Upregulation of Voluntary Alcohol Intake, Behavioral Sensitivity to Stress, and Amygdala *Crhr1* Expression Following a History of Dependence

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Background: A history of alcohol dependence recruits increased voluntary alcohol intake and sensitivity to stress. Corticotropin-releasing hormone (CRH) has been implicated in this transition, but underlying molecular mechanisms remain unclear.

Methods: A postdependent state was induced using intermittent alcohol exposure. Experiments were carried out following ≥3 weeks of recovery to eliminate contributions of acute withdrawal. Voluntary alcohol consumption was assessed in a two-bottle, free choice procedure. Behavioral sensitivity to stress was examined using fear suppression of behavior in a punished drinking (Vogel) conflict test. Effects of forced swim stress on voluntary alcohol intake were examined as a function of exposure history. Expression of Crh, Crhr1, and Crhr2 transcripts was analyzed by in situ hybridization histochemistry.

Results: Alcohol drinking was upregulated long-term following a history of dependence. Fear suppression of behavior was selectively potentiated in postdependent animals. This persisted 3 months after alcohol exposure and was reversed by the selective CRH-R1 antagonist 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine (MTIP) (10 mg/kg). Forced swim stress increased alcohol intake in postdependent animals but not in control animals. Behavioral changes were paralleled by an upregulation of Crhr1 transcript expression within basolateral (BLA) and medial (MeA) amygdala and Crh messenger RNA (mRNA) in central amygdala (CeA). In contrast, Crhr2 expression was down in the BLA.

Conclusions: Neuroadaptations encompassing amygdala CRH signaling contribute to the behavioral phenotype of postdependent animals.

Key Words: Alcoholism, animal model, conflict test, in situ hybridization, neuroadaptation

Alcoholism develops over years and requires prolonged periods of brain exposure to intoxicating levels of alcohol. Over the course of this process, recruitment of elevated anxiety, low mood, and increased sensitivity to stress, here collectively labeled negative affect, has been postulated as being critical for the transition from a nondependent to a dependent state (Breese *et al.* 2005a; Heilig and Egli 2006; Valdez and Koob 2004). Clinically, negative affect is most prominent during acute alcohol withdrawal but persists into protracted abstinence (Hershon 1977), as shown, for example, by potentiated startle responses (Krystal *et al.* 1997) and increased frequency of panic attacks (George *et al.* 1990). Environmental stressors constitute a major category of stimuli capable of triggering relapse in humans and experimental animals (Brownell *et al.* 1986; Shaham *et al.* 2003). Thus, increased sensitivity to stress in the postdependent state is likely to contribute to maintaining alcohol dependence. Identifying its neural substrates, therefore, is critical to developing novel alcoholism treatments.

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A history of alcohol dependence has been modeled in laboratory rats using prolonged exposure to alcohol vapor, which triggers long-lasting neural and behavioral plasticity that appear relevant for modeling human alcoholism. This type of manipulation produces persistently increased alcohol intake in genetically nonselected rats (Rimondini *et al.* 2002; Roberts *et al.* 2000). Exposure to repeated cycles of intoxication and withdrawal, which mimics the course of the clinical condition, is most effective for inducing increased alcohol drinking (O'Dell *et al.* 2004; Rimondini *et al.* 2002). Similar to the human condition, a minimum duration of dependence is required for lasting upregulation of alcohol preference (Rimondini *et al.* 2003). Elevated alcohol intake in postdependent rats is sensitive to the clinically effective compound, acamprosate, while alcohol intake of nondependent rats is unaffected by the same treatment (Egli 2005; Heyser *et al.* 1998; Rimondini *et al.* 2002; Spanagel and Zieglgansberger 1997). Furthermore, the postdependent state is characterized by a persistently upregulated behavioral sensitivity to stress (Valdez *et al.* 2002, 2003). Together, these findings indicate that neuroadaptive processes induced by a prolonged exposure to cycles of intoxication and withdrawal parallel those in human alcoholism and might be able to shed light on underlying neural mechanisms.

Corticotropin-releasing hormone (CRH) mediates behavioral stress responses through extrahypothalamic mechanisms. These actions of CRH are primarily mediated through the CRH-R1 receptor subtype, which has been proposed as an attractive target for medication development in anxiety, depression, and addiction (Heinrichs and Koob 2004; Holsboer 2003; Reul and Holsboer 2002; Sarnyai *et al.* 2001). Brain regions of particular importance for drug reward, including the medial prefrontal cortex (mPFC), the nucleus accumbens (NAcc), the bed nucleus of the stria terminalis (BNST), and several nuclei within the amygdala, including central (CeA), medial (MeA), and basolat-

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eral amygdala (BLA), are rich in CRH receptors, specifically of the R1 subtype (Potter *et al.* 1994; Van *et al.* 2000). Most of the CRH neurons targeting these regions originate from cortical interneurons or CeA (Swanson *et al.* 1983), a structure that mediates fear and anxiety (Davis *et al.* 1997; LeDoux *et al.* 1988; Möller *et al.* 1997). Anxious responding during acute alcohol withdrawal is attenuated by CRH antagonists, administered either systemically or directly into the CeA (Baldwin *et al.* 1991; Funk *et al.* 2007; Knapp *et al.*. 2004; Overstreet *et al.* 2004; Rassnick *et al.* 1993; Valdez *et al.* 2002). Furthermore, CRH antagonists block both elevated alcohol self-administration and potentiated anxiety-like responses to stressors seen during protracted abstinence following a history of dependence (Breese *et al.* 2005b; Valdez *et al.* 2002, 2003).

Recruitment of central CRH signaling thus underlies two key features of the postdependent state, namely, long-term increased voluntary consumption of alcohol and persistently upregulated behavioral sensitivity to stress. The molecular mechanisms for these changes are largely unknown. Recently, we reported that in the genetically selected Marchigian-Sardinian alcohol preferring (msP) rat line (Ciccocioppo *et al.* 2006), high alcohol preference has cosegregated with increased behavioral sensitivity to stress, creating a phenocopy of the postdependent phenotype. A screen for differential gene expression identified an innate upregulation of the *Crbr1* transcript in several brain regions of the msP line. Administration of the selective CRH-R1 antagonist, antalarmin, demonstrated a causal role of upregulated CRH-R1 receptors in the behavioral phenotype of msP rats (Hansson *et al.* 2006).

Here, we examined alcohol drinking, stress sensitivity, and expression of *Crh* and its receptors *Crbr1* and *Crbr2.* Our hypothesis was that, similar to what we have found in msP rats, an upregulation of the *Crbr1* transcript might be present in genetically nonselected rats following a history of dependence and that this upregulation would contribute to their behavioral phenotype.

Methods and Materials

Animals

Male Wistar rats (Mollegard, Denmark), weighing 225 g to 250 g at outset of experiments, were housed four per cage at 20°C to 22°C, 45% to 55% controlled humidity, and reverse 12:12 hour light/dark cycle (lights off at 11:00 AM) and tested during the dark phase. All procedures followed the *European Commission Council Directive for Care and Use of Laboratory Animals* (ethics permit S84/98, Stockholm South).

Overall Design

Animals were removed from alcohol or sham exposure after 4 or 7 weeks. After recovering for a period of 3 weeks, animals from each group were randomized to one of the three following experiments:

1. Voluntary Alcohol Drinking (7-week exposed: $n = 10$ vs. $n = 8$; 4-week exposed: $n = 7$ vs. $n = 8$, exposed and sham, respectively). After initial assessment of drinking, all 7-week animals continued voluntary alcohol drinking on the 24-hour access two-bottle free choice for 29 days, until assessed for effects of forced swim stress.
2. Stress Sensitivity in the Vogel Conflict Test (7-week only: $n = 20$ for exposed and sham, respectively). This experiment was carried out directly after the 3-week recovery period. Subjects were then kept for an additional 10 weeks, after which postdependent and control subjects were randomized to pretreatment with vehicle or CRH-R1 antagonist and retested ($n = 10$ per group).
3. Analysis of Gene Expression. Gene expression was assessed by in situ hybridization. Animals were sacrificed directly after the 3-week recovery period ($n = 7$ for both exposed and sham).

Table 1. Blood Alcohol Concentrations Resulting from the Alcohol Vapor Exposure Used to Induce Dependence

Exposure Week	BAC (mg/dL; mean ± SEM)	Range
1	194.4 ± 29.2	90–340
2	313.9 ± 15.0	227–377
3	237.3 ± 22.7	181–339
4	204.4 ± 14.6	152–293
5	309.8 ± 21.6	239–426
6	388.1 ± 38.6	257–574
7	303.0 ± 15.5	273–416

BAC, blood alcohol concentration.

Alcohol Vapor Exposure

Exposure was as described (Rimondini *et al.* 2002) in glass/steel chambers (1 × 1 × 1 m). High-performance liquid chromatography (HPLC) pumps (Knauer, Berlin, Germany) delivered alcohol into electrically heated stainless steel coils (60°C) connected to an airflow of 18 L per minute. Alcohol concentration was adjusted by changing pump flow and monitored via a spectrometer (Wilks, South Norwalk, Connecticut). Exposure was for 17 hours during each 24-hour period (on 4:00 PM; off 9:00 AM). Rats were allowed to habituate to the chambers for 1 week, then exposed to low alcohol concentration for 1 week, and finally exposed to alcohol vapor yielding blood alcohol concentrations as shown in Table 1. Control animals were kept in identical chambers with normal airflow. Weekly, rats were weighed and blood was collected from the lateral tail vein, serum extracted, and assayed for ethanol using an nicotinamide adenine dinucleotide phosphate dehydrogenase/spectrophotometric assay kit (Sigma Aldrich Inc., St. Louis, Missouri) according to the manufacturer's instructions.

Alcohol Consumption and Its Modulation by Stress

Alcohol consumption was measured as 24-hour access two-bottle free choice between 6% alcohol (wt/vol) in .2% saccharin solution, or vehicle, .2% saccharin solution only, as described (Rimondini *et al.* 2002). One week was used to fade in alcohol, and consumption was measured over the following 2 weeks.

To assess effects of stress on voluntary alcohol consumption, two-bottle free-choice drinking was continued for 29 days. Baseline data were obtained over a 3-day block on days 30 to 32 after initiation of drinking. The forced swim stress was carried out daily over a 3-day block on days 33 to 35 as described (Vengeliene *et al.* 2003). Briefly, around 3:00 PM on each test day, animals were removed from their home cage and placed for 10 min in a plastic cylinder (45 × 20 cm) filled up to 35 cm with 19°C water. After completion of the forced swim, subjects were returned to their home cages. Following completion of the stress block, poststress drinking measures were obtained over a final 3-day block (days 36 to 38).

Punished Drinking Test

A modified Vogel drinking test was used as described (Sommer *et al.* 2001), and punished licks during the 8-min conflict

interval were recorded. Unpunished licks during the preceding 4-min control interval were also recorded, as a control for possible effects on thirst or motor performance.

To assess whether potentiated fear suppression of behavior in the conflict test is mediated by CRH-R1 activation, a novel imidazopyridazine CRH-R1 antagonist, 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b] pyridazine (MTIP) (Lilly Research Laboratories, Indianapolis, Indiana) was used. The MTIP binds CRH-R1 receptors with nanomolar affinity, with no detectable activity at the CRH-R2 receptor or other common drug targets, and is highly brain penetrant. A 10 mg/kg was chosen because median effective dose (ED50) has been determined to approximately 1.5 mg, while 10 mg/kg produces a more than 90% blockade of several stress-induced behaviors (Gehlert *et al.*, 2007). Vehicle (10% Tween [Sigma Aldrich Inc.] 80 in distilled water) or MTIP in vehicle were administered intraperitoneally (IP) 30 min prior to testing.

In Situ Hybridization

Procedures were performed as described previously (Hansson *et al.* 2003, 2006). Rats were decapitated in the inactive phase (11:00 PM to 3:00 AM), and brains were removed, snap frozen in −40°C isopentane, and stored at −70°C until use. The 10-μm brain sections were taken at Bregma levels 1) +2.5 to +1.7 mm, 2) −.3 to −.4 mm, 3) −1.7 to 2.0 mm, and 4) −2.3 to −3.3 mm (Paxinos and Watson 1998). Following hybridization with riboprobes for *Crh*, *Crhr1,* or *Crhr2* messenger RNA (mRNA), sections were exposed to Fuji BAS-5000 Phosphorimager plates (Fujifilm, Tokyo, Japan). Digital images were analyzed using AIS Image Analysis Software (Imaging Research Inc., St. Catharines, Ontario, Canada). Regions of interest were chosen based on available functional data with microinjections of CRH receptor ligands within the amygdala and BNST. Values were converted to nCi/g using carbon 14 (^{14}C) standards.

Statistical Analysis

Daily alcohol intake was averaged over 2 weeks. The 4-week and 7-week sham-exposed control groups did not differ from each other and were, therefore, pooled. Following one-way analysis of variance (ANOVA), each of the exposed groups was compared against the pooled control group by Dunnett's post hoc test. In the swim-stress experiment, daily intake was averaged over each of the three blocks (baseline, stress, poststress), each of which was 3 days in duration. Drinking data were analyzed using two-way ANOVA, with history of dependence as between-subjects and the three drinking blocks as a within-subjects factor. Post hoc comparison was performed using the Newman-Keuls test.

Punished licks violated assumptions of homogenous variances and were rank-transformed prior to analysis. In the initial experiment, a one-way ANOVA was carried out, with history of dependence as between-subjects factor. In the pharmacological experiment, a two-way analysis was carried out, with history of dependence and treatment (MTIP vs. vehicle) as factors. To assess whether any fear suppression by the conflict remained following MTIP treatment, unpunished and punished licks were both recalculated as rates (licks/min) to be directly comparable, and equality was tested using the confidence interval method. In each experiment, unpunished licks were separately analyzed as a control for nonspecific effects on thirst or motor performance.

Gene Expression. Data were compared by region-wise one-way ANOVAs, followed by the reverse Holm-Bonferroni correction (Holm 1979; Dow 2003).

Results

Increased Alcohol Consumption Following a History of Dependence and Selective Increase of Alcohol Intake by Stress in Postdependent Rats

Average daily intake of 7-week and 4-week exposed animals was compared with that of their pooled control animals, since the latter did not differ. A more than twofold increase was observed in 7-week but not 4-week exposed animals when compared with control animals [3.8 ± .35 and 1.12 ± .42, respectively, vs. 1.44 ± .28; $F(2,30) = 17.5$, $p < .001$, Dunnett's post hoc test $p < .001$, 7-week exposed vs. control animals; Figure 1]. Vehicle intake was unaffected (data not shown).

In the stress experiment, animals with a history of dependence continued to consume higher amounts of alcohol [main effect of exposure history: $F(1,16) = 12.7$, $p = .003$]. There was a significant overall effect of stress exposure [main effect: $F(2,32) = 9.0$, $p = .0008$], but this affected the two groups differentially, as shown by a significant interaction term [$F(2,32) = 4.1$, $p = .027$]. Post hoc analysis revealed that within the postdependent group, drinking both during the stress and the poststress block was significantly higher than during the prestress baseline block ($p < .001$). In contrast, within the sham-exposed control group, drinking during the stress and the poststress block was indistinguishable from that measured during the prestress baseline block (Figure 2). Vehicle intake did not differ between groups and was unaffected by stress exposure (data not shown).

Potentiated Fear Suppression of Behavior Following a History of Dependence and Its Reversal by the Selective CRH-R1 Antagonist MTIP

After 3-week recovery, fear suppression followed a history of dependence, as shown by markedly lower rates of punished responding observed in 7-week exposed animals compared with their corresponding control group [$F(1,36) = 14.3$, $p = .0006$; Figure 3]. Unpunished responding did not differ between the groups.

Potentiation of fear suppression by a history of dependence persisted when tested 13 weeks after recovery [main effect of

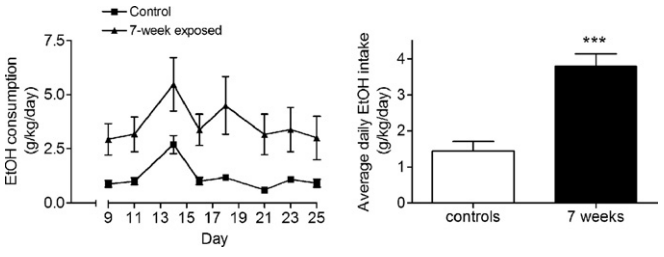

Figure 1. Long-lasting increases in voluntary alcohol consumption in rats with a history of dependence. Two-bottle, free-choice, continuous access alcohol in .2% saccharin versus .2% saccharin only was assessed after a 3-week resting period that followed the last exposure cycle. One week was allowed to gradually increase the alcohol concentration to 6% (wt/vol), and testing was over the following 2 weeks. Left panel: Consumption over the 2-week period. Right panel: Average daily consumption over the same period. There was a highly significant increase in alcohol consumption in the 7-week exposed group compared with their sham-exposed control group. Values are expressed as mean daily consumption ± SEM of 6% (vol/vol) alcohol consumption. ***$p < .001$. For detailed statistics, see Results.

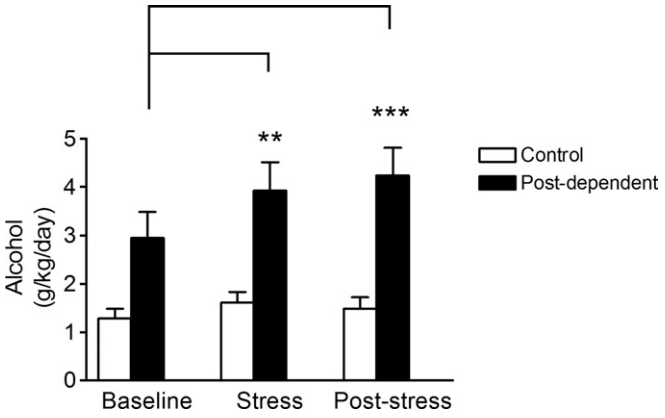

Figure 2. Increase in voluntary alcohol consumption in response to forced swim stress in animals with a history of dependence but not in nondependent animals. Following 7 weeks of intermittent alcohol vapor exposure and tested for voluntary alcohol drinking for 2 weeks (Figure 1), subjects were tested over three blocks (prestress baseline, stress, poststress), each of 3-day duration. During the stress block, animals were subjected to daily forced swim stress. $^{**}p < .01$, $^{***}p < .001$ versus prestress baseline value.

exposure history: $F(1,35) = 5.4$, $p = .025$]. At this time, pretreatment with the CRH-R1 antagonist, MTIP, had a robust anticonflict effect [main effect: $F(1,35) = 13.2$, $p = .0009$]. The MTIP eliminated the difference in conflict behavior related to exposure history. In fact, in both animals with and without a history of dependence, MTIP fully eliminated any fear suppression by conflict (equality between punished and unpunished lick rates within a 10% indifference interval: $p < .05$; Figure 4).

Long-Lasting Increase of *Crhr1* and *Crh* Gene Expression in the Amygdala Following a History of Dependence

Postdependent animals had robustly elevated levels of *Crh1* transcript in BLA [$F(1,12) = 11.3$, $p < .01$] and MeA [$F(1,12) = 25.6$, $p < .001$] but not in CeA or BNST (Figure 5C). The *Crhr1* transcript was not reliably detected in the hypothalamic paraventricular nucleus (PVN).

Expression of the *Crhr2* transcript was less affected in the

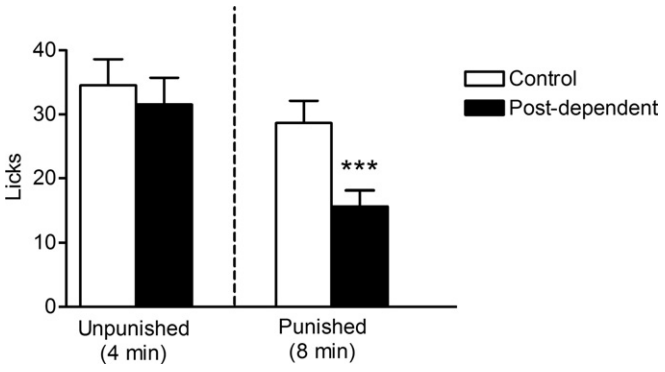

Figure 3. Long-term upregulated behavioral sensitivity to stress in rats with a history of dependence, induced by a 7-week exposure to daily cycles of intoxication and withdrawal in alcohol vapor chambers. To eliminate effects of acute withdrawal, fear-induced suppression of behavior in the punished drinking test was assessed after a 3-week resting period that followed the last exposure cycle. Punished licks recorded over an 8-min conflict period are given (mean ± SEM). Conflict testing was preceded by a 4-min period of unpunished drinking to control for potential nonspecific effects on thirst or motor performance. $^{***}p < .001$. For detailed statistics, see Results.

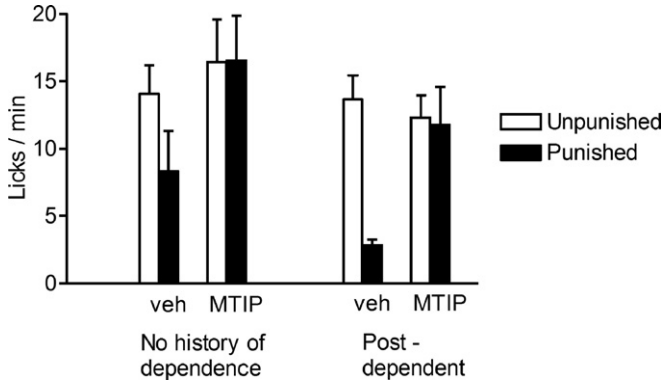

Figure 4. Persistent increased behavioral sensitivity to stress following a history of dependence as measured in the conflict test, when animals were retested 13 weeks after completion of alcohol exposure (main effect of history of dependence: $p = .025$). The selective, brain penetrant CRH-R1 antagonist MTIP (10mg/kg) produced a robust overall anticonflict effect (main treatment effect: $p < .001$). The MTIP eliminated the increased sensitivity to stress in the postdependent group and, in fact, eliminated any fear suppression of behavior by conflict, as shown by punished lick rates becoming virtually identical to unpunished rates. Data are licks/min, mean ± SEM; equality of punished and unpunished lick rates within an indifference interval of 10%: $p < .05$ for both animals without a history of dependence and postdependent subjects. CRH, corticotropin-releasing hormone; MTIP, 3-(4-Chloro-2-morpholin-4-yl-thiazol-5-yl)-8-(1-ethylpropyl)-2,6-dimethyl-imidazo[1,2-b]pyridazine.

extrahypothalamic regions studied, with the exception of a moderate decrease in the BLA [$F(1,11) = 6.7$, $p = .025$; Figure 5D]. Postdependent animals did not differ from control animals in *Crhr2* expression within the PVN (22.5 ± 1.43 vs. 23.3 ± 1.17 nCi/g, mean ± SEM, respectively).

Expression of the *Crh* transcript was elevated in CeA of postdependent animals [$F(1,12) = 7.1$, $p = .02$], while no difference was seen within the BNST (Figure 5B). Postdependent animals did not differ from control animals within the PVN (103.5 ± 3.5 vs. 107.4 ± 4.7).

Discussion

We report elevated voluntary alcohol consumption and potentiated stress sensitivity during protracted abstinence following a history of alcohol dependence. Postdependent animals were selectively sensitive to upregulation of alcohol consumption by a stressor. Long-term upregulation of the transcript encoding the CRH-R1 receptor was found within MeA and BLA of postdependent animals, while CRH-R1 antagonism eliminated the increased behavioral sensitivity to stress in the postdependent state.

Our drinking data replicate prior reports (O'Dell *et al.* 2004; Rimondini *et al.* 2002; Rimondini *et al.* 2003; Roberts *et al.* 2000). In the present study, alcohol intake more than doubled in animals with a history of dependence. This increase was seen after a 3-week recovery following completion of alcohol exposure and persisted for more than an additional month. It is therefore related to long-term neuroadaptations rather than acute withdrawal. A previously reported temporal threshold was replicated, so that a 7-week exposure upregulated alcohol intake, while a 4-week exposure did not. We have independently found that increased consumption in postdependent animals is not due to altered alcohol metabolism (Sommer *et al.*, in preparation).

Our conflict data show a long-lasting decrease in punished water drinking. This effect is not due to altered thirst, as shown by the unaffected rates of unpunished licks. Furthermore, it is

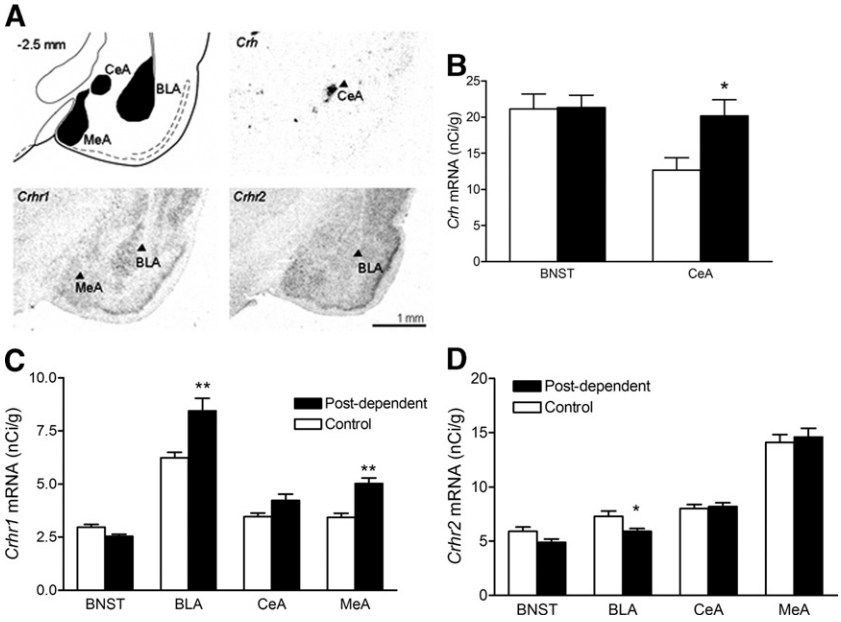

Figure 5. (A) Distribution of *Crh* transcript, encoding the CRH precursor, and the *Crhr1* and *Crhr2* transcripts, encoding the respective receptor subtype. Representative sections from the amygdala of rats without a history of dependence are shown at Bregma −2.5 mm. CeA: central; MeA: medial; and BLA: basolateral amygdala. Scale bar is 1 mm. Quantification of expression levels (nCi/g, mean ± SEM) for the respective transcript in postdependent rats versus rats without a history of dependence is shown in panels **(B)** through **(D)**. **(B)** *Crh* expression was upregulated within CeA, which was the only amygdala region where measurable levels of this transcript were present. **(C)** *Crhr1* message was robustly upregulated within BLA and MeA but not in CeA or BNST. **(D)** Expression of *Crhr2* transcript was unaffected within the extended amygdala, with the exception of BLA, where a moderate decrease was seen. For all panels, $*p < .05$, $**p < .01$, $***p < .001$ corrected for multiple tests. For detailed statistics, see Results. BLA, basolateral amygdala; BNST, bed nucleus of stria terminalis; CeA, central amygdala; CRH, corticotropin-releasing hormone; MeA, medial amygdala.

also unlikely to be caused by altered nociception, since we have independently established that a history of dependence does not affect pain thresholds in the hot plate test (Heilig *et al*., unpublished data). Central administration of CRH has previously been shown to produce potentiated fear suppression in the Geller-Seifter test, while both prototypical benzodiazepine anxiolytics and intracerebroventricular administration of the nonselective CRH receptor antagonist α-helical-CRH_{9-41} reversed this potentiation (Britton *et al*. 1985, 1986). More recently, potent anticonflict effects were shown in the classical Vogel test with the nonpeptide CRH-R1 antagonists antalarmin and SSR125543A (Griebel *et al*. 2002). The potentiated suppression of punished drinking observed in the present study is therefore likely to reflect a specific, CRH-mediated increase in behavioral sensitivity to stress. This finding is in line with prior reports that have demonstrated increased behavioral stress responses in postdependent rats (Breese *et al*. 2005b; Overstreet *et al*. 2002; Rasmussen *et al*. 2001; Valdez *et al*. 2002, 2003). A history of multiple withdrawals, paralleling the clinical course of alcoholism, seems most effective in inducing anxiety-like behavior. For instance, rats subjected to three cycles of withdrawal from an alcohol diet, but not those exposed to a single withdrawal, showed reduced social interaction during early abstinence (Overstreet *et al*. 2002). Although this acute withdrawal effect subsided within 48 hours, a remaining recruitment of negative affect systems was demonstrated by the observation that following a history of three withdrawal episodes, a single future withdrawal from reexposure to chronic ethanol in control animals was anxiogenic. Interestingly, in this model, administration of a CRH antagonist during the first two cycles of withdrawal blocked the increase in anxiety behavior (Knapp *et al*. 2004; Overstreet *et al*. 2004). Elevated anxiety has also been reported on the elevated plus-maze 4 weeks after a liquid alcohol diet (Rasmussen *et al*. 2001) or alcohol vapor exposure (Valdez *et al*. 2002). In the latter case, no overt phenotype was seen in postdependent rats, but when testing was preceded by a restraint stress challenge, an exaggerated anxiogenic response was seen (Valdez *et al*. 2003). Our present conflict data are similar in nature, as the conflict model itself is a stressor sufficient to recruit CeA activity and, in fact, relies on an activation of cell bodies in this structure for fear suppression of behavior (Möller *et al*. 1997). The fact that a stress-sensitive phenotype persists for more than 3 months after completion of alcohol exposure has not been reported previously, and the duration of this state is remarkable. It may be noted that all anxiety-like behaviors discussed here are unconditioned. In contrast, impaired associative fear learning has recently been reported in young human binge drinkers, presumably as a result of repeated cycles of intoxication and withdrawal (Stephens *et al*. 2005). This report proposed a "saturation" mechanism, whereby synaptic plasticity induced by a history of dependence reduces capacity for future learning, while allowing unconditioned stimuli access to neuronal pathways underlying conditioned fear.

Elevated alcohol consumption and increased sensitivity to stress have previously been reported in postdependent rats, but little data exist to link these phenomena. A transient increase in voluntary alcohol intake has been reported in nonselected Wistar rats in response to the forced swim stress used here, but these animals had been consuming alcohol for 4 months and had experienced alcohol deprivation during the course of this history, features that set the scene for neuroadaptive changes (Vengeliene *et al*. 2003). In the present study, nondependent Wistar rats did not increase their alcohol intake in response to repeated forced swim stress. In contrast, postdependent animals started out with elevated alcohol intake and showed a highly significant further increase in response to the stressor. Thus, the increased sensitivity to stress in the postdependent state translates into elevated motivation to consume alcohol in response to external stressors.

Our data provide a putative molecular mechanism for the postdependent behavioral phenotype. Recently, we reported an innate upregulation of *Crhr1* expression in the amygdala of msP rats that causes a behavioral phenotype similar to that seen in postdependent rats. This work also demonstrated that elevated *Crhr1* transcript within the amygdala is strongly correlated with increased binding density (Hansson *et al*. 2006). Here, we found that the postdependent phenotype is accompanied by a very similar upregulation of *Crhr1* receptors within the BLA and MeA. Elimination of fear suppression in the conflict test by the CRH-R1 antagonist MTIP strongly suggests that, similar to what we found in the msP line, the upregulated *Crhr1* expression in the postdependent state is causally related to the behavioral phenotype.

This conclusion is further supported by our recent findings, in a separate study, that MTIP fully and dose-dependently blocks the increase in alcohol intake found in the postdependent state (Gehlert *et al.*, 2007). These data in are in agreement with previous studies, which have demonstrated that both stress responses and elevated voluntary alcohol consumption in the postdependent state are blocked by CRH antagonism or specifically blockade of CRH-R1 receptors (Breese *et al.* 2005b; Valdez *et al.* 2002, 2003). Our findings are also in line with a recent study that showed a recruitment of local amygdala CRH circuits but not those in BNST or NAcc following a history of dependence (Funk *et al.* 2006). Collectively, these findings suggest that a long-lasting upregulation of CRH-R1 receptors within the amygdala constitutes a shared neurobiological substrate underlying the behavioral phenotype of msP and postdependent rats. It remains to be determined to what extent CRH-R1 receptors in CeA, MeA, or BLA are involved.

In the postdependent subjects, the upregulated *Crhr1* expression was accompanied by an increase of *Crh* mRNA in the CeA. This finding confirms and expands on a prior report of increased amygdala CRH immunoreactivity in postdependent rats 6 weeks after termination of a liquid alcohol diet (Zorrilla *et al.* 2001). Elevated tissue peptide content can result either from increased synthesis or decreased release. The observation of increased peptide levels in the previous report, together with elevated transcript in the present study, jointly establish that CRH synthesis is increased in CeA in the postdependent state. Thus, a presynaptic and a postsynaptic signaling component may act in concert to recruit the CRH system in postdependent animals. Finally, the *Crhr2* transcript, although less affected, was oppositely regulated to *Crhr1* within the BLA, in which a decrease was seen. This is in line with prior data showing that activation of CRH-R2 receptors is also capable of reversing the increased sensitivity to stress and elevated alcohol self-administration during the postdependent state (Valdez *et al.* 2004).

Our expression analysis suggests that the dysregulation of CRH systems found during the postdependent state is relatively restricted to extrahypothalamic systems, while the PVN and control of the hypothalamic-pituitary-adrenal (HPA) axis seem unaffected. A limitation of our study is that we were not able to analyze pituitary expression of CRH receptor transcripts. However, our findings are in agreement with prior reports that stable, long-term changes in basal serum corticosterone levels are not found in postdependent rats (Rimondini *et al.* 2002; Zorrilla *et al.* 2001). The precise mechanism by which a parallel increase in CRH and CRH-R1 expression occurs within the amygdala in the postdependent state is unclear. The increased vulnerability of this structure may result from its unique organization, characterized by close proximity or intertwining of CRH synthesis and target sites (Swanson and Petrovich 1998). Also, CRH in CeA mimics actions of alcohol to potentiate local gamma-aminobutyric acid (GABA) transmission through a CRH-R1 mediated mechanism, possibly mediating an autoregulatory loop through recurrent collaterals (Nie *et al.* 2004), while GABA responses to alcohol in CeA are upregulated in the postdependent state (Nie *et al.* 2004; Roberto *et al.* 2004). Intermittent exposure to alcohol intoxication and withdrawal may be particularly effective in driving an allostatic shift of the amygdala CRH system to a higher functional set point (Valdez and Koob 2004), since *Crhr1* gene expression regulation is expected to be on a slower scale than *Crh* itself. Ultimately, mechanisms regulating transcription must be recruited. An intriguing possibility is that these might be similar to those suggested in the phenomenon recently labeled "incubation of craving," which refers to increased propensity for relapse-like behavior over time and which has been shown to rely on signaling through the extracellular signal-regulated kinase (ERK)-pathway, known to be capable of regulating transcription (Grimm *et al.* 2001; Lu *et al.* 2005).

In summary, a recruitment of intra-amygdala but not hypothalamic CRH systems seems to be driving the postdependent phenotype. An increased stress sensitivity in this state may not be overt but is pronounced following a stress challenge. Postdependent upregulation of the CRH system gives rise to excessive rates of alcohol self-administration. Together, these data provide compelling evidence that a blockade of hyperactive signaling at CRH-R1 receptors in the postdependent state inhibit heavy drinking and reduced relapse risk.

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