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ORIGINAL INVESTIGATION

Effects of acute ethanol on corticotropin-releasing hormone and β-endorphin systems at the level of the rat central amygdala

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Abstract
Rationale The endogenous opioid and corticotropin-releasing hormone (CRH) systems, present in the central amygdala (CeA), are implicated in alcohol consumption.
Objectives The purpose of this study is to investigate the hypothesis that, in CeA, alcohol stimulates CRH release, which then stimulates β-endorphin release.
Materials and methods Rats were unilaterally implanted with a guide cannula to aim microdialysis probes in CeA. Experiment 1: rats received an intraperitoneal (IP) injection of various ethanol doses (0.0, 2.0, 2.4, or 2.8 g ethanol/kg body weight) and microdialysates were sampled at 30-min intervals to determine the effects over time of acute alcohol on the extracellular CRH concentrations in CeA. Experiment 2: phosphate-buffered saline, CRH, or CRH receptor (CRHR) antagonists (antalarmin or anti-sauvagine-30) was microinjected into CeA followed by a saline or 2.8 g/kg ethanol IP injection to determine the effects of CRHR activation or blockade in CeA on the basal and alcohol-stimulated release of β-endorphin. CRH and β-endorphin dialysate contents were determined using specific radioimmunoassays.
Results Acute alcohol induced a delayed increase in the extracellular CRH levels in CeA. Behavioural data showed no difference in locomotion between alcohol- and saline-treated rats. However, a transient increase in grooming was observed which did not correspond with alcohol-induced changes in CRH. Local CRH microinjections increased the extracellular β-endorphin concentrations in CeA. CRHR1 and CRHR2 blockade with microinjections of antalarmin and anti-sauvagine-30, respectively, attenuated the alcohol-induced increase of extracellular β-endorphin in CeA.
Conclusions Acute alcohol exerts indirect actions on CRH release and induced interactions of the CRH and β-endorphin systems in CeA.

Keywords Alcohol · Addiction · Beta-endorphin · Central amygdala · Corticotropin-releasing hormone · In vivo microdialysis · Opioid

Abbreviations

3V	3rd ventricle
ANOVA	Analysis of variance
ACTH	Adrenocorticotropin hormone
BLA	Basolateral amygdala
BSA	Bovine serum albumin
BSTIA	Bed nucleus of the stria terminalis intra-amygdaloid division
CA3	Field CA3 of hippocampus
CeA	Central amygdala
CPU	Caudate putamen
CRH	Corticotropin-releasing hormone
CRHR	Corticotropin-releasing hormone receptors
CRHR1	Corticotropin-releasing hormone receptors type 1

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CRHR2	Corticotropin-releasing hormone receptors type 2
DG	Dentate gyrus
INJ	Injection
IP	Intraperitoneal
LH	Lateral hypothalamus
MicroINJ	Microinjection
PBS	Phosphate-buffered saline
PIR	Piriform cortex
RIA	Radioimmunoassay

Introduction

Alcohol abuse involves multiple brain neurotransmitter and neuropeptide systems (Cowen et al. 2004; Koob 2009; Koob and Volkow 2009). Among these, the endogenous opioid and the corticotropin-releasing hormone (CRH) systems have been demonstrated to influence alcohol consumption in rodent models of alcohol abuse. In addition, the activity of both opioid and CRH peptides is altered by alcohol consumption in distinct brain regions including the central amygdala (CeA), effects suggesting the possibility of an interaction between the opioid and CRH systems in mediating some of the effects of alcohol (Funk et al. 2006; Heyser et al. 1999; Lam et al. 2008).

The endogenous opioid system consists of the opioid peptide ligands enkephalin (Hughes et al. 1975a, b), dynorphin (Goldstein et al. 1979) and β-endorphin (Bradbury et al. 1976) and the μ-, δ- and κ-opioid receptors (Lord et al. 1977; Martin et al. 1976). The β-endorphin peptide binds with equal affinity with the μ- and δ-opioid receptors (Chang and Chang 1983; Garzon et al. 1983). Several animal studies using opioid receptor antagonists have indicated a role of the endogenous opioid system in alcohol consumption (Altshuler et al. 1980; Froehlich et al. 1990). As well, on the basis of several clinical studies, the opioid receptor antagonist naltrexone has been established as a drug treatment for alcohol abuse and dependence in humans (O'Malley et al. 1992; Volpicelli et al. 1992). Moreover, the endogenous opioid system at the level of CeA has been implicated in alcohol consumption (Criado and Morales 2000; Heyser et al. 1999; Kang-Park et al. 2007; Lam et al. 2008).

The CRH system localised in the hypothalamus is a critical component of the hypothalamic–pituitary–adrenal axis stress response (Charmandari et al. 2005). In addition to the hypothalamic CRH, several studies have demonstrated the presence of extrahypothalamic CRH in numerous brain regions including CeA, which could act locally as a neuromodulator (Merchenthaler et al. 1982; Vale et al. 1981). The presence of both CRH receptor type 1 (CRHR1) and type 2 (CRHR2) has been demonstrated in the CeA (Chalmers et al. 1995). In addition, CRH has been demonstrated to stimulate β-endorphin release at the level of the pituitary gland (Keith et al. 1986; Vale et al. 1981). As well, in vitro studies using dissected hypothalamus from mice demonstrated that CRH stimulated β-endorphin release (de Waele and Gianoulakis 1993). Thus, both the endogenous opioid and CRH systems are present in CeA and have been implicated in alcohol consumption, suggesting a possible interaction between the CRH and β-endorphin systems at the level of CeA in response to alcohol exposure. However, to the best of our knowledge, there are no published reports on the effects of acute systemic alcohol administration on the CeA CRH and its possible interactions with the CeA β-endorphin.

Thus, the objectives of the current study were to investigate the hypotheses that (1) acute alcohol treatment would stimulate a dose- and time-dependent increase in CRH release in CeA and alter the behavioural activity of the rats; (2) local application of CRH at the level of CeA by microinjection would increase the extracellular concentrations of β-endorphin and (3) microinjection of the selective CRHR1 and CRHR2 antagonists, antalarmin and anti-sauvagine-30, respectively, would attenuate the alcohol-induced increase in β-endorphin release in CeA. To test these hypotheses, rats received microinjections in CeA of either phosphate-buffered saline (PBS), or 0.25 μg of one of the following: CRH, antalarmin or anti-sauvagine-30, followed by a systemic intraperitoneal (IP) injection of either saline or various doses of ethanol 5 min later. Dialysate samples were collected from the CeA using the technique of in vivo microdialysis and changes in extracellular CRH and β-endorphin levels were estimated with solid-phase radioimmunoassays (RIA). Changes in locomotor and grooming behaviours were scored from video recordings of the microdialysis sessions.

Materials and methods

Animals

Adult alcohol-naïve male Sprague–Dawley rats weighing approximately 250 g on arrival to the animal facility were used (Charles River, St.-Constant, QC). Rats were individually housed in cages in a temperature-regulated environment on a 12-h light–dark cycle (0800 lights on). Food and water were available ad libitum. Principles of laboratory animal care were followed in accordance with McGill University's Policy on the handling and treatment of laboratory animals and the guidelines of the Canadian Council on Animal Care.

Guide cannula implantation

Surgical implantation of the guide cannulae was performed as described previously (Lam et al. 2008) 1 to 2 weeks after the rats arrived at the animal facility. Briefly, the guide cannula (15 mm shaft length, Bioanalytical Systems, West Lafayette, IN) was unilaterally implanted above the CeA according to established coordinates (−2.3 mm anteroposterior from bregma, 4.0 mm mediolateral from the midline and 7.4 mm dorsoventral from the dura).

In vivo microdialysis experiments

After 3 to 5 days of post-surgical recovery, rats were placed in the same type of black, plastic containers as those used for microdialysis (diameter, 31 cm and height, 30 cm) on three separate occasions and were habituated to experimenter handling and IP injections using saline. The microdialysis setup and experiments have been previously described (Lam et al. 2008; Marinelli et al. 2003). In brief, on the evening before the experiment the microdialysis probe was implanted and artificial cerebral spinal fluid was pumped at a rate of 0.2 μl/min overnight. On the morning of the microdialysis session, the flow rate was increased to 2.0 μl/min and following a 2-h equilibration period dialysate samples were collected at 30-min intervals.

Experiment 1: effects of acute alcohol on extracellular CRH in CeA and on behavioural activity

The effects of alcohol on extracellular CRH concentrations were tested using in vivo microdialysis experiments consisting of IP injections of either saline or various doses of ethanol (2.0, 2.4 and 2.8 g ethanol/kg body weight) (Lam et al. 2008). The choice of the ethanol doses was based on previously published studies on β-endorphin release (Lam et al. 2008) and pilot studies indicating no effect on CRH release in response to ethanol doses lower than 2.0 g/kg. Microdialysate samples were collected from the CeA at 30 min intervals before and after IP injections. Samples were quickly frozen on dry ice and were stored at −70°C.

During the microdialysis experiments, the rat's behaviour in response to treatments with saline or the various doses of ethanol were recorded with a video camcorder (Panasonic) and later scored by an observer blind to the experimental treatments (Paul et al. 2007). For locomotor activity scoring, the microdialysis cage floor was divided into four quadrants and scores were given for each 5-s time interval the rat spent ambulating across quadrant boundaries. Grooming activity scores were given for each 5-s interval the rats spent engaged in repetitive brushing movement of the paws or nose.

Experiment 2: effects of local application of CRH or CRHR antagonists on alcohol-induced increases in extracellular β-endorphin levels in CeA

The effects of activation or blockade of CRHR1 and CRHR2 were also tested via microdialysis experiments consisting of microinjections in CeA of a total volume of 0.5 μl of either 0.25 μg CRH (Phoenix Pharmaceuticals, Burlingame, CA), 0.25 μg antalarmin hydrochloride (Sigma-Aldrich, St Louis, MO) or 0.25 μg anti-sauvagine-30 (Phoenix Pharmaceuticals) using a syringe pump at a rate of 0.5 μl/min (Harvard Apparatus, Holliston, MA, USA) with a Hamilton gas tight syringe (Hamilton, Reno, NV) connected by fluorinated ethylene propylene tubing to the microinjection port on the microdialysis probe. All microinjected compounds were dissolved in 0.5× PBS. The dose of 0.25 μg of CRH, antalarmin and anti-sauvagine-30 was chosen based on pilot studies using a range of doses from 0.25 to 0.35 μg for each compound. In the pilot experiments all doses of CRH used induced a 130% increase or higher in the extracellular concentration of β-endorphin in CeA. The lowest dose of CRH used (0.25 μg) which fulfilled the set criteria (at least a 130% increase of extracellular β-endorphin concentration) was chosen for the remaining experiments. The pilot experiments in the current study on groups receiving antalarmin or anti-sauvagine-30 microinjection in CeA followed by IP injection of ethanol (2.8 g/kg) demonstrated that both doses of the CRHR antagonists (0.25 and 0.35 μg) produced comparable effects on the alcohol-induced changes in extracellular β-endorphin concentrations (data not shown). The dose of 0.25 μg was chosen for the remaining experiments since previous studies demonstrated that this dose produced consistent behavioural responses (Heinrichs and Joppa 2001; Robison et al. 2004). Control animals received microinjection of 0.5 μl of PBS. At 5 min post-microinjection, an IP injection of either saline or 2.8 g/kg ethanol (previously been shown to induce the maximum increase in the extracellular concentrations of β-endorphin) was given (Lam et al. 2008). Microdialysate samples were collected from the CeA at 30-min intervals before and after the IP injection, quickly frozen on dry ice and were stored at −70°C.

Anatomical confirmation of probe placement

At the end of the microdialysis experiments, rats were euthanized in a CO_2 chamber. Brains were removed, snap frozen in isopentane and kept at −70°C. Coronal brain

slices (40 μm) were obtained using a cryostat. Photomicrographs of coronal sections showing the cannula and probe tract were taken with a Canon A620 digital camera in macro mode and the appropriate coronal diagram taken from The Rat Brain Atlas (Paxinos and Watson 1996) was superimposed onto the photomicrograph by computer software (Bert et al. 2004).

Solid-phase radioimmunoassay

The concentrations of immunoreactive β-endorphin and CRH peptides in the dialysate were determined by solid-phase RIA as previously described (Lam et al. 2010; Olive et al. 2001), using polyclonal antibodies for β-endorphin (Gianoulakis and Gupta 1986) and CRH (Bachem/Peninsula Laboratories, Torrance, CA). The specificity of the β-endorphin antibody was previously reported (Gianoulakis and Gupta 1986) and of the CRH antibody was provided by Bachem/Peninsula and presented 100% cross reactivity with rat CRH and 5% with sauvagine. The RIA detection limits for both CRH and β-endorphin were 0.5 pg/well and the IC50 values were 51.81±9.46 and 19.75±2.58 pg, respectively. The intra- and inter-assay coefficients of variance of the RIAs were, respectively, 8.04% and 19.86% for CRH and 8.79% and 12.63% for β-endorphin.

Statistical analysis

The data obtained from the RIAs were in picograms per 50 μl dialysate. To compare the effects of the treatments on the concentrations of β-endorphin or CRH in the dialysate, the data were expressed as a percent change from the baseline with the CRH or β-endorphin concentration in the dialysate immediately prior to the microinjection or IP injection serving as the 100% baseline value. Mixed two- and three-way analysis of variance (ANOVA) were performed to examine the effects of various treatments on extracellular CRH and β-endorphin concentrations. Simple effects and Tukey's honestly significant difference tests were used as post hoc tests. All statistical analyses were performed using Datasim (PC). The data presented in the figures (GraphPad Prism) are expressed as mean±SEM. Statistical significance was set at $p<0.05$.

Results

Histological confirmation of probe placement

Figure 1a presents a photomicrograph of a representative coronal brain section showing the neuroanatomical location of the microdialysis cannula and probe membrane in the CeA. Rounded bars indicate the location of microdialysis probe membrane implanted in CeA from multiple rats from which the data were used for statistical analysis (Fig. 1b). The criterion for inclusion in the data analysis was that approximately 50% or more of the active membrane should fall inside CeA. In experiments 1 and 2, data from seven and four animals, respectively, were excluded from statistical analysis due to the active membrane showing localization mostly or exclusively outside of CeA boundaries.

Experiment 1: effects of acute alcohol on the extracellular concentrations of CRH in CeA and on behavioural changes

The baseline concentration of CRH in the dialysate samples collected from the CeA was obtained by calculating the mean CRH concentration in the dialysate samples collected immediately prior to the IP injection of all animals used for the statistical analysis and was found to be 157.8±17.68 pg/50 μl of dialysate.

Effects of the various doses of ethanol and saline on the extracellular concentrations of CRH are shown in Fig. 2a–c. A two-way ANOVA on the percent change data revealed a significant main effect of dose [$F(3,29)=3.43$, $p<0.05$] and time [$F(6,174)=7.61$, $p<0.0001$], but did not reveal a significant interaction of dose × time ($p>0.05$). The significant main effect of dose was further analysed using post hoc tests and revealed that the mean concentrations of CRH in dialysates following the IP injections of 2.4 and 2.8 g/kg ethanol collapsed over all time points (154.7±14.49 and 145.7±9.60, respectively) were significantly higher than those following the saline IP injection (106.8±3.03; $p<0.05$). Post hoc tests conducted on the main effect of time collapsed over dose revealed that following the IP injection of 2.4 and 2.8 g/kg ethanol the extracellular CRH concentrations were significantly elevated from baseline at 120, 150 and 180 min post-IP injection (Fig. 2b, c; $p<0.05$).

Prior to the treatments, the locomotor and grooming activities of the rats were minimal because this period corresponded with the animals' sleep phase, therefore locomotor (Fig. 3a–c) and grooming (Fig. 3d–f) activity scores are presented from 30-min post-injection onwards. A two-way ANOVA on the locomotor activity scores over all time points (Fig. 3a–c) revealed a significant main effect of time [$F(5,150)=26.63$, $p<0.0001$]; however, it did not reveal a significant main effect of dose or an interaction of dose × time ($p>0.05$). A two-way ANOVA on the grooming activity scores over all time points (Fig. 3d–f) revealed a significant main effect of time [$F(5,140)=12.78$, $p<0.0001$] and a significant interaction of dose × time [$F(15,140)=1.76$, $p<0.05$]; however, it did not reveal a significant main effect of dose. The significant interaction of dose × time was further analysed with post hoc tests and revealed that the rats treated with 2.0 and 2.8 g/kg ethanol

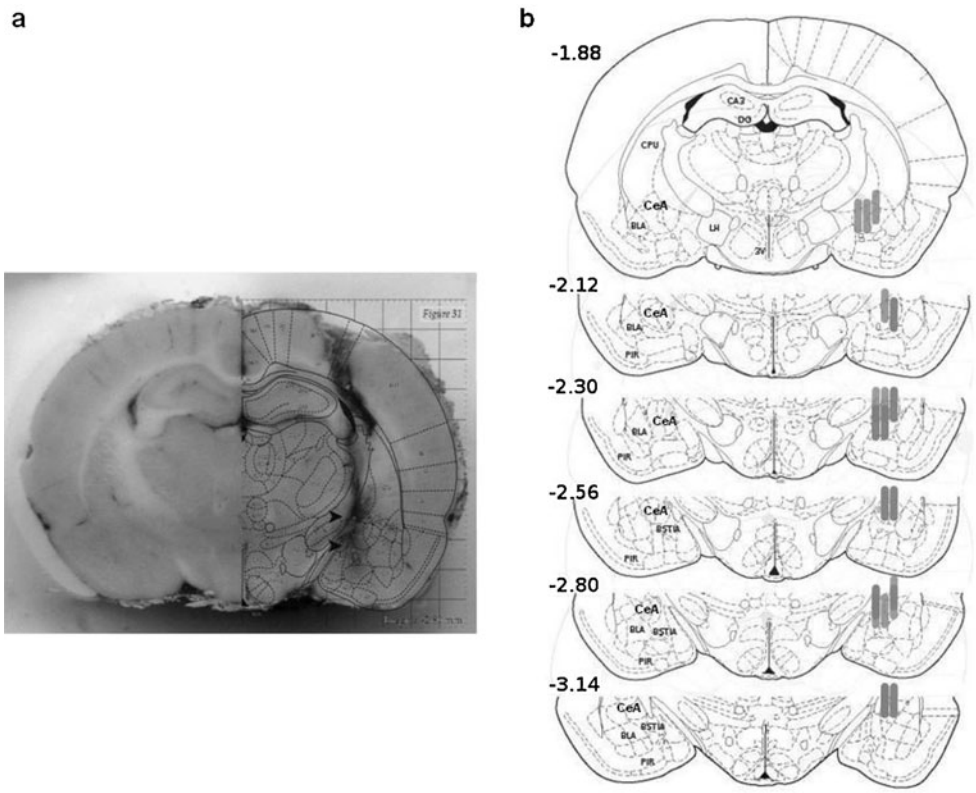

Fig. 1 Neuroanatomical verification of the microdialysis probe membrane placement. **a** The schematic coronal diagram based on the rat brain atlas (Paxinos and Watson 1996) was superimposed on a photomicrograph of a typical coronal brain section showing the active membrane of the microdialysis probe located in the central amygdala. *Arrowheads* indicate the site of the microdialysis probe membrane. **b** Coronal diagrams showing sites of active probe membrane location in the central amygdala of the rats from which statistical analysis on the data were performed. *Grey rounded bars* indicate location of the microdialysis probe membrane from multiple rats. *Numbers* indicate anterior–posterior position from bregma. Abbreviation of brain structures: *3V* third ventricle, *BLA* basolateral nucleus of amygdala, *BSTIA* bed nucleus of the stria terminalis intra-amygdaloid division, *CA3* field CA3 of hippocampus, *CeA* central amygdala, *CPU* caudate putamen, *DG* dentate gyrus, *LH* lateral hypothalamus, *PIR* piriform cortex

showed higher grooming activity at 30-min post-injection compared to those treated with saline at the same time point (Fig. 3d, f; $p<0.05$). In addition, post hoc tests revealed that rats treated with 2.4 g/kg body ethanol showed higher grooming activity at 60-min post-injection compared to those treated with saline at the same time point (Fig. 3e; $p<0.05$).

Experiment 2: effects of local application of CRH or CRHR antagonists on the alcohol-induced increase in the extracellular β-endorphin levels in CeA

The baseline concentration of dialysate β-endorphin collected from the CeA was obtained by calculating its mean concentration in the dialysate samples collected immediately prior to the microinjection of all animals used for the statistical analysis and was found to be 2,235±174.7 pg/50 μl of dialysate.

Figure 4 shows the changes in the extracellular concentrations of β-endorphin in CeA following the microinjection of either CRH or PBS followed 5 min later by an IP saline injection. A two-way ANOVA revealed a significant main effect of dose [$F(1,13)=13.76$, $p<0.01$], time [$F(6,78)=4.57$, $p<0.001$] and a significant interaction of dose × time [$F(13,78)=4.21$, $p<0.01$]. Post hoc tests revealed that following the CRH microinjection the dialysate β-endorphin concentrations at 30, 60, 90 and 120 min were significantly elevated from the corresponding baseline levels ($p<0.05$) and at 30, 60, 90, 120 and 180 min the dialysate β-endorphin concentrations were significantly higher than the concentrations at the same time points following the PBS microinjection ($p<0.05$).

Figure 5 shows the response of β-endorphin to the IP injection of either 2.8 g/kg ethanol or saline administered 5 min following the microinjection of PBS at the level of CeA. A two-way ANOVA on the percent change data revealed a significant main effect of dose [$F(1,15)=23.69$, $p<0.001$] and a significant interaction of dose × time [$F(6,90)=2.24$, $p<0.05$], but did not reveal a significant main effect of time ($p>0.05$). Post hoc tests revealed that the dialysate β-endorphin concentrations at 60, 90, 120, 150 and 180 min post-ethanol were significantly higher than the concentrations after the IP saline treatment ($p<0.05$).

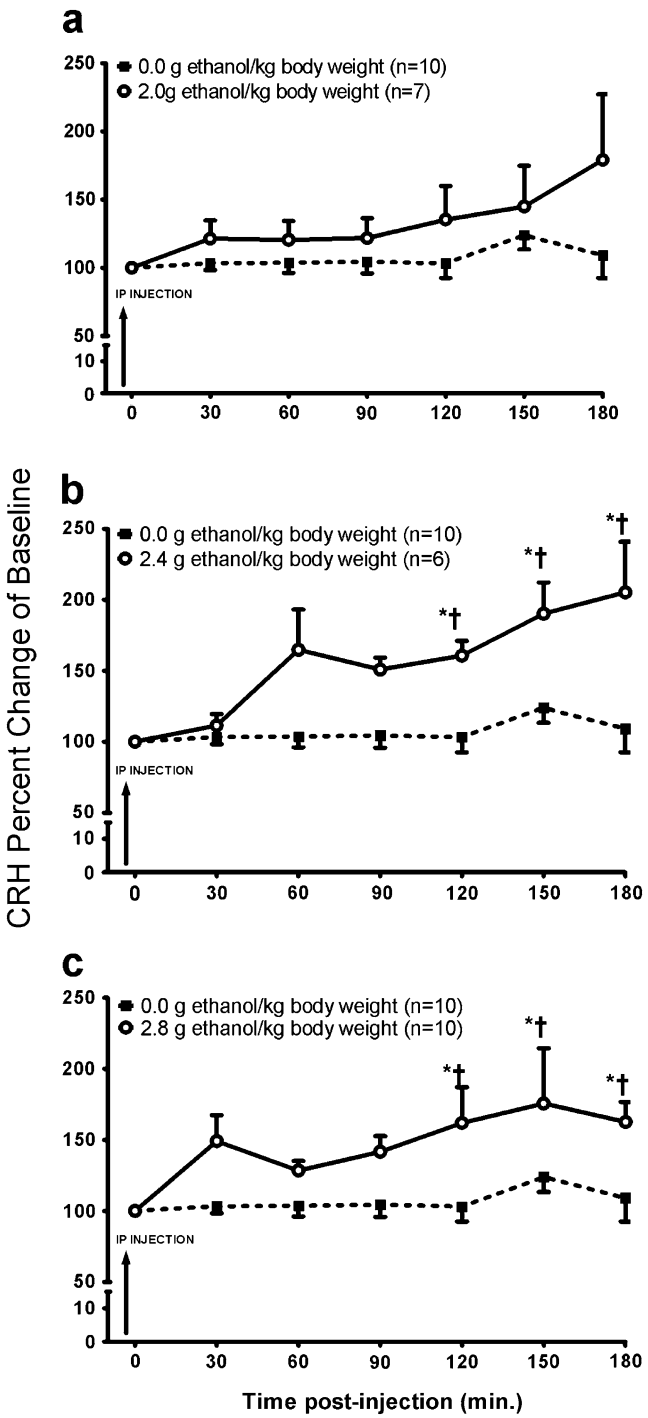

Fig. 2 Effects of IP injection of **a** 2.0, **b** 2.4 and **c** 2.8 g ethanol/kg body weight compared to the effect of saline on the dialysate CRH concentrations in CeA expressed as a percent change of the baseline. *n* number of rats in each dose group. *Arrow* indicates the time of the intraperitoneal (*IP*) injection. *Cross* indicates significant difference from saline control at the same time point ($p<0.05$); *asterisks* indicates significant difference from the baseline value of the same dose group ($p<0.05$)

Microinjection of antalarmin, a CRHR1-selective antagonist, 5 min prior to the IP injection of 2.8 g/kg ethanol attenuated the increases in the extracellular β-endorphin concentrations observed in the animals that received a microinjection of PBS followed by the IP injection of 2.8 g/kg ethanol (Fig. 6a). A three-way ANOVA revealed a significant interaction between microinjection × IP injection treatments [$F(1,25)=14.89$, $p<0.05$]; however, it did not reveal any other significant main or interaction effects of other factors ($p>0.05$). The significant interaction between microinjection × IP injection treatments was further analysed with post hoc tests and revealed that the CeA β-endorphin response was significantly lower in animals microinjected with antalarmin followed 5 min later by an IP injection of 2.8 g/kg ethanol than animals microinjected with PBS followed 5 min later by an IP injection of 2.8 g/kg ethanol (Fig. 6a; $p<0.05$). In addition, post hoc tests did not reveal a significant difference in the CeA β-endorphin response between animals microinjected with PBS followed 5 min later by a saline IP injection and animals microinjected with antalarmin followed 5 min later by a saline IP injection (Fig. 6b; $p>0.05$).

Microinjection of anti-sauvagine-30, a CRHR2-selective antagonist, 5 min prior to the IP injection of 2.8 g/kg ethanol also attenuated the increases in the extracellular β-endorphin concentrations observed in the animals that received a microinjection of PBS followed by the IP injection of 2.8 g/kg ethanol (Fig. 7a). A three-way ANOVA revealed a significant main effect of the microinjection treatment [$F(1,6)=5.37$, ($p<0.05$)], a significant interaction between IP injection × microinjection treatments [$F(1,6)=9.27$, ($p<0.05$)] and a significant interaction between IP injection × microinjection × time [$F(6,162)=2.47$, ($p<0.05$)]. The significant IP injection × microinjection × time was further analysed with post hoc tests and revealed that from 60 to 180 min the CeA β-endorphin response was significantly attenuated in animals microinjected with anti-sauvagine-30 followed 5 min later by an IP injection of 2.8 g/kg ethanol compared to animals microinjected with PBS followed by an IP injection of 2.8 g/kg ethanol (Fig. 7a; $p<0.05$). In addition, post hoc tests did not reveal significant differences between animals microinjected with PBS followed 5 min later by an IP injection of saline compared to animals microinjected with anti-sauvagine-30 followed 5 min later by an IP injection of saline (Fig. 7b; $p<0.05$).

Discussion

The current study demonstrated that acute systemic administration of various doses of alcohol induced a delayed increase in the extracellular concentrations of CRH at the level of CeA, an effect suggesting indirect actions of alcohol on the CeA CRH system. Although both the 2.4

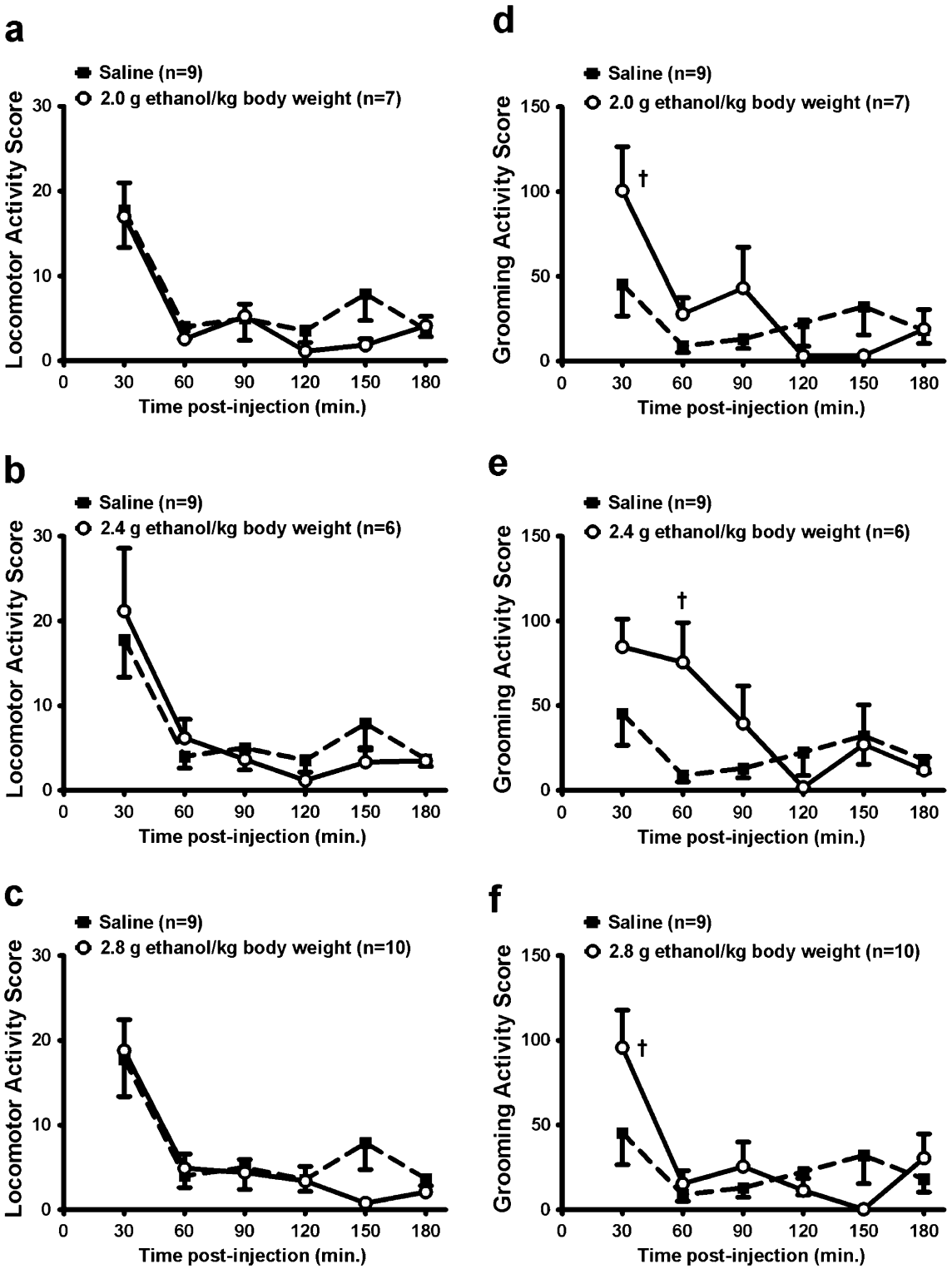

Fig. 3 Effects of IP injection of **a**, **d** 2.0, **b**, **e** 2.4 and **c**, **f** 2.8 g ethanol/kg body weight compared to the effect of saline on locomotor (**a**–**c**) and grooming (**d**–**f**) activity scores of the rats used for microdialysis. *n* number of rats in each dose group. *Cross* indicates significant difference from saline control at the same time point ($p < 0.05$)

and 2.8 g/kg ethanol doses induced a significant increase in extracellular CRH concentrations in CeA, the 2.8 g/kg ethanol dose was chosen in experiment 2 so that a maximum increase of extracellular β-endorphin concentrations could make the effect of the CRH antagonists more apparent. In addition, the changes in extracellular CRH in

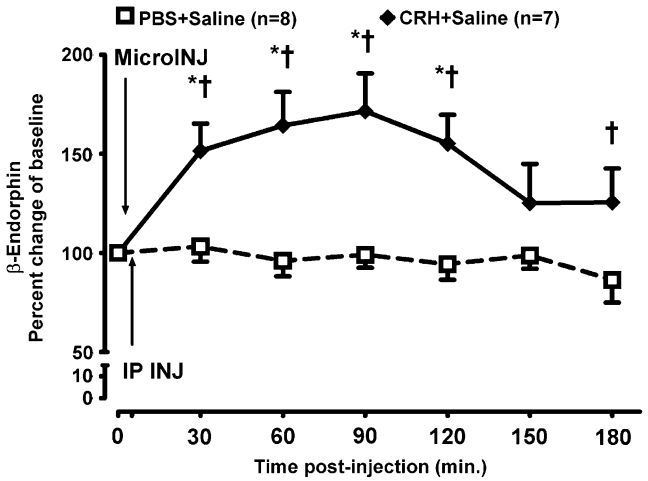

Fig. 4 Effects of phosphate-buffered saline (*PBS*) or corticotropin-releasing hormone (*CRH*) peptide microinjections into the central amygdala followed by an intraperitoneal (*IP*) injection of saline on the dialysate β-endorphin content expressed as percent change of baseline. *Arrows* indicate the time of the PBS or CRH microinjection (*microINJ*) and of the intraperitoneal saline injection (*IP INJ*). *n* number of rats in each treatment group. *Cross* indicates significant difference from the group receiving the PBS microinjection and IP saline injection at the same time points ($p<0.05$); *asterisks* indicates significant difference from the baseline value of the same treatment group ($p<0.05$)

response to alcohol were specific to the CeA whereby animals in which microdialysis probes were located beyond the boundaries of CeA did not show an increase in CRH response (data not shown). Moreover, activation of CRH receptors at the level of CeA by CRH microinjection significantly enhanced the extracellular concentrations of β-endorphin in CeA. Furthermore, blocking either CRHR1

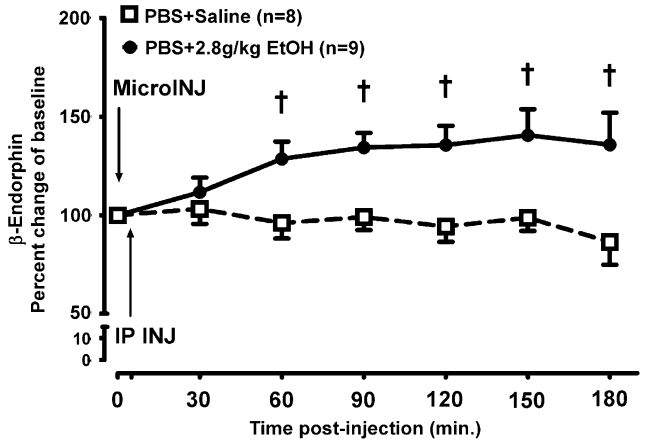

Fig. 5 Effects of phosphate-buffered saline (*PBS*) microinjection into the central amygdala (*CeA*) followed by an intraperitoneal (*IP*) injection of 2.8 g ethanol/kg body weight or saline on dialysate β-endorphin content expressed as percent change of baseline. *Arrows* indicate the time of microinjection (*microINJ*) and intraperitoneal injection (*IP INJ*). *n* number of rats in each treatment group. *Cross* indicates significant difference from the group receiving the PBS microinjection and IP saline injection at the same time points ($p<0.05$)

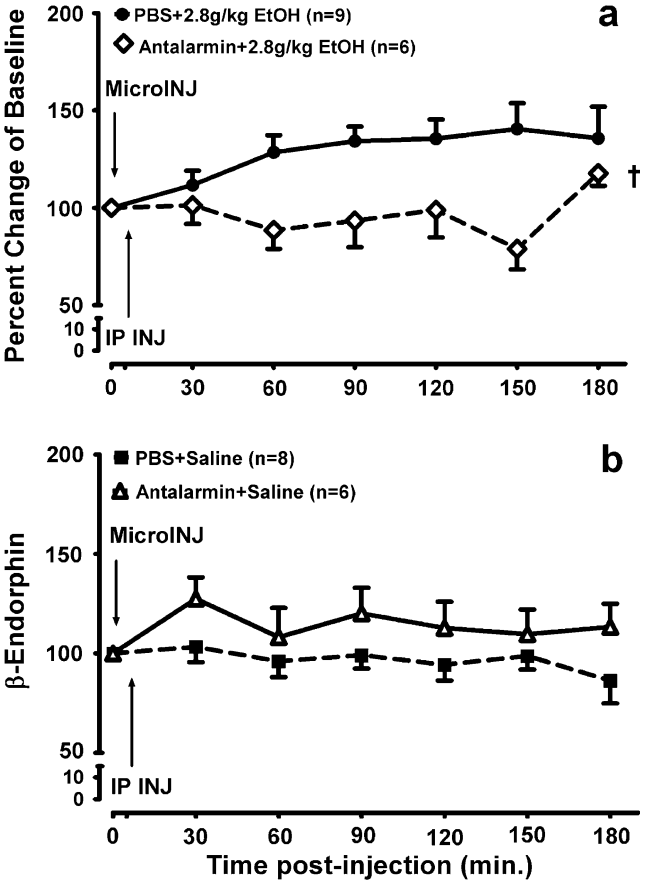

Fig. 6 A total volume of 0.5 μl of either phosphate-buffered saline (*PBS*) or 0.25 μg of antalarmin, a corticotropin-releasing hormone receptor type 1 antagonist, was microinjected into the central amygdala 5 min prior to an intraperitoneal (*IP*) injection of **a** 2.8 g ethanol/kg body weight or **b** saline, and the concentrations of β-endorphin in dialysates from CeA were determined. Data are expressed as percent change of baseline. *Arrows* indicate time of microinjection (*microINJ*) and of intraperitoneal injection (*IP INJ*). *n* number of rats in each treatment group. *Cross* indicates significant group difference from the group receiving PBS microinjection and IP injection of 2.8 gethanol/kg body weight ($p<0.05$)

with antalarmin or CRHR2 with anti-sauvagine-30 in CeA significantly attenuated the alcohol-induced increase in the extracellular β-endorphin levels. These results suggest an interaction of acute alcohol exposure with both the CRH and β-endorphin systems at the level of CeA.

The pattern of β-endorphin release at the level of CeA in response to the 2.8 g/kg ethanol dose observed in the current study is in accordance with that observed in the previous microdialysis studies investigating the interactions of acute alcohol with opioid peptides in CeA (Lam et al. 2008). However, in the current study, the maximum increase in the extracellular β-endorphin levels was lower compared to the previous microdialysis study. This difference in the magnitude could be due to the use of microdialysis probes with 1 mm membrane in the current study compared to 2 mm membrane in the previous study.

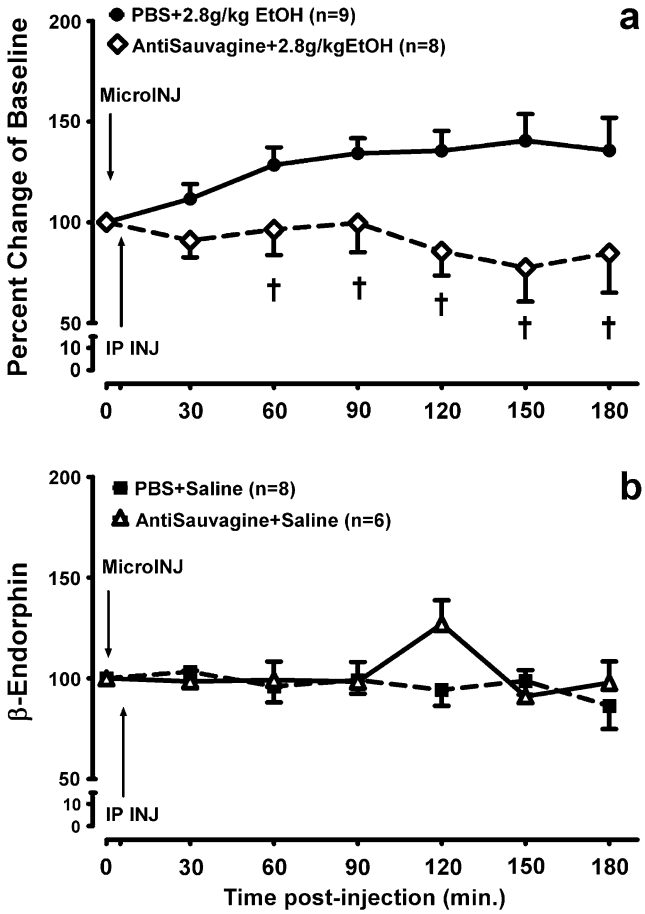

Fig. 7 A total volume of 0.5 μl of either phosphate-buffered saline (*PBS*) or 0.25 μg of anti-sauvagine-30, a corticotropin-releasing hormone receptor type 2 antagonist, was microinjected into the central amygdala 5 min prior to an intraperitoneal (*IP*) injection of **a** 2.8 gethanol/kg body weight or **b** saline, and the concentrations of β-endorphin in dialysates from CeA were determined. Data are expressed as percent change of baseline. *Arrows* indicate time of microinjection (*microINJ*) and of intraperitoneal injection (*IP INJ*). *n* number of rats in each treatment group. *Cross* indicates significant difference from the group receiving PBS microinjection and IP injection of 2.8 gethanol/kg body weight at the same time points ($p<0.05$)

The results in the current microdialysis studies suggest that the CRH system at the level of CeA could modulate local β-endorphin release in response to acute alcohol. Previous in vitro studies using extracted hypothalamus perifused with an ethanol-containing solution demonstrated that (1) acute alcohol could stimulate CRH release at the level of the hypothalamus and (2) CRH could stimulate hypothalamic β-endorphin release (de Waele and Gianoulakis 1993). Together, these findings provide support that acute alcohol could stimulate CRH release, which could subsequently stimulate β-endorphin release. In vitro studies have shown that low concentrations of β-endorphin could stimulate CRH release in the hypothalamus, while high β-endorphin concentrations inhibited CRH release (Buckingham 1986). Subsequent in vitro studies have demonstrated that β-endorphin could interact with the μ- but not with the δ-opioid receptors to stimulate hypothalamic CRH release (Buckingham and Cooper 1986). Since μ-opioid receptors are present in CeA, it may be speculated that the initial increase of β-endorphin in response to alcohol could stimulate CRH release which could stimulate a further increase of β-endorphin leading to high concentrations of β-endorphin which then could inhibit CRH release. Therefore, the microinjections of the CRHR antagonists in the current studies could attenuate the initial and sustained alcohol-induced β-endorphin release.

The behavioural data in these experiments showed that there is an initial increase of locomotor activity immediately after either the alcohol or the saline IP injections, followed by a general decrease of locomotor activity over time. The absence of differences in locomotor activity between the ethanol- and saline-treated rats may be due to the fact that the study was performed during the rat's sleep cycle. Therefore, following the initial activation of locomotion due to handling and IP injection (0 to 30 min) all the animals presented very low levels of locomotion for the next period (30–60 min) as well as the remaining time (60–180 min). If the study was performed during the rat's wake cycle, the locomotor activity of the saline-treated rats would have been greater than that of the alcohol-treated animals and would indicate the presence of alcohol-induced sedation. Since other tests of sedation such as loss of righting reflex were not performed (Sato et al. 2005), the presence of some sedating effects of alcohol in the current studies cannot be excluded. Behavioural effects of alcohol were clearly demonstrated in the current study when grooming activity was examined. Studies have shown that systemic injections of SKF 38393, a dopamine 1 receptor (D1) agonist, induced grooming in a naloxone-sensitive manner (Stoessl 1994). Thus, acute alcohol-induced increases in extracellular DA and opioids in brain regions such as the NAC (Imperato and Di Chiara 1986; Marinelli et al. 2005; Marinelli et al. 2003) could mediate the alcohol-induced increase in grooming activity observed following all doses of alcohol. The initial transient increase of grooming activity in the alcohol but not saline-treated rats, corresponds with the ascending limb of the blood alcohol concentration–time curve (Lam et al. 2008), a time when the increase in NAC DA activity is observed (Imperato and Di Chiara 1986). However, comparing the profiles of the alcohol-induced changes in behavioural activities with those in extracellular CRH and β-endorphin concentrations in CeA, it was evident that there was no correspondence between the increases in the extracellular CRH and β-endorphin concentrations in CeA and the increase in grooming. Further behavioural studies are required to determine whether changes in extracellular

CRH and β-endorphin concentrations in CeA in response to acute ethanol could be associated with changes in anxiety or stress levels.

The in vivo microdialysis technique allows the monitoring of neuropeptide level changes in distinct brain regions of awake and freely moving animals by collecting microdialysate samples at several time points prior to and following specific treatments (Kendrick 1990). However, the low recovery rates of peptides using the microdialysis technique coupled with solid-phase RIA pose some limitations on the detection limits of CRH and β-endorphin peptides making it necessary to collect dialysate samples at 30-min intervals, thus decreasing the temporal resolution (Maidment et al. 1989; Maidment et al. 1991). Nevertheless, previous studies using the in vivo microdialysis technique and 20 to 30 min sample collection intervals have demonstrated changes in the extracellular concentrations of CRH and β-endorphin peptides at the level of CeA (Lam et al. 2008; Merlo-Pich et al. 1995; Richter et al. 2000). Thus, the microdialysis technique can be used to effectively detect changes in the extracellular neuropeptide concentrations in the CeA in response to alcohol or other treatments.

In summary, the present study demonstrated that acute alcohol could stimulate β-endorphin release and indirectly stimulate CRH release in CeA, while local CRH microinjection resulted in increased β-endorphin release in CeA. Furthermore, the alcohol-induced increase in the extracellular concentrations of β-endorphin in CeA could be significantly attenuated by blocking CRHR1 with antalarmin and CRHR2 with anti-sauvagine-30. Therefore, interactions between the CRH and β-endorphin systems at the level of CeA following acute alcohol administration may be important in mediating some of the pharmacological effects of alcohol at the level of the brain.

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References

Altshuler HL, Phillips PE, Feinhandler DA (1980) Alteration of ethanol self-administration by naltrexone. Life Sci 26:679–688

Bert L, Favale D, Jego G, Greve P, Guilloux J-P, Guiard BP, Gardier AM, Suaud-Chagny MF, Lestage P (2004) Rapid and precise method to locate microdialysis probe implantation in the rodent brain. J Neurosci Methods 140:53–57

Bradbury AF, Smyth DG, Snell CR (1976) C fragment of lipotropin has a high affinity for brain opiate receptors. Nature 260:793–795

Buckingham JC (1986) Stimulation and inhibition of corticotrophin releasing factor secretion by beta endorphin. Neuroendocrinology 42:148–152

Buckingham JC, Cooper TA (1986) Pharmacological characterization of opioid receptors influencing the secretion of corticotrophin releasing factor in the rat. Neuroendocrinology 44:36–40

Chalmers DT, Lovenberg TW, De Souza EB (1995) Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. J Neurosci 15:6340–6350

Chang JK, Chang KJ (1983) The role of amino-terminal sequence of beta-endorphin and dynorphin in the determination of opiate receptor type selectivity. Life Sci 33:267–269

Charmandari E, Constantine T, Chrousos G (2005) Endocrinology of the stress response. Annu Rev Physiol 67:259–284

Cowen MS, Chen F, Lawrence AJ (2004) Neuropeptides: implications for alcoholism. J Neurochem 89:273–285

Criado JR, Morales M (2000) Acute ethanol induction of c-Fos immunoreactivity in pre-pro-enkephalin expressing neurons of the central nucleus of the amygdala. Brain Res 861:173–177

de Waele JP, Gianoulakis C (1993) Effects of single and repeated exposures of ethanol on hypothalamic beta-endorphin and CRH release by the C57BL/6 and DBA/2 strains of mice. Neuroendocrinology 57:700–709

Froehlich JC, Harts J, Lumeng L, Li TK (1990) Naloxone attenuates voluntary ethanol intake in rats selectively bred for high ethanol preference. Pharmacol Biochem Behav 35:385–390

Funk CK, O'Dell LE, Crawford EF, Koob GF (2006) Corticotrophin-releasing factor within the central nucleus of the amygdala mediates enhanced ethanol self-administration in withdrawn, ethanol-dependent rats. J Neurosci 26:11324–11332

Garzon J, Sanchez-Blazquez P, Hollt V, Lee NM, Loh HH (1983) Endogenous opioid peptides: comparative evaluation of their receptor affinities in the mouse brain. Life Sci 33:291–294

Gianoulakis C, Gupta A (1986) Inbred strains of mice with variable sensitivity to ethanol exhibit differences in the content and processing of beta-endorphin. Life Sci 39:2315–2325

Goldstein A, Tachibana S, Lowney LI, Hunkapiller M, Hood L (1979) Dynorphin-(1–13), an extraordinarily potent opioid peptide. Proc Natl Acad Sci 76:6666–6670

Heinrichs SC, Joppa M (2001) Dissociation of arousal-like from anxiogenic-like actions of brain corticotropin-releasing factor receptor ligands in rats. Behav Brain Res 122:43–50

Heyser CJ, Roberts AJ, Schulteis G, Koob GF (1999) Central administration of an opiate antagonist decreases oral ethanol self-administration in rats. Alcohol Clin Exp Res 23:1468–1476

Hughes J, Smith T, Morgan B, Fothergill L (1975a) Purification and properties of enkephalin—the possible endogenous ligand for the morphine receptor. Life Sci 16:1753–1758

Hughes J, Smith TW, Kosterlitz HW (1975b) Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature 258:577–579

Imperato A, Di Chiara G (1986) Preferential stimulation of dopamine release in the nucleus accumbens of freely moving rats by ethanol. J Pharmacol Exp Ther 239:219–228

Kang-Park MH, Kieffer BL, Roberts AJ, Siggins GR, Moore SD (2007) Presynaptic delta opioid receptors regulate ethanol actions in central amygdala. J Pharmacol Exp Ther 320:917–925

Keith LD, Crabbe JC, Robertson LM, Kendall JW (1986) Ethanol-stimulated endorphin and corticotropin secretion in vitro. Brain Res 367:222–229

Kendrick KM (1990) Microdialysis measurement of in vivo neuropeptide release. J Neurosci Methods 34:35–46

Koob GF (2009) Brain stress systems in the amygdala and addiction. Brain Res 1293:61–75

Koob GF, Volkow ND (2009) Neurocircuitry of addiction. Neuropsychopharmacology 35:217–238

Lam MP, Marinelli PW, Bai L, Gianoulakis C (2008) Effects of acute ethanol on opioid peptide release in the central amygdala: an in vivo microdialysis study. Psychopharmacology (Berl) 201:261–271

Lam MP, Nurmi H, Rouvinen N, Kiianmaa K, Gianoulakis C (2010) Effects of acute ethanol on β-endorphin release in the nucleus accumbens of selectively bred lines of alcohol-preferring AA and alcohol-avoiding ANA rats. Psychopharmacology (Berl) 208:121–130

Lord JAH, Waterfield AA, Hughes J, Kosterlitz HW (1977) Endogenous opioid peptides: multiple agonists and receptors. Nature 267:495–499

Maidment NT, Brumbaugh DR, Rudolph E, Erdelyi E, Evans CJ (1989) Microdialysis of extracellular endogenous opioid peptides from rat brain in vivo. Neuroscience 33:549–557

Maidment NT, Evans CJ, Robinson TE, Justice JRJ (1991) Measurement of extracellular neuropeptides in the brain: microdialysis linked to solid-phase radioimmunoassays with sub-femtomole limits of detection microdialysis in the neurosciences. Elsevier, New York, pp 275–302

Marinelli PW, Gianoulakis C, Quirion R (2003) A microdialysis profile of beta-endorphin and catecholamines in the rat nucleus accumbens following alcohol administration. Psychopharmacology (Berl) 169:60–67

Marinelli PW, Bai L, Quirion R, Gianoulakis C (2005) A microdialysis profile of Met-enkephalin release in the rat nucleus accumbens following alcohol administration. Alcohol Clin Exp Res 29:1821–1827

Martin WR, Eades CG, Thompson JA, Huppler RE, Gilbert PE (1976) The effects of morphine- and nalorphine-like drugs in the nondepedent and morphine-dependent chronic spinal dogs. J Pharmacol Exp Ther 197:517–532

Merchenthaler I, Vigh S, Petrusz P, Schally AV (1982) Immunocytochemical localization of corticotropin-releasing factor (CRF) in the rat brain. Am J Anat 165:385–396

Merlo-Pich E, Lorang M, Yeganeh M, Rodriguez de Fonseca F, Raber J, Koob GF, Weiss F (1995) Increase of extracellular corticotropin-releasing factor-like immunoreactivity levels in the amygdala of awake rats during restraint stress and ethanol withdrawal as measured by microdialysis. J Neurosci 15:5439–5447

Olive MF, Koenig HN, Nannini MA, Hodge CW (2001) Stimulation of endorphin neurotransmission in the nucleus accumbens by ethanol, cocaine, and amphetamine. J Neurosci 21:RC184

O'Malley SS, Jaffe AJ, Chang G, Schottenfeld RS, Meyer RE, Rounsaville B (1992) Naltrexone and coping skills therapy for alcohol dependence: a controlled study. Arch Gen Psychiatry 49:881–887

Paul CA, Beltz B, Berger-Sweeney J (2007) Observational methods used to assess rat behavior: general activity. Cold Spring Harb Protoc 2007: pdb.prot4799

Paxinos G, Watson C (1996) The rat brain in stereotaxic coordinates. Academic, New York

Richter RM, Zorrilla EP, Basso AM, Koob GF, Weiss F (2000) Altered amygdalar CRF release and increased anxiety-like behavior in Sardinian alcohol-preferring rats: a microdialysis and behavioral study. Alcohol Clin Exp Res 24:1765–1772

Robison CL, Meyerhoff JL, Saviolakis GA, Chen WK, Rice KC, Lumley LA (2004) A CRH1 antagonist into the amygdala of mice prevents defeat-induced behavior. Ann NY Acad Sci 1032:324–327

Sato Y, Seo N, Kobahashi E (2005) The dosing-time dependent effects of intravenous hypnotics in mice. Anesth Analg 101:1706–1708

Stoessl AJ (1994) Dopamine D1 receptor agonist-induced grooming is blocked by the opioid receptor antagonist naloxone. Eur J Pharmacol 259:301–303

Vale W, Spiess J, Rivier C, Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and β-endorphin. Science 213:1394–1397

Volpicelli JR, Alterman AI, Hayashida M, O'Brien CP (1992) Naltrexone in the treatment of alcohol dependence. Arch Gen Psychiatry 49:876–880