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A CRF₂ agonist administered into the central nucleus of the amygdala decreases ethanol self-administration in ethanol-dependent rats

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Abstract

Alcohol dependence is characterized by excessive consumption, loss of control over intake and the presence of a withdrawal syndrome, including both motivational and physical symptoms. Previous studies have implicated the brain corticotropin-releasing factor (CRF) stress systems in mediating the negative emotional state associated with ethanol withdrawal. CRF₁ receptor-specific antagonists, administered systemically, and CRF receptor subtype nonspecific antagonists, administered into the central nucleus of the amygdala (CeA), selectively decrease the anxiety-like behaviors and increased ethanol self-administration associated with ethanol withdrawal. In the present study, we investigated the role of CRF₂ receptors within the CeA in mediating ethanol self-administration in ethanol-dependent and nondependent animals. Male Wistar rats were made dependent on ethanol using an intermittent ethanol vapor exposure paradigm. Nondependent animals received similar conditions but were exposed to air only. Following 2 h of withdrawal from ethanol vapors, ethanol and water self-administration were measured following administration of urocortin 3, a highly selective CRF₂ agonist, in the CeA. In dependent rats, urocortin 3 (0.1 µg/µl and 0.5 µg/µl) decreased ethanol self-administration, with no effect on water self-administration. In nondependent rats, urocortin 3 (0.5 µg/µl) increased ethanol self-administration, with no effect on water self-administration. These data demonstrate an opposing role of the CRF₂ receptor subtype within the CeA in mediating ethanol self-administration in withdrawn, dependent and nondependent rats.

Keywords

Alcohol; Dependence; Withdrawal; Corticotropin-releasing factor receptor; Self-administration; Urocortin

1. Introduction

Alcoholism is a chronic relapsing disorder characterized by a compulsive use of alcohol and a loss of control over intake. As dependence develops, there is a shift from controlled use to uncontrolled, excessive consumption of alcohol, which has been argued to be a shift from positive to negative reinforcement ultimately driving continued alcohol use (Koob, 2003; Koob et al., 2004). Cessation of chronic alcohol use often is accompanied by negative emotional symptoms, such as increased anxiety. Alleviation of these negative emotional states is hypothesized to be a major force driving continued alcohol consumption (Hershon, 1977; Koob, 2003). Understanding the brain systems underlying these withdrawal-induced behaviors

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is an important step toward the development of novel pharmacotherapies for the treatment of alcohol dependence.

Ethanol-dependent rats also display enhanced anxiety-like behaviors and excessive ethanol self-administration during periods of withdrawal (Roberts et al., 2000; Rasmussen et al., 2001; Overstreet et al., 2002; Rimondini et al., 2002; Becker and Lopez, 2004; O'Dell et al., 2004). Multiple studies have implicated the brain corticotropin-releasing factor (CRF) stress system in mediating the motivational changes associated with dependence (Menzaghi et al., 1994; Valdez and Koob, 2004). CRF, a 41 amino-acid peptide residue involved in mediating the physiological and behavioral responses to stress, is distributed throughout the brain, with high concentrations of cell bodies in the paraventricular nucleus of the hypothalamus and in areas of the extended amygdala (Vale et al., 1981; Bloom et al., 1982; Dunn and Berridge, 1990). During ethanol withdrawal in dependent rats there is an increase in extracellular CRF within the extended amygdala, specifically within the central nucleus of the amygdala (CeA) (Merlo Pich et al., 1995). Administration of CRF antagonists within the CeA attenuates the enhanced anxiety-like behaviors and increased ethanol self-administration associated with withdrawal (Rassnick et al., 1993; Funk et al., 2006) suggesting an important role of CRF within this brain region in mediating ethanol dependence. Further, lesions of the CeA reduce anxiogenic-like behaviors resulting from restraint stress and reduce voluntary ethanol intake (Moller et al., 1997), demonstrating the importance of this brain region in mediating anxiety and ethanol consumption.

Within the brain, the cellular effects of CRF are mediated by two subtypes of high affinity receptors, CRF₁ and CRF₂ (Vale et al., 1981; Bloom et al., 1982; Dunn and Berridge, 1990; Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993; Lovenberg et al., 1995). Previous studies have demonstrated that CRF₁ antagonists, administered systemically, reduce ethanol self-administration in ethanol-dependent animals (Funk et al., 2007); effects likely mediated within the CeA (Funk et al., 2006). Interestingly, studies have demonstrated an opposing role for the CRF₂ receptor subtype in mediating ethanol withdrawal-induced behaviors (i.e., activation of CRF₂ receptors decreases both the enhanced anxiety-like behavior and ethanol self-administration in withdrawn, dependent rats when administered intracerebroventricularly (i.c.v.) (Valdez et al., 2004). Thus, in the present study we tested the hypothesis that CRF₂ receptors within the CeA may also mediate excessive ethanol self-administration in withdrawn, ethanol-dependent and nondependent rats.

2. Results

2.1. Effects of the CRF₂-specific agonist Ucn 3 on ethanol and water self-administration in dependent and nondependent rats

For the ethanol-dependent group ($n=8$), animal weights at the end of the experiment were 560.3 ± 36.5 grams. For the nondependent group ($n=7$), animal weights were 603.2 ± 54.9 grams. The mean BAL across the entire period of ethanol vapor exposure was 193 ± 31.7 mg%. Fig. 1 shows the effects of Ucn 3 (0.0, 0.02, 0.1 and 0.5 $\mu\text{g}/\mu\text{l}$) on ethanol and water self-administration in dependent versus nondependent animals.

In the 30-min test session, following vehicle injection (0.5 $\times$ PBS), the dependent animals pressed approximately 75 times (1.5 g/kg) for 10% ethanol, compared to 20 ethanol presses (0.41 g/kg) in nondependent animals. For ethanol self-administration, the two-way ANOVA revealed a significant effect of ethanol exposure ($F_{1,13}=40.5$, $p < 0.0001$), a significant effect of Ucn 3 dose ($F_{3,39}=6.5$, $p < 0.001$) and a significant interaction between ethanol exposure and Ucn 3 ($F_{3,39}=15.1$, $p < 0.0001$). Further analysis revealed a significant reduction in ethanol self-administration in ethanol-dependent animals at the 0.1 and 0.5 $\mu\text{g}/\mu\text{l}$ doses of Ucn 3

compared to the 0 µg/µl dose ($p < 0.001$). There was also a significant increase in ethanol self-administration in nondependent animals at the 0.5 µg/µl dose of Ucn 3 ($p < 0.05$).

For water self-administration, the two-way ANOVA revealed no effect of ethanol exposure ($F_{1,13} < 1.0$), no effect of Ucn 3 dose ($F_{3,39} < 1.0$) and no interaction between ethanol exposure and Ucn 3 dose ($F_{3,39} < 1.0$).

A potential caveat is that the site-specific injections may diffuse to other, surrounding brain regions. Thus, degree of spread of the agonist was determined by an injection of 0.5 µl cresyl violet, using the same injection procedure as outlined in the methods. The dye was localized within the CeA. Further, in a few animals the cannulae were misplaced (these animals were not included in statistical analyses) and in these animals, there was little to no reduction in ethanol withdrawal-induced drinking following administration of Ucn 3, suggesting that the CeA is an important site for the agonist's action. Three animals were removed from this study due to misplaced cannulae. One of these animals was dependent and two were nondependent. The following are the data (average±SEM) for ethanol lever responding in the 1 dependent animal: 0.0 µg/µl dose (67), 0.02 µg/µl (72), 0.1 µg/µl (69) and 0.5 µg/µl (70); and the data from the 2 nondependent animals: 0.0 µg/µl dose (17.5±1.5), 0.02 µg/µl (20.5±.5), 0.1 µg/µl (18±1) and 0.5 µg/µl (20±2).

2.2. Verification of injection site

Fig. 2 illustrates representative light micrographs demonstrating site specificity of the cannulae. Probe placements were verified using the Paxinos and Watson (1998) stereotaxic atlas. Only animals with bilateral probe placements in the intended brain regions were used for statistical analysis.

3. Discussion

A negative emotional state, especially enhanced anxiety and stress, experienced during ethanol withdrawal is a main factor eliciting relapse and binge drinking during periods of abstinence (Hershon, 1977). In humans alcoholics, anxiety has been shown to persist for up to 9 months, and in some cases for several years, post-withdrawal (Roelofs, 1985). Animal models for ethanol dependence have been developed which mimic excessive drinking and anxiety-like behaviors during withdrawal (Roberts et al., 2000; O'Dell et al., 2004). Here, male Wistar rats were made dependent on ethanol via exposure to intermittent ethanol vapors for 4 weeks. The highly selective CRF₂ agonist Ucn 3 significantly reduced ethanol self-administration in ethanol-dependent rats when administered directly into the CeA. Ucn 3, at the highest dose, increased ethanol self-administration in ethanol-nondependent animals. These data suggest an important role of the CRF₂ receptor subtype in mediating ethanol self-administration.

Regions of the extended amygdala (including the CeA) comprise part of the "extrahypothalamic" CRF-stress system. These nuclei contain high amounts of CRF terminals, cell bodies and receptors (Merchenthaler et al., 1982; Van Pett et al., 2000), and numerous studies have demonstrated the involvement of the extended amygdala in mediating the behavioral responses associated with anxiety (LeDoux et al., 1988; Walker and Davis, 1997). During ethanol withdrawal, extrahypothalamic CRF systems become hyperactive, with an increase in extracellular CRF within the CeA of dependent rats (Merlo Pich et al., 1995; Zorrilla et al., 2001; Funk et al., 2006). Subtype nonselective CRF receptor antagonists, administered i.c.v. or directly into the CeA, reduce both ethanol withdrawal-induced anxiety-like behaviors as well as ethanol self-administration in dependent rats (Baldwin et al., 1991; Rassnick et al., 1993; Valdez et al., 2002b; Funk et al., 2006), suggesting an important role for CRF, primarily within the CeA, in mediating ethanol withdrawal-induced behaviors.

Two types of high affinity CRF receptors are expressed within the brain, CRF₁ (Chang et al., 1993; Chen et al., 1993) and CRF₂ (Lovenberg et al., 1995). CRF₁ receptors are located throughout the brain (Van Pett et al., 2000) and play an important role in mediating anxiety-like behavior (Liebsch et al., 1995, 1999; Heinrichs et al., 1997; Smith et al., 1998; Timpl et al., 1998; Muller et al., 2003; Zorrilla and Koob, 2004). Studies have also demonstrated the importance of the CRF₁ receptor in mediating ethanol withdrawal-induced behaviors (Timpl et al., 1998; Breese et al., 2004; Overstreet et al., 2004; Funk et al., 2007).

CRF₂ receptors are not as widespread as CRF₁ within the brain, with expression mainly localized to the dorsal raphe, lateral septum, ventral hypothalamus and extended amygdala (Van Pett et al., 2000). The discovery of a putative endogenous selective CRF₂ agonist (Ucn 3) provided new insights into the functional significance of brain CRF systems (Fekete and Zorrilla, 2007). Ucn 3 exhibits subnanomolar functional activity at both rodent CRF₂ receptor splice variants, but virtually no activity at the CRF₁ receptor or binding protein (Fekete and Zorrilla, 2007). The role of the CRF₂ receptor subtype in mediating anxiety-like behaviors appears to be more complex than that of CRF₁ (Bale and Vale, 2004). While some studies report an anxiolytic-like effect of CRF₂ activation, others report an anxiogenic-like action. In genetic knockout lines, CRF₂ receptor-deficient mice display enhanced anxiety-like behavior (Bale et al., 2000; Kishimoto et al., 2000). CRF₂ agonists produce anxiolytic-like behaviors in rats (Valdez et al., 2002a, 2003) and in mice (Venihaki et al., 2004). However, other studies report an anxiogenic-like role for CRF₂ activation in mice (Pelley-mounter et al., 2002, 2004; Risbrough et al., 2003, 2004) and in rats (Ho et al., 2001). Further, low doses of the CRF₂ antagonist antisauvagine-30 in mice produce anxiolytic-like effects, but at some brain sites in rats it produces anxiogenic-like effects (Bakshi et al., 2002). These differences likely reflect the opposing functional role of CRF₂ in different brain regions (Liu et al., 2004).

In terms of ethanol withdrawal-induced behaviors, it appears CRF₂ receptors facilitate mainly anti-stress effects. Valdez et al. (2004) have reported previously that Ucn 3, administered i.c.v., reduces both the enhanced anxiety-like behaviors and the increased ethanol self-administration associated with withdrawal in ethanol-dependent rats (Valdez et al., 2004). Administration of Ucn 3 within the CeA produced similar effects in the present study (i.e., a decrease in ethanol intake in acutely withdrawn, ethanol-dependent rats). These data suggest that activation of CRF₂ receptors within the CeA leads to a reduction in ethanol self-administration via a decrease in anxiety-like behaviors. However, other mechanisms may be involved, such as a decrease in ethanol positive reinforcement, and future studies will be important to determine whether Ucn 3 within the CeA also decreases anxiety-like behaviors.

In nondependent rats, Ucn 3 also altered ethanol intake, increasing ethanol self-administration, an effect opposite to that observed in dependent rats. However, this effect was only observed at the highest dose and similar results have been observed with the benzodiazepine diazepam (Schmitt et al., 2002). While diazepam's effects in nondependent animals may be explained by its role in mediating appetite, we suggest that the effects of Ucn 3 on ethanol self-administration in nondependent animals are a result of its effects on anxiety-like behaviors and not of its appetite-reducing effects, which may be mediated mainly within the medial amygdala and hypothalamic nuclei (Fekete et al., 2007).

Although the mechanisms underlying the opposing actions of Ucn 3 in dependent and nondependent animals remain unknown, ethanol withdrawal may have a similar effect on CRF₂ receptor signaling as that previously reported for cocaine withdrawal (Liu et al., 2005). Within the lateral septum, activation of CRF₂ receptors depressed glutamatergic synaptic transmission in control animals, but facilitated transmission in animals undergoing acute cocaine withdrawal (Liu et al., 2005). The data reported here suggest that a similar switch in CRF₂ signaling may occur in the CeA in response to ethanol withdrawal.

The data presented here, taken together with earlier studies demonstrating that CRF antagonists administered in the CeA (Funk et al., 2006) and CRF₁ antagonists administered systemically (Funk et al., 2007), reduce ethanol self-administration in ethanol-dependent animals, produce a clearer picture for the underlying mechanisms of CRF mediating ethanol withdrawal-induced behaviors. These data suggest that CRF₁ and CRF₂ receptors may have opposing actions in the basal forebrain, and this interaction could be hypothesized to be in series or in parallel at specific forebrain sites. Within the CeA, decreased or compromised CRF₁ function likely participates in mediating the anxiogenic-like effects of ethanol withdrawal, whereas CRF₂ activation may serve as a compensatory mechanism to oppose this action. These data provide important clinical implications for the treatment of alcohol dependence in humans, although it will be important for future studies to also address the effectiveness of these CRF₂ receptor agonists in reducing ethanol self-administration during periods of protracted abstinence. Dysregulation of CRF stress systems may represent a long-lasting change within the brain resulting from chronic ethanol consumption and, as the present data indicate, the CRF₂ receptor may be a novel target in the development of new treatments for alcohol dependence.

4. Experimental procedures

4.1. Animals

Eighteen adult male Wistar rats weighing 180–200 grams at the start of the experiment were obtained from Charles River Laboratory (Kingston, NY). Animals were housed two to three per cage with food and water available *ad libitum*. Lights were on a 12-h light/dark cycle, with lights on at 8:00 PM. All procedures met the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

4.2. Drugs

Ethanol (10% w/v), for oral self-administration, was prepared using 95% ethyl alcohol and water. Murine urocortin 3 (Ucn 3; kindly provided by Dr. Jean Rivier, The Salk Institute for Biological Studies, La Jolla, CA) was aliquoted, parafilm and stored at –70 °C upon arrival. Immediately prior to injections, Ucn 3 was dissolved in 0.5× phosphate buffered saline (PBS; pH 7.4) and kept on ice.

4.3. Operant ethanol self-administration

Ethanol self-administration was established in standard operant chambers (Coulbourn Instruments, Allentown, PA) housed in sound-attenuated ventilated cubicles. Animals were trained to orally self-administer ethanol or water in a concurrent, two-lever, free-choice paradigm. Syringe pumps (Razel Scientific Instruments, Stamford, CT) dispensed ethanol or water into two stainless steel drinking cups mounted 4.0 cm above the grid floor in the middle of one side panel. Two retractable levers were located 4.5 cm to either side of the drinking cups. Fluid delivery and recording of operant self-administration were controlled by a microcomputer. Lever presses were not recorded during the 0.5 s in which the pumps were active. A continuous reinforcement fixed ratio-1 (FR1) schedule was used such that each response resulted in the delivery of 0.1 ml of fluid.

Rats were trained to press a lever for ethanol using a modification of the sweetened solution fading procedure (Samson, 1986). No fluid or food restriction period was employed. This training method culminates in rats consuming sufficient unsweetened 10% ethanol to produce pharmacologically relevant blood alcohol levels (BAL) (Roberts et al., 1999). Rats were initially trained to press a lever for a sweetened solution containing glucose (3% w/v) and saccharin (0.125% w/v) (Sigma Chemical Co., St. Louis, MO). Ethanol self-administration was initiated by adding ethanol (10% w/v) to the sweetened solution for 4–5 days, followed by 4–5 days of 10% ethanol + 0.125% saccharin only. Finally, the animals received the 10%

ethanol solution alone. During all training sessions, rats also were allowed to press for water on the opposite lever. The lever that produced water or ethanol was alternated daily to prevent selecting rats biased toward one lever. The animals received daily (5 days per week) 30-min access to ethanol for 20 to 25 days until stable rates of intake were observed. The criterion for stable baseline intake was $\pm 20\%$ across three consecutive sessions. Testing was performed at 8:00 AM (lights off at 8:00 AM).

4.4. Ethanol vapor exposure procedure

To induce ethanol dependence, two standard rat cages were housed in separate, sealed, clear plastic chambers into which ethanol vapor was intermittently introduced. Ethanol vapor was created by dripping 95% ethanol into 2000-ml Erlenmeyer vacuum flasks kept at 50 °C on a warming tray. Air was blown over the bottom of the flask at 11 L/min to vaporize the ethanol. The concentration of ethanol vapor was adjusted by varying the rate at which ethanol was pumped into the flasks and ranged from 22 to 27 mg/L. The chambers were connected to a timer that would turn the ethanol vapor on (4:00 PM) and off (6:00 AM) every day allowing animals to receive ethanol vapor for 14 h and control air for 10 h (O'Dell et al., 2004). Blood samples were taken at 6:00 AM for BAL determination every 3 days during vapor exposure. Target BALs were 150–200 mg% across a 4-week exposure period. This paradigm has been shown to produce physical dependence, demonstrated by the appearance of somatic withdrawal signs upon removal from the chambers (Roberts et al., 2000; O'Dell et al., 2004).

4.5. Intracerebral cannulations and drug infusions

Rats were anesthetized with an isoflurane-oxygen mixture and 26 gauge, 7.5 mm stainless steel guide cannulae (Plastics One; Roanoke, VA) aimed 2 mm above the desired brain regions were stereotactically implanted bilaterally. With the incisor bar set at -3.3 from interaural zero (flat skull), the coordinates for the CeA were anterior/posterior= -2.6 mm, medial/lateral= ± 4.2 mm, dorsal/ventral= -5.2 from dura (Paxinos and Watson, 1998). The guide cannulae were secured to the skull with dental cement and anchor screws, and guide cannulae were inserted with stylet wires to protect the brain tissue. Rats were allowed to recover at least 5 days before continuation of experiments.

Intracerebral (IC) injections were administered with the use of injectors (33 gauge; Plastics One, Roanoke, VA) that projected 2 mm past the guide cannula into the desired brain region. The injectors were attached to 70 cm of calibrated polyethylene-10 tubing preloaded with drug solution. Injection volumes were 0.5 μ l/side, infused over 1 min using Hamilton microsyringes connected to the injectors with polyethylene tubing. Drug delivery was controlled by a Harvard Apparatus infusion pump (Helliston, MA). Following drug delivery, the injectors were left in place for 60 s, and then replaced with the protective wire stylets. Following a 5-min preincubation period, the animals were placed into the self-administration chambers for testing.

4.6. Histology

At the completion of the experiment, animals were sacrificed with an overdose of pentobarbital and perfused transcardially, first with saline and then with a 4% paraformaldehyde solution. Brains were subsequently removed from the skull and frozen. The brains were sectioned in 60 μ m slices, mounted and stained with cresyl violet. Injection sites were verified under a light microscope. Only animals with correct, bilateral cannula placements were used for statistical analysis. Three animals had incorrect probe placements and were removed (1 dependent, 2 nondependent).

4.7. Experimental procedures

Rats first were trained to self-administer 10% ethanol on an FR1 schedule of reinforcement. Once stable baseline responding was attained, rats were surgically implanted with bilateral intracerebral cannulae aimed at the CeA. After recovery from surgery, self-administration sessions were resumed for about 2 weeks to reestablish baseline ethanol self-administration and subsequently the animals were transferred to ethanol (dependent group) or control (nondependent group) vapor chambers for a 4-week exposure period. At the end of the 4-week period of dependence induction, rats were re-tested for ethanol self-administration following a 2-h withdrawal period from ethanol vapors. At this time point, dependent animals display a significant increase in ethanol lever pressing (Valdez et al., 2002b). A Latin square design was used to test the effects of intracerebral administration of Ucn 3 (0.0, 0.02, 0.1 and 0.5 µg/µl). Test sessions were separated by 3–4 days, during which time the animals were returned to the ethanol or control vapor chambers.

4.8. Statistical analyses

Data were analyzed using a mixed two-way analysis of variance (ANOVA) with vapor treatment (ethanol or control) as the between-subjects factor and agonist dose (Ucn 3) as a within-subjects factor. Tukey's (A) honestly significant test was used for *post hoc* analysis of individual means ($p < 0.05$).

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Fig. 1.

Effects of Ucn 3 on ethanol and water self-administration in ethanol-dependent and nondependent rats. Ethanol dependence was induced by intermittent exposure to ethanol vapors for 4 weeks and animals were subsequently tested for ethanol and water self-administration following 2 h of acute withdrawal. Withdrawn, ethanol-dependent animals displayed a significant increase in ethanol lever pressing compared to nondependent animals. Ucn 3 significantly decreased ethanol self-administration in withdrawn, dependent rats. Ucn 3 significantly increased ethanol self-administration in ethanol-nondependent rats. Neither ethanol vapor exposure nor Ucn 3 treatment altered water responding. * $p < 0.05$ compared to

same drug dose in nondependent animals. [#] $p < 0.001$ compared to vehicle treatment in ethanol-dependent animals. [†] $p < 0.05$ compared to vehicle treatment in nondependent animals.

Central Nucleus of the Amygdala

Fig. 2.

Representative cannula site verification micrograph. At the completion of the experiments, brains were sectioned at 60- μ m intervals, mounted and stained with cresyl violet. Injection sites were verified under a light microscope. Only animals with correct, bilateral cannula placement were used for statistical analysis. The figure displays representative slides from the CeA. The number in the diagram represents the distance from bregma, based on the rat brain atlas by Paxinos and Watson (1998).