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The role of endogenous beta-endorphin and enkephalins in ethanol reward

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Abstract

Substantial evidence has implicated the endogenous opioid system in alcohol reinforcement. However, the role of each opioid peptide in alcohol reinforcement and, particularly, reward is not fully characterized. In this study, using the conditioned place preference (CPP) paradigm as an animal model of reward, we determined the role of endogenous β -endorphin and enkephalins in the rewarding action of ethanol. Female mice lacking beta-endorphin and/ or the proenkephalin gene as well as their respective wild-type controls were tested for baseline place preference on day 1, conditioned with ethanol versus saline on days 2 to 4 and were then tested under a drug-free state for postconditioning place preference on day 5. On each test day, mice were placed in the central neutral chamber and allowed to freely explore all three CPP chambers. The amount of time that mice spent in each chamber was recorded. We also studied the effect of naloxone, a non-selective opioid receptor antagonist, on ethanol CPP, in which wild-type mice were treated with saline or naloxone 10 min prior to ethanol or saline conditioning. Our results showed that the absence of β -endorphin or enkephalins alone failed to alter the acquisition of ethanol-induced CPP. However, the absence of both β -endorphin and enkephalins significantly reduced the CPP response. Interestingly, high but not low dose naloxone blunted ethanol CPP. These findings provide the first evidence illustrating that ethanol induces its rewarding action, at least in part, via a joint action of β -endorphin and enkephalins, possibly involving both mu and delta opioid receptors.

Keywords

Ethanol; Conditioned place preference; β -endorphin; enkephalins; Endogenous opioids; Knockout mice

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Conflict of interest

The authors declare no conflict of interest.

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1. Introduction

Alcoholism is a progressive brain disorder involving a transition from moderate or social use to loss of control over consumption and continued use despite negative consequences. This is because alcohol is a powerful reinforcer, where its chronic use leads to addictive behaviors including compulsive drug-seeking and drug-taking (Lewis, 1990). Alcohol, unlike other drugs of abuse, does not interact with a specific receptor or target system in the brain. Instead, there appears to be a complex interaction with multiple neurotransmitter/neuromodulator systems [for review, see (Clapp et al., 2008)]. As the neurobiological mechanisms underlying alcohol reward are not fully understood, the accompanying developments in pharmacotherapy have only proven to be of limited efficacy. Therefore, research on the effects of alcohol in the brain and how it interacts with these substances continues in hopes of developing more effective therapies to treat alcoholism and alcohol-related disorders.

Several lines of evidence support a role of the endogenous opioid system in ethanol reinforcement and consumption. There was a focus on the endogenous opioid system because it was shown that direct injection of β -endorphin and enkephalins produced conditioned place preference (CPP) (Phillips et al., 1983, Amalric et al., 1987) and mu opioid receptor agonists increased extracellular dopamine levels in the nucleus accumbens (Di Chiara and Imperato, 1988). In accord with these postulations, earlier studies using pharmacological approaches showed that blockade of opioid receptors by nonspecific opioid receptor antagonists, such as naltrexone and naloxone, decrease alcohol consumption in both humans and animals (Altshuler et al., 1980, Volpicelli et al., 1992, Stromberg et al., 1998, O'Malley et al., 2002). Consistent with these pharmacological studies, ethanol intake was also reduced in mu opioid receptor knockout mice (Roberts et al., 2000, Hall et al., 2001), suggesting that the endogenous opioid system, particularly the mu opioid receptor, is involved in alcohol reinforcement/consumption.

The role of the endogenous opioid system in alcohol reward is, however, somewhat controversial. For example, earlier studies demonstrated that systemic administration of naltrexone (Middaugh and Bandy, 2000) or local injection of methylnaloxonium into the ventral tegmental area (Bechtholt and Cunningham, 2005) reduced ethanol reward. Furthermore, a recent report showed that naloxone blocked both the acquisition and expression of ethanol-induced CPP in mice without motivational properties of its own (Wrobel, 2011). However, another study demonstrated that naloxone blocked these processes at a dose that induced aversion (Kuzmin et al., 2003). There is even evidence showing that naloxone failed to alter ethanol CPP (Cunningham et al., 1995) and this response was not altered in mice lacking the proenkephalin gene compared to their wild-type controls (Koenig and Olive, 2002). However, naloxone blocks various classes of opioid receptors at different doses. Therefore, we hypothesized that beta-endorphin and enkephalins jointly play a functional role in ethanol reward, leading to an intact CPP response in mice lacking only one peptide or in mice treated with low doses of naloxone, which may be selective in blocking the action of only one endogenous opioid peptide. To test our hypothesis, we used mice lacking β -endorphin and/or enkephalins to determine if the absence of both peptides jointly would regulate ethanol reward. Considering that compensatory changes may develop in knockout mice and that we hypothesized that naloxone may block ethanol CPP only at higher doses, we also assessed the effect of a high (10 mg/kg) and low (1 mg/kg) dose of naloxone on ethanol CPP in wild-type mice in order to provide complementary pharmacological evidence to that obtained from the knockout studies. It has been suggested that certain parameters in the experimental design of place conditioning studies can affect the CPP outcome, in particular the issue of apparatus bias (Bardo and Bevins, 2000, Tzschentke, 2007). In a biased apparatus, a group of naïve animals

will show a strong preference for one context over the other during the preconditioning test. With a biased apparatus, it has been argued that the ability of a drug to produce CPP does not reflect its rewarding value, but rather its anxiolytic effects, since animals show an unconditioned, initial bias for one of the CPP chambers (Cunningham et al., 2003). Therefore, we used both biased and unbiased CPP paradigms to determine the role of endogenous beta-endorphin and/or enkephalins in ethanol CPP and to assess whether the CPP response would be differentially regulated by the endogenous opioid peptide(s) in the two CPP paradigms.

2. Materials and methods

2.1. Subjects

Female mice between the ages of 2-4 months at the time of experiments were used throughout. We used female mice because they exhibit a significantly greater CPP response compared to male mice following an ethanol conditioning protocol similar to that used in the current study (Nguyen et al., 2012). Mice lacking β -endorphin (Rubinstein et al., 1996) and the proenkephalin gene (Konig et al., 1996) and their wild-type controls, fully backcrossed on a C57BL/6J mouse strain, were originally obtained from the Jackson Laboratory (Bar Harbor, ME). We crossed the null mice with each other as well as with their wild-type controls to generate mice heterozygous for one or both line(s). Male and female heterozygous mice of one or the two line(s) were then mated to generate mice lacking β -endorphin, enkephalins or β -endorphin and enkephalins and their respective wild-type littermates/controls. Pups were weaned and genotyped between the ages of 21 – 24 days. For genotyping, standard polymerase chain reaction (PCR) protocol was followed using samples obtained from ear snips. The following primers were used: 5'-ATGACCTCCGAGAAGAGCCAG (wild-type β -end), 5'-GAGGATTGGGAAGACAATAGC (knockout β -end), 5'-GCTGGGGCAAGGAGGTTGAGA (common β -end), 5'-TCCTTCACATTCCAGTGTGC (wild-type pENK), 5'-CAGCAGCTTCTGTTCCACATACACTTCAT (knockout pENK) and 5'-CATCCAGGTAATTGGCAGGAA (common pENK). Fig. 1 shows representative gels from the same animals genotyped for wild-type and knockout bands for each genotype. As can be observed, only wild-type bands are present and the knockout bands are absent for each genotype for lanes 1, 2, 7 and 8. Thus, these mice are wild-type for both genotypes. On the other hand, the knockout bands are present and wild-type bands are absent for both genotypes for lanes 3-6, confirming that these were double knockout mice. Mice were housed up to 4 mice per cage, with food and water available *ad libitum* throughout the study. They were kept in a temperature- and humidity-controlled room with standard 12h light/dark cycle. All experiments were carried out during the light phase. Mice were habituated to the testing room at least 1 day prior to the start of the experiment and remained there until the end of the experiment. All procedures were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences (Pomona, CA).

2.2. Drugs

Alcohol solutions were prepared from ethyl alcohol (200 Proof, OmniPur, EM Science; Gibbstown, NJ) and saline to the appropriate concentrations, and injected via intraperitoneal (i.p.) route in a volume of 10 ml/ kg body weight. Naloxone hydrochloride (Sigma; St. Louis, MO) was dissolved in saline and administered subcutaneously (s.c.). The primers were purchased from Bioneer (Alameda, CA).

2.3. Experimental Procedure

2.3.1. The dose-response relationship of ethanol-induced CPP—The CPP paradigm was used because it is believed to parallel drug-seeking and cue-elicited craving in humans, and has been used to evaluate the rewarding properties of several drugs of abuse including cocaine, opiates and alcohol [for review, see (Bardo and Bevins, 2000, Tzschentke, 2007)]. We first determined the dose-response relationship of ethanol CPP. A 3-chambered CPP apparatus (ENV-3013, Med Associates Inc., St. Albans, VT) was used. The details of the CPP apparatus are provided elsewhere (Nguyen et al., 2012). The conditioned place preference procedure consisted of three distinct phases conducted over a 5-day period: preconditioning (day 1), conditioning (days 2-4) and postconditioning (day 5). On the preconditioning test day, wild-type mice were placed in the central neutral gray chamber and allowed to freely explore the entire apparatus for 15 min under a drug-free state. The amount of time that mice spent in each chamber was recorded. During the conditioning days, animals received morning/afternoon injection of ethanol/saline or saline/ethanol (1, 2 or 4 g/kg) each day for three consecutive days (days 2-4). The number of animals was 7, 10 and 6 for the low, middle and high dose, respectively. Immediately after injection, mice were confined to the corresponding ethanol-paired or saline-paired chamber for 15 min. The morning and afternoon conditioning sessions were separated by 4 h. The injection order was counterbalanced such that mice received either ethanol or saline in the morning, and then the alternate treatment in the afternoon. On the postconditioning day, mice were again placed in the central chamber and allowed to freely explore the entire apparatus for 15 min. The amount of time that mice spent in each chamber was recorded in the same manner as for the preconditioning test.

2.3.2. The role of endogenous β -endorphin and enkephalins in ethanol reward—We then determined ethanol CPP in mice lacking β -endorphin or enkephalins and their respective wild-type counterparts. Considering that one peptide might compensate for the absence of the other, we also assessed the rewarding action of ethanol in mice lacking both β -endorphin and enkephalins and their wild-type controls. The conditioned place preference procedure was the same as described above. As stated above, we used both a biased and unbiased design in our study. In the biased procedure, mice received ethanol in the originally non-preferred side and saline in the preferred side. In the unbiased procedure, assignment of the drug-paired chamber was counterbalanced. The number of beta-endorphin wild-type and knockout mice was 11 and 16 for the biased paradigm, and 17 and 15 for the unbiased CPP paradigm, respectively. The number of wild-type and enkephalin knockout mice was 11 and 12 for the biased paradigm, and 24 and 20 for the unbiased paradigm, respectively. The number of mice for the double wild-type and double knockout mice was 10 and 15 for the biased paradigm, and 10 and 8 for the unbiased paradigm, respectively. During the conditioning days, animals received morning/afternoon injection of ethanol/saline or saline/ethanol (2 g/kg) each day for three consecutive days (days 2-4). Two additional cohorts of mice lacking beta-endorphin and enkephalins and their wild-type controls were also conditioned with a higher dose of ethanol (4 g/kg) using the unbiased CPP paradigm ($n = 7$ wild-type mice and $n = 5$ double knockout mice). However, this dose of ethanol caused lethality in both wild-type (1/7) and double knockout (1/5) mice. Therefore, the number of mice reported in each group is 6 and 4, respectively. Every attempt was made to include knockout mice with their respective wild-type controls in each cohort. Additionally, each experiment was conducted using at least three cohorts of 2-5 mice per genotype. On the postconditioning day, mice were again placed in the central chamber and allowed to freely explore the entire apparatus for 15 min. The amount of time that mice spent in each chamber was recorded in the same manner as for the preconditioning test.

2.3.3. The effect of naloxone on ethanol-induced CPP—Considering that compensatory changes could occur in knockout mice, we also studied the effect of naloxone, a non-selective opioid receptor antagonist, on ethanol CPP in wild-type mice. Control groups were injected with saline. The procedure was identical as described above except saline or naloxone (1 or 10 mg/kg, s.c.) was injected 10 min prior to saline or ethanol (2 g/kg) on each conditioning day. The number of animals per group was 10, 9, 7, 5, 6 and 6 mice for saline-EtOH, saline-saline, naloxone (1 mg/kg)-EtOH, naloxone (1 mg/kg)-saline, naloxone (10 mg/kg)-EtOH and naloxone (10 mg/kg)-saline, respectively.

2.4. Data Analysis

Data are expressed as mean ($\pm$ SEM) of the amount of time that mice of each genotype spent in the saline- and ethanol-paired chambers on preconditioning (D1) and postconditioning (D5) test days. Data were analyzed using repeated measures analysis of variance (ANOVA). To determine whether there was a difference between knockout and wild-type mice of each genotype, the amount of time that null mice spent in the ethanol-paired chamber on the preconditioning (D1) and postconditioning (D5) test days was compared to that of their respective wild-type controls using repeated measures ANOVA. Bonferroni *post-hoc* test was used to reveal significant differences between the genotypes and between pre- and postconditioning test days. Changes in body weight between null mice and their respective wild-type controls were analyzed by unpaired student's *t* test. A $p < 0.05$ was used to indicate a significant difference between groups.

3. Results

3.1. The dose-dependency of ethanol-induced CPP

The amount of time that mice spent in the saline- and ethanol-paired chambers is shown in Figure 2. The low dose of ethanol (1 g/kg) failed to produce CPP (Fig. 2A), as evidenced by no significant effect of the amount of time that mice spent in the CPP chambers ($F(1,12) = 0.07$, $p > 0.05$), no significant effect of test day ($F(1,12) = 0.12$, $p > 0.05$) and no significant interaction between the two factors ($F(1,12) = 0.08$, $p > 0.05$). On the other hand, the middle dose of ethanol (2 g/kg) induced a robust CPP (Fig. 2B). Repeated measures ANOVA revealed no significant effect of test day ($F(1,18) = 1.58$, $p > 0.05$), but there was a significant effect of the amount of time that mice spent in the CPP chambers ($F(1,18) = 26.91$, $p < 0.0001$) and a significant interaction between the two factors ($F(1,18) = 35.47$, $p < 0.0001$). *Post-hoc* analysis showed that conditioning with ethanol resulted in a significant preference for the ethanol-paired chamber in these mice (Fig. 2B). The high dose (4 g/kg) also induced CPP (Fig. 2C), as evidenced by a significant interaction between the two factors ($F(1,10) = 8.88$, $p < 0.02$). However, it appeared that the CPP response was reduced to some extent compared to the response obtained with the middle dose (compare Fig. 2B vs. 2C).

3.2. Ethanol-induced CPP was not altered in mice lacking β -endorphin compared to their wild-type littermates/controls

Considering that the high dose of ethanol (4 g/kg) caused lethality in 1 out of 7 mice and the CPP response was not as robust as with the middle dose (2 g/kg), we used the middle dose of ethanol for the rest of the studies. Figure 3 depicts the amount of time that mice lacking β -endorphin and their wild-type littermates/controls spent in the saline- and ethanol-paired chambers before (D1) and after (D5) conditioning in the biased (left panels) and unbiased (right panels) CPP paradigms. Wild-type mice exhibited a significant preference for the ethanol-paired chamber following ethanol conditioning in the biased paradigm (Fig. 3A). Repeated measures ANOVA revealed no significant effect of test day ($F(1,20) = 0.15$, $p > 0.05$), but there was a significant effect of the amount of time that mice spent in the CPP chambers ($F(1,20) = 4.45$, $p < 0.05$) and a significant interaction between the two factors

($F(1,20) = 49.44, p < 0.0001$). *Post-hoc* analysis showed that ethanol induced a robust CPP, as evidenced by a significant increase in the amount of time that mice spent in the ethanol-paired chamber on the post-conditioning (D5) test day (Fig. 3A). A similar result was observed in wild-type mice in the unbiased CPP paradigm (Fig. 3D). Repeated measures ANOVA revealed no significant effect of test day ($F(1,32) = 0.70, p > 0.05$), but there was a significant effect of the amount of time that mice spent in the CPP chambers ($F(1,32) = 11.83, p < 0.005$) and a significant interaction between the two factors ($F(1,32) = 35.62, p < 0.0001$). Ethanol conditioning induced a significant CPP in mice lacking beta-endorphin in the biased CPP paradigm (Fig. 3B). Repeated measures ANOVA revealed no significant effect of test day ($F(1,30) = 0.01, p > 0.05$) and no significant effect of the amount of time that mice spent in the CPP chambers ($F(1,30) = 1.21, p > 0.05$), but there was a significant interaction between the two factors ($F(1,30) = 51.81, p < 0.0001$). *Post-hoc* analysis showed that conditioning with ethanol resulted in a significant preference for the ethanol-paired chamber in these mice (Fig. 3B). A similar result was observed in mice lacking beta-endorphin in the unbiased CPP paradigm (Fig. 3E). Repeated measures ANOVA revealed no significant effect of test day ($F(1,28) = 0.23, p > 0.05$), but there was a significant effect of the amount of time that mice spent in the CPP chambers ($F(1,28) = 4.95, p < 0.05$) and a significant interaction between the two factors ($F(1,28) = 37.03, p < 0.0001$).

In order to determine whether the CPP response was different between the two genotypes in the biased paradigm, we compared the amount of time that mice lacking β -endorphin and their wild-type littermates/controls spent in the ethanol-paired chamber on preconditioning (D1) and postconditioning (D5) test days (Fig. 3C). Repeated measures ANOVA revealed a significant effect of test day ($F(1,25) = 47.41, p < 0.0001$) but no significant effect of genotype ($F(1,25) = 1.07, p > 0.05$) and no significant interaction between the two factors ($F(1,25) = 1.87, p > 0.05$), showing that the rewarding action of ethanol is not altered in mice lacking β -endorphin compared to their wild-type littermates/controls. A similar result was obtained in the unbiased CPP paradigm (Fig. 3F). Repeated measures ANOVA revealed a significant effect of test day ($F(1,30) = 34.88, p < 0.0001$) but no significant effect of genotype ($F(1,30) = 0.77, p > 0.05$) and no significant interaction between the two factors ($F(1,30) = 1.26, p > 0.05$). These results suggest that the rewarding action of ethanol was not altered in mice lacking beta-endorphin compared to their wild-type littermates/controls.

3.3. Ethanol-induced CPP was not altered in mice lacking enkephalins compared to their wild-type littermates/controls

Figure 4 depicts the amount of time that mice lacking the proenkephalin gene and their wild-type littermates/controls spent in the saline- and ethanol-paired chambers before (D1) and after (D5) conditioning in the biased (left panels) and unbiased (right panels) CPP paradigms. As expected, wild-type mice exhibited a robust CPP in the biased CPP paradigm (Fig. 4A), as evidenced by no significant effect of test day ($F(1,20) = 0.04, p > 0.05$) or the amount of time that mice spent in the CPP chambers ($F(1,20) = 2.26, p > 0.05$) but a significant interaction between the two factors ($F(1,20) = 45.73, p < 0.0001$). A similar result was observed in wild-type mice in the unbiased CPP paradigm (Fig. 4D). Repeated measures ANOVA revealed no significant effect of test day ($F(1,46) = 0.00, p > 0.05$), but there was a significant effect of the amount of time that mice spent in the CPP chambers ($F(1,46) = 13.63, p < 0.001$) and a significant interaction between the two factors ($F(1,46) = 25.03, p < 0.0001$).

Analysis of the data in proenkephalin knockout mice in the biased CPP paradigm revealed no significant effect of test day ($F(1,22) = 0.01, p > 0.05$) or the amount of time that mice spent in the CPP chambers ($F(1,22) = 2.06, p > 0.05$), but there was a significant interaction between the two factors ($F(1,22) = 28.47, p < 0.0001$). *Post-hoc* analysis showed that conditioning with ethanol resulted in a significant preference toward the ethanol-paired

chamber in these mice (Fig. 4B). A similar result was observed in mice lacking enkephalins in the unbiased CPP paradigm (Fig. 4E). Repeated measures ANOVA revealed no significant effect of test day ($F(1,38) = 0.25$, $p > 0.05$), but there was a significant effect of the amount of time that mice spent in the CPP chambers ($F(1,38) = 9.11$, $p < 0.005$) and a significant interaction between the two factors ($F(1,38) = 11.67$, $p < 0.005$).

Analysis of the amount of time that null mice and their wild-type littermates/controls spent in the ethanol-paired chamber on preconditioning (D1) and postconditioning (D5) test days in the biased CPP paradigm (Fig. 4C) revealed a significant effect of test day ($F(1,21) = 31.10$, $p < 0.0001$), but there was no significant effect of genotype ($F(1,21) = 0.10$, $p > 0.05$) and no interaction between the two factors ($F(1,21) = 0.67$, $p > 0.05$). As can be observed, there was virtually no difference in the amount of time that knockout mice spent in the ethanol-paired chamber on the preconditioning or postconditioning test day compared to their wild-type littermates/controls (Fig. 4C). The amount of time that mice lacking enkephalins and their wild-type littermates or age-matched controls spent in the ethanol-paired chamber was also comparable in the unbiased CPP paradigm (Fig. 4F). Repeated measures ANOVA revealed a significant effect of test day ($F(1,42) = 13.36$, $p < 0.001$) but no significant effect genotype ($F(1,42) = 0.41$, $p > 0.05$) and no significant interaction between the two factors ($F(1,42) = 0.05$, $p > 0.05$). These results suggest that the rewarding action of ethanol was not altered in mice lacking enkephalins compared to their wild-type littermates/controls.

3.4. Mice lacking both β -endorphin and enkephalins exhibited reduced CPP compared to their wild-type controls

The amount of time that mice lacking both β -endorphin and enkephalins and their wild-type littermates/controls spent in the CPP chambers before (D1) and after (D5) conditioning in the biased (left panels) and unbiased (right panels) CPP paradigms is shown in Figure 5. Repeated measures ANOVA of the data in wild-type mice in the biased CPP paradigm (Fig. 5A) revealed no significant effect of test day ($F(1,18) = 0.29$, $p > 0.05$), but there was a significant effect of the amount of time that mice spent in the CPP chambers ($F(1,18) = 5.23$, $p < 0.05$) and a significant interaction between the two factors ($F(1,18) = 93.14$, $p < 0.0001$). *Post-hoc* analysis showed that wild-type mice spent more time in the ethanol-paired chamber after (D5) but not before (D1) conditioning with ethanol (Fig. 5A). A similar result was observed in double wild-type mice in the unbiased CPP paradigm (Fig. 5D). Repeated measures ANOVA revealed no significant effect of test day ($F(1,18) = 1.11$, $p > 0.05$) but there was a significant trend for the amount of time that mice spent in the CPP chambers ($F(1,18) = 4.22$, $p < 0.06$), and a significant interaction between the two factors ($F(1,18) = 8.26$, $p < 0.05$). Analysis of the data in the double knockout mice in the biased CPP paradigm (Fig. 5B) revealed no significant effect of test day ($F(1,28) = 0.37$, $p > 0.05$) and no significant effect of the amount of time that mice spent in the CPP chambers ($F(1,28) = 0.21$, $p > 0.05$), but there was a significant interaction between the two factors ($F(1,28) = 20.50$, $p < 0.0001$), showing that ethanol conditioning modestly increased preference of double knockout mice for the ethanol-paired chamber (Fig. 5B). On the other hand, the double knockout mice failed to exhibit any CPP response in the unbiased CPP paradigm (Fig. 5E). Repeated measures ANOVA revealed no significant effect of test day ($F(1,14) = 0.33$, $p > 0.05$), no significant effect of the amount of time that mice spent in the CPP chambers ($F(1,14) = 0.05$, $p > 0.05$) and no significant interaction between the two factors ($F(1,14) = 1.65$, $p > 0.05$).

Comparing the amount of time that mice of both genotypes spent in the ethanol-paired chamber on pre- and postconditioning test days in the biased CPP paradigm revealed a significant effect of test day ($F(1,23) = 33.16$, $p < 0.0001$) and a significant effect of genotype ($F(1,23) = 5.71$, $p < 0.03$), but there was no significant interaction between the two factors

($F(1,23) = 1.80, p > 0.05$). Repeated measures ANOVA of the data in the unbiased paradigm revealed a significant effect of test day ($F(1,17) = 10.22, p < 0.05$) and a significant effect of genotype ($F(1,17) = 7.03, p < 0.05$), but no significant interaction between the two factors ($F(1,17) = 0.67, p > 0.05$). A similar result was obtained when a higher dose of ethanol (4 g/kg) was used (Fig. 6). Ethanol induced CPP in wild-type mice (Fig. 6A), as evidenced by no significant effect of test day ($F(1,10) = 1.26, p > 0.05$) or the amount of time that mice spent in the CPP chambers ($F(1,10) = 0.86, p > 0.05$) but a significant interaction between the two factors ($F(1,10) = 8.88, p < 0.05$). On the other hand, analysis of the data in mice lacking both beta-endorphin and enkephalins revealed no significant interaction between the amount of time that mice spent in the CPP chambers and test day ($F(1,6) = 0.29, p > 0.05$) (Fig. 6B). These results suggest that the rewarding action of ethanol was reduced in mice lacking β -endorphin and enkephalins compared to their wild-type controls.

3.5. Ethanol-induced CPP was blocked by a higher (10 mg/kg) but not lower (1 mg/kg) dose of naloxone

Figure 7 illustrates the amount of time that mice spent in the conditioning chambers on the preconditioning (D1) and postconditioning (D5) test days. As expected, ethanol induced a significant CPP in wild-type mice (Fig. 7A). Repeated measures ANOVA revealed a significant interaction between the amount of time that mice spent in the CPP chambers and test day ($F(1,18) = 35.47; p < 0.0001$). *Post-hoc* test showed a significant ($p < 0.0001$) increase in the amount of time that mice spent in the ethanol-paired compared to the saline-paired chamber on day 5 (Fig. 7A). In contrast, conditioning with saline failed to induce CPP (Fig. 7B). A lower dose of naloxone (1 mg/kg) failed to block ethanol CPP (Fig. 7C). Repeated measures ANOVA revealed a significant interaction between the two factors ($F(1,12) = 40.52; p < 0.0001$). *Post-hoc* test showed a significant increase in the amount of time that mice spent in the ethanol-paired compared to the saline-paired chamber on day 5 ($p < 0.0001$). Although this dose of naloxone failed to alter ethanol CPP, it induced aversion on its own (Fig. 7D). Repeated measures ANOVA revealed a significant interaction between the two factors ($F(1,8) = 20.38; p < 0.002$). *Post-hoc* test showed a significant decrease in the amount of time that mice spent in the naloxone-paired compared to the saline-paired chamber on day 5 ($p < 0.0001$). In contrast, the higher dose of naloxone (10 mg/kg) completely blocked ethanol CPP (Fig. 6E). Repeated measures ANOVA revealed no significant interaction between the amount of time that mice spent in the CPP chambers and test day ($F(1,10) = 0.02; p > 0.05$). However, this dose of naloxone alone also induced a significant aversion (Fig. 7F), as evidenced by a significant interaction between the two factors ($F(1,10) = 10.22; p < 0.01$). *Post-hoc* test showed that mice spent a significantly less amount of time in the naloxone-paired compared to the saline-paired chamber following ($p < 0.01$) but not before ($p > 0.05$) conditioning.

4. Discussion

The main findings of the present study are that ethanol-induced CPP was not altered in mice lacking enkephalins or β -endorphin. Nevertheless, the CPP response was blunted in the unbiased and showed a reduced trend in the biased CPP paradigm in mice lacking both β -endorphin and enkephalins. Additionally, the rewarding action of ethanol was blocked by a high (10 mg/kg) but not low dose (1 mg/kg) of naloxone, suggesting that alcohol exerts its rewarding action, at least in part, via a joint action of β -endorphin and enkephalins. In the absence of one peptide, the other peptide may compensate, leading to an intact CPP response in mice lacking only beta-endorphin or enkephalins.

Naltrexone and naloxone, non-selective opioid receptor antagonists, have been shown to reduce alcohol consumption in both humans and animals (Altshuler et al., 1980, Volpicelli et al., 1992, Stromberg et al., 1998, O'Malley et al., 2002), suggesting that the endogenous

opioid system plays a critical role in the reinforcing actions of alcohol. Existing literature has supported a prominent role of the mu opioid receptor in alcohol consumption. For example, the mu opioid receptor antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP) progressively decreased ethanol intake in alcohol-preferring AA rats when administered for 3 consecutive days (Hyytia, 1993). Similarly, the irreversible mu opioid receptor antagonist, β -funaltrexamine (β -FNA), reduced ethanol consumption [(Stromberg et al., 1998) but see (Le et al., 1993)]. Reduced ethanol intake has also been observed in mu opioid receptor knockout mice (Roberts et al., 2000, Hall et al., 2001). However, it is not known whether the opioid system alters the rewarding action of ethanol or the consummatory aspect of alcohol intake. Earlier, we reported that ethanol CPP was not altered in mice lacking dynorphins under a drug-free state, a paradigm used in the current study (Nguyen et al., 2012). Thus, the present study was designed to test the hypothesis that endogenous beta-endorphin and/or enkephalins are involved in ethanol reward. Considering that beta-endorphin has a high affinity for the mu opioid receptor and induces reward (Amalric et al., 1987), we first tested the hypothesis that ethanol induces its rewarding action via the release of beta-endorphin. In fact, ethanol administration has been shown to increase β -endorphin and enkephalin levels in the nucleus accumbens (Olive et al., 2001, Marinelli et al., 2003, Marinelli et al., 2005). Moreover, a correlation between β -endorphin and increased sensitivity to alcohol in both genetically-bred alcohol-preferring animals as well as in humans with a predisposition to alcoholism has been reported (Gianoulakis et al., 1989, Lam et al., 2010). Thus, we used mice lacking β -endorphin and their wild-type littermates/controls to assess the role of beta-endorphin in this process. This is the first study to assess the role of β -endorphin in ethanol reward using the CPP paradigm. Our results showed no difference in ethanol CPP between mice lacking β -endorphin and their wild-type controls. Mice lacking β -endorphin have been reported to express either increased (Grahame et al., 1998, Grisel et al., 1999) or decreased (Racz et al., 2008) ethanol consumption. However, it should be pointed out that while ethanol CPP assesses the motivational value of contextual cues that gain saliency following pairing with ethanol, alcohol consumption deals with reinforcement and consummatory aspects of ethanol intake. Thus, these results suggest that beta-endorphin alone is not critical in ethanol CPP under the current experimental paradigm.

Enkephalins, although considered the endogenous ligands of the delta opioid receptor, also have affinity for mu opioid receptors, raising the possibility that enkephalins either alone or in conjunction with β -endorphin may be important in this response. We found no difference in ethanol CPP in mice lacking enkephalins compared to their wild-type controls, suggesting that proenkephalin-derived peptides may not be involved in ethanol reward. This result is in agreement with an earlier report showing that ethanol CPP was not altered in male mice lacking the proenkephalin gene and their wild-type controls (Koenig and Olive, 2002). Given that high doses of naloxone reduced ethanol CPP (Kuzmin et al., 2003, Wrobel, 2011), we then proposed that beta-endorphin might compensate for the absence of enkephalins in mice lacking enkephalins and vice versa in beta-endorphin-deficient mice. Thus, if both opioid peptides were absent, we might be able to observe a reduction in ethanol-induced CPP. Accordingly, we next examined whether ethanol-induced CPP would be altered in mice lacking both β -endorphin and enkephalins compared to their wild-type controls. Interestingly, we discovered a significant reduction in ethanol-induced CPP in double knockout mice compared to their wild-type controls. As double knockout mice showed some CPP in the biased design with the low dose of ethanol (2 g/kg), it was not clear whether the rewarding action of ethanol was reduced or enhanced in these animals. To address this issue, we assessed ethanol CPP in double knockout mice and their wild-type controls using a higher dose of ethanol (4 g/kg). Our result showed that wild-type but not double knockout mice exhibited CPP (Fig. 6), suggesting that ethanol CPP was reduced in null mice. However, considering that 2 versus 4 g/kg represents a wide dose range in terms of assessment of ethanol sensitivity and behavioral responses, it may be possible to observe

a different result if a closer dose range had been used. Nevertheless, as wild-type mice showed more robust CPP with the lower (2 g/kg) compared to higher (4 g/kg) dose of ethanol and that a similar pattern was observed in double knockout mice, we believe that this possibility may be less likely. Thus, the current results provide evidence illustrating that ethanol reward is mediated, at least in part, via a joint action of β -endorphin and enkephalins. However, it is not known how the two peptides regulate the action of ethanol. We propose that the ability of alcohol to alter the function of the mesolimbic reward circuit is altered in these mice, but further studies are needed to address this possibility.

Certain procedural differences may affect the CPP outcome (Cunningham et al., 2003), in particular the issue of apparatus bias (Bardo and Bevins, 2000, Tzschentke, 2007). For example, with a biased apparatus, it has been argued that the ability of a drug to produce CPP does not reflect its rewarding value, but rather its anxiolytic effects, since animals show an unconditioned, initial bias for one of the CPP chambers (Cunningham et al., 2003). For example, clonidine, an α_2 adrenergic receptor agonist, produced CPP using a biased apparatus when paired with the initially non-preferred side but failed to do so in an unbiased apparatus (Cervo et al., 1993). However, it is important to distinguish between a biased apparatus and a biased design, in which the drug is paired with the initially non-preferred side. In other words, a biased design refers only to the procedure of assigning the drug to a (non-preferred) context. Mice in our study do not show an initial bias for either conditioning chamber (i.e., approximately half spent more time on one side during preconditioning, and half in the other). Given the robustness of the CPP response in the biased paradigm, in a first series of experiments we used a biased assignment procedure and conditioned mice in their non-preferred chamber. We found that the absence of both beta-endorphin and enkephalins is necessary to reduce the CPP response. It has been argued that conditioning in the preferred side may represent a ceiling effect, while conditioning in the non-preferred side may provide greater opportunity to observe a difference or the anxiolytic rather than the rewarding actions of drugs. In order to clarify these issues, we subsequently assessed ethanol-induced CPP using an unbiased design, in which assignment of the drug-paired chamber was counterbalanced. Consistent with our results in the biased CPP paradigm, we observed no difference in the CPP response between mice lacking beta-endorphin or enkephalins alone compared to their respective wild-type littermates or age-matched controls in the unbiased paradigm (Figs. 3 and 4, right panels). On the other hand, we observed a blunted CPP response in mice lacking both beta-endorphin and enkephalins compared to their wild-type controls in the unbiased CPP paradigm (Figs. 5 and 6, right panels). Although we observed a small but significant CPP in double knockout mice using the biased design, the response was nevertheless reduced compared to their wild-type controls. This response was completely abolished in the double knockout mice in the unbiased CPP paradigm. Together, these results suggest that beta-endorphin and enkephalins jointly regulate ethanol reward. We speculate that the absence of beta-endorphin and enkephalin may lead to a reduction in the activity of mesolimbic dopaminergic neurons, which are known to be an essential component of the reward circuit, thereby leading to reduced CPP in mice lacking the two opioid peptides compared to their wild-type controls. However, these null mice and their wild-type controls exhibit a comparable cocaine-induced CPP (Lutfy et al., unpublished data), raising the possibility that other mechanisms may be involved. Thus, further studies are needed to delineate the underlying mechanisms leading to the reduced CPP response in these null mice.

Considering that compensatory developmental changes could occur in knockout mice, we also used two different doses of naloxone, a non-selective opioid receptor antagonist, to provide complementary pharmacological evidence to that obtained in the knockout mice studies. Naloxone at low doses is relatively selective at blocking the mu opioid receptor, but at higher doses the drug blocks all subtypes of opioid receptors. We took advantage of this

characteristic of naloxone and assessed its effect on ethanol reward. We found that high (10 mg/kg) but not low dose (1 mg/kg) naloxone blocked ethanol CPP. This result is consistent with earlier studies measuring ethanol reward [(Kuzmin et al., 2003, Wrobel, 2011) but see (Cunningham et al., 1995)] and reinforcement/consumption (Hubbell et al., 1986, Froehlich et al., 1990). Both doses of naloxone were effective pharmacologically because either dose of the drug induced aversion in the current study, raising the possibility that the action of high dose naloxone on ethanol CPP may not simply be due to its aversive effects. Thus, we propose that naloxone at higher doses antagonizes both mu and delta opioid receptors and therefore blocks the binding of endogenous enkephalins and beta-endorphin, thereby leading to blunted ethanol reward. It would be interesting to assess whether the effect of high dose naloxone on ethanol reward would be mimicked by a selective mu opioid receptor antagonist administered in conjunction with a delta selective antagonist. Further studies are needed to determine this possibility. Additionally, given that we used female mice for the current study, further studies are needed to assess the impact of estrous cycle on ethanol-induced CPP, because it has been shown that basal levels of dopamine in the prefrontal cortex vary during different phases of the estrous cycle and that ethanol-induced changes in extracellular dopamine in this region also differ during various phases of the estrous cycle (Dazzi et al., 2007). However, considering that wild-type littermates served as controls for the knockout mice wherever possible, we do not believe that differences in ethanol CPP between double knockout and wild-type mice would be attributable to fluctuations in beta-endorphin and enkephalin levels during different phases of the estrous cycle. Furthermore, because we used multiple cohorts of mice for each experiment, we would have observed more variability in our results had estrous cycle contributed to any differences. Likewise, we should have seen alterations in CPP response not only in double knockout mice but also in mono knockout mice.

5. Conclusion

The results of the current study showed that the rewarding action of ethanol was not altered in mice lacking enkephalins or β -endorphin as well as in mice treated with the low dose naloxone. On the other hand, mice lacking both opioid peptides exhibited reduced ethanol reward. Similarly, mice treated with a relatively higher dose of naloxone (10 mg/kg) failed to exhibit CPP. Accordingly, drugs that target more than one opioid peptide or its receptor(s) may provide a broader spectrum of inhibitory effect on the rewarding and reinforcing actions of ethanol.

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Highlights

- The role of opioid peptides in alcohol reward is not fully characterized.
- We assessed the role of β -endorphin and enkephalin in ethanol CPP in female mice.
- We also assessed the effect of naloxone on ethanol CPP in female mice.
- CPP was reduced in mice lacking both opioids or treated with high dose naloxone.
- Both peptides may jointly regulate ethanol reward.

Fig. 1. Representative wild-type (+/+) and knockout (-/-) bands for each genotype

Pups separated on days 21-24 were lightly anesthetized and a small piece of ear was cut. The DNA was extracted and assayed for the presence and absence of wild-type and knockout bands for each genotype using standard PCR protocol. Representative gels were taken from the same animals genotyped for wild-type and knockout bands for each genotype.

Fig. 2. The dose-response relationship of ethanol-induced CPP in C57BL/6 mice

Mice were tested for baseline place preference on day 1 (D1), received saline/ethanol (1, 2 or 4 g/kg) or ethanol/saline conditioning twice daily on days 2-4 and were then tested for postconditioning place preference on day 5 (D5). Data are mean ($\pm$ SEM) of 6-10 mice per dose. Data represent time spent in CPP chambers on preconditioning (D1) and postconditioning (D5) test days for the low (**A**; $n = 7$), middle (**B**; $n = 10$) and high (**C**; $n = 6$) dose ethanol. * $p < 0.05$, **** $p < 0.0001$ vs. saline-paired chamber on the same test day.

Fig. 3. Ethanol-induced CPP in mice lacking β -endorphin (β -end $^{-/-}$) and their wild-type (β -end $^{+/+}$) littermates

Mice were tested for baseline place preference on day 1 (D1), received saline/ethanol (2 g/kg) or ethanol/saline conditioning twice daily on days 2-4 and were then tested for postconditioning place preference on day 5 (D5) in the biased (left panels) and unbiased (right panels) CPP paradigms. Data are mean ($\pm$ SEM) of 11-17 mice per genotype for each paradigm. Data represent time spent in CPP chambers on preconditioning (D1) and postconditioning (D5) test days in wild-type (A; biased, $n = 11$ and D; unbiased, $n = 17$) and null (B; biased, $n = 16$ and E; unbiased, $n = 15$) mice. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ vs. saline-paired chamber on the same test day; C and F) Data represent time spent in ethanol-paired chamber on D1 and D5 in null vs. wild-type mice.

Fig. 4. Ethanol-induced CPP in mice lacking the proenkephalin (pENK^{-/-}) gene and their wild-type (pENK^{+/+}) littermates

Mice were tested for baseline place preference on day 1 (D1), received saline/ethanol (2 g/kg) or ethanol/saline conditioning twice daily on days 2-4 and were then tested for postconditioning place preference on day 5 (D5) in the biased (left panel) and unbiased (right panel) CPP paradigms. Data are mean ($\pm$ SEM) of 11-24 mice per genotype for each paradigm. Data represent time spent in CPP chambers on preconditioning (D1) and postconditioning (D5) test days in wild-type (A; biased, $n = 11$ and D; unbiased, $n = 24$) and null (B; biased, $n = 12$ and E; unbiased, $n = 20$) mice. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs. saline-paired chamber on the same test day; C and F) Data represent time spent in ethanol-paired chamber on D1 and D5 in wild-type vs. null mice.

Fig. 5. Ethanol-induced CPP in mice lacking β -endorphin and enkephalins (β -end $^{-/-}$ / pENK $^{-/-}$) and their wild-type (β -end $^{+/+}$ / pENK $^{+/+}$) littermates

Mice were tested for baseline place preference on day 1 (D1), received saline/ethanol (2 g/kg) or ethanol/saline conditioning twice daily on days 2-4 and were then tested for postconditioning place preference on day 5 (D5) in the biased (left panels) and unbiased (right panels) CPP paradigms. Data are mean ($\pm$ SEM) of 8-15 mice per genotype for each paradigm. Data represent time spent in CPP chambers on preconditioning (D1) and postconditioning (D5) test days in double wild-type (A; biased, $n = 10$ and D; unbiased, $n = 10$) and double knockout (B; biased, $n = 15$ and E; unbiased, $n = 8$) mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ vs. saline-paired chamber on the same test day; C and F) Data represent the amount of time that mice of both genotypes spent in the ethanol-paired chamber on D1 and D5. * $p < 0.05$ vs. double knockout mice

Fig. 6. The rewarding effect of a higher dose of ethanol (4 g/kg) in mice lacking β -endorphin and enkephalins (β -end $^{-/-}$ / pENK $^{-/-}$) and their wild-type (β -end $^{+/+}$ / pENK $^{+/+}$) littermates using the unbiased CPP paradigm

Mice were tested for baseline place preference on day 1 (D1), received saline/ethanol (4 g/kg) or ethanol/saline conditioning twice daily on days 2-4 and were then tested for postconditioning place preference on day 5 (D5). Data are mean ($\pm$ SEM) of 4-6 mice per genotype. Data represent time spent in CPP chambers on preconditioning (D1) and postconditioning (D5) test days in double wild-type (**A**; $n = 6$) and double knockout mice (**B**; $n = 4$). * $p < 0.01$ vs. saline-paired chamber on the same test day.

Fig. 7. Ethanol-induced CPP in wild-type mice treated with naloxone

Mice were tested for baseline place preference on day 1 (D1), received saline or naloxone 10 min prior to saline/ethanol (2 g/kg) or ethanol/saline conditioning twice daily on days 2-4 and were then tested for postconditioning place preference on day 5 (D5). Data are mean ($\pm$ SEM) of 5-10 mice per treatment. Data represent time spent in CPP chambers on preconditioning (D1) and postconditioning (D5) test days in mice treated with saline followed by ethanol (A; $n = 10$) or saline (B; $n = 9$), naloxone (1 mg/kg) followed by ethanol (C; $n = 7$) or saline (D; $n = 5$) and naloxone (10 mg/kg) followed by ethanol (E; $n = 6$) or saline (F; $n = 6$). ** $p < 0.01$, **** $p < 0.0001$ vs. saline-paired chamber on the same test day.