater than in control rats (Figure 3E and 3F vs. Figure 3B and 3C). Also, in CeA of dependent rats the three antagonists induced a significantly (p < .05) larger (by 25%) mean increase in PPF of evoked IPSCs (for antalarmin, 24% ± 3%, n = 10, p < .05; for NIH-3, 25% ± 2.5%, n = 5, p < .05; for R121919, 26% ± 4%, n = 8, p < .05; not shown), compared with that of naive rats (by 10%–15%, see above).

The greater effect of CRF1 antagonists on basal IPSCs of dependent rats may reflect increased tonic release of endogenous CRF, constitutive CRF1 activation, increased receptor number, and/or sensitization of CRF1 in CeA of dependent rats. Because CRF (like ethanol) decreased PPF of evoked IPSCs in rat
CeA and increased mIPSC frequency in rat and mouse (14,15), increased vesicular GABA release in dependent rats is likely. Conversely, CRF1 antagonists directly increased PPF of IPSCs, consistent with decreased GABA release, thus opposing ethanol actions. These combined findings suggest an important ethanol–CRF interaction on GABAergic transmission in the CeA that markedly increases during development of ethanol dependence.

Microdialysis

Increased Sensitivity to a CRF1 Antagonist on CeA GABA Release. We measured the neurochemical effects of a CRF1 antagonist, R121919, on baseline and ethanol-stimulated dalyseate GABA levels in the CeA of freely moving rats. We collected dialysate samples from ethanol-dependent rats during early ethanol withdrawal (2–8 hours). Baseline dialysate GABA levels were significantly higher in CeA of ethanol-dependent rats (n = 9) than in that of ethanol-naive rats [Figure 4A; n = 7; 118 ± 20 vs. 33 ± 4 nmol/L respectively, F(1,14) = 15.169, p < .005]. Local administration of ethanol (1 mol/L) via reverse dalyysis significantly increased dialysate GABA levels in both ethanol-depen- dent [F(1,8) = 20.747, p < .005] and control [F(1,6) = 63.16, p < .0005] rats (Figure 4A). Two-way ANOVA revealed a significant effect of ethanol history [F(1,28) = 21.288, p < .0001] and perfusate ethanol [F(1,28) = 8.88, p < .01] on dialysate GABA, although there was no significant interaction between these two variables [F(1,28) = 2.374, p > .05], consistent with our electrophysiologic evidence of a lack of tolerance to ethanol-induced increases in CeA GABA following chronic ethanol exposure (12).

In contrast, there was an influence of ethanol history on the effects of 300 nmol/L R121919 alone on dialysate GABA, as indicated by a significant interaction between these variables in a two-way ANOVA [F(1,28) = 4.749, p < .05]. Whereas dialysate GABA levels in ethanol-naive rats were unaltered by reverse-dialysis with R121919 [F(1,6) = 6.744, p > .05], GABA levels in ethanol-dependent rats were significantly reduced by perfusion with R121919 [F(1,8) = 17.141, p < .005; Figure 4Al. The results suggest that increased CRF1 function or number during acute ethanol withdrawal may contribute to the higher baseline CeA dialysate GABA levels in these animals. Co-perfu-

**Figure 3.** Corticotropin releasing factor-1 (CRF₁) antagonists block the ethanol-induced increase of evoked inhibitory postsynaptic currents (IPSCs) in central amygdala (CeA) neurons of naive rats. (A) Ethanol (44 mmol/L, 10- to 12-min application) significantly (*p < .05) increases evoked IPSCs with recovery in washout. (B) The CRF₁ antagonists slightly decrease the baseline gamma-aminobutyric acid (GABAergic transmission and prevent the ethanol-induced increase of IPSCs in naive CeA neurons from naive rats. Representative evoked IPSCs recorded before and during superfusion of CRF₁, antagonists 10 μmol/L antalarmin (Anta), 10 μmol/L NIH3 and 1 μmol/L R121919) and during and after the coaplication of the CRF₁ antagonists with ethanol and washout. (C) Pooled data of the experiments from Figure 3B: superfusion of CRF₁ antagonists prevented the ethanol-induced increase of IPSCs in naive CeA neurons. (D) IPSC recordings from CeA of dependent rats. As in CeA of naive rats, ethanol (44 mmol/L, 10- to 12-min application) significantly (*p < .05) augments evoked IPSCs with recovery on washout. (E) The CRF₁ antagonists significantly decrease basal GABA transmission and block ethanol effects in CeA neurons from ethanol-dependent rats. Representative recordings of evoked IPSCS recorded before and during superfusion of CRF₁ antagonists, during coapplication of the CRF₁ antagonists and ethanol, and washout. (F) Pooled data of the experiments in Figure 3E: superfusion of the three CRF₁ antagonists significantly (*p < .05) decreased the mean amplitudes of evoked IPSCs and also blocked the ethanol-induced augmentation of IPSCs. * Indicates that the CRF₁ antagonist effects in ethanol-dependent rats are significantly larger (p < .05) than the antagonist effects in naive rats (Panel F vs. Panel C).
sion of R121919 with ethanol completely blocked ethanol-induced increases in dialysate GABA in ethanol-naive rats \( F(1,6) = .081, p < .05 \). In ethanol-dependent animals local CeA ethanol administration significantly decreased dialysate GABA levels when coadministered with R121919 \( F(1,8) = 8.478, p < .05 \), relative to levels obtained during perfusion with R121919 alone.

**Molecular Biology**

To further probe changes in the CRF system within CeA of dependent rats, we used quantitative real-time PCR to measure CRF and CRF\(_1\) mRNA levels. CRF mRNA levels were significantly higher in CeA punches from ethanol-dependent rats \( n = 8 \) compared with those from naive controls \( n = 11 \); \( t(17) = 2.04, p < .05 \; \) (Figure 4B). In the same ethanol-dependent rats, there was also a trend for increased \( 25\% \); not shown) CeA CRF\(_1\) mRNA levels. We observed no group differences in CRF or CRF\(_1\) mRNA expression in basolateral amygdala or nucleus accumbens punches \( n = 8 \) compared with naive \( n = 11 \) controls, as measured by quantitative real-time polymerase chain reaction.

**Behavioral Studies**

**Operant Ethanol Intake During Transition to Ethanol Dependence.** To determine the role of CRF\(_1\) in the transition to alcohol dependence, we chronically injected rats with R121919 during dependence induction via alcohol vapor inhalation. Chronic R121919 injections and operant alcohol self-administration tests occurred throughout the dependence induction phase at the same acute withdrawal time point of 6 to 8 hours into daily withdrawal, but injections and operant tests were always separated by no less than 24 hours. Dependence induction via chronic intermittent alcohol vapor inhalation reliably produces increases in operant alcohol self-administration tested during withdrawal, and these increases are blocked by acute intra-CeA (25) or systemic injections of CRF antagonists (9,21,26,27) in the absence of effects on self-administration in nondependent non-deprived rats. Figure 5A shows ethanol intake (for body weight; g/kg) by rats during 30-min operant sessions conducted 6 to 8 hours into withdrawal every fourth day during the first 23 days of intermittent vapor exposure. As expected, a three-way ANOVA (Vapor Treatment \( \times \) Dose \( \times \) Test Day) indicated that alcohol vapor exposure significantly increased alcohol intake relative to air-exposure controls in vehicle-injected rats across the 23 days [Vapor: \( F(1,25) = 5.5, p < .05 \)] and that rats drank more alcohol over the course of the vapor exposure period [Test Day: \( F(6,150) = 8.47, p < .001 \)]. As predicted, however, rats chronically injected with 10 mg/kg R121919 exhibited significantly lower ethanol intake (g/kg) than vehicle-injected controls: [Dose: \( F(1,25) = 5.09, p < .05 \)]. There were no significant two-way or three-way interactions on ethanol intake (g/kg), indicating that R121919 reduced ethanol intake both during the transition to ethanol dependence (vapor subjects) and during intermittent ethanol self-administration testing (air controls). A separate two-way

![Figure 4. Acute and chronic ethanol increase dialysate levels of gamma-aminobutyric acid (GABA) in rat central amygdala (CeA) in vivo. (A) In ethanol-dependent rats, the CRF\(_1\) antagonist R121919 administration into the CeA significantly \( (**p < .005) \) decreased mean local dialysate GABA levels. In both naive and ethanol-dependent rats, R121919 administration significantly \( (p < .05) \) blocked the ethanol-induced increase in GABA release. Furthermore, the mean baseline dialysate GABA level was significantly \( (\#p < .001) \) increased in ethanol-dependent rats, compared with naive rats. (B) In ethanol-dependent \( n = 8 \) rats, the levels of CRF mRNA, normalized to cyclophilin A, were significantly increased in CeA punches \( (p < .05) \), compared with naive \( n = 11 \) controls, as measured by quantitative real-time polymerase chain reaction.](https://www.sobp.org/journal)

![Figure 5. (A) Chronic R121919 treatment blocks the development of alcohol dependence-induced increases in alcohol consumption (g/kg) and also blocks moderate increases in alcohol drinking by nondependent rats over time. Rats were injected with R121919 (10 mg/kg subcutaneous) or vehicle on even-numbered days of vapor exposure (indicated by arrows), and tested for responding at 6- to 8-hour withdrawal on Days 3, 7, 11, 15, 19, and 23 of vapor exposure. (B) Cumulative ethanol intake (g/kg) across all operant test sessions during the vapor exposure period. * Indicates significance \( (p < .05) \) suppression by R121919 relative to vehicle, and # indicates significantly \( (p < .05) \) higher ethanol intake by dependent rats relative to nondependent controls.](https://www.sobp.org/journal)
Dysregulation of the CRF system may contribute to several stress-induced psychiatric disorders such as human alcoholism (28); numerous reports suggest that stressful life events and maladaptive responses elicit alcohol drinking and relapse behavior (29–31). Increased anxiety in humans is a putative risk factor for relapse and excessive drinking during abstinence (32). As with human alcoholics, alcohol-dependent animals display enhanced anxiety-like behaviors and excessive alcohol self-administration during withdrawal (22,33–35). Substantial evidence suggests that extrahypothalamic brain CRF systems are activated during the development of alcohol dependence, and this activation has motivational significance (1). The experiments described here reveal that a dysregulation of CRF signaling contributes to alterations in GABAergic function in the CeA of alcohol-dependent rats and that this altered CRF–GABA interaction participates in the development of dependence-related excessive alcohol consumption. Our data demonstrate that alcohol dependence is associated with increased CRF₁, influence on GABA release in the CeA and that prolonged CRF₁ blockade normalizes this dysregulation of CeA GABA signaling and prevents the development of excessive alcohol consumption normally associated with dependence.

Our previous electrophysiologic data showed that alcohol enhanced GABAergic transmission at both pre- and postsynaptic sites in rat CeA slices (11) and that a similar alcohol augmentation of GABA IPSCs in mouse CeA required activation of presynaptic CRF₁ (13,15), suggesting that interactions between the CRF and GABAergic systems in CeA play an important role in acute alcohol actions. However, this interaction has not been studied in the context of ethanol dependence. Therefore, in this study, we used a multidisciplinary approach to define the cellular site of CRF action on GABAergic transmission in rat CeA; most importantly, we found neuroadaptations in the CeA CRF system during dependence. As in the CeA of naive mice, here we show that CRF₁, like acute alcohol, increases evoked IPSC amplitudes, decreases PPF of IPSCs, and increases mIPSC frequencies in CeA neurons from naive rats, suggesting a presynaptic action leading to increased GABA release. Three specific CRF₁ antagonists blocked these alcohol effects, indicating mediation by CRF₁. Notably, in CeA neurons from alcohol-dependent rats, the efficacy, but not the apparent affinity, of CRF₁ to augment IPSCs and mIPSC frequencies was increased, and the CRF₁ antagonists alone significantly diminished basal IPSCs (in contrast to insignificant effects in naive CeA) and blocked alcohol effects on IPSCs. In dependent rats, the CRF-induced increase of both evoked IPSC amplitudes and mIPSC frequencies was significantly enhanced compared with those increases in CeA of naive rats; however, the decrease in PPF of evoked IPSCs was similar in the two groups. This lack of increased efficacy on PPF may suggest some postsynaptic change associated with chronic ethanol exposure, although lack of change in mIPSC amplitude suggests otherwise.

To determine more directly whether CRF₁ mediates alcohol-induced increases in CeA GABA release, we measured dialysate GABA levels in CeA in vivo. In agreement with our previous studies (12), we found elevated basal GABA levels in the CeA of ethanol-dependent rats relative to naive controls. Local administration of the CRF₁ antagonist R121919 reduced baseline dialysate GABA levels in ethanol-dependent rats but not in nondependent controls. This is consistent with our cellular physiology experiments and suggests an increased CRF₁ influence on basal GABA release in the CeA of ethanol-dependent rats. In further agreement with the electrophysiologic experiments, R121919 prevented ethanol-induced increases in CeA dialysate GABA levels in both ethanol-dependent and nondependent rats. The molecular studies show that CRF mRNA levels were increased in CeA (but not in accumbens or basolateral amygdala) from alcohol-dependent rats, suggesting a molecular basis for the upregulation of the CRF system in the CeA during the development of alcohol dependence, consistent with the electrophysiologic and microdialysis data. Our concentration–response data in Figure 1A (showing little change in the EC₅₀ for CRF but an increase in the CRF maximal effect during dependence) suggest that receptor number is upregulated, not apparent receptor sensitivity (or affinity). Notably, the mIPSC frequency data also show an increased maximal CRF effect during dependence, suggesting increased receptor numbers on GABAergic terminals. Although the mRNA levels for CRF₁ only tended to be increased, our electrophysiologic data may suggest posttranscriptional and/or posttranslational CRF₁ increases (e.g., receptor trafficking) in dependent animals.

In Marchigian Sardinian (msP) alcohol-preferring rats, increased expression of CRF₁ in the amygdala is associated with increased sensitivity to stress and to CRF₁ antagonists (36). An upregulation of CRF₁ in CeA was also found in rats in the postdependent state, and CRF₁ antagonists blocked the increased alcohol intake associated with protracted abstinence (37). Collectively, these results agree with past findings that acute withdrawal is marked by increased CRF release within the amygdala that exhibits strong temporal correlation with the emergence of a high-anxiety behavioral state (5,25,38).

Acute systemic administration of CRF₁ antagonists (8,21, 26,27) and more specifically R121919 (9), block dependence-induced increases in alcohol intake during acute withdrawal without affecting alcohol intake in nondependent nondeprived controls. Suppression of alcohol drinking in dependent rats by CRF₁ antagonists is not contingent on the presence of acute withdrawal symptoms during testing, because a CRF₁ antagonist blocks operant alcohol self-administration as well as stress-induced reinstatement of previously extinguished alcohol responding in postdependent rats (39). These effects are likely mediated by CeA because acute infusion of a CRF antagonist into CeA selectively blocks dependence-induced increases in alcohol intake during acute withdrawal (25). Here, we found that chronic R121919 treatment blocks the development of excessive alcohol consumption characteristic of the transition to alcohol dependence and also blocks moderate alcohol drinking by nondependent rats. A key aspect of these findings is that the alcohol intake by rats chronically injected with R121919 during the transition to alcohol dependence resembled that of nondependent rats injected with vehicle. This critical observation suggests that increased activation of CRF systems actually mediates the development of excessive drinking associated with development of dependence.

Three additional aspects of repeated R121919 treatment are worth noting. First, repeated R121919 administration not only blocked the development of excessive alcohol consumption...
during dependence induction, but also tempered the moderate increase in alcohol consumption following periods of abstinence in nondependent rats. Thus, like chronic administration of the clinically efficacious compound acamprosate (39, 40), prolonged CRF antagonist administration may prevent excessive alcohol consumption under a variety of behavioral and physiologic conditions. Second, the present data demonstrate that rats do not exhibit tolerance to the suppressive effects of R121919 on alcohol drinking and may even become more sensitive to its effects over time, pointing to efficacy of this type of compound in a prolonged treatment context likely in a clinical setting. Finally, the behavioral effects of systemically administered R121919 persist for 24 hours postinjection. R121919 has a relatively short plasma half-life (1.7 hours) in rats (41) but dissociates from the CRF1 with a half-life of approximately 11 to 12 hours (D. Grigoriadis, Personal Communication), resulting in prolonged occupancy of CRF1 (42). Heinrichs et al. (40) reported 50% and 100% occupancy 1 hour following oral administration of 2.5 and 20 mg/kg doses of R121919, respectively, consistent with reports that the in vivo efficacy of R121919 outlasts plasma levels of the drug following systemic administration in rats (41).

Our combined findings suggest an important CRF-alcohol interaction on GABAergic transmission in CeA that becomes more pronounced during alcohol dependence. CRF antagonists block alcohol actions in CeA neurons from alcohol-dependent rats and also block excessive alcohol intake. Thus, we hypothesize that the motivational effects of CRF1 antagonists in alcohol dependence are mediated by the inhibitory GABAergic system in CeA. Most CeA neurons from which we record are putative GABAergic inhibitory interneurons with inhibitory recurrent or feed-forward connections, as well as GABAergic projection neurons targeting downstream nuclei (13, 14, 42, 43). Therefore, effects on the local inhibitory CeA circuits by alcohol and CRF1 antagonists may gate and regulate the GABAergic inhibitory information flow to downstream brain regions.

Our findings that both alcohol and CRF presynaptically enhance GABAergic transmission in a brain region known to be involved in stress-related behaviors provide a possible mechanism linking stress and depression/anxiety with alcohol reinforcement in dependence. These results suggest that CRF1 antagonists may selectively suppress alcohol self-administration by reversing a key cellular process that drives excessive drinking in dependent animals, and they thus support the potential of CRF1 antagonists in treating alcoholism (44).

We thank Dr. Floyd E. Bloom for critical comments on the manuscript and Maury Cole for the assistance with the ethanol vapor chambers. Supported by National Institutes of Health Grant Nos. AA06459, AA015566, AA06420, AA016985, AA014619, AA016731, AA13498, AA17447, DA023680, the Pearson Center for Alcoholism and Addiction Research; and the Harold L. Dorris Neurological Research Institute at the Scripps Research Institute (to MK and EP2). LWH63, antalarmin, and R121919 were synthesized and generously contributed by Dr. Kenner Rice at the Neurological Research Institute at the Scripps Research Institute for Alcoholism and Addiction Research; and the Harold L. Dorris Neurological Research Institute at the Scripps Research Institute.

The authors report no biomedical financial interests or potential conflicts of interest.

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