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Operant self-administration of ethanol in C57BL/6 mice lacking β-endorphin and enkephalin

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Abstract

To test whether endogenous opioid peptides are necessary for the rewarding effects of ethanol, we examined operant oral self-administration of ethanol in mice congenic to the C57BL/6J strain but lacking expression of β-endorphin, enkephalin or both peptides. The influences of prandial state, schedule interval and type, and ethanol concentration were all examined. Food-restricted subjects were tested in postprandial and preprandial states and subsequently at normal body weight when feeding ad libitum (ad lib). Operant studies were conducted using fixed ratio (FR) intervals of 2 and 8 as well as a progressive ratio (PR) interval of 2. The main significant effect relevant to our hypothesis was increased responding by female mice lacking β-endorphin under ad lib feeding conditions and only for lower ethanol concentrations (3% and 6%). Importantly, all subjects including those lacking both β-endorphin and enkephalins learned to self-administer ethanol similarly to wild-type mice and maintained responding for ethanol under a variety of procedural variables. Consequently, the two opioid peptides believed to be the endogenous ligands for the μ-opioid receptor (MOR) were not necessary to shape or perpetuate ethanol-reinforced operant responding. These results suggest that the rewarding effects of ethanol do not require β-endorphin or enkephalin signaling.

Keywords: Alcohol; Operant behavior; β-Endorphin; Enkephalin; Knockout mice

1. Introduction

Evidence from a variety of experimental paradigms in numerous animal models and humans has implicated the endogenous opioid system in the modulation of ethanol reward (reviewed in Herz et al., 1997; Swift, 1995; Ulm et al., 1995). The strongest pharmacological evidence supports a predominant role of the μ-opioid receptor (MOR) and its natural ligands β-endorphin and the enkephalins. However, selective delta receptor (DOR) antagonists can also reduce ethanol intake in preference tests in rodents (Franck et al., 1998; Froehlich et al., 1991; June et al., 1999; Krishnan-Sarin et al., 1995a; Krishnan-Sarin et al., 1995b; Le et al., 1993). Although enkephalins are often regarded as the endogenous ligand for DOR, β-endorphin has equivalent affinities for this receptor subtype and the MOR while enkephalin also has similar affinities for both receptors (Raynor et al., 1994). Thus, no pharmacological agent can selectively discriminate among the endogenous opioid peptides and so it is not clear which, if any, of these endogenous ligands mediate ethanol's reinforcing effects through the MOR and possibly DOR.

We have previously investigated the putative role of β-endorphin in ethanol intake using a mutant mouse strain developed in our lab by homologous recombination in embryonic stem cells (Rubinstein et al., 1996). Homozygous β-endorphin-deficient mice exhibited the unexpected phenotype of mildly increased ethanol preference and intake

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for a 7% solution in a two-bottle free-choice drinking paradigm (Grisel et al., 1999). There were no differences between genotypes for either measure when the ethanol concentration was increased to 10%. However, the heterozygous mice from the same mutant strain had increased ethanol drinking compared to wild types at both ethanol concentrations. A follow-up study was generally consistent with these data and also demonstrated that restricted access to the ethanol bottle for a period of 2 h per day disproportionately stimulated increased drinking in the KO mice, with the effect waning over time (Grahame et al., 2000). Naltrexone decreased ethanol intake in an orderly dose–response pattern with equal potency in wild-type and β-endorphin-deficient mice. A third study using operant intravenous self-administration of ethanol demonstrated persistent ethanol intake in the β-endorphin-deficient mice (Grahame et al., 1998). However, the latter study was somewhat confounded by very low operant responding for ethanol in the wild-type control population compared to historical controls, even though the mutant strain was seven generations backcrossed to the C57BL/6 background.

Together, these previous studies suggest that the relatively MOR selective opioid peptide β-endorphin is not required for ethanol reward in mice using two standard models of reward behavior, two-bottle free-choice drinking and instrumental responding for ethanol reinforcers. However, other investigators demonstrated that MOR knockout mice did not self-administer oral ethanol following a saccharin-fading procedure (Roberts et al., 2000). In contrast, DOR knockout mice had an increased ethanol self-administration phenotype (Roberts et al., 2001), similar to the β-endorphin knockout mice.

Notwithstanding the possibility of developmental compensations in non-opioid systems, a number of other possible scenarios can explain these data in the framework of existing models for opioid involvement in neural pathways subserving reward. One hypothesis is that enkephalins, either independently or interactively with β-endorphin, are the endogenous peptides mediating the reinforcing actions of ethanol. Alternatively, β-endorphin may actually play only a minor role in the acute reinforcing properties of ethanol. Instead, levels of β-endorphin may have a much more complex involvement in determining the onset of a drinking episode or the frequency of such episodes. This second hypothesis is supported by data indicating that high doses of naltrexone are less effective than lower doses and intermittent opioid receptor blockade is more effective than chronic blockade to decrease ethanol consumption (Middaugh and Bandy, 2000; Phillips et al., 1997) or attenuate the deprivation effect associated with increased ethanol intake (Holter et al., 1999).

The experiments presented here addressed these theories from several perspectives. First, the mutant mice described herein represent a second establishment of congenic C57BL/6J strains distinct from those used in our earlier studies. The absence of β-endorphin alone on ethanol reinforcement was further tested under a range of different procedural variables that have been previously shown to influence ethanol consumption in wild-type C57BL/6J mice (Middaugh and Kelley, 1999). Testing of enkephalin-deficient mice that produce normal levels of β-endorphin addressed a specific role for enkephalin peptides in ethanol reward. Finally, a comparison of phenotypes among the individual opioid peptide mutants and the double homozygous mutants tested whether β-endorphin and enkephalins have complementary, opposing, or interdependent roles in modulating the reinforcing properties of ethanol.

2. Methods

2.1. Animals

A total of 64 mice, 8 males and 8 females from each genotype ($ENK^{+/+}$, $END^{+/+}$; $ENK^{+/+}$, $END^{-/-}$; $ENK^{-/-}$, $END^{+/+}$ and $ENK^{-/-}$, $END^{-/-}$) congenic to the C57BL/6J background were generated from double heterozygote ($ENK^{+/-}$, $END^{+/-}$) mating pairs. Originally, heterozygous β-endorphin mutants (Rubinstein et al., 1996) at N9 on the C57BL/6 background were crossed to heterozygous enkephalin mutants (F2 hybrids of 129S8 and C57BL/6) (Nitsche et al., 2002; Ragnauth et al., 2001). Subsequently, double heterozygotes were backcrossed for at least 11 consecutive generations, alternating genders, to C57BL/6J mice newly acquired from The Jackson Laboratories (Bar Harbor, ME) to produce the congenic double heterozygous mice. Genotyping was performed as previously described (Hayward et al., 2002). All subjects were individually housed to accommodate restricted access to food and water as described below and were housed in a 12:12 light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee and followed the Public Health Service guidelines for the humane care and use of experimental animals.

The study was conducted in two blocks of 15 weeks each with the group (cohort) compositions balanced for genotype and gender between blocks (32 mice/cohort). This design was necessary to accommodate 64 mice with eight operant chambers and four sessions of 30 min each per chamber per day. The composition of the two cohorts differed significantly in age at the beginning of the study as detected by ANOVA [$F(1,48)=47.668$, $p<0.0001$]; a post hoc analysis found that ages in four groups were significantly different between the two cohorts. The range in ages (in weeks) for these four groups was as follows: female $Enk^{+/+}$, $End^{+/+}$ (cohort 1=9–16, cohort 2=21–25), female $Enk^{-/-}$, $End^{-/-}$ (cohort 1=11–18, cohort 2=16–25), male $Enk^{+/+}$, $End^{-/-}$ (cohort 1=6–13, cohort 2=12–24) and male $Enk^{-/-}$, $End^{-/-}$ (cohort 1=9–15, cohort 2=12–25). The ages of the other four groups were balanced between cohorts. The large difference in ages was unavoidable due to the low frequency of the

individual genotypes in offspring of $Enk^{+/-}$, $End^{+/-}$ mating pairs.

2.2. Equipment

Four 16×14×13 cm and four 22×18×13 cm modular operant chambers (Med Associates, Georgia, VT), designed specifically for use by mice and equipped with ultra-sensitive, retractable response levers and liquid dippers, were used in these experiments. There was a single active lever that counted towards a reinforcer and a single inactive lever that served as a control for specificity of activity in the operant chamber. When a session began the levers extended into the chamber and a house light (100 mA) was illuminated. Completion of the instrumental contingency on the active lever turned off the house light and turned on a dim white LED stimulus light above the liquid dipper receptacle for the 10-s duration of availability of the 20 μl liquid dipper cup, following which the house light turned back on and the stimulus LED turned off as the dipper retracted. The positions of the active and inactive levers were counterbalanced between mice but remained consistent between sessions for each mouse. The dipper delivered the reinforcing solution from a trough located between the two levers. The chambers were individually enclosed in ventilated, light and sound attenuating cabinets. Operation of the machines was fully controlled through a computer interface and software designed by Med Associates (MedPC for Windows).

2.3. Experimental protocol

The shaping and testing trials are summarized in Table 1 and were adapted from the procedures of Middaugh and Kelley (1999) who studied wild-type C57BL/6J mice. Mice were housed individually and all training and experimental trials were conducted in daily (Mon–Fri) 30-min sessions from 10:00 to 13:00. During training phase 1 mice were food-restricted to 80% of ad lib feeding body weights and habituated to the chambers. In all subsequent phases labeled "postprandial," the daily food allotment was provided 1 h prior to testing except the testing in experimental phase 3, labeled "preprandial," which was immediately followed by provision of the daily food allotment. Water was restricted during the first 6 days of shaping, so that subjects were given 2 h of access to water at the end of the operant session. Subsequent to this shaping period, water was freely available except during the 1-h feeding period prior to testing (or the 1-h immediately preceding the preprandial and ad lib test sessions) and the time in the operant chambers. The active and inactive levers were introduced in training phase 2 and the reinforcement schedule increased from FR1 to FR2 on the 12th day of training phase 2. During training phase 3, mice were exposed to each new ethanol concentration for four consecutive days every week.

Experimental phase 1 was a response stabilization period of five continuous days of 12% ethanol self-administration under an FR2. During the experimental phases 2–6, the subjects were presented with ethanol concentrations that increased each day beginning with water on the first day. For experimental phases 2–4 and 6, sessions were 30 min long just as during shaping. For experimental phase 5, a PR2 reinforcement schedule was used whereby the number of responses required for each reinforcer increased by an increment of 2 (i.e. 2,4,6, etc.). The sessions terminated when 90 s passed with no responses on the active lever (i.e. "breaking point"). At the end of the session, the levers retracted and the total number of responses was recorded. Following experimental phase 5, food and water were given in the home cage with ad lib access. During experimental phase 6, all subjects were tested under ad lib feeding and water access except that water was removed 1 h prior to testing and returned immediately following testing.

2.4. Blood ethanol concentration

Because we were unable to ensure that all ethanol reinforcers were consumed, we measured blood ethanol concentrations (BECs) at the conclusion of the FR2, FR8, and PR2 postprandial sessions for 12% ethanol reinforcement (experimental phases 1,4, and 5, respectively). Twenty microliters of tail blood was collected into heparinized capillary tubes immediately following the operant sessions. Blood samples were processed for BEC analysis on an Agilent 6890 gas chromatograph as described (Ponomarev et al., 2002).

Table 1
Summary of experimental phases

Phase	Experimental focus	Duration (week)	Reinforcement schedule	[EtOH] (%)	Testing time
Training 1	Habituation, Food Deprivation	1	None	None	Postprandial
Training 2	Lever & Reward Training	3	FR1, FR2	0	Postprandial
Training 3	EtOH Reward Training	4	FR2	3, 6, 9, 12	Postprandial
Experimental 1	Response Stabilization	1	FR2	12	Postprandial
Experimental 2	EtOH Concentration/Response	1	FR2	0, 3, 6, 9, 12	Postprandial
Experimental 3	EtOH Concentration/Response	1	FR2	0, 3, 6, 9, 12	Preprandial
Experimental 4	EtOH Conc./Response/inc. FR	1	FR8	0, 3, 6, 9, 12	Postprandial
Experimental 5	EtOH Conc./Response/PR	1	PR2	0, 3, 6, 9, 12	Postprandial
Experimental 6	EtOH Conc./Response/FR	1	FR2	0, 3, 6, 9, 12	Ad lib feeding

2.5. Statistical analysis

A mixed factorial analysis of variance with repeated measures (RMANOVA) was used on the dependent variables. Training phases 2 and 3 were analyzed separately with gender, genotype, and cohort as between-group factors and shaping day as a within-group factor. The number of reinforcers earned in experimental phases 2 and 3 was analyzed with gender, genotype, and cohort as between-group factors and ethanol concentration and prandial state as within-group factors. The number of responses on the active lever for Experiments 4 and 5 was analyzed with gender, genotype, and cohort as between-group factors and ethanol concentration and schedule as within-group factors. However, the dose of ethanol self-administered per kilogram body weight (g/kg EtOH) was analyzed separately for the two schedules using RMANOVAs with gender, genotype, and cohort as between-group factors and ethanol concentration (excluding water) as the within-group factor. In Experiment 6, the number of reinforcers and the dose of EtOH self-administered were analyzed separately in RMANOVAs with gender, genotype, and cohort as between-group factors and ethanol concentration (excluding water for g/kg EtOH data) as the within-group factor. All post hoc analyses used Fisher's protected least significant difference (PLSD). BEC measurements (mg%) were subjected to a multifactorial ANOVA with gender, genotype, and cohort as between-group factors. All statistical analyses were performed using StatView 5.0 for the Macintosh (SAS Institute, Cary, NC). In all tests the criterion for significance was set at $p \leq 0.05$.

3. Results

All groups learned equally well to lever press for a water reinforcer as shown in Fig. 1A and B. Increasing numbers of water reinforcers were earned with additional days of training until the schedule was changed from an FR1 to an FR2. RMANOVA detected no main effects other than the within-

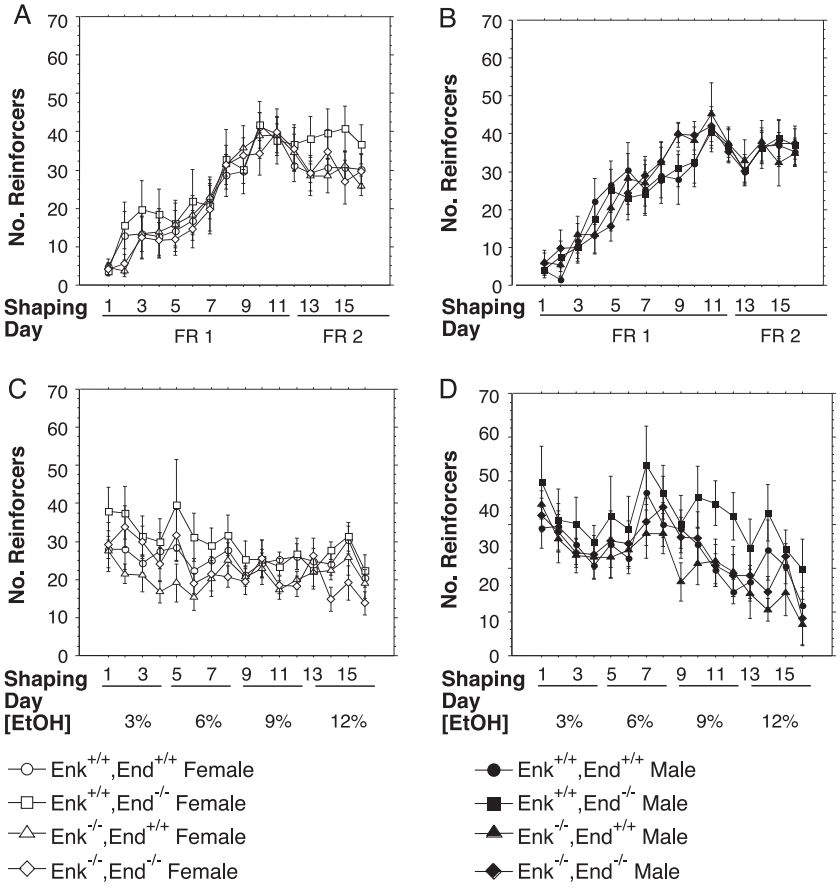

Fig. 1. Shaping of ethanol self-administration (training phases 2 and 3). Data represent the mean number of reinforcers earned±S.E.M. and are separated by gender and genotype as shown in the figure, open symbols represent females and closed symbols represent males. (A) The number of reinforcers earned by females during the first 16 days of operant responding for water reinforcement under an FR1 for the first 11 days and an FR2 for the last 5 days (training phase 2). (B) The number of reinforcers earned by males during the first 16 days of operant responding for water reinforcement under an FR1 for the first 11 days and an FR2 for the last 5 days (training phase 2). (C) The number of reinforcers earned during the first 16 days of operant responding for ethanol reinforcers by females of the given concentrations (training phase 3). Subjects were tested for four continuous days at each concentration. (D) The number of reinforcers earned during the first 16 days of operant responding for ethanol reinforcers by males of the given concentrations (training phase 3). Subjects were tested for four continuous days at each concentration.

group analysis of shaping day [$F(15,720)=67.0$, $p<0.0001$]. Once ethanol was introduced as a reinforcer, the subjects continued to maintain high levels of responding under an FR2 (Fig. 1C and D). However, female subjects (Fig. 1C) did not maintain as high a level of responding as males (Fig. 1D) to the increasing concentrations of ethanol until the highest concentration was reached. This was evidenced by a main effect of gender [$F(1,48)=17.74$, $p<0.0001$] as well as a Gender×Ethanol concentration interaction [$F(15,720)=4.272$, $p<0.0001$]. A main effect of shaping day was also detected [$F(15,720)=13.096$, $p<0.0001$].

The first experimental phase consisted of five continuous days of response stabilization under an FR2 to 12% ethanol. Analysis by RMANOVA of these data found a main effect of the testing days on the number of reinforcers earned [$F(4,192)=9.538$, $p<0.0001$], which could be largely attributed to an increase in the number of reinforcers earned during the first 3 days (Fig. 2A and B). No main effects by gender or genotype or interactions between these factors were detected.

Because we were unable to ensure that all ethanol reinforcers were consumed, we measured BECs. BEC measurements taken at the end of Day 5 in the first experimental phase revealed a main effect of genotype [$F(3,48)=7.65$, $p<0.001$] and post hoc analysis demonstrated that male Enk$^{-/-}$, End$^{+/+}$ were significantly lower than male Enk$^{+/+}$, End$^{+/+}$ mice and female Enk$^{-/-}$, End$^{+/+}$ and Enk$^{-/-}$, End$^{-/-}$ were significantly lower than female Enk$^{+/+}$, End$^{+/+}$ mice (Table 2). The number of reinforcers earned on Day 5 by females of each genotype was consistent with this trend, albeit not significant by post hoc analysis (Fig. 2A), but the responses by the males were not consistent with their BEC difference (Fig. 2B). These data suggested that the number of responses for ethanol were generally consistent with consumption and overall there was a significant correlation between the number of reinforcers

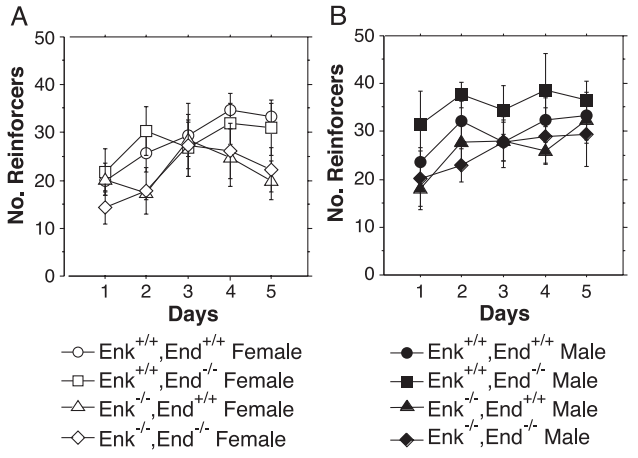

Fig. 2. Response stabilization for 12% ethanol under an FR2 (experimental phase 1). Data represent the mean number of reinforcers earned±S.E.M. and are separated by genotype as shown. All subjects responded for 12% ethanol on all 5 days. (A) The number of reinforcers earned by females. (B) The number of reinforcers earned by males.

Table 2
Blood ethanol concentrations (mg%) following 12% EtOH self-administration

	Exp. 1 FR2	Exp. 4 FR8	Exp. 5 PR2
Male Enk$^{+/+}$, End$^{+/+}$	273±32	174±23	89±34
Male Enk$^{+/+}$, End$^{-/-}$	218±18	187±25	108±25
Male Enk$^{-/-}$, End$^{+/+}$	189±13*	152±15	107±27
Male Enk$^{-/-}$, End$^{-/-}$	202±32	205±42	94±24
Female Enk$^{+/+}$, End$^{+/+}$	263±24	154±42	116±34
Female Enk$^{+/+}$, End$^{-/-}$	234±40	234±10	108±25
Female Enk$^{-/-}$, End$^{+/+}$	130±31*	186±23	86±23
Female Enk$^{-/-}$, End$^{-/-}$	92±31*	165±37	110±30

* $p<0.05$ compared to wild-type of the same gender.

earned and BEC for individuals ($r^2=0.21$, $p=0.001$ for experimental phase 1).

Following the response stabilization period, the subjects were tested for five continuous days with increasing concentrations of ethanol in a 1-h postprandial paradigm (experimental phase 2) and then for 5 days in a preprandial paradigm (experimental phase 3). These experiments found that increased thirst, produced by feeding with no water available 1 h before testing, produced higher responding than when the subjects were fed following testing (Fig. 3A and B). This was evidenced by a main effect of prandial state on responding for ethanol [$F(1,47)=104.85$, $p<0.0001$]. No main effect of genotype or an interaction with other factors was detected, however, a main effect of gender was detected [$F(1,47)=8.662$, $p=0.005$]. A simple main effect analysis determined that this effect was due primarily to males (Fig. 3B) having consistently higher responses than females (Fig. 3A) of the same genotype in both prandial states [Experiment 2, $F(1,48)=8.353$, $p=0.0058$; Experiment 3, $F(1,48)=5.341$, $p=0.0252$].

During experimental phases 4 and 5, the subjects were tested for five continuous days under an FR8 and then under a PR2. The subjects responded to the increased work requirement under a PR2 by increasing their number of active lever responses specifically for ethanol (Fig. 4A and B). This was evidenced by a main effect of schedule [$F(1,48)=8.713$, $p=0.0049$] and a Schedule×Ethanol concentration interaction [$F(4,192)=4.146$, $p=0.003$]. There were no main effects of genotype or gender or interactions between these or between the within-group factors. However, a main effect of the two cohorts in which the study was conducted was detected [$F(1,48)=10.1$, $p=0.0026$]. Further analyses of these two cohorts found a main effect of cohort on body weight throughout the entire study [$F(1,48)=22.439$, $p<0.0001$]. Since body mass can influence the amount of ethanol consumed, we also analyzed the dose of ethanol received in experimental phases 4 and 5, thus normalizing for body weight differences. During experimental phase 4 (Fig. 5A and B), we found no main effect of genotype, gender, and cohort or interactions among these factors but we found a main effect of ethanol concentration on the dose of ethanol received [$F(3,144)=126.784$, $p<0.0001$]. Samples were analyzed for BEC

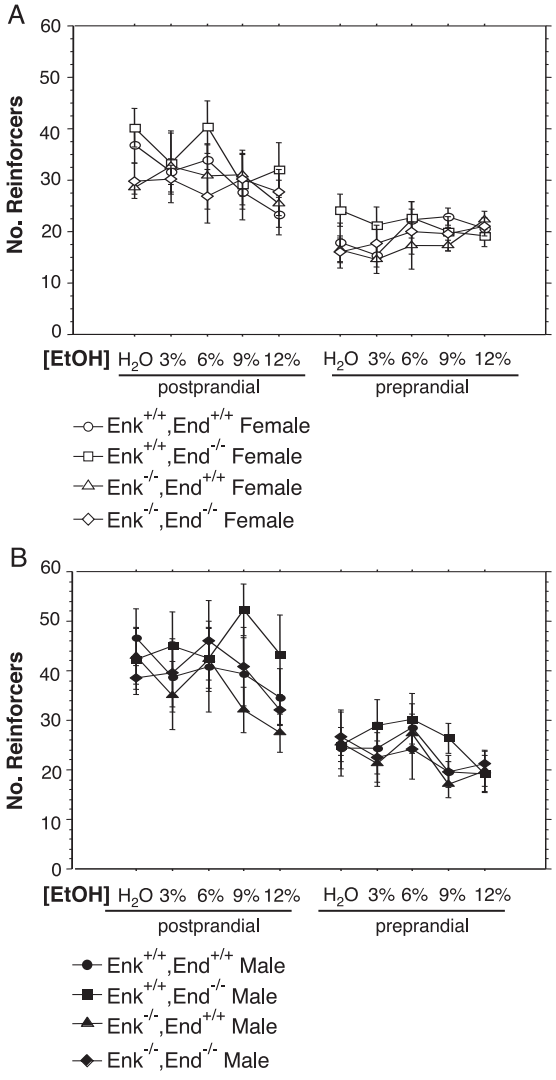

Fig. 3. Ethanol self-administration under an FR2 comparing a 1-h postprandial and preprandial procedure (experimental phases 2 and 3). Data represent the mean number of reinforcers earned±S.E.M. and are separated by gender and genotype as shown in the legend, open symbols represent females and closed symbols represent males. (A) Female results from 5 days of the 1-h postprandial procedure first (experimental phase 2) and 5 days of the preprandial procedure last (experimental phase 3). (B) Male results from 5 days of the 1 h postprandial procedure first (experimental phase 2) and 5 days of the preprandial procedure last (experimental phase 3).

immediately following the 12% ethanol sessions under the FR8 and similar to the lever response data we found no main effect of gender or genotype (Table 2). A main effect of ethanol concentration on the dose of ethanol received was also detected during experimental phase 5 [$F(3,144)$= 209.508, $p<0.0001$] (Fig. 5C and D). However, no main effect of gender, genotype, and cohort, or interactions among these factors was detected. Samples were analyzed for BEC immediately following the 12% ethanol sessions under the PR2 and similar to the lever response data there was no main effect of gender or genotype (Table 2).

Following the experiments conducted under food restriction, we conducted a similar experiment with the mice having free access to food and water. In experimental phase 6, the subjects were tested under a FR2 (Fig. 6). Analysis of the number of reinforcers earned found main effects of genotype [$F(3,48)=3.61$, $p<0.05$], cohort [$F(1,48)=12.57$, $p<0.001$], and ethanol concentration [$F(4,192)=6.21$, $p=0.0001$]. A post hoc analysis found that for 3% and 6% ethanol concentrations female $Enk^{+/+}$, $End^{-/-}$ earned more reinforcers than any other group (Fig. 6A and B). Interestingly, responses by female $Enk^{-/-}$, $End^{-/-}$ were identical to their wild-type counterparts. However, there was no interaction of genotype and gender [$F(3,48)=2.21$, $p=0.10$ n.s.] and no three-way interaction of genotype, gender, and ethanol concentration [$F(12,192)=1.35$, $p=0.20$ n.s.]. Since

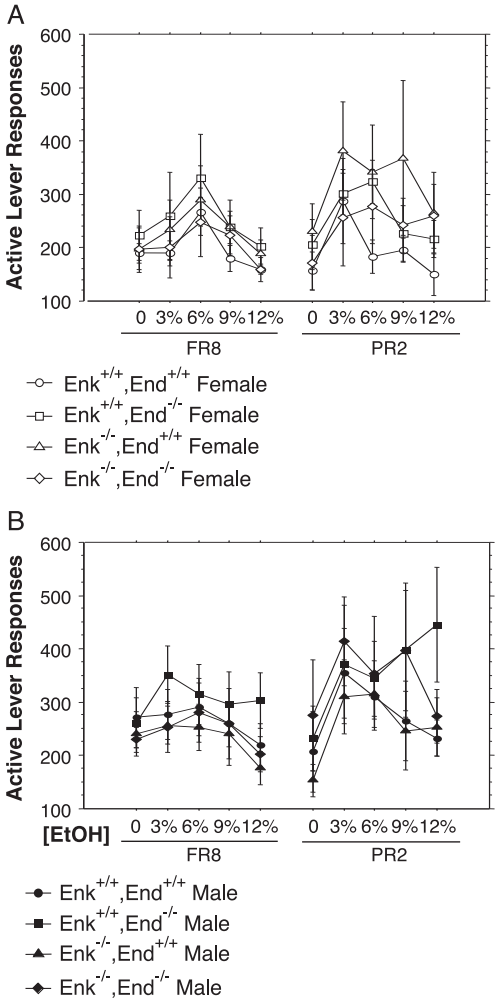

Fig. 4. Ethanol self-administration comparing an FR8 (experimental phase 4) and a PR2 schedule (experimental phase 5) using a 1-h postprandial procedure. Data represent the mean±S.E.M. and are separated by gender and genotype as shown in the figure, open symbols represent females and closed symbols represent males. (A) The number of female responses on the active lever for increasing concentrations of ethanol first under an FR8 and then a PR2. (B) The number of male responses on the active lever for increasing concentrations of ethanol first under an FR8 and then a PR2.

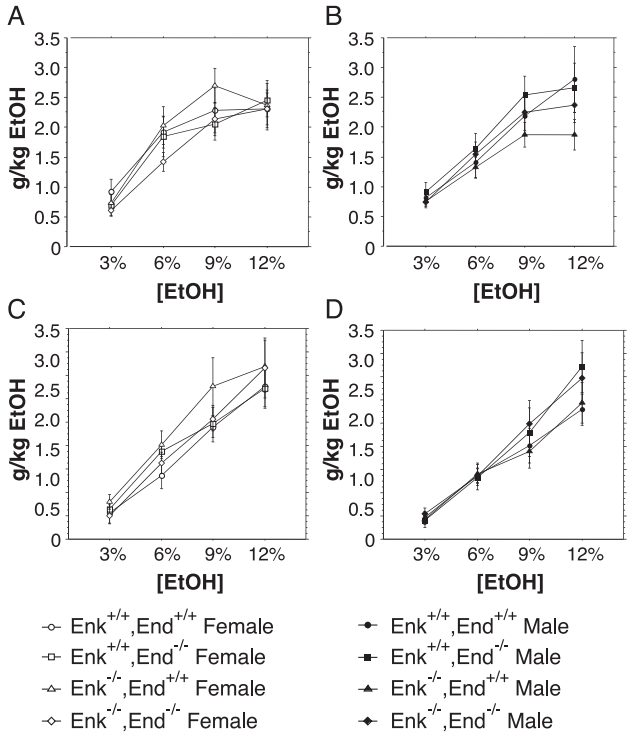

Fig. 5. Dose of ethanol received during self-administration sessions comparing an FR8 (experimental phase 4) and a PR2 schedule (experimental phase 5) using a 1-h postprandial procedure. Data represent the mean±S.E.M. and are separated by gender and genotype as shown in the figure, open symbols represent females and closed symbols represent males. (A) The dose of ethanol received by females under the FR8 procedure. (B) The dose of ethanol received by males under the FR8 procedure. (C) The dose of ethanol received by females under the PR2 procedure. (D) The dose of ethanol received by males under the PR2 procedure.

there was a main effect of the cohort on this measure, a separate post hoc analysis conducted on each cohort also found significant differences between female $Enk^{+/+}$, $End^{-/-}$ and the other groups. Importantly, there were no interactions between cohort and either gender [$F(1,48)=1.03$, $p=0.42$ n.s.] or genotype [$F(3,48)=0.85$, $p=0.47$ n.s.], nor was there a three-way interaction among these factors [$F(3,48)=0.81$, $p=0.49$ n.s.], indicating that $Enk^{+/+}$, $End^{-/-}$ females behaved similarly in the two cohorts.

Since body weight was likely one of the factors contributing to the differences between the two cohorts, we also analyzed the dose of ethanol received (Fig. 6C and D) and again detected a main effect of cohort [$F(1,48)=19.18$, $p<0.0001$] and ethanol concentration [$F(3,144)=64.45$, $p<0.0001$]. No main effects of gender [$F(1,48)=2.5$, $p=0.12$ n.s.], genotype [$F(3,48)=2.24$, $p=0.095$] or Gender×Genotype interactions [F (3,48)= 1.24, $p=0.30$ n.s.] were detected. A post hoc analysis found that for 3% and 6% ethanol concentrations, female $Enk^{+/+}$, $End^{-/-}$ responded for more reinforcers than any other group (Fig. 6A and B) even when the two cohorts were analyzed separately, consistent with the number of reinforcers earned. Importantly, there were no interactions between cohort and either gender [$F(1,48)=1.75$, $p=0.19$ n.s.] or genotype [$F(3,48)=1.34$, $p=0.27$ n.s.], nor was there a three-way interaction among these factors [$F(3,48)=1.02$, $p=0.39$], indicating that $Enk^{+/+}$, $End^{-/-}$ females behaved similarly in the two cohorts. Under this ad lib feeding condition, the motivation to consume ethanol was apparently decreased compared to the restricted feeding conditions. A comparison of the number of reinforcers earned during restricted feeding conditions (Fig. 3A and B, postprandial section) and ad lib feeding conditions (Figs. 6A and 6B) illustrates that free access to food for the mice between testing sessions resulted in a dramatic drop in their ethanol self-administration under the same FR2 schedule.

4. Discussion

This study examined the role of endogenous opioids in oral ethanol self-administration by operant responding, a standard behavioral test that is ideally suited to measure the reinforcing efficacy of positive stimuli and has previously been shown to be modulated by endogenous opioid peptides in mice (Middaugh et al., 1999). To specifically assess the

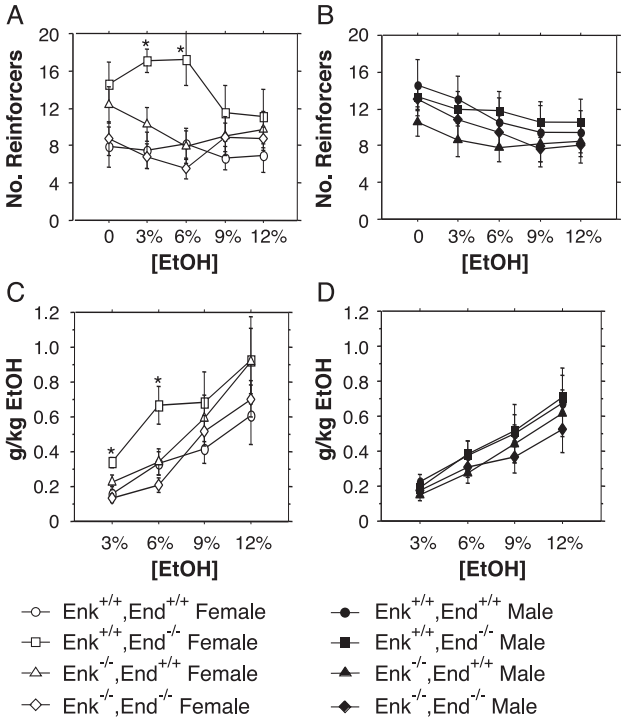

Fig. 6. Ethanol self-administration under an FR2 schedule using ad lib feeding subjects (experimental phase 6). Data represent the mean±S.E.M. and are separated by gender and genotype as shown in the figure, open symbols represent females and closed symbols represent males. (A) The number of reinforcers earned by females for increasing concentrations of ethanol. (B) The number of reinforcers earned by males for increasing concentrations of ethanol. (C) The dose of ethanol received by females in the same experiment. (D) The dose of ethanol received by males in the same experiment. *$p<0.05$ compared to all other groups by post hoc Fisher's PLSD.

role of β-endorphin and enkephalin, we used mice completely lacking either of these peptides individually or together. Additionally, we used mutant mice congenic to the C57BL6/J background, an alcohol preferring strain. This unique approach allowed us to potentially discriminate between the relative contributions of two classes of endogenous opioid modulation, something that standard opioid receptor antagonists do not allow. These two peptides are the primary endogenous ligands for the MOR, a receptor previously shown to modulate ethanol self-administration in numerous studies. We have previously published data from male β-endorphin knockout mice and these studies surprisingly found that β-endorphin knockout mice responded more for ethanol and consumed more ethanol in two-bottle free-choice drinking and intravenous self-administration studies (Grahame et al., 1998; Grahame et al., 2000; Grisel et al., 1999). However, no study has examined ethanol drinking in enkephalin knockout mice or mice lacking expression of both peptides.

An important focus of this study was the role of procedural variables on ethanol self-administration in the mutant genotypes. The data comparing prandial states on ethanol self-administration confirm that the subjects were likely not consuming ethanol for its caloric value but that the postprandial paradigm did significantly increase thirst, consistent with previous observations (Elmer et al., 1986). If the increased thirst produced by prefeeding masked any phenotype associated with increased motivational state of the subjects, we might have expected to detect differences between the genotypes in the preprandial condition. For example, postprandial conditions support self-administration of ethanol in C57BL/6J and BALB/cJ mice but preprandial conditions only support ethanol self-administration in C57BL/6J mice (Elmer et al., 1987). The data comparing the FR8 and PR2 postprandial tests demonstrated that the subjects increased responding under a PR2 in response to increased requirements for reinforcers, especially when responding for ethanol but not for water (Fig. 4A and B). However, the overall dose received under a PR2 was less than under an FR8, indicating that the subjects had not reached a ceiling of ethanol ingestion. In fact, our BEC data are consistent with these data since under an FR2 high levels of blood ethanol were attained, under an FR8 lower blood levels of ethanol were detected and under a PR2 still lower levels were attained (Table 2).

The ad lib feeding condition supported responding under an FR2 and was the only condition where a significant genotype difference in responding was detected. This effect of feeding state was consistent with our previous studies on food reward where decreased responding by the same opioid mutant strains was detected only under ad lib feeding conditions (Hayward et al., 2002). We previously suggested that food deprivation might suppress opioid action or actively override it. Considerable evidence suggests that food deprivation can reduce or completely block the effect of opioid antagonists on food reinforcement (Hayward and Low, 2001; Rudski et al., 1994), two-bottle free choice for saccharin (Lynch et al., 1983) and free feeding (Weldon et al., 1996). The fact that the food-responding and ethanol-responding phenotypes were in opposite directions in the β-endorphin knockouts is interesting in that it suggests that mice may not consume ethanol for its caloric value or its oral sensory stimulation (Bachmanov et al., 1996).

In our previous study on food reinforcement, we found that although operant responding was reduced in the same strains of opioid mutant mice, the relative preference for the reinforcers, which varied in palatability, was not altered. Additionally, preference for sucrose or saccharin in a two-bottle free-choice paradigm was also unchanged in the knockout mice (Hayward et al., 2002). Thus, β-endorphin and enkephalin knockout mice do not appear to have a general deficit in recognizing palatable flavors or an alteration in their preference for these flavors. Interestingly, β-endorphin knockout mice had increased preference for ethanol in the two-bottle free-choice procedure (Grisel et al., 1999) and increased lever pressing for ethanol here. Thus, it appears that the β-endorphin null mutation had opposing effects on food reinforcement and ethanol reinforcement, suggesting that at least β-endorphin may contribute to food and ethanol reinforcement via different neuronal pathways. In fact, we have evidence that β-endorphin is not involved in sucrose preference in the two-bottle free-choice test (Hayward et al., 2003).

A comparison of our data to previous studies on ethanol reinforced operant responding in C57BL/6J mice found general agreement with these studies. The differences in postprandial and preprandial responding under a FR2 (experimental phases 2 and 3) were consistent with those reported in several previous papers (Elmer et al., 1986; Middaugh et al., 1999; Middaugh et al., 2000). Responding was significantly higher in our study under the FR8 (experimental phase 4) than in Middaugh and Kelley (1999) but BECs in our wild-type mice were identical to theirs, suggesting that equipment differences may be responsible for the observed differences in responding but that amounts of ethanol ingested were actually consistent between the two studies.

One problem we had in interpreting our data was that the cohort factor interacted significantly with some of the other factors as we reported in the Results section. We were able to overcome this limitation by correcting the dose of ethanol received by body weight. Body weights varied significantly between cohorts throughout the entire study, likely because β-endorphin knockout males have an obese phenotype (Appleyard et al., 2003) and the differences in ages between the two cohorts (see Methods). The age of the subjects also likely contributed to the differences in behavior since previous studies have suggested that age of mice can influence ethanol drinking (Domiati-Saad et al., 1993). This condition was inevitable when we tried to balance mutant genotypes and genders in the two cohorts by using double heterozygote mating pairs. We were concerned that one

genotype could have a phenotype related to parenting (i.e. maternal effect) and so we chose to only use heterozygote mating pairs. The low Mendelian ratio of the useful genotypes from this breeding strategy was a tradeoff against the alternative use of parallel breeding colonies of homozygous mutant pairs.

The only data presented here that support a role for enkephalin in modulating ethanol self-administration were the BEC values at the end of the response stabilization period (experimental phase 1). The lower BECs in $Enk^{-/-}$, $End^{+/+}$ suggest that enkephalins may play a role in modulating ethanol self-administration at the highest concentration tested. However, the operant responses throughout the shaping period for ethanol or in the subsequent experimental phases do not support this conclusion, particularly when all the data are compared at the same ethanol concentration (12%).

The other significant genotype difference found in this study was that only female $Enk^{+/+}$, $End^{-/-}$ mice responded more for the lower ethanol concentrations (3% and 6%) compared to all other groups under an FR2 when maintained under ad lib feeding conditions. This difference is consistent with previous studies demonstrating that β-endorphin knockout mice responded more for ethanol and consumed more ethanol in two-bottle choice drinking and intravenous self-administration studies (Grahame et al., 1998; Grahame et al., 2000; Grisel et al., 1999). Our data are also consistent with observations on the differences in operant self-administration of ethanol between female and male mice (Middaugh and Kelley, 1999). Interestingly, the double mutant female $Enk^{-/-}$, $End^{-/-}$ mice did not behave similarly to the female $Enk^{+/+}$, $End^{-/-}$ mice. Possibly, the loss of enkephalin reversed the phenotype produced by the loss of β-endorphin.

A critical comparison to highlight between our current data and previously reported studies is that gene deletion of the MOR produced a significant reduction in operant responding for oral ethanol self-administration (Roberts et al., 2000). When considered with our results demonstrating no reduction in ethanol self-administration by β-endorphin and enkephalin deficient mice, we could infer that another endogenous MOR ligand subserves ethanol reinforcement. One obvious candidate is the high-affinity MOR peptide endomorphin, which has been demonstrated to support intracranial self-administration in rats (Zangen et al., 2002) but whose role in ethanol drinking or modulation of other reinforcers has not been reported. A less likely candidate could be dynorphin, which is usually considered to be the endogenous ligand for the kappa opioid receptor, but also has substantial affinity to the MOR (Chavkin et al., 1985; Raynor et al., 1994). We have previously shown that the β-endorphin knockout mouse specifically lacks expression of β-endorphin, leaving all of the other posttranslational products from the POMC prohormone intact except for full-length β-lipotropin hormone (Rubinstein et al., 1996). The enkephalin knockout was designed to eliminate all of the enkephalins, both met- and leu-enkephalins (Nitsche et al., 2002). While the three peptides from the prodynorphin prohormone contain the leu-enkephalin pentapeptide, these do not appear to produce detectable enkephalin levels in the enkephalin knockout mouse (unpublished observation). Thus, it is unlikely that any remaining opioids other than dynorphin or endomorphin could be still present in the lines of mice used here.

An alternative explanation for the conflicting observations between MOR knockout and opioid peptide knockout mice relates to the different paradigms used. Chronic food restriction has been shown to increase self-administration of many abused drugs (reviewed in Carr et al., 2002). Importantly, the study testing ethanol reinforcement in the MOR-1 knockout mice did not use food restriction (Roberts et al., 2000). Instead, the authors used a saccharin-fading procedure to establish ethanol consumption without increasing the motivational state of the subjects by caloric deprivation. As a consequence, ethanol intake in the Roberts et al. (2000) study was under 25% of that obtained in the current study based on a comparison of BECs. The final experimental phase. The final experimental phase of the current study (experimental phase 6) did examine ethanol self-administration under ad lib feeding conditions, where the dose of ethanol received by opioid peptide-deficient mice was similar to the study using the MOR knockout mice. Thus, if food restriction circumvented the opioid system, we would predict that the ad lib phase of our study would have had the same result as the MOR knockout if either β-endorphin or enkephalin was necessary for ethanol reinforcement. However, interpretation of the data from this final phase of the study is limited by the fact that these subjects had been maintained under food restriction for 3 months. Food restriction was shown to significantly affect behavior in different strains of mice even after they have been returned to ad lib feeding conditions (Cabib et al., 2000). Additionally, we did not determine BECs of these mice in the ad lib study.

A third explanation for the contradictory results could be that the differing genetic backgrounds of the mice in the two studies were responsible for the observed differences. Thus, MOR knockout mice made congenic to C57BL/6J might behave more similarly to the β-endorphin or enkephalin-deficient mice in this study. It should also be noted that the background of the mice used in this study differed considerably from our previous studies with the β-endorphin knockout mice (Grahame et al., 1998; Grahame et al., 2000; Grisel et al., 1999), in which incipient congenic or C57BL/6 mice from Simonsen Laboratories (Gilroy, CA) instead of Jackson Laboratories (Bar Harbor, ME) were used.

One interesting possibility is that the endogenous opioids may have differing affinities to the opioid receptor subtypes and that these are all involved in ethanol reward at different levels. Evidence to support this come from studies that have suggested the delta-2 receptor selectively reduces ethanol

consumption (June et al., 1999; Krishnan-Sarin et al., 1995a) but others have not found the same result using operant self-administration (Middaugh et al., 2000; Williams et al., 1998). Additionally, enkephalin's ability to inhibit forskolin-stimulated adenylyl cyclase was identical via the delta-1 and delta-2 receptors (Noble et al., 1996), suggesting that this endogenous opioid is not selective for one delta receptor subtype. Two forms of the mu receptor have also been suggested based on pharmacology (Pasternak, 2001) but selectivity for either of these two forms by endogenous opioids has not been demonstrated. It should also be pointed out that although pharmacological studies have indicated a possible heterogeneity within each class of opioid receptor (Reisine et al., 1995), the molecular cloning of the three opioid receptors has not found distinct genes encoding the receptor subtypes.

The results we present here confirm a paradoxical finding from our previous studies; the complete loss of β-endorphin expression had an unexpected phenotype of increased ethanol self-administration behavior under selective conditions (Grahame et al., 1998; Grahame et al., 2000; Grisel et al., 1999). Additionally, the loss of enkephalin expression seems to have had even less of an effect on ethanol self-administration behavior, and in subjects lacking both endogenous opioids, ethanol self-administration was unchanged. While our data do not necessarily contradict the large body of pharmacological evidence that suggests these opioids modulate ethanol ingestion, they do support a conclusion that neither of these peptides are necessary for animals to self-administer ethanol. A possible explanation for this lack of a dramatic effect may be that other nonopioid pathways have compensated for the loss of β-endorphin and enkephalin, resulting in the unpredicted phenotype. Compensatory changes resulting from the developmental consequence of β-endorphin deficiency are likely to be informative, if identified. Alternatively, endogenous opioids may modulate ethanol drinking when present but in their absence ethanol drinking is relatively unchanged.

In summary, the results presented here support the hypothesis that endogenous opioids can modulate ethanol ingestion under specific conditions, but perhaps the more apparent conclusion is that the endogenous opioids β-endorphin and enkephalin are not absolutely necessary to support oral ethanol self-administration. This conclusion is unexpected, given that MOR-deficient mice did not self-administer ethanol (Roberts et al., 2000). One interesting possibility is that β-endorphin may actually act through the delta receptor to inhibit ethanol drinking, which would be consistent both with the DOR knockout studies on ethanol self-administration (Roberts et al., 2001) and the results with the β-endorphin knockout mice reported here and elsewhere (Grahame et al., 1998; Grahame et al., 2000; Grisel et al., 1999). A final conclusion from this study is that β-endorphin and enkephalin apparently do not modulate ethanol self-administration in a similar manner to food self-administration (Hayward et al., 2002), suggesting that these endogenous opioids may contribute to food and ethanol reinforcement via different neuronal pathways.

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