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β-Endorphin influences sedative and ataxic effects of alcohol

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ABSTRACT

Beta-endorphin (β-E) is an opioid peptide linked to the behavioral effects of ethanol. For example, β-E provides negative feedback to inhibit the hypothalamic-pituitary-adrenal (HPA) stress axis, and neuroadaptation of this system to ethanol may facilitate sex differences in disordered drinking. Locomotor sensitivity to ethanol may also influence the risk for addiction; however, the role of β-E in psychomotor effects of ethanol is not fully understood. We examined the role of β-E and sex on locomotor effects of ethanol using adult male and female wild-type C57BL/6J and β-E deficient B6.129S2-Pomctm1Low/J mice in a parallel rod floor apparatus following 0.75 or 2.0 g/kg ethanol. Beginning 15 min after intraperitoneal injection, we recorded foot slips, distance traveled, slips per meter, first instance of immobility, and total time spent off-balance (lying on the floor) over 15 min, and collected blood for analysis of ethanol concentration 60 min after injection. Overall, β-E deficient mice were more sedated and ataxic following ethanol; at the lower dose they slipped more frequently and had a higher rate of slips per meter traveled. At the higher dose, β-E deficient mice were predominantly sedated, slipping less frequently, and traveling less, as well as spending more time off-balance and becoming immobile sooner. Genotype interacted with sex in that male β-E deficient mice slipped more frequently than their female counterparts, suggesting that β-E may elicit sex-dependent effects of ethanol-induced ataxia. Blood ethanol concentration did not differ between any group, suggesting that behavioral differences result from altered sensitivity to ethanol. Our data support the contention that β-E modulates the locomotor effects of ethanol and may influence ataxia in a sex-dependent manner. These findings help elucidate the role of β-E in diverging behavioral responses to ethanol and may aid the development of targeted treatments for alcohol use disorders.

Introduction

Alcohol influences a broad range of neurotransmitters and circuits throughout the brain, resulting in biphasic stimulatory and sedative drug effects. Differential sensitivity to these effects influences the risk for alcohol misuse and addiction. For example, increased sensitivity to stimulant effects and decreased sensitivity to sedation predict greater liability for future binge drinking and alcohol use disorder (AUD) (Addicott, Marsh-Richard, Mathias, & Dougherty, 2007; Hendler, Ramchandani, Gilman, & Hommer, 2012; King, de Wit, McNamara, & Cao, 2011). The opioid peptide β-endorphin (β-E) may play a role in the balance between arousal and sedation by mediating the stress response consequent to alcohol's influence on the hypothalamic-pituitary-adrenal (HPA) axis. Stressors initiate corticotropin-releasing hormone (CRH) release from the periventricular nucleus of the hypothalamus, resulting in cleavage of proopiomelanocortin (POMC) into β-E and adrenal corticotropic hormone (ACTH) in the anterior pituitary. ACTH potentiates the physiological stress response by promoting cortisol release from the adrenal glands, while β-E acts as negative feedback to inhibit further CRH release (Calogero, Gallucci, Gold, & Chrousos, 1988).

β-E is a potent agonist at μ-opioid receptors, and low β-E ligand activity is correlated with AUD (Méndez & Morales-Mulia, 2008; Roth-Deri, Green-Sadan, & Yadid, 2008). Low transient β-E and μ-opioid receptor levels are reported in alcohol-dependent humans and animals (Hermann et al., 2017; Méndez, Leriche, & Calva, 2001; Zalewska-Kaszubska & Czarnecka, 2005), and transgenic β-E deficient mice demonstrate increased alcohol self-administration (Grisel, Bartels, Allen, & Turgeon; Grisel et al., 1999). Alcohol-preferring rat strains exhibit greater basal POMC mRNA expression and β-E immunoreactivity in the arcuate nucleus compared to alcohol non-preferring strains (Gianoulakis, de Waele, & Kiianmaa, 1992). β-E activity at μ-opioid receptors directly

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Abbreviations

ACD	acetaldehyde
ACTH	adrenal corticotropic hormone
AUD	alcohol use disorder
β-E:	beta-endorphin
CRH	corticotropin-releasing hormone
CYP-2E1	cerebral cytochrome P450 2E1
HPA axis	hypothalamic-pituitary-adrenal axis
POMC	proopiomelanocortin
VTA	ventral tegmental area

influences locomotor activity and does so in a sex-dependent manner (Craft, Clark, Hart, & Pinckney, 2006; Dempsey & Grisel, 2012; Krzanowska & Bodnar, 2000). Female gonadectomized rats exhibit reduced whole-brain β-E concentrations, while gonadectomized males show less profound and more selective reductions (Pluchino et al., 2009). Not surprisingly then, there are sex differences in the interactions between β-E and alcohol. Sex-dependent hormonal modulation of β-E contributes to differences in locomotor responses to alcohol (Craft, 2008; Craft et al., 2006) and stress-induced drinking (Peltier et al., 2019; Satta, Hildebrand, & Lasek, 2018). These results may help explain increased vulnerability to the negative reinforcing, sedative, and anxiolytic effects of alcohol in women with AUD (Diehl et al., 2007; Gianaulakis, Dai, & Brown, 2003; Peltier et al., 2019).

β-E may also influence effects of alcohol via its interactions with the metabolite acetaldehyde (ACD). Alcohol is initially converted to ACD, primarily via oxidation with the product of brain catalase and hydrogen peroxide, accounting for most acetaldehyde production, as well as cerebral cytochrome P450 2E1 (CYP-2E1), which accounts for the remaining ~30 % (for review, see Font, Luján, & Pastor, 2013). ACD is psychoactive and reinforcing (Font et al., 2013; Yu & Han, 1989), and a locomotor stimulant at low doses (Correa, Arizzi-Lafrance, & Salamone, 2009), but is a sedative at high doses (Quertemont & Didone, 2006). The metabolite acts predominantly on the high-density network of POMC-expressing neurons in the arcuate nucleus of the hypothalamus, a region important for homeostatic regulation that relays with the nucleus accumbens, ventral tegmental area (VTA), and periaqueductal gray (PAG), central to the mesolimbic reward pathway, reward association and motivation, and locomotor initiation (Correa et al., 2009; Hood, Nagy, Leyrer-Jackson, & Olive, 2022; Yu & Han, 1989). Both alcohol and ACD induce locomotor stimulation at low doses when injected into the arcuate nucleus (Correa et al., 2009; Pastor & Aragon, 2008), an effect that is blocked by the opioid antagonist naltrexone, as well as inhibition of brain catalase (Pastor & Aragon, 2008). In addition, increasing CYP-2E1, as well as brain catalase and hydrogen peroxide expression, increases locomotor stimulation from alcohol (Ledesma, Miquel, Pascual, Guerri, & Aragon, 2014; Pastor, Sanchis-Segura, & Aragon, 2003). Notably, elevated expression of brain catalase decreases loss of righting reflex from alcohol consumption, while low catalase expression heightens loss of righting reflex, and inhibition of the arcuate nucleus diminishes the locomotor effects of alcohol (Sanchis-Segura, Correa, & Aragon, 2000; Sanchis-Segura, Correa, Miquel, & Aragon, 2005). This body of research supports the contention that brain acetaldehyde may influence alcohol's behavioral and locomotor effects by modulating β-E release.

Ataxia is frequently used as a measure of intoxication and, much like locomotor activity, is associated with other risk factors for addiction such as withdrawal severity (Shen, Dorow, Huson, & Phillips, 1996). In order to directly assess the influence of β-E on the locomotor effects of ethanol, and its putative interaction with sex, we evaluated male and female wild-type (C57BL/6J) and β-E deficient mice in a parallel rod apparatus following low and high doses of ethanol. Our results suggest that this opioid peptide reduces sedation and ataxia from ethanol and may reduce ataxia in a sex-dependent manner.

Materials and methods

Animals/husbandry

Experimentally naive male and female C57BL/6J (β-E +/+) and β-E deficient B6.129S2-Pomc^{tm1Low}/J (β-E −/−) mice between 50 and 100 days old were used in all trials. Transgenic mice contained a truncated POMC transgene unable to synthesize β-E while other protein products of POMC remain unchanged (Rubinstein et al., 1996). Mice were bred in-house from stock obtained through The Jackson Laboratories (Bar Harbor, Maine, United States) and group-housed after weaning on postnatal day 21 with at least one other mouse of the same litter and sex in Plexiglas cages with corn cob bedding and a 0.75 square-inch cotton square for nest material. Food and water were provided *ad libitum*. Mice were kept in homozygous breeding colonies and random samples of transgenic mice were genotyped by tail biopsy to confirm that the allele encoding β-E had been reintroduced. All home cages were kept in a ventilated colony room maintained at 22 ± 2 °C and 50 ± 20 % humidity and a continuous light–dark schedule of 12-h intervals with lights off at 0930 h.

We were primarily interested in genotypic influences on the locomotor effects of ethanol but included a limited number of saline controls in Experiment 1. We assessed the locomotor response of 34 mice following 0.75 g/kg ethanol with n = 8–10 per genotype/sex, 22 mice following saline with n = 4–7 per genotype/sex, and 61 mice after 2.0 g/kg ethanol, with n = 15–16 per genotype/sex, all during the active/dark phase of their circadian cycle.

Apparatus and measurements

We built a parallel rod floor apparatus in-house referencing dimensions from Crabbe and colleagues (Kamens & Crabbe, 2007). This apparatus assesses locomotor incoordination and sedation by how successfully mice balance on a series of parallel rods. Mice that are more ataxic will slip more frequently onto a solid lower floor beneath the parallel rods, and mice that are more sedated will travel less, have longer periods of immobility, and spend more time with their feet in contact with the lower floor when resting on the parallel rods. Our model featured a 15 × 15 cm^2 arena enclosed in a 20-cm high open-topped Plexiglas frame. The arena floor was composed of 1.6-mm diameter steel rods spaced 4 mm apart and 1 cm above a detached solid lower floor plate. An infrared beam grid using four pairs of Cutler Hammer PRISM series 18-mm thru-beam emitters and detectors were spaced in pairs 6 cm and 12 cm apart (Fig. 1A) to detect floor contact from the subject. Beam breaks at the surface of the solid lower floor were captured by interruptions of beam signal by the subject, causing a red LED onboard the detector(s) to switch off. The lower floor plate was fitted to the inner perimeter of the apparatus frame and kept stationary during trials, allowing the body of the apparatus to be lifted out for cleaning and put back directly over the floor plate at a precise position. An overhead camera recorded trials for analysis of distance traveled by AnyMaze software, and an apparatus-level camera recorded beam breaks indicated by LED shutoffs. In designs that automate slip data from counting circuit breaks, disruptions made by the tail can confound foot slip measurements (Crabbe et al., 2003). To account for this, animal trials were

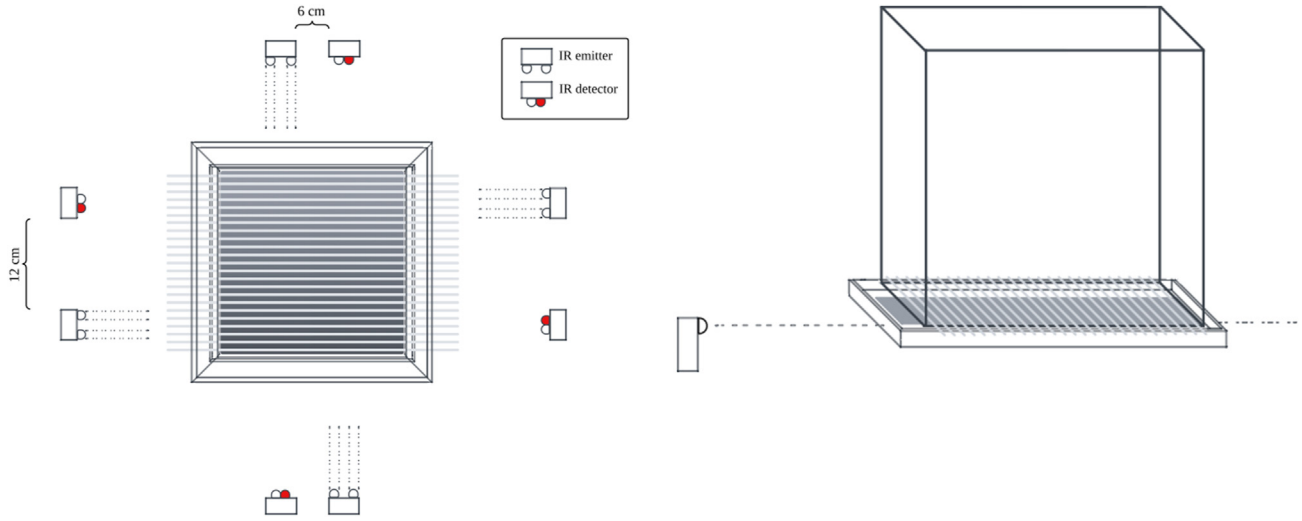

Fig. 1. Parallel Rod Floor Apparatus (PRF). From above (**A**). The infrared beam grid consisted of four single-channel closed circuits between each IR emitter and the detector on the opposite side. Breaking a beam caused the detector red LED to switch off; LED shutoffs were recorded and tallied as beam breaks. (**B**) PRF apparatus from the side. IR beams contacted the surface of the lower floor. Foot slips were determined by tallying beam breaks made by the tail in trial recordings and subtracting them from total beam breaks. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

recorded from the side, and beam breaks made by the tail were tallied from trial recordings and subtracted from the total number of beam breaks to yield foot slips for each animal (Griffin et al., 2013). The first instance of immobility was defined as the first ≥5-s interval where the animal remained in one place with all four feet stationary. Time spent off-balance (in contact with the lower floor) and the first instance of immobility were timed from trial recordings.

Experimental design

Subjects were brought to the testing site adjacent to the colony rooms in home cages and assessed one at a time. Those left in the home cage remained in a separate dark room until their trial commenced. Each trial began as subjects were weighed and intraperitoneally (i.p.) injected with 0.75 or 2.0 g/kg of 20 % ethanol or an equivolume dose of normal saline, then habituated in the dark for 15 min in individual Plexiglas holding cages with corn cob bedding. Subjects were then brought to the testing room and gently lowered into the parallel rod floor apparatus where their behavior was recorded for 15 min by video camera as described in the apparatus and measurements section. Experiment 1 assessed the sensitivity of the apparatus to low-dose ethanol by comparing behavior in mice injected with 0.75 mg/kg ethanol to saline-injected mice. Experiment 2 followed up on genotype differences by assessing locomotor effects after 2.0 g/kg ethanol.

All trials were counterbalanced for sex and genotype by cage and took place at least 1 h apart from light cycle changes, between 10:30 and 19:30. The experimenter was blind to subject order during data collection, and subject order was re-randomized before measuring each dependent variable. In one instance where subject order failed to be randomized before tallying slip counts in Experiment 1, a subset of trial recordings from each experimental group was randomly selected to be re-scored by a second blind rater; the correlation coefficient between raters was 0.99.

Blood ethanol concentration

Following Experiment 2, subjects were returned to single cages for 30 min, then anesthetized with isoflurane and sacrificed via rapid decapitation 1-h post-injection. Trunk blood was collected and centrifuged to analyze blood ethanol concentration in plasma (Analox Alcohol Analyzer, Analox Instruments; Stourbridge, United Kingdom). Blood samples were immediately put on ice until being centrifuged on the day of collection, and plasma was subsequently stored at −20 °C for a period not exceeding 60 days before analysis.

Statistical analysis

We evaluated the number of slips (measured as total beam breaks minus tail-generated beam breaks), the distance traveled, slips per meter, duration to first immobile period, and the total time spent off-balance in each experiment using SPSS 28.0.1. Results in Experiment 1 were evaluated by 3-way ANOVAs (dose × genotype × sex). Group differences in Experiment 2 were assessed by 2-way ANOVAs, by sex and genotype. The criterion for significance was set at $p < 0.05$.

Results

Trials were broken up into two experiments. Experiment 1 evaluated 0.75 g/kg ethanol or an equivolume dose of saline by sex/genotype, and Experiment 2 evaluated all groups at 2.0 g/kg ethanol. For each experiment we assessed body weight in order to eliminate genotypic weight differences as a potential confound. In Experiment 1, weight did not differ between genotype ($F_{(1,55)} = 2.650$, $p > 0.05$) but unsurprisingly depended on sex ($F_{(1,55)} = 212.284$, $p < 0.001$) with males weighing more than females. In addition, there were no differences in body weight by injection group ($F_{(1,55)} = 0.748$, $p > 0.05$) or interaction between sex and genotype ($F_{1,55} = 0.285$, $p > 0.05$), sex and dose ($F_{(1,55)} = 3.661$, $p > 0.05$), genotype and dose ($F_{(1,55)} = 0.824$, $p > 0.05$), or sex, genotype, and dose ($F_{(1,55)} = 0.000$, $p > 0.05$). In Experiment 2, weight similarly did not depend on genotype ($F_{(1,57)} = 1.558$, $p > 0.05$) and males weighed more than females ($F_{(1,57)} = 180.369$, $p < 0.001$). Again, there was no interaction between sex and genotype ($F_{(1,57)} = 0.328$, $p > 0.05$) on body weight.

Experiment 1

The results of Experiment 1 indicate that β-E −/− mice are more sensitive to locomotor ataxia following a low dose (0.75 g/kg) of

alcohol and suggest that the influence of β-E deficiency on ataxia may be more pronounced in males. There were more slips in the alcohol group than in saline-injected controls ($F_{(1,52)} = 62.939$, $p < 0.001$) as well as main effects of genotype ($F_{(1,52)} = 29.452$, $p < 0.001$) and sex ($F_{(1,52)} = 12.148$, $p = 0.001$) where β-E deficient and male mice slipped more, respectively (Fig. 2A). There were interactions between genotype and sex ($F_{(1,52)} = 3.945$, $p = 0.05$) suggesting that β-E −/− males were particularly prone to slips. There was also an interaction between genotype and dose ($F_{(1,52)} = 14.428$, $p < 0.001$), demonstrating increased sensitivity in β-E −/− mice. The alcohol group traveled less distance ($F_{(1,52)} = 12.148$, $p = 0.001$; Fig. 2B) and there was an interaction between sex and dose on distance traveled ($F_{(1,52)} = 6.449$, $p = 0.014$). The amount of slips per meter traveled depended on dose ($F_{(1,48)} = 54.970$ $p < 0.001$; Fig. 2C) as well as genotype ($F_{(1,48)} = 8.881$ $p = 0.005$), and males tended to slip more per meter than females ($F_{(1,48)} = 3.433$ $p = 0.070$). There was an interaction between genotype and dose on slips per meter ($F_{(1,48)} = 5.089$ $p = 0.029$) and no other significant interactions (Sex × Geno, $F_{(1,48)} = 1.755$, $p > 0.05$; Sex × Dose, $F_{(1,48)} = 1.125$, $p > 0.05$; Sex × Geno × Dose, $F_{(1,48)} = 0.796$, $p > 0.05$). Though males tended to travel less than females the sex difference was not significant ($F_{(1,52)} = 3.101$ $p = 0.085$). There were no other influences on distance traveled (Geno, $F_{(1,52)} = 0.847$, $p > 0.05$; Sex × Geno, $F_{(1,52)} = 0.000$, $p > 0.05$; Geno × Dose, $F_{(1,52)} = 1.419$, $p > 0.05$; Sex × Geno × Dose, $F_{(1,52)} = 0.758$, $p > 0.05$). The total number of beam breaks did not differ between any group or show any interactions (Fig. 2D); (Sex, $F_{(1,52)} = 0.843$, $p > 0.05$; Geno, $F_{(1,52)} = 0.031$, $p > 0.05$; Dose, $F_{(1,52)} = 0.513$, $p > 0.05$; Sex × Geno, $F_{(1,52)} = 0.077$, $p > 0.05$; Sex × Dose, $F_{(1,52)} = 0.001$, $p > 0.05$; Geno × Dose, $F_{(1,52)} = 0.084$, $p > 0.05$; Sex × Geno × Dose, $F_{(1,52)} = 0.262$, $p > 0.05$). We accessed time to first immobility (Fig. 2E) and time spent off-balance (Fig. 2F) in 2-way ANOVAs between sex and genotype in the alcohol-injected mice. At this low dose, time to first immobility did not depend on genotype ($F_{(1,30)} = 0.347$, $p > 0.05$) or sex ($F_{(1,30)} = 0.999$, $p > 0.05$), and did not

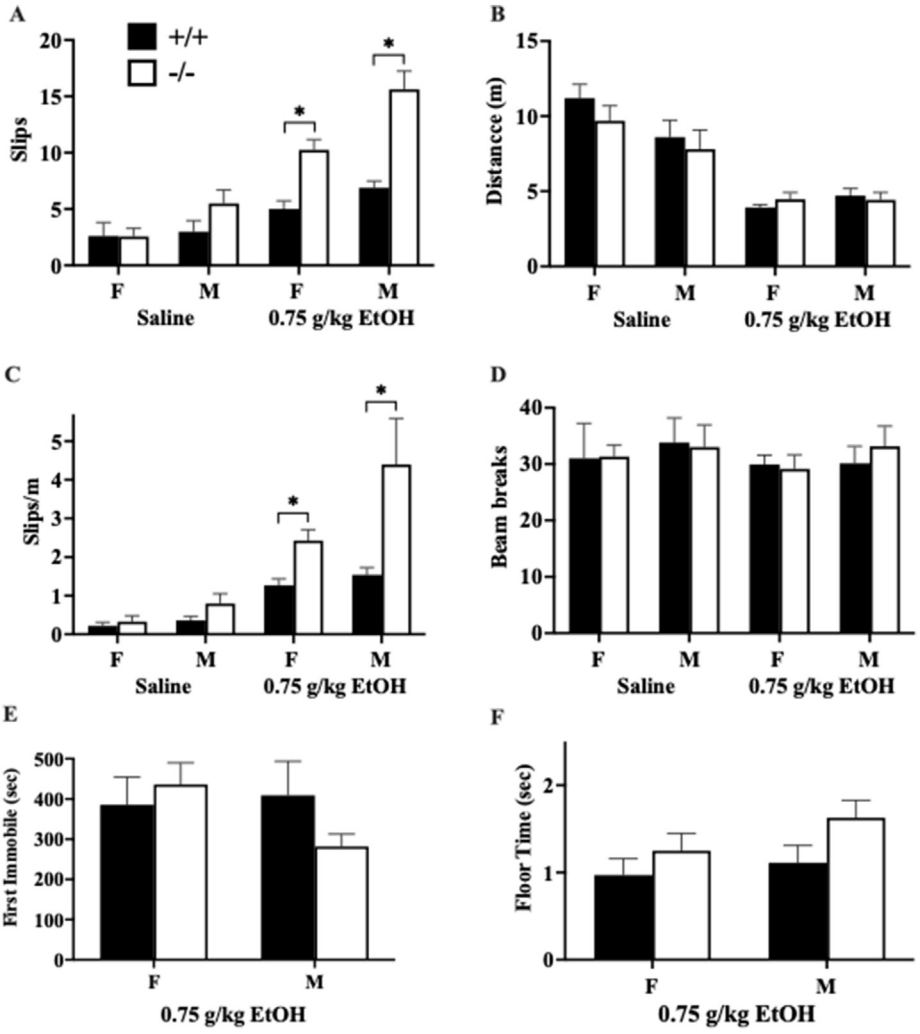

Fig. 2. Ataxic effects of low dose alcohol. (**A**) Both male and female, wild-type and β-E deficient subjects slipped more following 0.75 ethanol than saline. Males slipped more than females, and β-E −/−mice slipped more than wild-types. In addition, the effect of alcohol depended on genotype, as β-E −/−mice were more impaired by the drug than wild-type controls (interactions indicated by *; see results for full details). (**B**) Both genotypes traveled less following alcohol. (**C**) Alcohol increased slips per meter traveled. β-E −/− mice slipped more per meter traveled than wild-type. (**D**) There was no difference in beam breaks between groups, suggesting that beam breaks made by the tail obscured evidence of ataxia at this low dose. (**E**) Duration to the first immobile period did not differ between groups in the alcohol treatment. (**F**) There was a trend for β-E −/−mice to spend more time off-balance, but the effect was not significant. Bars represent group means ± SEM, and asterisks depict selected significant interactions. There were 11 subjects of each genotype in the saline condition. Sample sizes: 6 +/+ male saline; 5 +/+ female saline; 4 −/− male saline; 7 −/− female saline; 8 +/+ male 0.75 ethanol; 10 +/+ female 0.75 ethanol; 8 −/− male 0.75 ethanol; 8 −/− female 0.75 ethanol.

show an interaction between genotype and sex ($F_{(1,30)} = 1.858$, $p > 0.05$). There was a non-significant tendency for β-E −/− mice to spend more time off-balance ($F_{(1,30)} = 3.873$, $p = 0.058$), but time spent off-balance did not depend on sex ($F_{(1,30)} = 1.679$, $p > 0.05$) or show an interaction between genotype and sex ($F_{(1,30)} = 0.349$, $p > 0.05$).

Experiment 2

The results of Experiment 2 indicate that β-E −/− mice are more sedated following a high dose (2.0 g/kg) of alcohol and suggest that the influence of β-E deficiency on sedation may be more pronounced in males. Perhaps because they spent more time immobilized, β-E deficient mice slipped less often than wild-type controls following 2.0 g/kg ethanol ($F_{(1,57)} = 25.105$, $p < 0.001$; Fig. 3A) and there was a significant interaction between sex and genotype ($F_{(1,57)} = 8.737$, $p = 0.005$) on slip count, but no main effect of sex ($F_{(1,57)} = 0.000$, $p > 0.05$). β-E deficient mice traveled less ($F_{(1,57)} = 4.172$, $p = 0.046$; Fig. 3B) and distance traveled did not depend on sex ($F_{(1,57)} = 0.532$, $p > 0.05$) or show an interaction ($F_{(1,57)} = 1.025$, $p > 0.05$). β-E deficient mice had fewer beam breaks as evidenced by a main effect of genotype ($F_{(1,57)} = 19.609$, $p < 0.001$; Fig. 3D). There was no main effect of sex on beam breaks ($F_{(1,57)} = 0.309$, $p > 0.05$); however, there was an interaction between genotype and sex ($F_{(1,57)} = 6.231$, $p = 0.015$). β-E −/− mice became immobile sooner ($F_{(1,56)} = 19.906$, $p < 0.001$; Fig. 3E), but there was no main effect of sex on time to first immobility ($F_{(1,56)} = 1.354$, $p > 0.05$) or interaction between sex and genotype ($F_{(1,56)} = 2.482$, $p > 0.05$). Time spent off-balance depended on genotype ($F_{(1,57)} = 18.678$, $p < 0.001$; Fig. 3F), where β-E −/− mice spent more time balancing on the lower floor, and there was no main effect of sex ($F_{(1,57)} = 0.131$, $p > 0.05$) or interaction between sex and genotype ($F_{(1,57)} = 0.776$, $p > 0.05$). Slips per meter traveled did not depend on sex ($F_{(1,57)} = 0.344$, $p > 0.05$; Fig. 3C) or genotype ($F_{(1,57)} = 0.598$, $p > 0.05$) and did not show an interaction ($F_{(1,57)} = 0.859$, $p > 0.05$).

We evaluated BEC from trunk blood samples collected 1 h after administration to assess for variability in ethanol administration at the highest dose. There was no difference in BEC between sex ($F_{(1,53)} = 0.162$, $p > 0.05$; Fig. 4) or genotype ($F_{(1,53)} = 0.514$, $p > 0.05$)

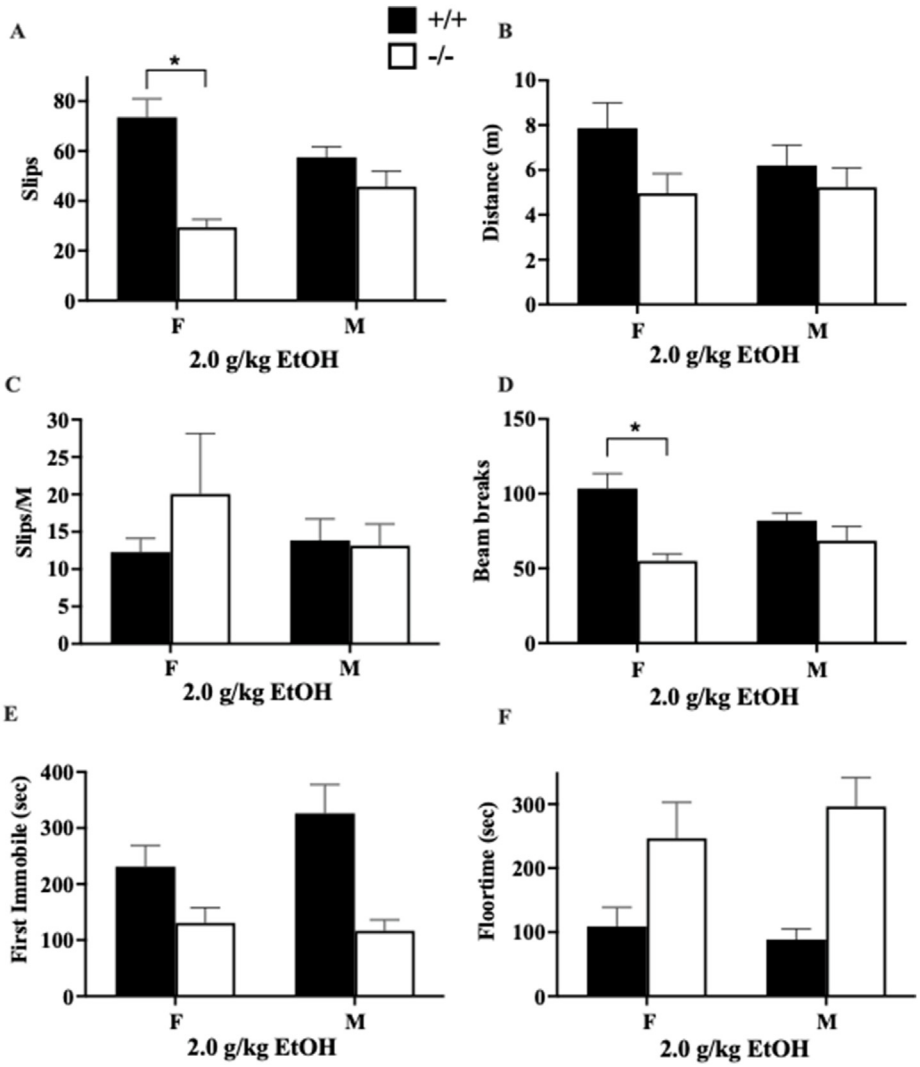

Fig. 3. Ataxic effects of 2.0 g/kg ethanol. β-E influenced sedation at a high dose of alcohol. (**A**) β-E −/− mice slipped less than C57BL/6J controls. There was an interaction between genotype and sex indicating that female, but not male, β-E −/− mice slipped less than their wild-type counterparts. (**B**) β-E −/− mice traveled less. (**C**) Slips per meter traveled did not differ between groups. (**D**) β-E −/− mice were less active as measured by fewer beam breaks. (**E**) These data reflect more time in a prone position: β-E −/− mice became immobile sooner than wild-type (**F**) and spent more time immobile than wild-type controls. Bars represent group means ± SEM and asterisks designate significant interactions. Sample sizes: 15 +/+ male 2.0 ethanol; 15 +/+ female 2.0 ethanol; 16 −/− male 2.0 ethanol; 15 −/− female 2.0 ethanol.

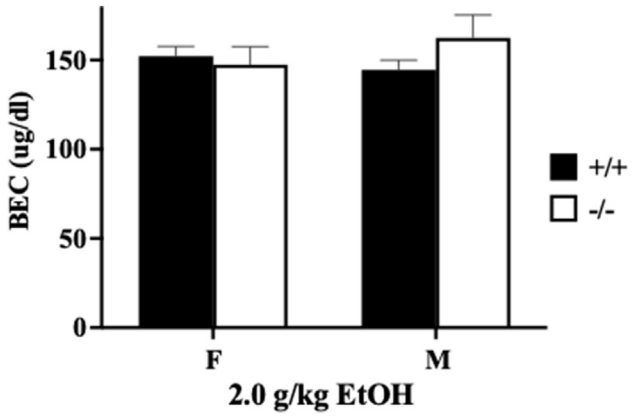

Fig. 4. Ethanol metabolism does not depend on genotype or sex. 1 h after 2.0 g/kg ethanol. Bars depict means ± SEM from 14 +/+ male, 14 +/+ female, 14 −/− male, and 15 −/− female mice.

and no interaction between genotype and sex ($F_{(1,53)} = 1.566$, $p > 0.05$).

Discussion

Alcohol misuse persists as a leading cause of preventable health emergencies and societal harm across the globe. The opioid peptide β-endorphin influences the behavioral and motivational effects of alcohol, and conversely endogenous opioids, including β-E, adapt to chronic alcohol misuse, contributing to dependence (for review, see Palm & Nylander, 2016). The complex interactions between alcohol and endogenous opioid systems have made β-E of interest for further understanding the heterogeneity of AUD risk factors, onset, and outcomes, as well as developing effective pharmaceutical interventions. We found that alcohol increased sedation and ataxia in mice lacking β-E, and that these effects were more pronounced in males than in females.

Alcohol's influence on locomotion is multifaceted, and different aspects of locomotion such as ataxia and arousal may be more pronounced at different doses. In Experiment 1, the parallel rod floor (PRF) apparatus was sensitive to changes in locomotion following a low dose (0.75 g/kg) of alcohol. Males and β-E −/− mice slipped more than wild-type mice, and β-E −/−males had the highest slip count and rate of slips per meter. The alcohol group traveled less, initially suggesting increased sedation; however, there were no differences between alcohol and saline groups in time spent off-balance or duration to first immobile period. Others have found that rats exhibit locomotor sedation only at doses of 1 g/kg or more, suggesting that lower doses of alcohol do not reliably produce sedation (Karlsson & Roman, 2016). Therefore, the lower distance traveled by the alcohol group may represent heightened ataxia due to an increased difficulty with maintaining balance and subsequent difficulty moving freely on the parallel rods. β-E is released in the nucleus accumbens from doses as little as one standardized drink in humans (Mitchell et al., 2012), and is released in the hypothalamus at doses ranging from 10 to 120 mM in rats (Gianoulakis, 1990). However, the contribution of β-E to dose-dependent effects of alcohol is unclear. Results from Experiment 1 suggest that β-E modulates ataxia and may interact with sex to influence ataxic effects at a low dose of alcohol. The PRF apparatus may help further elucidate dose-dependent effects in future studies as a sensitive assay to multiple measures of locomotion at low doses.

In Experiment 2, there were pronounced sedative effects that were increased in β-E deficient mice. Interestingly, β-E −/− mice slipped less than wild-type mice, in contrast to Experiment 1. This is likely due to overall less movement in the more sedated group. β-E −/− mice spent more than twice as much time off-balance and became immobile sooner than wild-type mice, indicating longer and more frequent intervals of propping at least one foot on the lower floor while remaining stationary. β-E −/− females slipped less and had fewer beam breaks than any other group but did not differ from β-E −/− males in any other measure. This may suggest the continuation of greater ataxia in β-E −/− males than females that was found at the lower dose; however, more data are needed to substantiate this idea. Results from Experiment 2 collectively suggest that β-E reduces sedative effects from high doses of alcohol. In Experiment 2 we found no group differences in blood alcohol concentration, but we only evaluated one time point following a single dose. Others have previously reported no sex differences in area under the curve or peak BAC for C57BL/6J mice across multiple doses (Pruett, Tan, Howell, & Nanduri, 2020; White et al., 2023), suggesting that pharmacokinetic factors are unlikely to play a role in the behavioral differences exhibited here.

The mechanism by which alcohol facilitates β-E release is not fully understood. Acetaldehyde (ACD) has been suggested to play a role; however, the rapid conversion of ACD to acetate in the brain has limited direct approaches of study. Biobehavioral effects of the metabolite following alcohol are primarily studied by modulating production of brain catalase and CYP-2E1, enzymes that convert ethanol into ACD, and aldehyde dehydrogenase, which converts ACD into acetate (Deng & Deitrich, 2008). ACD is self-administered in rats (Peana, Muggironi, & Diana, 2010) and elicits conditioned place preference in alcohol-preferring rats and place aversion in alcohol non-preferring rats (Quintanilla & Tampier, 2003), suggesting that it influences behavior through circuits involved in alcohol reinforcement. In addition, simultaneous administration of alcohol and cyanamide, an aldehyde dehydrogenase inhibitor, results in greater CRH expression in the HPA axis and POMC mRNA expression in the arcuate nucleus than from alcohol alone (Kinoshita et al., 2001). Another study found that inhibiting brain catalase blocks β-E secretion from hypothalamic neurons *in vitro* (Reddy, Boyadjieva, & Sarkar, 1995). This body of research suggests that ACD induces β-E release in the HPA axis, perhaps in response to elevated CRH; however, more research is needed to confirm the direct involvement of ACD and elucidate how the metabolite produces its effects.

Transgenic animal models enable researchers to evaluate the contributions of a single gene to behavior, but also place these contributions in the context of interdependent networks in the brain. Single gene modifications, especially those of highly distributed products such as β-E, can indirectly modulate gene expression in affected circuits during development, and these background modulations may independently affect behavior (for reviews, see Doetschman, 1999; Gingrich & Hen, 2000; Gorelick, 1996; Nebert & Duffy, 1997; Nelson & Young, 1998). Circuits affected by a missing gene may compensate for the lost gene function, and such changes have been useful in elucidating the circuit relationships and function (Doetschman, 1999; Gingrich & Hen, 2000; Gorelick, 1996). For example, the transgenic line used in this study is more analgesic than wild-type controls when exposed to cold water, suggesting that additional non-opioid pathways are upregulated to compensate for an absence of β-E and contribute to analgesia (Rubinstein et al., 1996). Likewise, the differences we observed between wild-type and β-E deficient mice may be influenced by latent adaptations to a lack of β-E. However, others have argued that compensatory adaptations obscure the role of the missing gene (Crawley, 2006; Matthaei, 2007), and it would be useful to study inducible and reversible knockouts as well. Nonetheless, our lab has previously found evidence for a

hyperactive HPA axis in β-E deficient mice with increased sensitivity to anxiolysis from alcohol (Barfield et al., 2010; Grisel, Bartels, Allen, & Turgeon, 2008) and indeed, genetic and epigenetic markers of HPA overactivation are present in humans with a family history of AUD and vulnerable populations such as veterans (Hill et al., 2022; Sinha, 2022; Siomek-Gorecka, Dlugosz, & Czarnecki, 2021; Szabo et al., 2020). Thus, while traditional knockouts may not isolate the direct role of β-E, they help inform how the disruption of a gene product may affect a system as a whole and may better reflect the resulting differences in behavior (Doetschman, 1999; Gingrich & Hen, 2000). Future studies employing inducible β-E knockouts to our assay may then be useful for distinguishing the direct effects of β-E from background adaptations to a lack of peptide.

Greater synthesis and release of β-E and heightened sensitivity in the mesolimbic reward pathway are well documented in alcohol-preferring and low β-E rodent strains (Hayward, Pintar, & Low, 2002; Herz, 1997; Hood et al., 2022; Méndez et al., 2001; Molina-Martínez & Juárez, 2021; Turton & Lingford-Hughes, 2016). Release of β-E from the arcuate nucleus disinhibits GABAergic downregulation of dopamine transmission in the VTA, nucleus accumbens, amygdala, and prefrontal cortex (Juárez & Molina-Martínez, 2019), eliciting increased motivation and sensitivity to positive reinforcing effects. This mechanism, perhaps along with induction by acetaldehyde, may help explain increased drug-seeking behavior (Charbogne, Kieffer, & Befort, 2014) and alcohol self-administration in these strains (Grisel et al., 1999; Nentwig, McGonigle, Wilson, Rhinehart, & Grisel, 2017). Alcohol's negative reinforcing effects, however, also are amplified in animals and humans with low β-E (for reviews, see Juárez & Molina-Martínez, 2019; Topel, 1988). These effects are believed to manifest when self-medicating a chronically overactive HPA axis, effectively supplementing inadequate negative feedback from low basal levels of β-E with alcohol-induced β-E release (Barry & Grisel, 2012; Bolton, Cox, Clara, & Sareen, 2006; Gianoulakis, 1998; Gianoulakis et al., 1989; Koob & Le Moal, 2008a, 2008b; Molina-Martínez & Juárez, 2021; Topel, 1988). Indeed, chronic alcohol use promotes POMC, expressing cell death and further upregulating the HPA axis (Gianoulakis, Dai, & Brown, 2003; Sarkar, Kuhn, Marano, Chen, & Boyadjieva, 2007), and CRH antagonists block anxiety-like symptoms of withdrawal and decrease binge drinking (Kiefer, Horntrich, Jahn, & Wiedemann, 2002; Koob, 2003; Müschen et al., 2019). The transition in increased sensitivity from positive to negative reinforcing effects in progressing addiction is thought to contribute to initial risk factors and dependence (Cho et al., 2019; Koob, 2004, 2013; Koob & Le Moal, 2008a, 2008b) and promoted by neuroadaptation of the endogenous opioid system. Considering that the transgenic line of mice used in this study are more anxious than wild-type mice (Brown, Corey, & Moore, 1999; Grisel et al., 2008; Seibenhener & Wooten, 2015) and show increased sensitivity in the HPA axis to the effects of alcohol (De Waele, Papachristou, & Gianoulakis, 1992), as well as exhibit greater anxiolytic effects from alcohol (Barfield et al., 2010; Grisel et al., 2008), we surmise that our data represent differences in predominantly negative reinforcing drug effects, and that increased ataxia and sedation may rely on similar mechanisms as the increased anxiolytic effects of ethanol seen in transgenic mice.

In recent years there has been increasing interest in investigating the role of sex in neurobiological, genetic, and sociocultural perspectives of addiction. Alcoholism in women is historically understudied (Del Carmen Miguez & Permuy, 2017; Mulia & Bensley, 2020; Wilke, 1994), yet the rate of alcohol misuse is increasing more rapidly in women (Grant et al., 2017; Mulia & Bensley, 2020), and the gap between female and male rates of misuse is narrowing (Keyes, Li, & Hasin, 2011). Here we found that slip counts depended on an interaction between β-E and sex at both doses, where female β-E deficient mice were less ataxic than their male counterparts. Overall, males slipped more and also tended to travel less at the lower dose. Together these results suggest that the influence of β-E on locomotion following alcohol may depend on sex. Our lab has previously shown sex-dependent effects of β-E in alcohol reward (Scopano, Jones, Stea, Freeman, & Grisel, 2023), binge drinking (Nentwig, Wilson, Rhinehart, & Grisel, 2017), and *in vivo* neuronal activation in response to alcohol (Rhinehart et al., 2020). Others have found this interaction to influence alcohol consumption (Leyrer-Jackson, Hood, & Olive, 2022) and the efficacy of naltrexone on alcohol dependency (Matzeu, Terenius, & Martin-Fardon, 2018). A growing body of literature contends that sex disparities in AUD involve sex-dependent modification of the HPA axis (Logrip et al., 2013; Ogilvie & Rivier, 1997; Przybycien-Szymanska, Gillespie, & Pak, 2012; Przybycien-Szymanska, Rao, & Pak, 2010) and subsequent vulnerability to stress-induced drinking and craving (Fox & Sinha, 2009; Guinle & Sinha, 2020; Keyes, Martins, Blanco, & Hasin, 2010; Piazza, Vrbka, & Yeager, 1989). Gonadal hormones may underlie these dimorphisms — for example, females and castrated males show increased blood levels of adrenal corticotropic hormone (ACTH) and corticosterone following alcohol (Ogilvie & Rivier, 1997). An elegant series of experiments by Pak and colleagues also revealed that 17β-estradiol is necessary to elicit sexually dimorphic effects of chronic alcohol exposure in the HPA axis (Przybycien-Szymanska et al., 2012). Others have found that estradiol as well as testosterone influence alcohol seeking (Bertholomey & Torregrossa, 2019), and that alcohol-driven ACTH release reduces testosterone in men but raises it in women (Frias, Torres, Miranda, Ruiz, & Ortega, 2002), suggesting that gonadal hormones both influence and adapt to alcohol use via the HPA axis. Another contributing factor may be stress itself; women are thought to experience more stress (Matud, 2004; for review, see Bale & Epperson, 2015) and exhibit greater CRH neuronal sensitivity to stress in the HPA axis and the locus coeruleus (for review, see Bangasser & Valentino, 2012). Women also experience higher rates of childhood sexual abuse and domestic violence (Oram, Khalifeh, & Howard, 2017), which are among the strongest predictors of AUD and stress-related mood disorders that correlate with AUD (for review, see Guinle & Sinha, 2020). Our data may help inform the neurobiological underpinnings of these relationships by suggesting that β-E deficiency in females leads to an inherently more sensitive HPA axis, reducing anxiolytic and sedative effects of alcohol.

Alcohol addiction varies substantially in its onset, trajectory, and outcomes between individuals. Interdisciplinary research efforts have revealed that alcohol affects a broad array of neurotransmitters and pathways, contributing to differential patterns of sensitivity in those predisposed to genetic and environmental risk factors. Among these, the literature has found an increasingly important role of alcohol-induced β-E release, not only for its well-characterized rewarding and reinforcing properties, but also through mediation of the neuroendocrine stress response. Here we have shown that β-E regulates the sedative and ataxic effects of alcohol. Our findings contribute to the understanding of β-E's involvement in the behavioral effects of alcohol and support the growing evidence for its role in stress regulation as a site for AUD risk factors.

Author contributions

SGS contributed to conceptualization, data acquisition and curation, Formal analysis, wrote the original draft, and edited the final manuscript. JEG provided preliminary data, and helped with conceptualization, Formal analysis, and writing.

Declaration of competing interest

The authors have no conflict of interest.

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