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Sex differences in binge-like EtOH drinking, corticotropin-releasing hormone and corticosterone: effects of β-endorphin

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ABSTRACT

Binge drinking is an increasingly common pattern of risky use associated with numerous health problems, including alcohol use disorders. Because low basal plasma levels of β-endorphin (β-E) and an increased β-E response to alcohol are evident in genetically at-risk human populations, this peptide is thought to contribute to the susceptibility for disordered drinking. Animal models suggest that the effect of β-E on consumption may be sex-dependent. Here, we studied binge-like EtOH consumption in transgenic mice possessing varying levels of β-E: wild-type controls with 100% of the peptide (β-E +/+), heterozygous mice constitutively modified to possess 50% of wild-type levels (β-E +/−) and mice entirely lacking the capacity to synthesize β-E (−/−). These three genotypes and both sexes were evaluated in a 4-day, two-bottle choice, drinking in the dark paradigm with limited access to 20% EtOH. β-E deficiency determined sexually divergent patterns of drinking in that β-E −/− female mice drank more than their wild-type counterparts, an effect not observed in male mice. β-E −/− female mice also displayed elevated basal anxiety, plasma corticosterone and corticotropin-releasing hormone mRNA in the extended amygdala, and all of these were normalized by EtOH self-administration. These data suggest that a heightened risk for excessive EtOH consumption in female mice is related to the drug's ability to ameliorate an overactive anxiety/stress-like state. Taken together, our study highlights a critical impact of sex on neuropeptide regulation of EtOH consumption.

Keywords BNST, CeA, CRF, HPA axis, POMC, stress.

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INTRODUCTION

The causes and consequences of drug use differ between men and women, although the mechanisms determining sex-dependent trajectories remain poorly understood. For example, a longstanding gap between the sexes in alcohol use disorders and their consequences has been shrinking as problematic incidence in women has risen over the past few years (Keyes *et al.* 2011). One possible contribution to this rise is an increase in female rates of binge drinking, a pattern that predicts negative health outcomes, including alcohol use disorders (Jennison 2004). According to the National Institute on Alcohol Abuse and Alcoholism, binge drinking is a pattern of excessive alcohol consumption that results in blood ethanol concentrations (BECs) of 80 mg/dl or above, usually a result of a woman having four, or man having five, drinks within about 2 hours (National Institute on Alcohol and Alcoholism Advisory Council 2004). Researchers hypothesize that women may be particularly susceptible to negative reinforcing effects of alcohol consumption because they are more prone to stress and anxiety (Lehavot *et al.* 2014).

The endogenous opioid peptide, β-endorphin (β-E), has long been implicated in EtOH consumption (Herz 1997). β-E, derived from the precursor proopiomelanocortin (POMC), is an agonist with high affinity for μ-opioid and δ-opioid receptors (Raffin-Sanson *et al.* 2003) where it modulates EtOH reward (Gass & Olive

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2007; Roth-Deri *et al.* 2008). In the clinic, basal levels of plasma β-E, as well as a rise in this peptide following alcohol administration, correlate with a heritable risk for high consumption (Dai *et al.* 2005; Kiefer *et al.* 2006). Our laboratory and others have demonstrated that β-E-deficient mice exhibit altered patterns of EtOH consumption, which depend upon sex (Williams *et al.* 2007; Zhou *et al.* 2017), and a *Pomc* haplotype marker has been associated with human alcoholism in women, but not men (Racz *et al.* 2008). However, the mechanisms responsible for this interaction remain unknown.

β-Endorphin also regulates hypothalamic–pituitary–adrenal axis (HPA-axis) activity via μ-opioid mediated inhibition (Buckingham 1986; Wynne & Sarkar 2013), influences basal anxiety-like behavior and EtOH-mediated anxiolysis (Grisel *et al.* 2008; Barfield *et al.* 2010; Barfield *et al.* 2013), and interacts with CRH in brain regions associated with stress and anxiety (Reyes *et al.* 2006; Lam & Gianoulakis 2011). Moreover, CRH signaling in the extended amygdala—comprised the nucleus accumbens shell (NAc), bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA)—mediates binge-like EtOH consumption (Lowery-Gionta *et al.* 2012; Pleil *et al.* 2015; Rinker *et al.* 2017), and we recently observed increased *Crh* mRNA in the extended amygdala of β-E −/− female mice (McGonigle *et al.* 2016). Therefore, we hypothesized that binge-like drinking is regulated by sex and β-E through alterations in *Crh* expression and glucocorticoid secretion affecting stress circuitry and anxiety-like behavior.

METHODS

Animals

Adult male and female C57BL/6J (β-E +/+), B6.129S2-Pomc^{tm1Low}/J (stock number: 003191; β-E −/− mice) and heterozygous (β-E +/−) mice were either bred in-house and weaned at 21 days from stock obtained from Jackson Laboratories (Bar Harbor, ME) or purchased as adults from Jackson Laboratories, in which case they were acclimated at least 10 days prior to the onset of any experimental procedures. The β-E-deficient mice were developed in the laboratory of Malcolm Low and are now fully backcrossed onto a C57BL/6J background. Transgenic mice harbor a truncated *Pomc* transgene that prevents synthesis of β-E, although other POMC protein products remain unchanged, such that homozygotes cannot synthesize β-E and heterozygotes produce ~50% of wild-type levels (Rubinstein *et al.* 1996). β-E −/− male mice have been shown to exhibit an overweight phenotype that increases with age, although we observed no differences in weight across genotypes of either sex in the present study (supplemental results and Fig. S1). The mice were group-housed by sex and genotype before the start of the experiment, and individually during the experiment, in Plexiglas® cages with corncob bedding and *ad libitum* access to chow and water. The animal colony and experimental room were maintained at ~21°C with a 12-hour/12-hour reverse light/dark cycle (lights off at 0930). All procedures were in accordance with the National Institute of Health guidelines and approved by the Bucknell University Institutional Animal Care and Use Committee.

Drinking in the dark procedures

A two-bottle, 4-day drinking in the dark (DID) procedure was performed (Giardino & Ryabinin 2013) with water continuously available in one bottle for all mice. The mice were acclimated to individual housing for at least 7 days prior to the 4-day DID testing. On days 1–3 of DID testing, for 2 hours beginning 3 hours into the dark cycle, the mice had access to two 25-ml graduated cylinders containing either 20% EtOH in tap water (*v*/*v*) or tap water alone, while water control groups received tap water in both bottles. On day 4, access to EtOH or the additional water tube was extended to a 4-hour binge test (BT) session. Sucrose control experiments were performed to assess whether differences in consumption between +/+ and −/− female mice were specific to EtOH. The animals underwent the same DID procedures, except that a 10% sucrose solution (*w*/*v*) was presented instead of a 20% EtOH solution. Fluid intake levels were measured by a trained observer blind to experimental condition by reading gradations on bottles with accuracy to the nearest 0.1 ml.

Blood and tissue collection

Immediately following the 4-hour BT on day 4, the subjects were individually transported to an adjacent room, anesthetized by using isoflurane and rapidly decapitated. Trunk blood was collected and centrifuged to analyze plasma for blood EtOH concentrations (BEC; Analox Alcohol Analyzer, Analox Instruments, Stourbridge, UK) or stored at −20°C for subsequent processing of corticosterone (CORT) by using an ELISA. Simultaneously, the brains were removed, frozen on dry ice and stored at −80°C for gene expression by using quantitative real-time polymerase chain reaction (qRT-PCR). Finally, left and right adrenal glands from each mouse were harvested and weighed. To control for potential effects of individual housing stress on CORT, a separate cohort of group-housed mice was sacrificed at the same time of day to assess homecage CORT.

Post-DID anxiety-like behavioral testing

To assess the behavioral effects of voluntary consumption and normalized CORT in β-E −/− female mice, we

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evaluated a separate cohort of β-E +/+ and β-E −/− female mice for anxiety-like behavior in the light–dark box (LDB) following the EtOH DID procedure. Immediately following the BT on day 4, the subjects were transported to an adjacent room and placed in a LDB for 5 minutes (Grisel *et al*. 2008). Experimentally blind observers scored time spent in the light and dark compartments, and crossings between compartments were tallied as a measure of general locomotor activity. Immediately following LDB testing, the animals were anesthetized and rapidly decapitated, and trunk blood was collected for analysis of BECs and CORT as described in the preceding texts.

Brain punch protocol and qRT-PCR

Frozen tissue was sliced on a Thermo Fisher HM 550 cryostat (Thermo Fisher Scientific, Waltham, MA), and bilateral 1.5-mm cylindrical punches were taken of the NAc (AP: +1.94 mm to +0.86 mm; ML: ±0.75 mm; DV: +1.0 mm), BNST (AP: +0.62 mm to −0.22 mm; ML: ±1.0 mm; DV +1.35 mm) and CeA (AP: −0.82 mm to −1.82 mm, ML: ±2.35 mm; DV: +1.25 mm), relative to bregma, and immediately submerged in Qiazol lysis buffer (Qiagen GmbH, Hilden, Germany). Each sample tube containing one brain region from one mouse was homogenized immediately after sectioning. Total RNA was extracted by using the Qiagen RNeasy Lipid Tissue Minikit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Concentration and purity of eluted RNA were verified by using the NanoDrop Lite UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and 500 ng of total RNA was reverse-transcribed by using the iScript™ cDNA Synthesis Kit (BioRad, Hercules, CA) also according to the manufacturer's instructions. qRT-PCR was performed by using FastStart Essential DNA Probes Master Mix (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. PrimeTime® XL qRT-PCR Assays designed by IDT (Integrated DNA Technologies, Coralville, IA) were performed by using *Crh* (Assay ID: Mm. PT.58.32061593) and the reference gene, *glyceraldehyde-3-phosphate dehydrogenase* (Assay ID: Mm. PT.58.12733669) in duplicate on a LightCycler 96 (Roche Diagnostics, Indianapolis, IN). All assays had similar optimum PCR efficiencies. For all qRT-PCR experiments, *glyceraldehyde-3-phosphate dehydrogenase* gene expression was used as the reference gene and relative changes in gene expression determined by using the $2^{-\Delta\Delta CT}$ method (Schmittgen & Livak 2008).

Corticosterone ELISA

Corticosterone was measured by ELISA (Enzo Life Sciences, Farmingdale, NY), according to the manufacturer's instructions. Blood plasma was diluted 1:40 with assay buffer. Absorbance was read at 405 nm by using an iMark microplate reader (BioRad, Hercules, CA). Sample concentrations for CORT were calculated from a standard curve by using GRAPHPAD PRISM version 7.0 software (GraphPad, La Jolla, CA). Assay sensitivity was 27 pg/ml with a range of detection up to 20 000 pg/ml. All samples were assayed in duplicate.

Statistical analysis

Repeated measures ANOVAs with sex and genotype as factors were used to analyze EtOH consumption and preference and sucrose consumption (only female mice) across days 1–3 of the DID paradigm. Two-way ANOVAs with genotype and sex as factors were used to analyze EtOH consumption and preference on the day 4 BT, average EtOH consumption and preference, average weight and homecage CORT. Three-way ANOVAs with sex, genotype and treatment as factors were used to analyze CORT, adrenal weights and *Crh* expression in the NAc, BNST and CeA. Simple linear regression was used to analyze relationships between BECs and anxiety-like behavior. Unpaired two-tailed *t*-tests were used to analyze genotype differences in anxiety-like behavior and locomotor activity in the LDB and average and BT sucrose consumption. An *F*-test for equality of variances was used to compare variances in CORT levels in β-E −/− female mice based on intoxication threshold. Bonferroni *post hoc* tests were used to correct for multiple comparisons following significant main effects and interactions. Degrees of freedom may differ between groups/brain regions due to unquantifiable tissue or blood plasma samples. All data were analyzed by using SPSS 24.0 software and GRAPHPAD PRISM 7.0. Data are presented as mean ± SEM. Effects were considered statistically significant at $P \leq 0.05$.

RESULTS

β-E deficiency promotes enhanced binge-like EtOH consumption in female, but not male, mice

We evaluated male and female β-E +/+, β-E +/− and β-E −/− mice in the DID model of binge drinking to test the hypothesis that β-E regulates binge-like EtOH consumption in a sexually dimorphic manner. Binge EtOH consumption across the 2-hour periods (days 1–3), the 4-hour BT (day 4) and average consumption are summarized in Fig. 1 (n = 15–20/group). A repeated measures ANOVA on days 1–3 revealed a significant sex by genotype interaction ($F_{(2,97)}$ = 5.648, P = 0.005). All other main effects and interactions were not significant: day ($F_{(2,194)}$ = 1.803, P = 0.167), day by genotype ($F_{(4,194)}$ = 2.197, P = 0.071), day by sex ($F_{(2,194)}$ = 1.724, P = 0.181), day by genotype by sex

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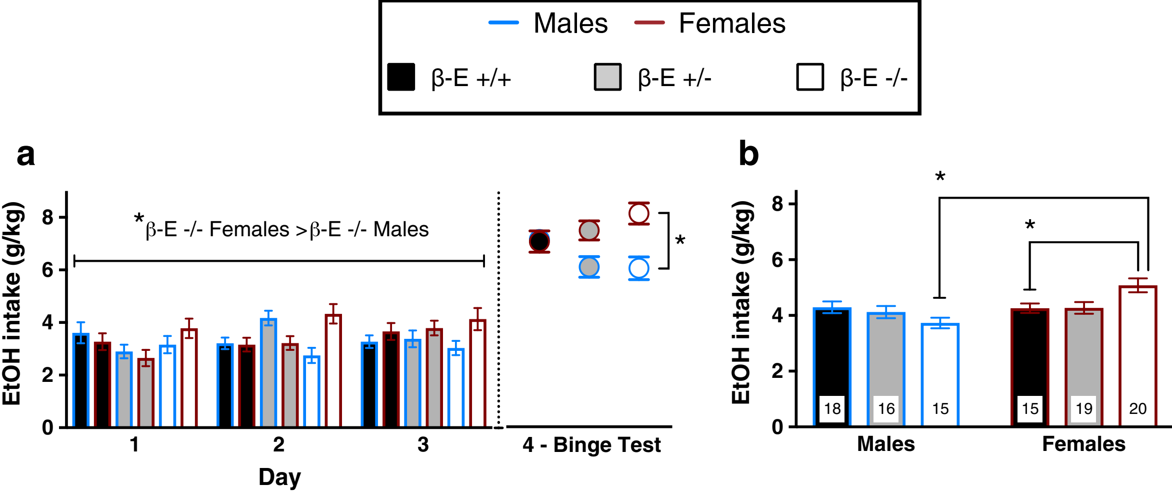

Figure 1 β-Endorphin (β-E) masks sex differences in binge-like EtOH drinking. (a) Daily consumption of 20% EtOH versus water across the 2-hour sessions (days 1–3) and 4-hour binge test (day 4) of the drinking in the dark procedure. A repeated measures ANOVA across days 1–3 revealed a significant sex by genotype interaction, and *post hoc* analysis indicated that β-E −/− female mice consumed more EtOH than β-E −/− male mice. A two-way ANOVA on the day 4 binge test (BT) revealed a main effect of sex (female mice > male mice) and a sex by genotype interaction. There was a main effect of sex and a sex by genotype interaction for the 4-hour BT. *Post hoc* analysis indicated that β-E −/− female mice consumed more EtOH than β-E −/− male mice. (b) These data are summarized as average consumption across the 4-day paradigm. There was a main effect of sex (female mice > male mice), and a sex by genotype interaction with *post hoc* analysis indicating that β-E −/− female mice consumed more EtOH overall than β-E +/− and β-E +/+ female and β-E −/− male mice. Data are presented as means ± SEM; *$P < 0.05$ (Bonferroni corrected); *n* for each group are displayed within their respective bar

($F_{(4,194)} = 1.476$, $P = 0.211$), genotype ($F_{(2,97)} = 0.426$, $P = 0.654$) and sex ($F_{(1,97)} = 2.532$, $P = 0.115$). *Post hoc* analysis following the sex by genotype interaction indicated that β-E −/− female mice consumed more EtOH than β-E −/− male mice across days 1–3 ($P < 0.05$). A two-way ANOVA on day 4 BT revealed a main effect of sex ($F_{(1,97)} = 12.317$, $P = 0.001$) and a sex by genotype interaction ($F_{(2,97)} = 3.853$, $P = 0.025$) but no main effect of genotype ($F_{(2,97)} = 0.394$, $P = 0.675$). *Post hoc* analyses following the sex by genotype interaction indicated that β-E −/− female mice consumed more EtOH than β-E −/− male mice during the BT ($P < 0.05$; Fig. 1a). To determine the overall effect of β-E on binge-like EtOH consumption, we analyzed average EtOH consumption across the 4-day DID paradigm. A two-way ANOVA revealed a main effect of sex ($F_{(1,97)} = 7.631$, $P = 0.006$) and a sex by genotype interaction ($F_{(2,97)} = 5.962$, $P = 0.003$) but no main effect of genotype ($F_{(2,97)} = 0.476$, $P = 0.622$). *Post hoc* analyses following the sex by genotype interaction indicated that β-E −/− female mice consumed more EtOH than β-E +/+ female mice ($P < 0.05$) and β-E −/− male mice ($P < 0.05$; Fig. 1b). EtOH preference data from these experiments can be found in the supplemental results and Fig. S2. We also conducted sucrose control experiments in a separate cohort of β-E +/+ and −/− female mice to assess whether differences in consumption were specific to EtOH. These data are presented in the supplemental results and Fig. S4.

Binge-like EtOH consumption normalizes elevated CORT levels in female, but not male, β-E −/− mice

To determine the role of HPA-axis activity in the sexually divergent pattern of EtOH consumption, we assessed plasma CORT levels immediately following water or EtOH consumption on the day 4 BT. A three-way ANOVA revealed the main effects of genotype ($F_{(2,159)} = 30.688$, $P < 0.001$) and sex ($F_{(1,159)} = 14.730$, $P < 0.001$), but not treatment ($F_{(1,159)} = 3.854$, $P = 0.051$). There were also significant interactions of genotype by sex ($F_{(2,159)} = 3.470$, $P = 0.033$), genotype by treatment ($F_{(2,159)} = 3.440$, $P = 0.034$), sex by treatment ($F_{(1,159)} = 8.721$, $P = 0.004$) and genotype by sex by treatment ($F_{(2,159)} = 3.934$, $P = 0.022$). *Post hoc* analysis following the three-way interaction indicated that water control β-E −/− female mice have higher CORT levels than β-E +/+ water control female mice ($P < 0.05$) and water control β-E −/− male mice ($P < 0.05$). However, CORT levels are reduced by EtOH drinking in β-E −/− female mice to levels similar to wild-type female mice ($P > 0.05$; Fig. 2). To control for potential effects of individual housing, we also measured CORT levels in a separate cohort of naïve, water-drinking group-housed male and female β-E +/+ and −/− mice (supplemental results and Fig. S3).

Anxiolytic effects of EtOH in female mice depend upon β-E

Previous research had demonstrated an inverse relationship between β-E levels and anxiety-like behaviors in

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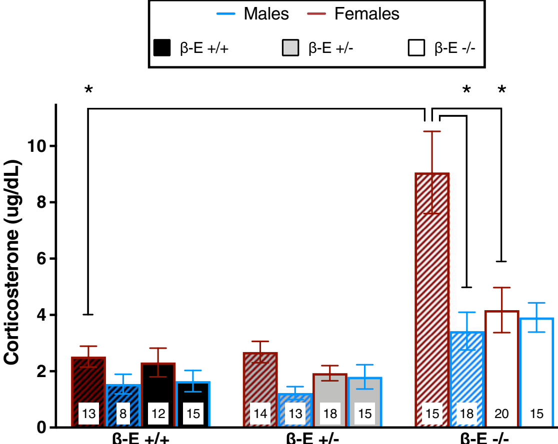

Figure 2 Binge-like EtOH consumption normalizes corticosterone (CORT) in female, but not male, β-endorphin (β-E) −/− mice. Plasma CORT levels obtained immediately following the day 4 binge test from β-E +/+, +/− and −/− female and male mice consuming either EtOH and water (solid bars) or water controls (hatched bars). A three-way ANOVA revealed a significant genotype by sex by treatment interaction. *Post hoc* analysis indicated that β-E −/− female mice exhibit greater CORT than β-E +/+ female and β-E −/− male mice under basal conditions, but voluntary binge-like EtOH consumption significantly reduces β-E −/− female CORT levels to near wild-type levels. Data are presented as means ± SEM; **P* < 0.05 (Bonferroni corrected); *n* for each group are displayed within their respective bar

these lines (Grisel *et al.* 2008; Barfield *et al.* 2010; Barfield *et al.* 2013), but in order to assess the behavioral relevance of the physiological changes in endocrine measures, we tested a separate group containing only female +/+ and −/− mice for anxiety-like behavior in the LDB after the 4-day DID procedure. Linear regression revealed a significant relationship between BECs and preference for the light side of a LDB in β-E −/− female mice ($F_{(1,8)}$ = 12.02, P = 0.0085, R^2 = 0.60), but not β-E +/+ female mice ($F_{(1,5)}$ = 0.008, P = 0.93), supporting the contention that β-E −/− female mice exhibit heightened sensitivity to the anxiolytic effects of EtOH (Fig. 3a). One-sample *t*-tests revealed that mean light side preference was significantly less than 50% for both genotypes [β-E +/+ ($t_{(7)}$ = 5.71, P = 0.0007); β-E −/− ($t_{(9)}$ = 4.432, P = 0.0016)], and an unpaired *t*-test revealed no differences between genotypes ($t_{(16)}$ = 0.423, P = 0.6779), suggesting that both groups exhibited similar anxiety-like behavior in the LDB on average (Fig. 3c). An unpaired *t*-test also indicated that there were no genotype differences in locomotor activity, as assessed by crossings between light/dark compartments ($t_{(15)}$ = 0.5176, P = 0.612; Fig. 3d).

To further explore the relationship between binge-like drinking and CORT, we ran a linear regression that revealed a significant relationship between BECs and CORT solely in β-E −/− female mice ($F_{(1,29)}$ = 5.721, P = 0.023, R^2 = 0.16; not shown). All other regressions were non-significant [β-E −/− male mice ($F_{(1,13)}$ = 1.187, P = 0.295), β-E +/+ male mice ($F_{(1,12)}$ = 3.04, P = 0.10) and female mice ($F_{(1,17)}$ = 0.568, P = 0.461), and β-E +/− male mice ($F_{(1,13)}$ = 0.043, P = 0.837) and female mice ($F_{(1,16)}$ = 1.595, P = 0.224)]. We next split β-E −/− female mice based on level of intoxication, those with BECs <80 or >80 mg/dl, and analyzed CORT as a function of pharmacological intoxication. An unpaired *t*-test with Welch's correction revealed a strong trend for reduced CORT only in β-E −/− female mice that achieved intoxication ($t_{(10.54)}$ = 2.105, P = 0.060), although this did not reach statistical significance. However, an *F*-test to compare variances indicated that variance in β-E −/− female mice achieving intoxicating BECs (mean ± SEM = 3.294±0.463, n = 20) was significantly reduced relative to β-E −/− female mice that did not achieve intoxication (mean ± SEM = 6.802 ± 1.601, n = 10; $F_{(9,19)}$ = 5.968, P = 0.001), suggesting that intoxicating doses of EtOH serve to reduce the variability of glucocorticoid secretion in β-E −/− female mice (Fig. 3b).

β-E −/− mice have elevated BNST *Crh* expression, which is normalized by binge-like EtOH consumption

To determine a potential mechanism underlying the enhanced EtOH consumption and elevated CORT levels in β-E −/− female mice, we used qRT-PCR to analyze *Crh* mRNA in the NAc, BNST and CeA in a subset of male and female β-E +/+, β-E +/− and β-E −/− mice from the water and EtOH DID experiments. Our hypothesis was focused on the BNST and CeA, and the NAc was included as a neuroanatomical control region. However, we did observe changes in this forebrain region that were dependent upon sex (supplemental results and Fig. S5).

A three-way ANOVA on BNST *Crh* revealed significant main effects of genotype ($F_{(2,82)}$ = 7.020, P = 0.002) and sex ($F_{(1,82)}$ = 10.857, P = 0.001), but not treatment ($F_{(1,82)}$ = 0.531, P = 0.468). There was also significant genotype by treatment ($F_{(2,82)}$ = 9.242, P < 0.001) and sex by treatment ($F_{(1,82)}$ = 3.979, P = 0.049) interactions, but not genotype by sex ($F_{(2,82)}$ = 1.226, P = 0.299) or genotype by sex by treatment ($F_{(2,82)}$ = 1.741, P = 0.182). *Post hoc* analysis following the genotype by treatment interaction indicated that β-E −/− water controls have higher *Crh* expression than β-E +/+ water controls (P < 0.05). However, in EtOH drinking, β-E −/− mice *Crh* expression is reduced, relative to β-E −/− water controls (P < 0.05), to levels similar to β-E +/+ EtOH drinking mice (P > 0.05), suggesting

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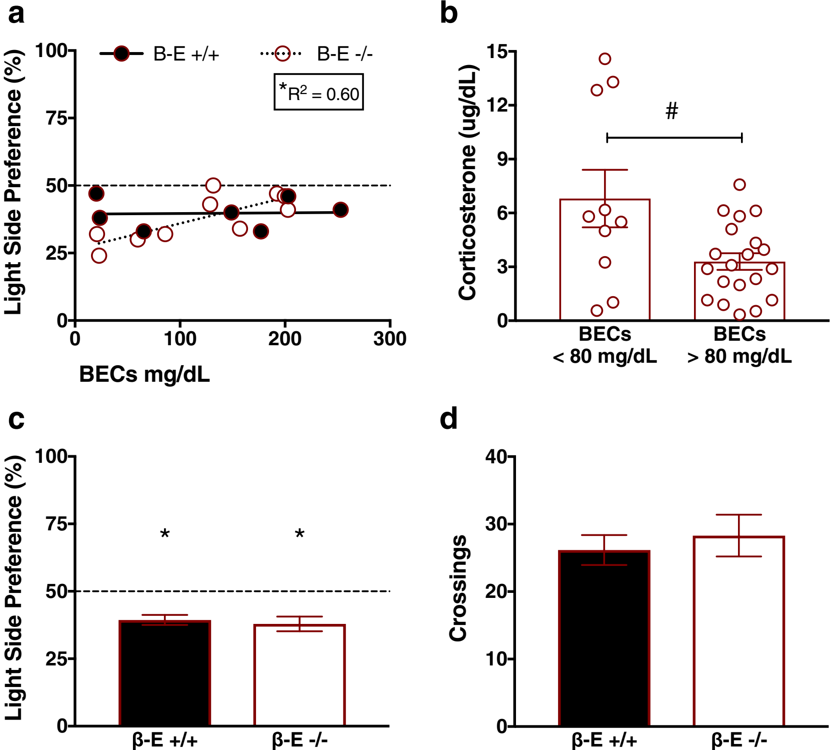

Figure 3 β-Endorphin (β-E) −/− female mice exhibit increased sensitivity to EtOH's anxiolytic effects. (a) Linear regressions depicting the relationship between blood ethanol concentrations (BECs) and preference for the light side of the light–dark box (LDB) in β-E +/+ and −/− female mice immediately following the binge test. There was a significant positive relationship between degree of intoxication (BECs) and reduced anxiety-like behavior in β-E −/−, but not β-E +/+, female mice, suggesting that β-E −/− female mice exhibited greater EtOH-mediated anxiolysis. The dashed line represents the value at which equal time is spent in both compartments of the LDB; the asterisk denotes a significant regression and corresponding goodness of fit value. (b) Because the only significant regressions between BECs and corticosterone (CORT) were observed in β-E −/− female mice (see section), we further explored the effect of EtOH consumption on CORT. All β-E −/− female mice were split into two groups based on intoxication threshold (BECs < 80 mg/dl and BECs > 80 mg/dl), and CORT levels were compared. β-E −/− female mice that achieved intoxication tended to exhibit reduced CORT, relative to β-E −/− female mice that did not, although this did not reach statistical significance (P = 0.060). However, an *F*-test comparing variances indicated that β-E −/− female mice with BECs >80 mg/dl exhibited significantly reduced variability in CORT levels, relative to β-E −/− female mice with BECs <80 mg/dl (#P = 0.05). (c) Mean summary of data in (a) indicating that, overall, both β-E +/+ and −/− exhibited anxiety-like behavior in the LDB; however, there was no significant difference between genotypes. The asterisk denotes mean light side preference significantly lower than the null hypothesis of 50%. (d) There was also no significant genotype difference in locomotor activity, assessed by crossings, during LDB testing. Data are presented as means ± SEM

that β-E −/− mice exhibit elevated BNST *Crh* under basal conditions, but binge-like EtOH drinking normalizes *Crh*. *Post hoc* analyses following the sex by treatment interaction indicated that male water controls have higher BNST *Crh* than female water controls ($P < 0.05$), suggesting that under basal conditions, female mice have less *Crh* mRNA in the BNST than male mice (Fig. 4).

β-E levels negatively correlate with CeA *Crh* expression in female, but not male, mice

A three-way ANOVA on CeA *Crh* revealed significant genotype by sex ($F_{(2,82)}$ = 4.478, P = 0.014) and genotype by treatment ($F_{(2,82)}$ = 4.577, P = 0.013) interactions. All other main effects and interactions were not significant: genotype ($F_{(2,82)}$ = 3.040, P = 0.053), sex ($F_{(1,82)}$ = 0.287, P = 0.594), treatment ($F_{(1,82)}$ = 2.508, P = 0.117), sex by treatment ($F_{(1,82)}$ = 0.917, P = 0.341) and genotype by sex by treatment ($F_{(2,82)}$ = 1.673, P = 0.194). *Post hoc* analyses following the genotype by sex interaction indicated that β-E −/− and β-E +/− female mice had greater CeA *Crh* expression than β-E +/+ female mice ($P < 0.05$), suggesting that β-E negatively correlates with CeA *Crh* in female, but not male, mice. *Post hoc* analyses following the genotype by treatment interaction indicated that EtOH drinking β-E −/− mice had lower *Crh* than β-E −/− water controls ($P < 0.05$), suggesting that in β-E −/− mice, binge-like EtOH consumption reduces *Crh* expression in the CeA (Fig. 5).

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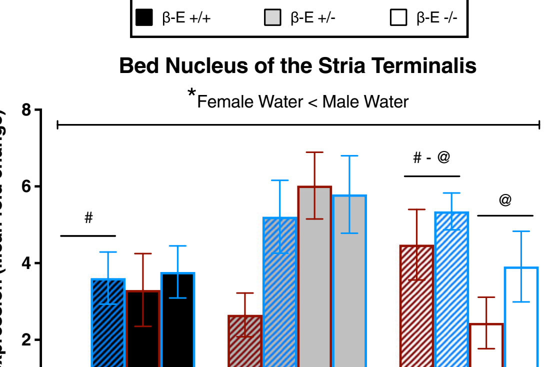
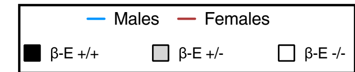


Figure 4 β-Endorphin (β-E) −/− mice have elevated bed nucleus of the stria terminalis (BNST) *Crh* under basal conditions, but binge-like ethanol consumption normalizes *Crh* expression. *Crh* mRNA expression from mice consuming either EtOH and water (solid bars) or water controls (hatched bars) is represented as mean fold change (±SEM) normalized to β-E +/+ female water mice as indicated by the dashed line. A three-way ANOVA revealed a genotype by treatment interaction and a sex by treatment interaction. *Post hoc* analysis following the genotype by treatment interaction indicated that β-E −/− water controls exhibit increased *Crh* expression, relative to β-E +/+ water controls. However, binge-like EtOH consumption reduced *Crh* in β-E −/−, relative to water controls, such that there was no longer a genotype difference in BNST *Crh* expression. Note that these effects are primarily driven by differences in female mice; however, the lack of a significant 3-way interaction precluded our ability to statistically assess such comparisons. Although, *post hoc* analysis following the sex by treatment interaction indicated that, overall, female water controls have lower BNST *Crh* mRNA than male water controls (*), which provides novel evidence for basal sex differences in BNST *Crh* expression. Like symbols indicate significant differences between groups ($P < 0.05$; Bonferroni corrected); *n* for each group are displayed within their respective bar



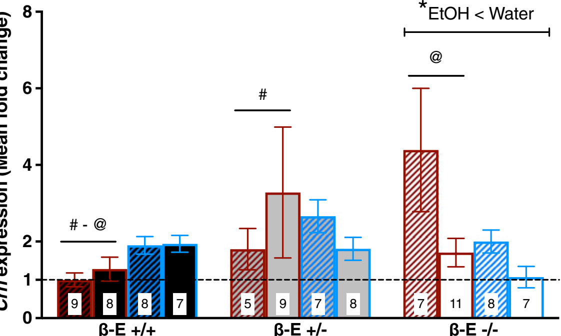


Figure 5 Sex differences in CeA *Crh* mRNA: β-Endorphin (β-E) is inversely associated with CeA *Crh* in female, but not male, mice. *Crh* mRNA expression from mice consuming either EtOH and water (solid bars) or water controls (hatched bars) is represented as mean fold change (±SEM) normalized to β-E +/+ female water mice as indicated by the dashed line. A three-way ANOVA revealed a genotype by sex interaction and a genotype by treatment interaction. *Post hoc* analysis following the genotype by sex interaction indicated that β-E −/− and β-E +/− female mice exhibit higher CeA *Crh* than β-E +/+ female mice; however, these effects were not observed in male mice. *Post hoc* analysis following the genotype by treatment interaction indicated that binge-like EtOH consumption reduced *Crh* only in β-E −/− mice (*), which was likely driven by the magnitude of reduction in β-E −/− female mice. Like symbols indicate significant differences between groups ($P < 0.05$; Bonferroni corrected); *n* for each group are displayed within their respective bar

Adrenal glands of β-E-deficient mice are more sensitive to EtOH-induced alterations

We previously observed increased adrenal gland weight in β-E deficient mice following EtOH exposure (Grisel *et al.* 2008; McGonigle *et al.* 2016) but did not explore basal or sex differences. Therefore, immediately following the BT on day 4, the left and right adrenal glands were harvested and total adrenal weight (normalized by body weight, g/kg) was calculated and is summarized in Fig. 6.

A three-way ANOVA revealed a main effect of sex ($F_{(1,144)} = 103.975$, $P < 0.001$), indicating that female adrenals weighed more than male adrenals, and a significant genotype by treatment interaction ($F_{(2,144)} = 21.445$, $P < 0.001$). No other main effects or interactions were significant: genotype ($F_{(2,144)} = 1.251$, $P = 0.289$), treatment ($F_{(1,144)} = 2.662$ $P = 0.105$), genotype by sex ($F_{(2,144)} = 1.112$, $P = 0.332$), sex by treatment ($F_{(1,144)} = 0.015$, $P = 0.902$) and genotype by sex by treatment ($F_{(2,144)} = 1.712$, $P = 0.184$). *Post hoc* analysis following the genotype by treatment interaction indicated that water control β-E −/− mice have smaller adrenals than β-E +/+ water controls ($P < 0.05$). However, binge-like EtOH drinking increases adrenal size in β-E −/− mice ($P < 0.05$), such that EtOH drinking β-E −/− mice exhibit larger adrenals than EtOH drinking β-E +/+ mice ($P < 0.05$).

DISCUSSION

We investigated behavioral, neuroendocrine and genetic substrates of EtOH consumption in a binge-drinking model that reliably produces pharmacological intoxication (Giardino & Ryabinin 2013). Using transgenic mice (Rubinstein *et al.* 1996), we show that β-E deficiency results in sexually divergent patterns of EtOH consumption with corresponding alterations in HPA-axis activity and *Crh* expression in the extended amygdala.

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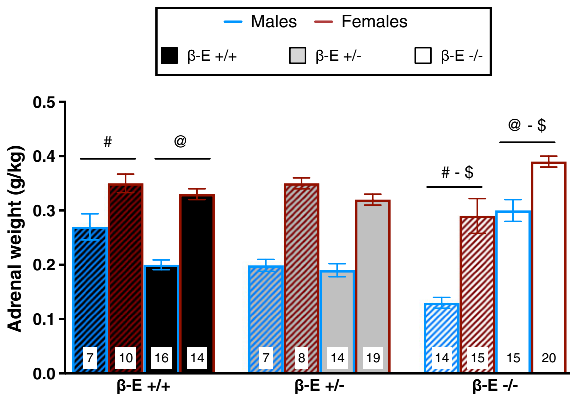

Figure 6 β-Endorphin (β-E) −/− mice exhibit heightened sensitivity to EtOH-induced changes in adrenal gland size. Total adrenal gland weight (mean ± SEM, normalized by body weight) from mice consuming either EtOH and water (solid bars) or water controls (hatched bars). A three-way ANOVA revealed a main effect of sex, indicating that, overall, female mice have larger adrenals than male mice. There was also a genotype by treatment interaction. *Post hoc* analysis following the genotype by treatment interaction indicated that β-E −/− water controls have smaller adrenals than β-E +/+ water controls. However, binge-like EtOH consumption increased adrenal size only in β-E −/− mice, relative to β-E −/− water controls, which resulted in β-E −/− EtOH mice possessing larger adrenals than β-E +/+ EtOH mice. Like symbols indicate significant differences between groups ($P < 0.05$; Bonferroni corrected); *n* for each group are displayed within their respective bar

β-Endorphin deficiency enhances voluntary binge-like EtOH consumption in female, but not male, mice. β-E −/− female mice also exhibit elevated CORT, which is normalized by voluntary EtOH drinking, coincident with a decrease in anxiety-like behavior. Under basal (i.e. water-drinking) conditions, β-E −/− mice exhibit increased BNST *Crh* expression, and EtOH consumption obviates this difference. Similarly, β-E levels negatively correlate with CeA *Crh* expression in female, but not male, mice and the elevated basal expression is normalized following EtOH consumption. β-E −/− mice also exhibit heightened sensitivity to EtOH-induced changes in adrenal gland size. These data suggest that genetic and neuroendocrine markers of exaggerated stress sensitivity correlate with binge-like EtOH drinking, perhaps contributing to sex-dependent trajectories in alcohol use disorders. We also provide novel evidence for sex differences in *Crh* expression. That is, female mice have lower basal BNST *Crh* than male mice, but this difference is abolished following binge-like EtOH consumption.

Previous research demonstrated that β-E influences EtOH consumption (Grisel *et al.* 1999; Racz *et al.* 2008) and that the peptide's impact may be sex dependent (Williams *et al.* 2007; Zhou *et al.* 2017). β-E is synthesized in both hypothalamic and pituitary neurons, but because we used a global knockout, the current study does not delineate the source of influence (Logan *et al.* 2015). However, Zhou *et al.* (2017) did not see an effect of pituitary-specific POMC knockout on drinking in the DID model. Variations in β-E also influence anxiety-like behavior sex-dependently, as β-E −/− female mice display heightened stress responses relative to other groups (Grisel *et al.* 2008; Barfield *et al.* 2010; Barfield *et al.* 2013). In the present study, we demonstrate that β-E −/− female mice also exhibit approximately two to three-fold increases in plasma CORT and *Crh* mRNA in the BNST and CeA compared with wild-type female mice. Because the BNST and CeA regulate stress, anxiety and alcohol-related behaviors, primarily through CRH signaling (Gilpin *et al.* 2015; Vranjkovic *et al.* 2017), these differences are likely to contribute to the high-drinking, high-stress phenotype of β-E −/− female mice. The BNST regulates HPA-axis activation via excitatory and inhibitory projections to the paraventricular nucleus of the hypothalamus (PVN), and the CeA can modulate these inputs via GABAergic projections to the BNST (Choi *et al.* 2007). Both CRH and EtOH can increase CeA GABAergic transmission onto inhibitory PVN-projecting BNST neurons, resulting in disinhibition of PVN CRH neurons and activation of the HPA-axis (Gilpin 2012). Thus, increased *Crh* in the CeA and/or BNST of β-E −/− female mice may explain elevations in basal CORT. While acute EtOH has also been shown to increase adrenal size, the increase in β-E −/− mice, following only 4 days of EtOH drinking, suggests they exhibit enhanced adrenal sensitivity to EtOH, which might be associated with their dysregulated HPA-axis activity (Rivier & Vale 1985; Rasmussen *et al.* 2000).

Elevated CRH in the BNST and CeA are hallmarks of a negative affective state that drives excessive EtOH consumption, presumably to alleviate the associated symptoms (Koob 2013). Toward that end, Olive and colleagues demonstrated that extracellular levels of CRH increase during EtOH withdrawal and that subsequent consumption following reintroduction of EtOH reduced CRH to basal levels (Olive *et al.* 2002), a pattern observed with CORT levels and Crh mRNA in our β-E −/− female mice following binge EtOH consumption. Moreover, chemogenetic inhibition of BNST CRH neurons reduces binge-like EtOH consumption, suggesting a causal role for BNST CRH signaling in regulating excessive drinking (Pleil *et al.* 2015; Rinker *et al.* 2017). Similarly, extracellular CRH in the CeA also increases during EtOH withdrawal, and immunoreactivity in the CeA is upregulated following binge-like EtOH consumption, but blockade of CRH1 receptors in the CeA prevents the excessive EtOH consumption associated with these effects (Funk *et al.* 2006; Lowery-Gionta *et al.* 2012). Taken together, these findings suggest a potential mechanism that puts β-E −/− female mice at increased risk for binge-like EtOH consumption.

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The tension reduction hypothesis proposes that the motivation to consume alcohol manifests from a desire to reduce stress and anxiety (Cappell & Herman 1972), a clinical phenomenon that is more pronounced in female subjects (Sinha *et al.* 1998). In addition, rodents displaying heightened anxiety-like behavior are more susceptible to the anxiolytic effects of EtOH (Stewart *et al.* 1993; Grisel *et al.* 2008) and anxiety-like behavior has been shown to predict high levels of EtOH consumption and preference (Spanagel *et al.* 1995). The present results suggest that low β-E confers elevated anxiety-like behavior and enhanced EtOH-mediated anxiolysis in female mice, which corresponds with clinical data correlating low plasma β-E with anxiety and negative affect-related psychopathologies (Dai *et al.* 2005; Merenlender-Wagner *et al.* 2009).

Similarly, stress also modulates EtOH intake, often leading to increases in consumption and instigating alcohol-seeking behavior (Spanagel *et al.* 2014). Stress-induced EtOH consumption is regulated by glucocorticoids (Ostroumov *et al.* 2016), suggesting that the elevated glucocorticoid tone in β-E −/− female mice may contribute to their increased EtOH consumption.

CONCLUSIONS

Women experience increased susceptibility for many stress-related psychiatric disorders, which often co-occur with alcohol use disorders and exacerbate excessive alcohol use (McLean *et al.* 2011). Despite formidable evidence that women are more sensitive to stress, alcohol and their interaction (Logrip *et al.* 2017), the neural mechanisms underlying sex-specific regulation of alcohol use have been poorly understood. The present findings help fill this gap by demonstrating sexually dimorphic neuropeptide regulation of EtOH sensitivity.

Our data suggest that sex and β-E interact to determine sexually dimorphic effects on excessive alcohol consumption and tie this tendency to heightened HPA-axis activity, enhanced EtOH-mediated anxiolysis and elevated *Crh* in the BNST and CeA. Given the importance of neuropeptide signaling in EtOH-related behaviors (Koob 2013), it seems plausible that other neuropeptides also demonstrate distinct sex-specific effects. Taken together, our observations shed light on the previously unknown impact of sex and β-E on EtOH consumption and suggest that understanding the trajectory toward disordered drinking may depend on better knowledge of sex-dependent substrates.

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Authors Contribution

T.B.N. performed data acquisition. D.E.W. assisted with qRT-PCR data collection. T.B.N., E.M.R. and J.E.G. designed the study and performed data analysis. T.B.N. and J.E.G. wrote the manuscript. All authors critically reviewed content and approved final version for publication.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. No differences in weight across genotype during the EtOH DID experiments.

Figure S2. β-E deficiency does not alter EtOH preference.

Figure S3. Elevated basal CORT in β-E −/− females is present in experimentally naïve mice.

Figure S4. β-E +/+ and −/− females do not differ in binge-like sucrose consumption.

Figure S5. Females exhibit lower *Crh* expression than males, under basal conditions, but increase expression following binge-like EtOH consumption.

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