β-Endorphin Mediates Behavioral Despair and the Effect of Ethanol on the Tail Suspension Test in Mice

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Background: The opioid peptide β-endorphin (β-E) is synthesized and released in response to stressful stimuli as well as acute alcohol administration. The release of β-E following exposure to an inescapable aversive situation may mediate behaviors that contribute to allostasis of the stress response. The present study examines the effects of β-E on immobility in assays involving inescapable stress, both under basal conditions and after acute administration of EtOH.

Methods: Female and male transgenic mice with varying capacities to translate β-E were subjected to either the forced swim (FST, Experiment 1) or the tail suspension test (TST, Experiment 2). In Experiment 3, mice were divided into three groups based on hormonal status (male, female-estrous, and female-nonestrous) and injected with either 1 g/kg EtOH or equivolume saline 14 minutes prior to behavioral assessment on the TST.

Results: Experiments 1 and 2 demonstrated a direct relationship between β-E levels and immobility. There were also sex differences in behavior in these tests, with males displaying more immobility than females. A main effect of genotype in Experiment 3 replicated findings in Experiments 1 and 2. There was also an effect of EtOH (increasing immobility) and a significant interaction reflecting a particularly robust effect of the drug in mice with low β-E. In addition, there were interactions between β-E, EtOH effects, and hormonal status.

Conclusions: These findings support the contention that β-E moderates behavioral responses to stressful stimuli and suggest a role for this peptide in coping behavior. Furthermore, the effects of EtOH on the response to stress may be mediated by β-E. Sex differences in this influence may contribute to sex differences in disease susceptibility and expression.

Key Words: Opioids, Depression, Anxiety, Transgenic, Alcohol.

THE ENDOGENOUS OPIOID peptide β-endorphin (β-E) is synthesized and released in response to stress and alcohol (EtOH) administration through activation of the hypothalamic-pituitary-adrenal (HPA) axis (Constanopoulos et al., 1995; Gianoulakis, 1990; Marinelli et al., 2004; Schedlowski et al., 1995). A member of the large family of endogenous opioids, β-E, possesses potent analgesic and addictive properties and serves a role in homeostatic functions (e.g., appetite, temperature) as well as in the rewarding and reinforcing properties of drugs of abuse, such as alcohol (Froehlich et al., 2000; Gianoulakis, 2004; Racz et al., 2008).

Following stimulation by corticotrophin releasing hormone (CRH), β-E is cleaved from the proopiomelanocortin (*POMC*) gene along with adrenocorticotropin hormone (ACTH). β-E contributes to the behavioral responses to stress (Amir, 1981; Grisel et al., 2008; Hunt and Zakhari, 1995; Janssen and Arntz, 2001) perhaps in part, by inhibiting secretion of CRH (Buckingham, 1986; Plotsky, 1991). Thus, release of β-E by acute activation of the HPA axis following exposure to an inescapable aversive situation may moderate the stress response and thereby facilitate endocrine and behavioral allostasis (McEwen, 2002).

Likewise, variations in sensitivity of the HPA axis and subsequent β-E production in response to stress exposure may underlie individual differences in coping behavior (Gianoulakis, 1998; Hunt and Zakhari, 1995). We have shown, for example, an inverse relationship between β-E levels and anxious behavior in mice (Grisel et al., 2008) suggesting decreased ability to behaviorally manage stressful stimuli with lower β-E levels, along with, physiologically, a blunted attenuation of the stress response (Gianoulakis, 1998; McEwen, 2001; Sarkar et al., 2007).

Compromised regulation of the stress response in low β-E subjects may have implications for a longstanding "opioid deficiency hypothesis" suggesting that those with low basal levels of β-E may be especially inclined to self-medicate with drugs of abuse (Gianoulakis, 2001; Koob and Le Moal,

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2008; Ulm et al., 1995; Zalewska-Kaszubska and Czarnecka, 2004). A number of experimental reports indicate that alcoholics, at-risk nonalcoholics, and animal models for these clinical groups have low β-E levels (Aguirre et al., 1995; Dai et al., 2005; Gianoulakis, 2001, 2004; Gianoulakis et al., 1996a,b; Grisel et al., 1999; Zalewska-Kaszubska and Czarnecka, 2004. Acute alcohol administration increases levels of pituitary β-E through activation of the HPA axis (Gianoulakis, 2001; Gianoulakis and Barcomb, 1987; Herz, 1997; Thiagarajan et al., 1989; de Waele and Gianoulakis, 1993). So by drinking, individuals with low β-E may be self-medicating a hyperactive stress axis along with its behavioral sequelae (Gianoulakis et al., 1989; Khantzian, 1985; Markou et al., 1998; Zalewska-Kaszubska and Czarnecka, 2004). Thus, it is hypothesized that in the absence of sufficient β-E for effective means of coping with stress, alcohol serves as an alternative coping mechanism.

In the present study, we investigated the role of β-E in moderating behavioral responses to an inescapable aversive stressor as well as the relationship between β-E and EtOH in the same situation. Because it is well documented that alcohol alters the opioid system (Gianoulakis, 1998; Gianoulakis et al., 1989) and that subjects with varying levels of β-E display varying effects of, as well as preference for, EtOH, (de Waele et al., 1992; Gianoulakis et al., 1992; Grisel et al., 1999, 2008; see Herz, 1997 for review) our study was aimed at determining the effects of β-E on coping capabilities in mice after acute administration of EtOH, using the forced swim and tail suspension tests.

MATERIALS AND METHODS

Subjects

Adult naïve male and female β-E-deficient (B6.129S2-*Pomc*^{tm1Low}/J; KO), heterozygous (HT), and wildtype (C57BL/6J; B6) mice were used in these experiments. These mice were developed over a decade ago in the laboratory of Malcolm Low (Rubinstein et al., 1996) by insertion of a premature stop codon into the *Pomc* gene. Homozygotes (KO) cannot synthesize β-E, though all other *Pomc* products show normal expression. Opioid receptor expression also remains unchanged (Rubinstein et al., 1996). HT mice produce 50% of B6 levels of β-E. Mice for these studies were bred in-house from stock purchased from Jackson Laboratories (Bar Harbor, ME). The gene mutation has been fully backcrossed to the C57BL/6J strain (>20 generations). HT mice were bred from KO males and B6 females; others were bred under identical conditions from genotype-matched pairs. They were group housed by sex with 4 to 5 per Plexiglas cage following weaning at 20 to 21 days and maintained in a 21 ± 2°C colony room with ad lib food and water on a reverse 12:12 light/dark cycle with lights on at 7 PM. Because β-E is known to help regulate energy homeostasis and to contribute to weight differences with increasing age (Low, 2004), all subjects were between 50 and 90 days old at time of testing and matched (within sex) for body weight. Pilot studies in our lab have shown no genotypic differences in brain or blood EtOH concentrations following a range of EtOH doses. Testing always occurred between 1000 and 1600 hours during the dark phase of the light/dark cycle, after at least 30 minutes habituation in a dimly lit testing room. During habituation, mice were weighed and tail marked according to experimental group. Testing order was counterbalanced across genotype, sex, and drug condition; and experimenters were blind to genotype and (as much as possible) drug injection. There were 8 to 10 subjects per genotype and sex, in each experiment, unless otherwise noted.

All procedures were carried out in accordance with the National Institutes of Health guidelines and were approved by the Animal Care and Use Committee of Furman University.

Experiment 1 and 2, Basal Immobility

Forced Swim Test. Mice were subject to a modified version of Porsolt and colleagues' (1977) forced swim test (FST) for 15 minutes in a white plastic 5 gallon bucket measuring 30 cm in diameter by 40 cm in height containing 20 cm of water maintained at 23°C. Mice were judged immobile when making no movements other than that required to stay afloat, for at least 5 seconds. Latency to immobility, total time spent immobile, and number of immobile segments were recorded.

Tail Suspension Test. Mice were hung by threading their tails through a 1-cm hole in a board measuring 10 cm by 20 cm. The board was suspended from a stand 30 cm in height. Tails were secured with lab tape, approximately 2 cm from the base of the tail to the opposite side of the board. Latency to immobility, number of immobile segments, and total time spent immobile were recorded during the 6-minute test.

Experiment 3, Tail Suspension Test With EtOH

Interactions between β-E and EtOH were evaluated using the Tail Suspension Test (TST). The FST and TST are thought to reflect the same substrates (Cryan et al., 2005; Kulkarni and Dhir, 2007) but at least in our hands, the TST is less variable and we were interested in minimizing the number of subjects required. During habituation, at the time of weight determination, females were visually checked for estrous by two independent experimenters using the basic strategy of Champlain and colleagues (1973). This procedure takes practice, but is relatively straightforward; the size, shape, and color of the vaginal opening differs between proestrous/estrous and nonestrous females. During experimenter training, we corroborated the validity of the visual method in our laboratory using cytology (vaginal smears) and demonstrating significant differences in body weight and behavior following experimenter-blind assessment. Only female mice that were scored identically (over 90%) were used in the study. There were 15 to 19 subjects per genotype and drug condition (saline or EtOH) to get a minimum of 5 subjects per genotype, condition, and hormonal state (male, estrous female, and nonestrous female). An intraperitoneal injection (i.p.) of either 1 g/kg EtOH (20% vol:vol) or equivolume saline was administered 14 minutes prior to TST evaluation, to evaluate behavior during the period when EtOH brain concentrations and effects are approaching peak levels. Mice were individually housed between injection and testing to control for differential interactions depending upon drug or saline. Latency to immobility, number of immobile segments, and total time spent immobile were recorded for 6 minutes.

Statistical Analysis

Data were analyzed separately for each experiment by factorial analysis of variance (ANOVA) in SPSS (SPSS, Inc., Chicago, IL): first by genotype, sex or hormonal status, and drug (where appropriate) and then, in the absence of significant interactions with sex/hormone status, collapsing across this factor. Significant main effects and interactions were investigated further using Tukey's HSD test for post hoc comparisons. In all cases, the criterion for significance (α level) was set at $p \leq 0.05$.

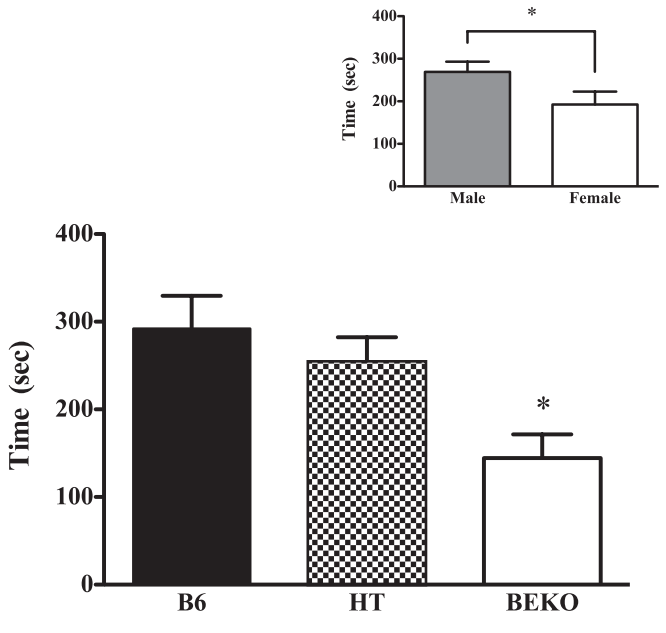

Fig. 1. Experiment 1 evaluated immobility in wildtype C57BL/6J (B6), heterozygote (HT), and β-E "KO" mice in the forced swim test. The *lower panel* shows the amount of time mice spent immobile during the 15-minute test (data show mean ± SE). βEKO mice differed significantly from B6's. The *inset panel* shows sex differences in time immobile, collapsed across strain. Significant differences were determined following ANOVA by post hoc analysis (Tukey's HSD) test and are designated by an asterisk (all p values ≤0.05).

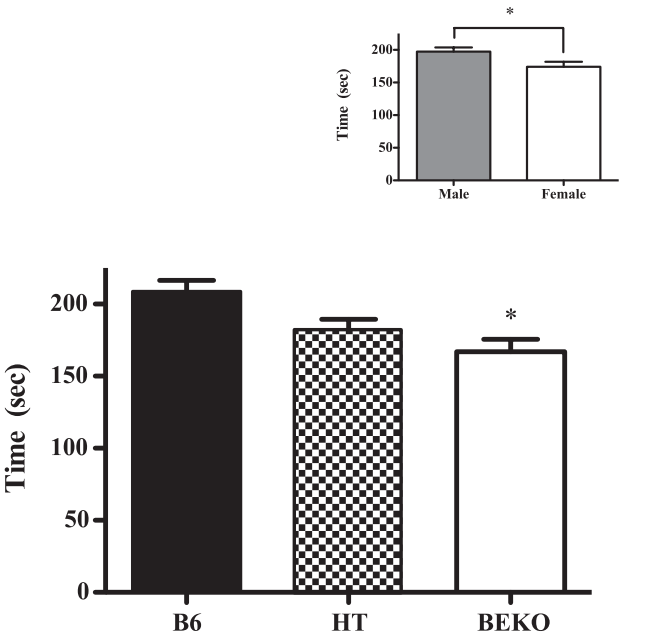

Fig. 2. Experiment 2 evaluated immobility of B6, heterozygote (HT), and βEKO mice in the tail suspension test. The *lower panel* shows the amount of time spent immobile during the 6-minute test (data show mean ± SE). There was a tendency for decreased immobility in HTs ($p = 0.068$ in Tukey's post hoc test). The inset panel shows sex differences in immobility time, collapsed across strain. Significant differences from control (B6) or between groups are designated with an asterisk following ANOVA by post hoc analysis (Tukey's HSD) test.

RESULTS

Experiment 1 and 2, Basal Immobility

β-endorphin genotype was directly related to immobility in both the FST and the TST. In the FST (see Fig. 1), B6 mice spent the most time immobile and the KO mice spent the least time immobile [$F(2,50) = 6.244$; $p < 0.005$]. Likewise, in the tail suspension test, increasing β-E increased immobility [Fig. 2; $F(2,49) = 8.77$; $p < 0.001$]. There were also significant effects of sex in both the FST [$F(1,50) = 4.469$; $p < 0.05$] and the TST [$F(1,49) = 6.658$; $p < 0.01$] with males spending more time immobile, though there were no significant interactions between genotype and sex on either of these tests ($p > 0.05$; see insets, Figs. 1 and 2). Despite a tendency toward increased latency to immobility as β-E levels decreased, neither this nor the total number of immobile segments depended upon genotype, sex, or their interaction (data not shown, $p > 0.05$).

Experiment 3

β-endorphin level was again directly correlated with the total time immobile in the TST [Fig. 3, left panel; $F(2,97) = 9.258$; $p < 0.001$] replicating the results of Experiment 1. There was also a main effect of drug on immobility, in that EtOH increased total time spent immobile [$F(1,97) = 3.888$; $p = 0.052$]. Moreover, the effect of EtOH depended upon genotype [$F(2,97) = 3.675$; $p < 0.05$] reflecting particularly robust drug effects in HT but not other genotypes (Tukey's HSD $p \leq 0.05$). These data are depicted in Fig. 3. There was no significant effect of sex (M/F) or hormone status (male, estrous female, and nonestrous female) nor was there any interaction with these factors (p's all >0.05).

In this study, although the number of immobile segments during the 6-minute test was no more prevalent as β-E levels increased [$F(2,97) = 0.181$; $p > 0.05$] or following EtOH [$F(1,97) = 0.966$; $p > 0.05$], EtOH increased the number of immobile segments in HT mice, as demonstrated by a significant interaction between genotype and drug [$F(2,97) = 3.594$; $p \leq 0.05$, and confirmed by Tukey's HSD; see Fig. 3, middle panel]. There were no significant effects of sex hormonal status or interactions with sex or hormonal status on the number of immobile segments displayed.

In terms of the latency to first demonstrate immobility (Fig. 3, right hand panel), there was a main effect of genotype [$F(2,95) = 3.914$; $p \leq 0.05$] and drug [$F(1,95) = 5.691$; $p \leq 0.05$] but no interaction [$F(2,95) = 0.141$; $p > 0.05$]. Thus, decreased β-E was associated with increased latency to become immobile, and EtOH generally decreased the latency to display immobility. However, for this measure, despite no significant main effect of sex or hormone status, there was a triple interaction between genotype, EtOH, and hormone/sex [$F(4,95) = 3.380$; $p \leq 0.05$; see Fig. 4]. In the left-hand panel of Fig 4, B6 mice demonstrate a tendency to assume immobility slightly more quickly with the administration of EtOH, independent of hormone status (though there is a nonsignificant trend for females in estrous to take longer to first become

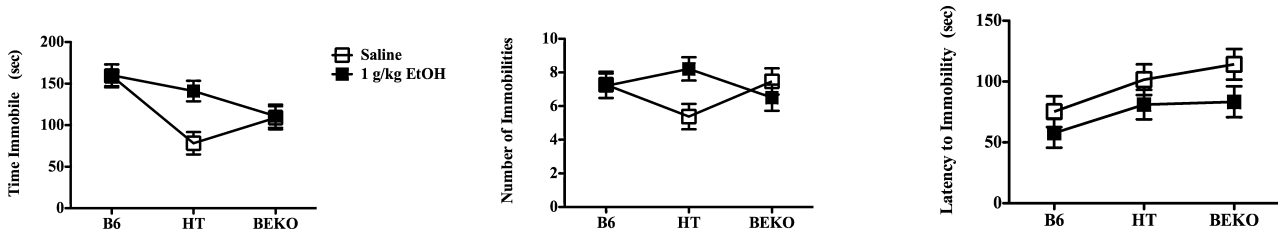

Fig. 3. Experiment 3 evaluated behavior of B6, heterozygote (HT), and βEKO mice in the tail suspension test (data show mean ± SE). The *left panel* shows time spent immobile during the 6-minute test in all genotypes following either saline or 1 g⁄kg EtOH, in which there were main effects of genotype and drug, as well as a significant interaction, reflecting enhanced drug efficacy in HT mice. The *middle panel* shows the number of immobilities in these groups during the same test, which did not depend upon genotype or drug, but again, a significant interaction between these factors reflects a drug effect in HT mice. The *right panel* shows the latency to become immobile during this test, which did depend upon genotype and drug. Significant differences between groups were determined following ANOVA by post hoc analysis (Tukey's HSD) and are discussed more fully in the text.

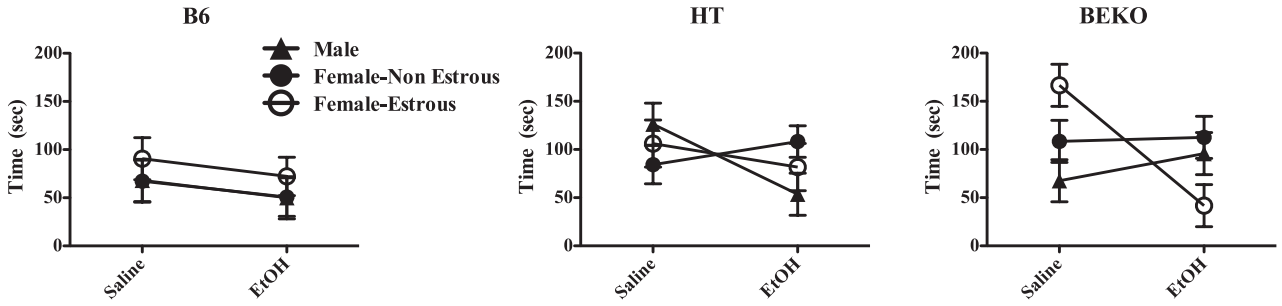

Fig. 4. A triple interaction between genotype, drug, and hormonal status on latency to first show immobility in Experiment 3 is shown here (data show mean ± SE). Effects of drug and genotype (depicted in Fig. 3) depended upon hormonal state; with decreased levels of β-E, EtOH effects became more genotype dependent.

immobile than either males or nonestrous females who are virtually identical each other). The far right panel of this figure shows the relationship between hormone status and drug effect in β-E deficient (KO) mice. Female mice in estrous that are entirely lacking β-E struggle the longest in the inescapable situation after saline injections but also show the most profound effect of EtOH in precipitating immobility.

DISCUSSION

Transgenic mice engineered with a modified capacity to transcribe the opioid peptide β-E showed altered behavioral responses in two murine assays of behavioral despair. The direct relationship found between β-E levels and amount of immobility in both the tail suspension and the forced swim tests (TST, FST) supports the idea that β-E contributes to the behavioral consequences of stress (Gianoulakis, 1998; Hunt and Zakhari, 1995; see Yamada and Nabeshima, 1995 and Ribeiro et al., 2005 for reviews). B6 mice assumed an immobile posture sooner and for a longer period of time in these assays than their counterparts with low or absent β-E. The TST and FST tests were used to subject mice to an inescapable aversive situation whereby failure to exhibit actions aimed at escape may represent an effective coping strategy in a despairing situation. Indeed, experimenters noted that mice deficient in β-E displayed frequent and intense struggling behavior rather than passive coping. Combined with previous results using the plus maze and light dark box assays, (Grisel et al., 2008) these data support the contention that β-E moderates behavioral responses to stressful stimuli.

A significant effect of low-dose EtOH (1 g⁄kg) on immobility in these tests was found primarily in the HT line of mice, suggesting a β-E-dependent behavioral response to the effects of EtOH. While HT mice behaved similarly to KO mice following a saline injection, HT mice injected with EtOH behaved similarly to B6 mice. This ability of EtOH to normalize behavior in mice with low levels of β-E may be related to their capacity to synthesize and release β-E in response to the drug. These results are consistent with previous findings that EtOH-induced anxiolysis is particularly strong in β-E-deficient mice (Grisel et al., 2008) and may help explain why HT mice, producing 50% of the wildtype amount of β-E, consistently self-administered more EtOH than β-E KO or B6 control mice (Grisel et al., 1999; Williams et al., 2007). The alcohol-seeking behavioral trend seen in mice with low levels of β-E (Grahame et al., 1998; Grisel et al., 1999) correlates with data showing that alcoholics and at-risk nonalcoholics have lower plasma and basal levels of β-E (Gianoulakis, 2001, 2004; Gianoulakis et al., 1996b; de Waele et al., 1992). These data support the hypothesis that increased preference for EtOH in HT mice may be due in part to increased sensitivity to the effects of EtOH (Froehlich et al., 1990; Gianoulakis et al., 1989; Williams et al., 2007; Zalewska-Kaszubska and Czarnecka, 2004). Furthermore, our data suggest that a

β-E deficiency may be indicative of a coping deficiency whereby insufficient attenuation of the stress response may be ameliorated by administration of EtOH. Thus, an individual who is less able to cope with stress (perhaps as a result of a genetic inability to produce sufficient β-E for allostasis of the stress response) may be inclined to self-medicate with alcohol as a substitute coping mechanism.

Transgenic models like the ones used in this study can provide insight into neural substrates of behavior, but data interpretation should evince an appreciation (if not understanding) of the complex, interactive brain systems underlying behavior. Indeed, we have argued elsewhere (Grisel et al., 2008) that a constitutive lack of β-E leads to an over-active stress axis (demonstrated behaviorally and physiologically) and that compensatory adaptation may involve changes in gene expression, chemical signaling, and consequent sensitivity to drugs and behavioral tests. In the present study, selective effects of EtOH in HT mice suggest a direct effect of β-E. This contrasts with our previous results showing augmented effects of EtOH in both HT and KO mice (Grisel et al., 2008). Contributions of both direct and indirect effects of β-E are probable and will be revealed with more empirical studies, but it is worth noting that transgenic models may enable insight about the functional interplay between contributing neural factors (e.g., Mogil and Grisel, 1998).

The combination of rewarding and positively reinforcing effects of EtOH is theorized to prompt alcohol drinking, while the negatively reinforcing effects of drinking are theorized to play a larger role in maintaining chronic alcohol drinking, leading in some cases to the development of alcoholism (see Koob and Le Moal, 2008 for a recent review). Acute administration of EtOH induces an increase in the synthesis and release of β-E in the hypothalamus and pituitary gland (Keith et al., 1986; de Waele and Gianoulakis, 1993) as well as dopamine in the nucleus accumbens leading to positive reinforcement (Gianoulakis, 1998; Goldowitz et al., 2006; Koob and Le Moal, 1997; Markou et al., 1998; de Waele and Gianoulakis, 1993). Negative reinforcing effects have been linked to anxiety-reduction following exposure to alcohol (Goldowitz et al., 2006; Kiefer et al., 2002). Thus, increased release of β-E by acute EtOH administration may encourage the acquisition of alcohol drinking, but decreased synthesis and release of β-E with chronic use may be partly responsible for the maintenance of alcohol drinking (Aguirre et al., 1990; Genazzani et al., 1982; Kiefer et al., 2002; Sarkar et al., 2007; Scanlon et al., 1992; de Waele and Gianoulakis, 1993). Chronic alcohol drinking has been hypothesized to produce tolerance to the effects of alcohol and desensitize the β-E system (Goldowitz et al., 2006; Koob, 2003). Chronic exposure, therefore, may lower levels of β-E and subsequently induce overactivation of the HPA axis (Wand, 2001) such that alcohol withdrawal induces anxiety. Thus, to reduce this anxiety and discomfort, alcohol drinking becomes negatively reinforcing and is maintained by the need to remove the aversive stimulus resulting, perhaps in part, from down-regulation of β-E (Aguirre et al., 1995; Becker and Lopez, 2004; Diana et al., 1993; Scanlon et al., 1992; Valdez et al., 2004).

Of the many factors influencing the development of alcoholism in humans (Froehlich et al., 1990), the genetically determined amount of β-E produced (Froehlich et al., 1990; Gianoulakis et al., 1996b; Wand et al., 1998) as well as environmental stressors and the ability to cope with those stressors is modeled in our study. In addition, though the majority of animal studies employ only male subjects, we included both sexes to assess hormonal effects that may underlie sex differences. For instance, the statistic that females have significantly lower alcoholism rates than males (Brady and Randall, 1999; Hettema et al., 2003; Hunt and Zakhari, 1995) suggests the possibility of sex-dependent coping mechanisms. Our experiments found that female mice were significantly less immobile than males in both the FST and the TST. After acute administration of EtOH, however, we found a triple interaction between strain, drug, and hormonal status (we evaluated three groups: male, female nonestrous, and female estrous) on the latency to become immobile in the TST. In general, with decreasing levels of β-E, EtOH's effect in the TST became more hormone dependent (Fig. 4). Female β-E-deficient mice in estrous were by far the most sensitive to EtOH, showing about a 4-fold reduction in latency to immobility relative to saline-injected controls. While the mechanisms underlying the relationship between EtOH, β-E, and sex hormones are unclear, our data suggest a role for sex hormones in coping abilities depending on levels of β-E. Numerous epidemiological and genetic studies (Grant et al., 2005b; Hasin et al., 2007; Hettema et al., 2003; Hunt and Zakhari, 1995; Racz et al., 2008) show significant sex differences in the risk and prevalence of disorders associated with chronic stress; for men, alcohol abuse and dependence, whereas for women, major depressive disorder. These findings suggest sexually dimorphic means of coping; however, future research is needed to elucidate the mechanisms responsible, which may lead to sex-specific approaches to understanding and treatment of anxiety, alcoholism, and depression.

The comorbidity of alcohol abuse and dependence, anxiety disorders, and major depressive disorder has long been observed (Brook et al., 2002; Grant and Harford, 1995; Helzer and Pryzbeck, 1988; Hettema et al., 2003; Kushner et al., 2000; Regier et al., 1990), but less is known of the shared neural mechanisms involved in all three disorders. While correlation does not prove causation, epidemiological studies (Grant et al., 2005a; Hasin et al., 2007; Nunes and Rounsaville, 2006; Nurnberger et al., 2001; Schuckit, 2006) consistently reveal links between genetic risk factors, substance use, and psychiatric disorders. For example, alcohol dependence reliably precedes the onset of major depression for males while anxiety disorders reliably precede the onset of alcoholism and depression (across both sexes; Hettema et al., 2003). Moreover, genetic studies have elucidated similar origins in the development of alcoholism and depression (Hettema et al., 2003; Nurnberger et al., 2001; Todd et al., 1996). Because stress and the ability to cope with stressful

stimuli are implicated as causal factors in the development of anxiety, alcoholism, and depression (Bastürk et al., 2000; Brown et al., 1995; Darko et al., 1992; Koob, 2006; Racz et al., 2008 for example), β-E may be a common mediator, either directly or indirectly, of these disorders. Our data support the notion that those with low β-E may be less able to effectively cope with stressful stimuli as a result of insufficient attenuation of the stress response, and thus be more inclined to suffer from anxiety, self-administer alcohol, and develop alcoholism and depression. The fact that most alcoholics are men and that most sufferers of major depression are women, in light of the present findings of sex-dependent influences of β-E, suggests that this peptide may help mediate these differences. Overall, the results of our study support the contention that β-E plays an active role in coping behavior and may be implicated in the complex interplay of stress-related disorders.

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