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Hnrnph1 IS A NOVEL REGULATOR OF ALCOHOL REWARD

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Abstract

Background: *Hnrnph1* is a validated quantitative trait gene for methamphetamine behavioral sensitivity that encodes for heterogeneous nuclear ribonucleoprotein H1 (hnRNP H1). This RNA-binding protein is involved in all stages of RNA metabolism that impacts mesocorticolimbic dopamine neurotransmission to influence addiction-related behavior.

Methods: We characterized the alcohol behavioral phenotypes of mice heterozygous for a deletion in the first coding exon of *Hnrnph1* (*Hnrnph1+/−*). We examined alcohol intake under both continuous- and limited-access procedures, as well as alcohol-induced place-conditioning. Follow-up studies examined genotypic differences in the psychomotor-activating and sedative-hypnotic effects of acute and repeated alcohol, and a behavioral test battery was employed to determine the effects of *Hnrnph1* deletion on the manifestation of negative affect during alcohol withdrawal.

Results: Relative to wild-type (WT) controls, *Hnrnph1+/−* males exhibited blunted intake of high alcohol concentrations under both drinking procedures. *Hnrnph1* deletion did not impact the conditioned rewarding properties of low-dose alcohol, but reversed the conditioned place-aversion elicited by higher alcohol doses (2 and 4 g/kg), with more robust effects in male versus female mice. No genotypic differences were observed for alcohol-induced locomotor activity. *Hnrnph1+/−* mice exhibited a modest increase in sensitivity to alcohol's sedative-hypnotic effects, but did not differ from WT mice with regard to tolerance to alcohol's sedative-hypnotic effects or alcohol

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Contributors

EFK, MAC, DL, CLJC and KKS conducted the experiments; CLJC and KKS analyzed the data; KKS supervised the research and graphically depicted the data; EFK composed the initial manuscript draft; KKS and CDB revised the manuscript; all authors contributed to the final editing of the manuscript prior to submission.

Conflict of Interest

Nothing declared

metabolism, Inconsistent effects of *Hnrnph1* deletion were observed in models for withdrawal-induced negative affect.

Conclusions: These data identify *Hnrnph1* as a novel, male-selective, driver of alcohol consumption and high-dose alcohol aversion that is potentially relevant to the neurobiology of alcohol abuse and alcoholism.

Keywords

hnRNP H1; binge-drinking; place-preference; intoxication; negative affect; ethanol; dysphoria

1. INTRODUCTION

hnRNP H1 (heterogenous nuclear ribonucleoprotein H1) is an RNA-binding protein (RBP) that is ubiquitously expressed in brain (Lein et al., 2007) and can regulate all aspects of RNA metabolism, including pre-mRNA splicing through binding at specific intron sites, mRNA stability, and translational regulation via 5'UTR and 3'UTR binding and poly-adenylation control (e.g., Han et al., 2010; Dreyfuss et al., 2002; Schaub et al., 2007). hnRNP H family proteins (including hnRNP H1) are considered critical regulators of neuron and oligodendrocyte differentiation (Aranburu et al., 2006; Tiruchiinapalli et al., 2008). Congenic mice harboring *Hnrnph1* polymorphisms associated with decreased methamphetamine sensitivity express a set of down-regulated genes involved in neurodevelopment, including a 1.5-fold decrease in the transcription factor *Nurr1/Nr4a2* (Yazdani et al., 2015). While RBPs, including hnRNP H1, are localized to the nucleus, exposure to extracellular stimuli (e.g., stressors, neuronal activity, drugs) can cause their translocation to cytoplasm where they can be positioned to regulate local translation underlying activity-dependent synaptic plasticity (Fukuda et al., 2009; Guil et al., 2006; Markmiller et al., 2018; Wall et al., 2020; Zhang et al., 2012).

As reviewed elsewhere (Bryant and Yazdani, 2016), there is a growing appreciation that RBPs play a pivotal role in addiction-related synaptic plasticity. Using an unbiased, forward genetic and fine mapping approach, we positionally cloned and validated *Hnrnph1* as a quantitative trait gene underlying sensitivity to the locomotor stimulant response to methamphetamine (Yazdani et al., 2015) that we subsequently showed is likely mediated by a set of four, 5' UTR variants that cause decreased 5' UTR usage and decreased hnRNP H protein expression (Ruan et al., 2020b). Homozygous deletion of *Hnrnph1* is lethal (Yazdani et al., 2015), however, we subsequently showed that a heterozygous mutation in the first coding exon of *Hnrnph1* also decreased the rewarding and reinforcing properties of methamphetamine (Ruan et al., 2020a). *Hnrnph1* contributes to post-transcriptional processing of *OPRM1,* including translational repression (Song et al., 2012) and splicing (Xu et al., 2014). *OPRM1* encodes the mu opioid receptor, which is the primary molecular target underlying the addictive and analgesic properties of opioid drugs (e.g., Matthes et al., 1996). Supporting a potential role for *Hnrnph1* in substance use disorders, an intronic variant in *HNRNPH1* was associated with the severity of heroin dependence and differential splicing of *OPRM1* in humans (Xu et al., 2014). Further, mice with a small frameshift deletion within the first coding exon of *Hnrnph1* (*Hnrnph1+/*) (Yazdani et al., 2015) self-administer less fentanyl than their wild-type (WT) counterparts, independent of any

observable effects of gene deletion on fentanyl-induced antinociception or physiological dependence (Bryant et al., 2020).

Decades of evidence from both human and laboratory animal studies implicate *OPRM1* polymorphisms in the etiology and treatment prognosis of alcoholism (see Berrentini 2016 for review). Moreover, a survey of 17 proteomic studies indicate an association between alcohol exposure and an increase in *Hnrnph* expression in the brains of laboratory rodents (Wang et al. 2011). To the best of our knowledge, the functional relevance of hnRNP H1 in alcohol drinking and dependence is unexplored. Thus, the present study characterized the effect of a heterozygous *Hnrnph1* deletion on AUD-related behaviors. Both female and male *Hnrnph1+/−* mice showed reduced sensitivity to the locomotor stimulant response to methamphetamine (Yazdani et al., 2015), while only *Hnrnph1+/−* females showed reduced fentanyl-induced locomotion (Bryant et al., 2020). Thus, we compared *Hnrnpnh1*+/− versus +/+ mice of both sexes for alcohol-induced locomotor activity and sedative-hypnotic effects. Prior co-administration studies indicated common neural adaptations contribute to both methamphetamine and alcohol intake and conditioned reward (Fultz et al., 2017; Fultz and Szumlinski, 2019; Sern et al., 2020). Thus, we also tested for genotypic differences in alcohol consumption and conditioned reward. Finally, as the severity of alcohol withdrawal correlates with the motivation to drink, we compared *Hnrnpnh1*+/− versus *+/+* mice in behavioral models for alcohol withdrawal-induced negative affect. These results identify select, sometimes sexually dimorphic, alcohol behavioral phenotypes that were modified by acute and repeated alcohol exposure in *Hnrnph1* mutant mice.

2. MATERIALS AND METHODS

2.1 Subjects.

Hnrnph1+/− and their wild-type (WT; +/+) littermates were originally generated on an isogenic C57BL/6J background using TALENs targeting the first coding exon which induced a small deletion and frameshift mutation resulting in a premature stop codon (Yazdani et al., 2015). *Hnrnph1*+/− mice were maintained at UCSB by mating *Hnrnph1+/−* males from a colony established at UC Santa Barbara with C57BL/6J females purchased from The Jackson Laboratory (Sacramento, CA). At weaning, offspring were housed with same-sex littermates (a minimum of 2 mice per cage) and genotyped as detailed below. Behavioral testing commenced no earlier than PND 50, and the mice ranged in age between PND50 and PND100, with a vast majority of mice aged PND56–70 at the start of testing. At least one week prior to commencement of experimental testing, mice involved in the place-conditioning or withdrawal-induced anxiety studies were relocated to a colony room maintained on a 12:12 h light–dark cycle (lights on at 0700 h), while those involved in the alcohol-drinking studies were relocated to a colony room maintained on a 12:12 h reverse light cycle (lights off: 1000 h). Food and water were available *ad libitum* in the home cage. All procedures were approved by the UC Santa Barbara Animal Care and Use Committee and were conducted in strict accordance with National Institute of Health guidelines for the care and use of laboratory animals.

2.2 Genotyping.

Genomic DNA was extracted from tail clips obtained upon weaning and used in a PCR reaction with primers amplifying approximately 100 base pairs upstream and downstream of the TALENs binding domains, as detailed in prior reports (Bryant et al., 2020; Ruan et al., 2018,2020; Yazdani et al., 2015). After PCR, samples were mixed with a restriction enzyme cocktail overnight (BstNI), run on a 2% ethidium bromide Tris-borate-EDTA gel for 1.2 hrs, and imaged with ultraviolet light. TALENs-edited mice were identified by bands that were uncut by the restriction enzyme due to the loss of the restriction enzyme binding site (Yazdani et al., 2015).

2.3 Alcohol Drinking Procedures:

Female and male (n=8/genotype) mice were single-housed under a 12-h reverse cycle (lights off: 1000 h) for at least 7 days prior to commencing alcohol drinking procedures, which began with an examination for genotypic differences in alcohol intake under continuous-access, followed by limited-access, conditions in the same mice. For the continuous-access procedure, mice were presented in the home cage with 4 sipper tubes containing 0, 5, 10 or 20% alcohol (v/v) for 14 consecutive days. This continuous-access procedure was employed by our group previously to generate a within-subjects dose-response function for alcohol intake and preferences (e.g., Lominac et al., 2006). Bottles were weighed daily at the same time each day and the volume consumed from each sipper tube was calculated to determine intake (expressed as a function of body weight, determined weekly). Then, following a 3-day respite, the same mice underwent testing for alcohol intake under limited-access conditions. For the limited-access procedure, we employed our 4-bottle-choice version of the Drinking-in-the-Dark (DID) paradigm (e.g., Cozzoli et al., 2014; Lee et al., 2015, 2016), in which mice were presented with 4 sipper tubes containing 5, 10, 20 and 40% alcohol (v/v) for 2 h/day, beginning at 3 h into the dark phase of the circadian cycle. Limited-access drinking procedures were conducted for 10 consecutive days. Alcohol intake was determined immediately at the end of each 2-h session as described for the continuous-access procedures.

2.4 Alcohol-induced place-conditioning:

A separate cohort of experimentally naïve female and male mice (n>8/genotype) underwent alcohol-induced place-conditioning procedures to determine how *Hnrnph1* deletion impacts the motivational valence of alcohol. The apparatus and procedures employed were similar to those described previously (e.g., Ary et al., 2012; Szumlinski et al., 2008). In brief, an unbiased place-conditioning procedure involving 8 pairings of alcohol (0.5, 1.0, 1.5, 2 or 4 g/kg) was conducted, with one compartment of a 2-compartment apparatus that differed in wall pattern (marbled vs. wood-paneled) and floor texture (rough vs. smooth). Random counterbalancing of the alcohol-paired side assignment was employed, irrespective of initial side preference. Conditioning commenced with a pre-conditioning test (PreTest) in which mice were allowed to explore both compartments for 15 min. This PreTest was conducted mid-day (around 1200 h) and mice were returned to the colony room. Then, in the morning (between 0900–1100 h), mice were injected IP with saline (vol=0.2 ml/10 g) and confined to one of the compartments for 15 min, with animals randomly assigned to the saline-

conditioned compartment. In the afternoon (between 1630–1830 h), mice were injected IP with their assigned dose of alcohol and confined to the opposite compartment. Following 8 conditioning days, mice were tested for preference for the alcohol-paired compartment in the absence of any injection and the total time spent in the alcohol- versus saline-paired side (CPP Score) served to index the motivational valence of alcohol (Post-Test). Similar to the PreTest, the Post-Test was conducted mid-day, approximately 18 h following the last alcohol- conditioning session. The locomotor activity of the mice was recorded during each of the alcohol-conditioning sessions to index drug-induced psychomotor activity and changes in psychomotor activity with repeated alcohol treatment. Both the time spent in the two compartments and the distance traveled during conditioning were tracked using AnyMaze™ tracking software (Stoelting Co., Wood Dale, IL, USA).

2.5 Alcohol withdrawal-induced negative affect:

To examine the possibility that genotypic differences in the direction of the alcohol-conditioned response under high-dose alcohol place-conditioning procedures might reflect differential sensitivity to withdrawal-induced anxiety, another separate cohort of experimentally naïve female and male mice were injected IP, once daily, with 4 g/kg alcohol for a total of 8 days of injections (to mimic the place-conditioning injection regimen). Alcohol was injected in this study to control for the precise amount of alcohol exposure and to avoid the interpretational confounds associated with genotypic differences in alcohol drinking (see Results). The day following the last injection, mice were then tested for alcohol withdrawal-induced anxiety using a behavioral test battery consisting of light-dark shuttle-box, marble-burying and forced swim tests. These paradigms were selected as they are pharmacologically-validated models for negative affect and are consistently sensitive to the negative affective state produced by withdrawal from alcohol drinking in C57BL/6J mice (e.g., Lee et al., 2016, 2017a,b; 2018a,b). Recently, we observed inconsistent effects of *Hnrnph1* deletion on indices of anxiety-like behavior expressed by male mice only (Bryant et al., 2020; but see Ruan et al., 2020). Thus, a subset of alcohol-naïve *Hnrnph1+/+* and *Hnrnph1−/−* mice were included to further examine the potential genotype by sex interaction in basal affective state.

The light/dark shuttle box test indexes anxiety-like behaviors (Bourin & Hascoet, 2003; Crawley, 1985) and involves placing mice into a polycarbonate box (46cm long×24cm high×22cm wide), which is equally subdivided into a white, uncovered compartment and a black, covered compartment, separated by a central divider with an opening. Testing began with the mice on the dark side and the latency to enter the light side, number of light-side entries, and total time spent in the light side of the shuttle box were recorded during the 15-min trial using Any-maze™ tracking software (Stoelting Co., Wood Dale, IL).

The marble-burying test provides an additional index for anxiety-like behavior (Nicolas et al., 2006). Here, we placed 10 square glass pieces (2.5 cm2 × 1.25 cm tall) in the animals' home cage, 5 at each end. The total number of marbles buried by at least 75% (i.e., at least ¾ of the marble was covered by bedding) at the end of the 20-min trial was recorded and video-recordings during the 20-min session were scored for the latency to begin burying and

the total time spent burying by experimenters, who were blinded to the treatment of the mice.

Behavioral testing ended in the Porsolt forced swim test, in which each mouse was placed into an 11-cm diameter cylindrical container and the latency to first exhibit immobility (defined as no horizontal or vertical displacement of the animal's center of gravity for ≥ 5 s), total time spent immobile, and the numbers of immobile episodes were monitored throughout the entire 6-min trial period using AnyMaze™ tracking software. All testing for negative affect was conducted during the animals' circadian light phase.

2.6 Alcohol-induced intoxication and sedation.

In another cohort of alcohol-naïve mice, genotypic differences in the intoxicating and sedative properties of alcohol were assayed, respectively, using rotarod and regain of righting reflex procedures. The rotarod procedures were similar to those employed previously to examine genotypic differences in basal motor coordination (Ruan et al., 2020a) and commenced with successive training of mice to walk on a fixed speed (10 rpm) rotarod for 2 min. The following day, mice were tested for baseline rotarod performance over a 3-min period and then were injected with either 2 or 3 g/kg alcohol (the doses were reported previously by our group to induce motor in-coordination in mice; see Quadir et al., 2016, 2017) and, 15 min later, the average time to fall from the rotarod was determined in 3 successive 3-min tests. To examine the development of tolerance to alcohol's intoxicating effects, mice were injected with their assigned dose of alcohol once daily (~1100 h) for 8 injections (i.e., the same number of injections as those employed in the place-conditioning study). Then, mice were assayed again for alcohol-induced changes in rotarod performance using procedures identical to the test for alcohol's acute intoxicating effects. For the righting reflex study, a distinct cohort of alcohol-naïve female and male mice (n=7–9/sex/genotype) were injected acutely (~1100 h) with 4 g/kg and placed in an empty home cage. Upon observing the loss of righting reflex (defined as the inability to turn over and place all 4 paws on the floor of the cage; occurred within 1–2 min post-injection), mice were placed in a supine position and the latency to right themselves was determined using a stop-watch by an observer who was blinded to the genotype of the mice. The mice tested for righting reflex were then injected once daily (~1100 h), with 4 g/kg alcohol, followed by testing for withdrawal-induced anxiety as described earlier.

2.7 Alcohol pharmacokinetics:

To test for the potential relationship between genotypic differences in behavior and alcohol metabolism, mice were injected IP with 1.5 g/kg and blood was sampled from the submandibular vein at 5, 15, 30 and 60 min post-injection. Samples were analyzed by gas chromatography due to its effectiveness and accuracy in determining ethanol levels in various substances, including blood (Tiscione et al., 2011). Blood alcohol concentrations (BACs) were determined using a Shimadzu GC-2014 gas chromatography system (Shimadzu, Columbia, MD) and GC Solutions 2.10.00 software. Samples were diluted at 1:9 with non-bacteriostatic saline (50 μl of sample). Acetone and dichloromethane were used as the pre-solvents due to their lower boiling point versus ethanol. Each sample was tested within 1-week of blood collection to reduce the potential for alcohol evaporation during

storage. The determination of ethanol concentration from each sample was derived using the standard curve equation determined prior to analyses of the samples. A new standard curve was formulated for each cohort of blood samples to ensure maximal accuracy. After the ethanol peak area was determined, the peak area was used to determine the ethanol concentration and subsequently the percent of ethanol in the blood (Campbell et al., 2019; Jimenez-Chavez et al., 2020).

2.8 Statistical Analyses:

The data were analyzing using multi-factorial ANOVAs, with sex and genotype included as between-subjects factors for all initial analyses. Failure to detect sex effects or interactions prompted removal of the factor and data re-analysis. Significant interactions were deconstructed along the relevant factor(s), followed by t-tests (when fewer than 3 comparisons were conducted), tests for simple main effects or LSD post-hoc tests, when appropriate. Alpha = 0.05 for all analyses.

3. RESULTS

3.1 Alcohol intake under continuous-access.

The effect of *Hnrnph1* deletion on consumption of 5, 10 and 20% alcohol (v/v) under free-access conditions was sex-dependent (Fig.1) [Sex X Genotype X Concentration X Day: $F(26,728)=1.81$, $p=0.008$]. Interestingly, *Hnrnph1* deletion did not produce any detectable effect on intake of alcohol at any concentration in female mice (Fig.1, left) [Dose effect: $F(2,364)=29.36$, $p<0.0001$; Dose X Day: $p=0.08$; Genotype effect and interactions: all p's>0.20]. In contrast, the effect of gene deletion in males varied as a function of alcohol concentration (Fig.1, right) [Genotype X Dose X Day: $F(26,364)=1.87$, $p=0.007$]. Specifically, gene deletion did not influence the intake of 5 or 10% alcohol in male mice (Fig.1B,D) [for 5% alcohol, Genotype X Day ANOVA: all p's>0.06; for 10% alcohol, Day effect: $F(13,182)=5.92$, $p<0.0001$; Genotype effect and interaction, p's>0.25]. However, *Hnrnph1* +/− males exhibited lower intake of the 20% solution, particularly during the 2nd week of testing (Fig.1F) [Genotype X Day: $F(13,182)=2.45$, $p=0.004$; *post-hoc* tests for simple main effects]. In contrast to alcohol intake, water intake declined in all groups over the course of testing [Day effect: $F(13,364)=1.91$, $p=0.03$], but we failed to observe any overt sex or genotypic difference in this regard (Fig.1G,H; Genotype or Sex effects/interactions: all p's>0.07)

The sex difference in the effect of *Hnrnph1* deletion on alcohol drinking was also apparent with respect to the total alcohol intake exhibited by the mice drinking under continuous-access conditions (Fig.2A) [Sex X Genotype interaction: $F(1,31)=8.11$, $p=0.008$]. Male *Hnrnph1+/−* mice exhibited lower alcohol intake than their male *Hnrnph1+/+* counterparts [$t(14)=2.99$, $p=0.01$], while no genotypic difference was apparent in females (t-test: $p=0.25$). Although the average water intake exhibited by male *Hnrnph1+/−* mice was also lower than their respective male controls (Fig.2B), this difference was not statistically reliable (Sex X Genotype ANOVA: all p's>0.10). Taken together, these data implicate *Hnrnph1* in regulating alcohol intake under continuous-access procedures, with the *Hnrnph1* mutation reducing alcohol consumption only in males.

3.2 Binge Alcohol intake under limited-access.

As reported previously (e.g., Lee et al., 2016), alcohol intake under limited-access procedures was stable across the 14 days of testing [Day effect and interactions, $F(13,364)<1.2$, p's>0.26] and mice of both genotypes consumed amounts of alcohol that are predicted to result in BAC's in excess of the 0.08 g/dL criterion for binge-drinking (e.g., Rhodes et al. 2005; Lee et al., 2016). Although we did not detect a significant Genotype X Sex X Dose interaction (p=0.53) for alcohol intake under DID procedures, *Hnrnph1* deletion reduced alcohol intake in this paradigm [Genotype effect: $F(1,28)=7.24$, p=0.01; Genotype X Dose interaction [$F(3,84)=4.18$, p=0.008], with a non-significant trend for a Sex X Dose interaction [$F(3,84)=2.38$, p=0.07]. Given the sex-specific effect of *Hnrnph1* deletion on alcohol intake under continuous-access procedures (Fig.1; Fig.2A), we deconstructed the data for binge-intake along the sex factor for re-analysis of potential sex-specific effects and confirmed no effect of *Hnrnph1* deletion on the dose-intake function for female mice drinking under DID procedures (Fig.2C) [Dose effect: $F(3,42)=68.34$, $p<0.0001$; Genotype effect: p=0.10; interaction: p=0.48]. In contrast, male *Hnrnph1+/−* mice tended to binge-drink less 20% and 40% alcohol than their WT counterparts, but the genotypic differences were statistically unreliable (Fig.2D) [Genotype X Dose: $F(3,42)=3.67$, p=0.02; *post-hoc* tests for simple main effects, p's>0.05]. Thus, while not as robust as the results observed for alcohol intake under continuous-access procedures, these data nonetheless are consistent with a sex-specific effect of *Hnrnph1* deletion also on binge alcohol-drinking.

3.3 Alcohol-induced locomotor activity.

No genotypic difference was apparent with respect to distance traveled during the Pre-Test, when mice had access to both compartments of the place-conditioning apparatus, although females tended to locomote more than males (Fig.3) [Sex effect: $F(1,185)=3.54$, p=0.06; Genotype effect: $F(1,185)=2.75$, p=0.09; interaction, p=0.61]. However, no sex or genotype differences were apparent with respect to the locomotor response to an acute saline injection (Genotype X Sex ANOVA, p's>0.10; data not shown).

Analysis of the dose-response function for acute alcohol-induced locomotion (0.5, 1, 1.5, 2 and 4 g/kg) indicated a shift upwards in females versus males (Fig.4A,B) [Dose effect: $F(4,185)=12.16$, $p<0.0001$; Sex effect: $F(1,185)=14.16$, $p<0.0001$], but no genotypic difference (Genotype X Sex X Dose ANOVA, all other p's>0.15). As no genotypic difference was noted for the acute locomotor response to alcohol, we next examined the effect of the *Hnrnph1* mutation on the change in alcohol-induced locomotion during the course of place-conditioning by subtracting the distance traveled on Injection 1 from that on Injection 8. While the analysis of this dose-response function revealed a significant Genotype X Sex X Dose interaction [$F(4,185)=2.89$, p=0.02], deconstruction of the interaction along the sex factor failed to detect any dose or genotype effect in females (data not shown; Genotype X Dose ANOVA, p's>0.10), and only a statistical trend for lower responding in male *Hnrnph1* mutants [data not shown; Dose effect: $F(1,96)=3.10$, p=0.02; Genotype effect: p=0.08; interaction, p>0.20]. Thus, in contrast to both methamphetamine (Yazdani et al., 2015; Ruan et al., 2020) and fentanyl (Bryant et al. 2020), *Hnrnph1* deletion does not significantly affect the acute or sensitized locomotor response to alcohol.

3.4 Alcohol-induced place-conditioning.

In contrast to sex-dependent genotypic differences in drinking, a robust genotypic difference was detected with respect to the dose-response function for alcohol-induced place-conditioning, irrespective of Sex [Genotype X Side X Dose: F(4,166)=4.45, p=0.002; 4-way interaction, p=0.24], with *Hnrnph1+/+* mice exhibiting a strong alcohol-conditioned place-aversion at the two highest doses tested, whereas *Hnrnph1+/−* mice did not show any significant aversion at either dose of alcohol (Fig.4C) [for 2 g/kg, Side X Genotype: F(1,37)=11.63, p=0.002; for 4 g/kg, Side X Genotype: F(1,28)=11.60, p=0.002; for other doses, Side X Genotype ANOVAs, all p's>0.17]. Further, direct comparisons of the time spent in the alcohol- versus saline-paired side during the conditioning test confirmed a place-aversion in *Hnrnph1+/+* controls at both the 2 g/kg [F(1,18)=11.21, p=0.004] and 4 g/kg doses [F(1,15)=6.77, p=0.02]. In contrast, *Hnrnph1*+/−mutants were place-ambivalent at the 2 g/kg dose (p=0.31) and instead, exhibited a significant place-preference at the 4 g/kg dose [F(1,13)=4.96, p=0.04]. While the results of the ANOVA failed to indicate any sex effect or interactions, the prior Sex by Genotype interactions that we observed for alcohol-drinking prompted a comparison of the dose-response functions for alcohol-induced place-conditioning between female versus male mice. As illustrated in Fig.4D, no signs of high-dose alcohol-conditioned place-aversion were apparent in either *Hnrnph1*+/− male or female mice. However, the large genotypic difference in CPP scores observed when the data are collapsed across sex (Fig.4C) is driven primarily by the larger, less variable genotypic differences in conditioning of the male mice, including a more robust preference in +/− males and a more robust aversion in +/+ males (Fig.4D). These data indicate that *Hnrnrph1* deletion reduces sensitivity to the aversive effects of high-dose alcohol, without impacting the rewarding properties of lower alcohol doses, which is a finding in line with our previous study indicating greater high-dose methamphetamine CPP compared to wild-types (Ruan et al., 2020a). However, in contrast to our previous methamphetamine study, the effect of *Hnrnph1* deletion on alcohol's motivational valence is more pronounced in males.

3.5 Alcohol Intoxication and Sedation.

No genotype or sex differences were noted in the number of trials required for alcohol-naïve mice to remain on the fixed speed rotarod for 2 min during training (*Hnrnph1+/+*: 3.0 ± 0.0 trials, n=11; *Hnrnph1+/−*: 3.1 ± 0.1 trials, n=11; Genotype X Sex ANOVA, all p's>0.30) and both alcohol-naïve *Hnrnph1*+/+ and +/− mice remained on the rotarod for the entire 3-min period prior to alcohol injection. Overall, the latency to fall from the rotarod appeared to be longer in mice injected repeatedly with 3 g/kg alcohol, with *Hnrnrph+/+* mice exhibiting better rotarod performance than *Hnrnph1+/−* mice (Fig.5A). However, an analysis of these data failed to support the development of tolerance to alcohol's intoxicating effects (no Injection effect or interactions, p's>0.20), nor did it indicate any overall effects of, or interaction between, the Genotype and Sex factors (all p's>0.20). Likewise, *Hnrnph1+/+* controls tended to right themselves in a shorter period of time than *Hnrnph1+/−* mice following an acute injection with 4 g/kg alcohol (Fig.5B), but no significant genotype or sex differences were detected for this variable (Genotype X Sex ANOVA, all p's>0.20). Thus, *Hnrnph1* deletion does not reliably alter the intoxicating or sedative effects of higher alcohol doses.

3.6 Blood Alcohol Levels.

We next tested for the relationship between genotypic differences in alcohol intake and alcohol aversion to alcohol metabolism. As expected, BACs declined over time following injection with 1.5 g/kg alcohol [Time effect: $F(2,32)=50.23$, $p<0.0001$], but there were no genotype or sex differences in this regard (Fig.5C; Genotype X Sex X Time ANOVA, other p's>0.40). These BAC data are consistent with no overt effect of gene deletion on the locomotor, intoxicating, and sedative properties of alcohol.

3.7 Alcohol Withdrawal-Induced Anxiety.

3.7.1 Light-Dark Box.—No group differences were observed in the latency to first enter the light side (data not shown; Genotype X Sex X Treatment ANOVA, all p's>0.16). Overall, alcohol withdrawal reduced the number of light-side entries (Fig.6A) [Treatment effect: $F(1,65)=16.14$, $p<0.0001$], indicative of anxiety-like behavior. However, this alcohol withdrawal effect was more pronounced in *Hnrnph1−/−* mice as indicated by a significant Genotype X Treatment interaction [$F(1,65)=3.96$, $p=0.05$] and the results of within-genotype comparisons between alcohol- and saline-experienced mice [for +/+: $t(28)=1.61$, $p=0.12$; for +/−: $t(34)=4.01$, $p<0.0001$]. Alcohol withdrawal also reduced the time spent in the light side [Treatment effect: $F(1,65)=44.46$, $p<0.0001$]; however, the magnitude of this effect did not vary significantly with Genotype (Fig.6B; Genotype effects and interactions, p's>0.50) or with Sex (Sex effects and interactions, p's>0.08).

3.7.2 Marble-burying.—Compared to alcohol-naïve controls, mice in alcohol withdrawal exhibited a significantly shorter latency to begin marble-burying (Fig.6C) [Treatment effect: $F(1,65)=10.22$, $p=0.002$], and buried more marbles than alcohol-naïve controls (Fig.6D) [Treatment effect: $F(1,65)=191.56$, $p<0.0001$]. While, *Hnrnph1−/−* mice tended to exhibit a shorter latency to bury overall (Fig.6C; Genotype effect, $p=0.07$), neither Genotype nor Sex significantly interacted with the Treatment factor for this variable (Genotype effect, $p=0.07$; other p's>0.20) or the number of marbles buried (Genotype X Sex X Treatment ANOVA, other p's>0.16]. In contrast, no group differences were observed regarding the time spent burying (data not shown; Genotype X Sex X Treatment ANOVA, all p's>0.30). Thus, while alcohol withdrawal-induced anxiety was also observed in the marble-burying test, the intensity of this state was not affected by *Hnrnph1* deletion.

3.7.3 Forced Swim.—Analysis of the latency to first float in the forced swim test revealed a modest Genotype X Sex X Treatment interaction [$F(1,65)=3.94$, $p=0.05$]. Deconstruction of this interaction along the Sex factor indicated a significant Genotype X Treatment interaction only in female mice (Fig.7A) [$F(1,30)=4.83$, $p=0.037$], but not for males (Fig.7B) [p's>0.65]. As illustrated in Fig. 7A, the interaction in females reflected a shorter latency to float in alcohol-withdrawn *Hnrnph1+/+* mice versus their alcohol-naïve controls [$t(17)=1.81$, $p=0.08$], while no alcohol withdrawal effect was apparent in the mutant females (t-test, $p=0.15$). While it appeared that alcohol-naïve female +/− mice also exhibited a shorter latency to float than their +/+ counterparts, follow-up analyses failed to indicate any significant genotypic differences in either alcohol-naïve or -experienced females (t-tests, all p's>0.10).

A sex difference was detected for the withdrawal-induced increase in the number of immobile episodes [Sex X Treatment: $F(1,65)=5.35$, $p=0.02$], but there was no effect of Genotype (all p's>0.07). Alcohol withdrawal doubled the number of immobile episodes exhibited by female mice [$t(29)=4.79$, $p<0.0001$], but had no effect on immobile episodes in males (Fig.7C; t-test, $p=0.13$). Alcohol-withdrawal also increased the time spent immobile (Fig.7D) [Treatment effect: $F(1,65)=36.31$, $p<0.0001$], but this effect did not vary with sex or genotype (all other p's>0.13).

Taken altogether, these data for withdrawal-induced negative affect provide little evidence that hnRNP H1 plays a key role in regulating the basal affective state or alcohol withdrawal-induced changes therein.

4. Discussion

The present study sought to characterize the alcohol-related behavioral phenotype of mice with a heterozygous deletion of *Hnrnph1*. When allowed 24-h concurrent access to water and alcohol (10, 20 and 40%, v/v), male *Hnrnph1+/−* mice consumed less alcohol than WT controls, while no effect of gene deletion on drinking was apparent in female mice, with a similar pattern of results being observed under limited-access drinking procedures. The larger genotypic difference observed in male drinking in the DID versus continuous access procedure likely reflects the timing of alcohol presentation in DID, which coincides with the time of peak fluid intake during the circadian cycle (Gill et al., 1996; Rhodes et al. 2005). However, the fact that *Hnrnph1* heterozygous males exhibited lower alcohol intake under two distinct drinking paradigms is consistent with the results of meta-analysis indicating a correlation between continuous-access alcohol drinking (when water is freely available) and DID drinking, both in WT and mutant mice (see Blednov et al., 2012) and suggests that *Hnrnph1* deletion impacts a common underlying psychobiological mechanism to curb alcohol intake in males.

Interestingly, while no overt Sex by Genotype interaction was observed with respect to the effects of *Hnrnph1* deletion on oral methamphetamine intake (Ruan et al., 2020b), for intake with the mu opioid receptor agonist fentanyl, only male *Hnrnph1+/−* mice exhibited lower operant self-administration (Bryant et al., 2020). Further, as reported previously for fentanyl intake (Bryant et al., 2020), the genotypic difference in alcohol intake observed herein was only observed at the higher alcohol concentrations tested. This result suggests that heterozygous *hnrnpn1* deletion induces a sex-specific shift in both alcohol and opioid sensitivity. Although the mechanism by which male *Hnrnph1+/−* mice exhibit reduced alcohol or fentanyl consumption is unclear, a gene homolog, *Hnrnph2*, is located on the X chromosome in both rodents and humans. Mutations in both *Hnrnph1* and *Hnrpnh2* are linked to a rare, x-linked neurodevelopmental disorder in females (Bain *et al.* 2016; Pilch *et al.* 2018; Harmsen *et al.* 2019). If *Hnrnph2* undergoes variable X-inactivation, heterozygous deletion of *Hnrnph1* could induce sex-dependent changes in *hnrnph2* expression to influence the self-administration of certain drugs of abuse by males. While cocaine (Reynolds et al., 2011) and opioids (Suder et al., 2009) are reported to alter hnRNP H2 expression in rodent brain, it remains to be determined if alcohol can also regulate hnRNPH 1/2 mRNA or protein expression. Alternatively, sex hormones are well-characterized to influence alcohol

intake in both humans and laboratory rodents (for recent reviews, Finn, 2020; Verplaetse et al., 2020) and may contribute to sex differences in the effect of *Hnrnph1* deletion on alcohol drinking. While the molecular mechanisms by which *Hnrnph1* deletion exerts sex-specific effects on alcohol (and fentanyl) intake are unknown, the present findings provide novel evidence that *Hnrnph1* function is necessary for alcohol drinking behavior in males.

We employed alcohol-induced place-conditioning procedures to relate genotypic differences in alcohol intake to the affective/motivational valence of alcohol, with the hypothesis that reduced alcohol intake by *Hnrnph1*+/− males would reflect either less sensitivity to the conditioned rewarding properties of alcohol (as reported for both low-dose methamphetamine- and low-dose fentanyl-induced place-conditioning; Bryant et al., 2020; Ruan et al., 2020a) and/or greater sensitivity to the conditioned aversive properties of the drug. While the results of the statistical analyses failed to indicate a significant sex difference in the effect of *Hnrnph1* deletion on the dose-response function for alcohol-induced place-conditioning, a comparison across sexes suggests that the marked genotypic difference in the direction of the conditioned response to high-dose alcohol was driven, in large part, by male subjects. While the direction of the observed *Hnrnph1*+/− effect on place-conditioning is opposite our original hypothesis, these data nevertheless provide additional support for a male-selective effect of *Hrnrnph1* deletion on measures of alcohol reward and argue instead that the low alcohol intake exhibited by male *Hnrnph1*+/− mice might reflect a compensation for their increased sensitivity to alcohol's positive interoceptive effects.

It is interesting to note that, akin to the present findings for alcohol, *Hnrnph1*+/− mice also exhibited blunted sensitivity to the conditioned aversive properties of 2 mg/kg methamphetamine, as indicated by a greater conditioned place-preference in mutant mice, relative to WT controls at this dose (Ruan et al., 2020a). A similar trend was also observed with fentanyl (Bryant et al., 2020) which together, raises the intriguing possibility that *Hnrnph1*+/− blocks the negative affective/motivational valence of a variety of drugs of abuse. At least in the case of methamphetamine, the attenuated aversion exhibited by *Hnrnph1*+/− mice cannot be readily explained by an effect of gene deletion on nucleus accumbens dopamine, as no genotypic difference is observed for basal dopamine content (Ruan et al., 2020a). Moreover, *Hnrnph1*+/− blunted the capacity of acute methamphetamine to elevate nucleus accumbens extracellular dopamine levels (Ruan et al., 2020a) and blunted dopamine release within the nucleus accumbens is reported to promote, rather than prevent, a methamphetamine-conditioned place-aversion (Lominac et al., 2014). Likewise, we know from prior our work that methamphetamine-induced place-conditioning is bidirectionally regulated by nucleus accumbens glutamate levels (Szumlinski et al., 2016); however, *Hnrnph1*+/− did not alter either basal, or acute methamphetamine-induced changes in, extracellular glutamate within the nucleus accumbens (Ruan et al., 2020a). Of relevance to the manifestation of place-preference/aversion, we have yet to determine the effects of *Hnrnph1*+/− on drug-induced neurotransmitter levels within the nucleus accumbens of mice following repeated drug exposure, nor do we know how gene deletion alters neurotransmitter levels following acute or repeated alcohol. Alcohol-induced place-aversion is linked to anomalies in glutamate plasticity within both the nucleus accumbens (Szumlinski et al., 2005) and bed nucleus of the stria terminalis (Campbell et al., 2019),

implicating the extended amygdala as at least one potential neurocircuit affected by *Hnrnph1* deletion.

Under taste-conditioning procedures, binge alcohol-drinking inversely correlated with magnitude of a conditioned taste aversion in WT mice across various genetic backgrounds (Blednov et al., 2012; Rhodes et al., 2007) and this relationship was disrupted in a number of different transgenic mutations (see Blednov et al., 2012). While we did not assay *Hnrnph1+/−* mice for alcohol-conditioned taste-aversion, our place-conditioning data indicate that heterozygous *Hnrnph1* deletion not only blocked, but reversed the negative affective/motivational valence of high-dose alcohol. As C57BL/6J mice are reported to exhibit weak alcohol-induced place-preference (Cunningham, 2014; Cunningham et al., 1992), the failure of alcohol to elicit a place-preference in our WT mice likely reflects their genetic background rather than the alcohol doses selected and it remains to be determined whether or not the *Hnrnph1+/−* mutation would exert a similar effect on alcohol place-conditioning or any of our other measures in mice of a different genetic background more prone to exhibit place-preference or less likely to consume alcohol (e.g., DBA/2J). Nevertheless, the incongruency in results between our drinking and place-conditioning studies indicates that heterozygous *Hnrnph1* deletion blurs the inverse relationship between the conditioned aversive properties of alcohol and alcohol intake. This “blurring” is consistent with the results of alcohol-conditioned taste aversion studies of other mutant mouse lines (Blednov et al., 2012). and highlights the importance of conducting multiple assays of drug reward when phenotyping mice of both sexes.

In humans, the perception of alcohol’s interoceptive effects as aversive or appetitive typically relates to individual variation in sensitivity to alcohol-induced intoxication (e.g., Krystal et al., 2003; Schuckit and Smith, 2000) or the severity of alcohol withdrawal (e.g., Anton and Becker, 1995; Schuckit et al., 1998), as well as individual differences in alcohol metabolism (c.f., Cederbaum, 2012). However, a number of results from the present study argue against these psychopharmacological factors as contributing to the alcohol reward phenotype of *Hnrnph1+/−* mice. For one, we did not detect any consistent effect of *Hnrnph1* deletion on acute alcohol-induced locomotor activity, locomotor sensitization, intoxication or sedation, nor did we detect differences in alcohol pharmacokinetcs. Thus, the alcohol reward phenotype of *Hnrnph1* mutants is unrelated to changes in sensitivity to any of alcohol’s effects on motor behavior or alcohol metabolism. Our findings for alcohol-induced locomotor activity contrast sharply with our prior results for both methamphetamine-(Yazdani et al., 2015; Ruan et al., 2020a, 2020b) and fentanyl-induced locomotion (Bryant et al. 2020), suggesting that hnRNP H1 does not play a universal role in regulating all drug-induced psychomotor activity. Alternatively, our lack of genotypic differences in alcohol-induced locomotion may reflect procedural differences related to the duration of locomotor testing as genotypic differences in methamphetamine-induced locomotion were most robust when saline and drug trials were conducted over a 1-h period compared to a 30-min period (Yazdani et al., 2015). This being said, we have successfully detected large genotypic differences in alcohol-induced locomotion and/or sensitization using our place-conditioning procedures (e.g., Ary et al., 2012; Campbell et al., 2019; Szumlinski et al., 2005; 2008). Unfortunately, the limited number of alcohol-naïve mice available at the time of study precluded further investigation of this procedural issue.

Notably, we also failed to detect consistent effects of *Hnrnph1* deletion on negative affect-like measures in alcohol-naïve mice – a finding replicating our results indicating that *Hnrnph1*+/− does not affect baseline emotionality in mice (Bryant et al., 2020; Ruan et al., 2020a). We have shown repeatedly that early withdrawal from a history of binge-drinking induces a negative affective state in mice (e.g., Jimenez Chavez et al., 2020; Lee et al., 2015, 2016, 2017a,b, 2018a,b; Szumlinski et al., 2019), raising the possibility that the marked genotypic differences in alcohol-induced place-conditioning could reflect reduced sensitivity to a withdrawal-induced negative affective state. Given group differences in place-conditioning, we opted to inject mice repeatedly with high-dose alcohol (4 g/kg) in a manner consistent with the regimen employed during place-conditioning procedures and showed that this injection regimen was sufficient to increase anxiety- and depression-like behaviors when assessed during early alcohol withdrawal. However, as reported for fentanyl withdrawal (Bryant et al., 2020), we did not detect a consistent Genotype effect or Sex by Genotype interactions in the alcohol withdrawal-induced negative affective state. Thus, there does not appear to be a relationship between either reduced alcohol intake or an absence of alcohol-conditioned place-aversion and the severity of alcohol withdrawal in *Hnrnph1*+/− mice.

In conclusion, heterozygous deletion of *Hnrnph1* reduced high-concentration alcohol intake under two distinct drinking paradigms in male mice only. *Hnrnph1*+/− profoundly reversed the negative affective/motivational valence of high-dose alcohol – an effect that was more pronounced and less variable in males. The effects of *Hnrnph1* deletion on these measures of alcohol reward were unrelated to changes in alcohol pharmacokinetics, sensitivity to the psychomotor-activating, intoxicating or sedative properties of the drug, or the severity of alcohol withdrawal. These findings further support a general and surprisingly selective role for *Hnrnph1* function specifically following exposure to multiple addictive substances, although the underlying mechanisms regarding the effect of *Hnrnph1*+/− on behavior are likely to differ among drug classes and sex.

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Highlights

- Heterozygous deletion of *Hnrnph1* (+/−) reduces alcohol intake by mice under continuous- and limited-access procedures.
- *Hnrnph1*+/− mice are resistant to the conditioned aversive properties of high-dose alcohol.
- The effects of *hnrnph1* deletion on alcohol reward are male-selective.
- *Hnrnph1* deletion does not alter alcohol metabolism, withdrawal-induced anxiety, or its sedative-hypnotic effects.
- *Hnrnph1* is a novel regulator of alcohol reward.

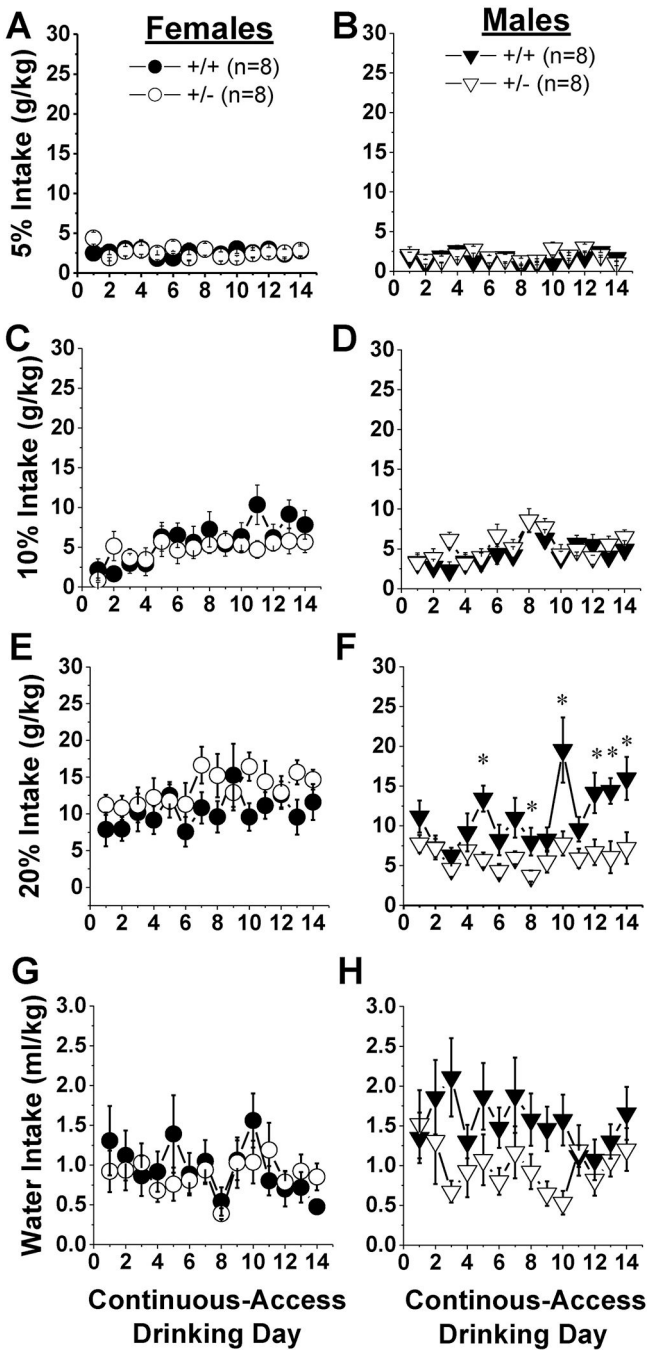

Figure 1: Male Hnrnph1+/− mice consume less high-concentration alcohol under continuous-access procedures.

Wild-type mice (+/+) and their littermates with a heterozygous deletion of *Hnrnph1* (+/−) were offered 24-h, concurrent, access to 0, 5, 10 and 20% alcohol (v/v) in the home-cage over the course of a 14-day period. No genotypic difference was detected for water intake in female ***(A)*** or male ***(B)*** mice. Likewise, no genotypic difference was detected for intake of 5% alcohol in either sex (**C,D**). Furthermore, for females, there was no genotypic difference in the intake of 10% ***(E)*** or 20% ***(G)*** alcohol. In contrast, male +/− mice exhibited blunted

intake of both 10% ***(F)*** and 20% alcohol ***(H)***. Data represent the means ± SEMs of the number of mice for each genotype (n) indicated in Panels A and B. *p<0.05 vs. +/+.

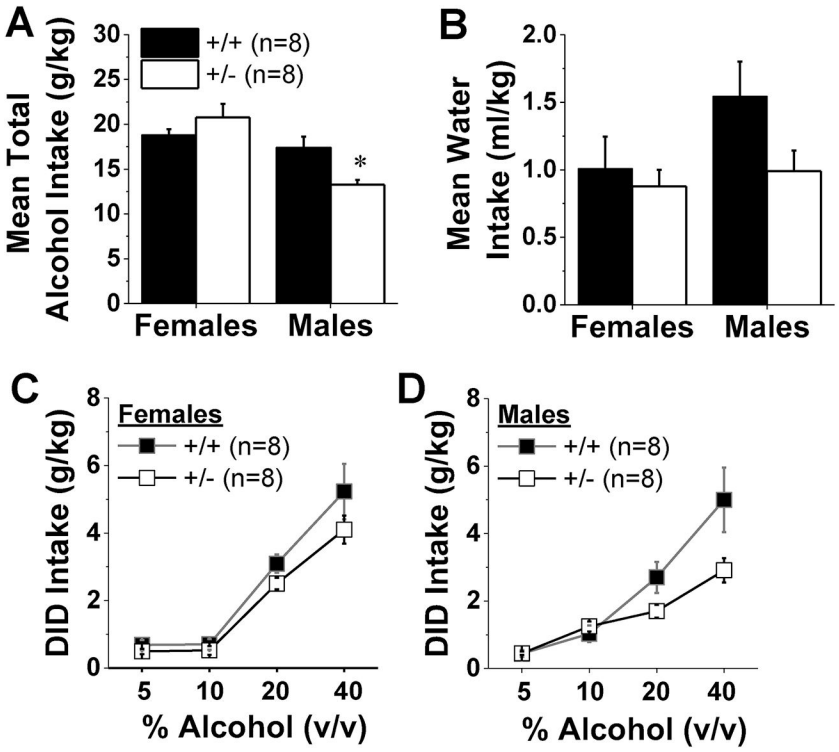

Figure 2: Male Hnrnph1+/− mice consume less high-concentration alcohol under limited-access procedures.

(A) The data from Fig. 1 are expressed as the average total alcohol intake over the 14-day course of drinking under continuous-access procedures and highlight the male-selective effect of gene deletion on alcohol-drinking. ***(B)*** No significant genotypic difference was observed for the average water intake during continuous-access procedures in either female or male mice. In assessing alcohol intake under Drinking-in-the-Dark (DID) procedures (concurrent access to 10, 20 and 40% alcohol v/v for 2 h/day), no genotypic difference was detected in female mice ***(C)***, while male *Hnrnph1+/−* mice consumed less high-concentration alcohol than their +/+ counterparts ***(D)***. The data represent the means ± SEMs of the number of mice indicated in Panel A. *$p<0.05$ vs. +/+.

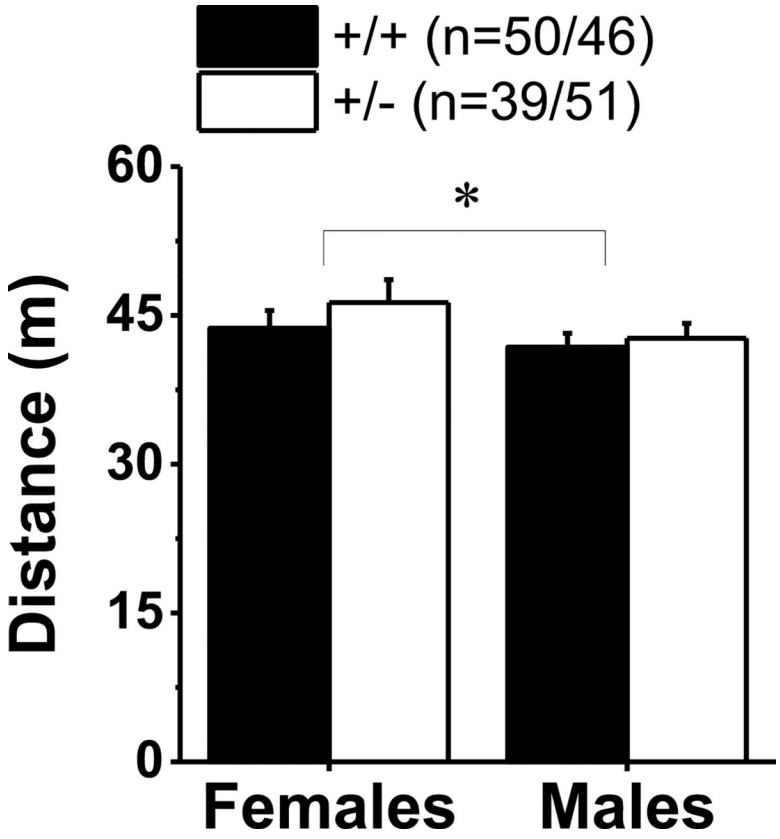

Figure 3:

Hnrnph1 deletion does not alter spontaneous locomotion. Wild-type mice (+/+) and their littermates with a heterozygous deletion of Hnrnph1 (+/−) were allowed to habituate to the place-conditioning apparatus for 15 min. Females locomoted more than males, irrespective of genotype with no effect of gene deletion detected. The data represent the means ± SEMs of the number of mice indicated. *$p < 0.05$ vs. males.

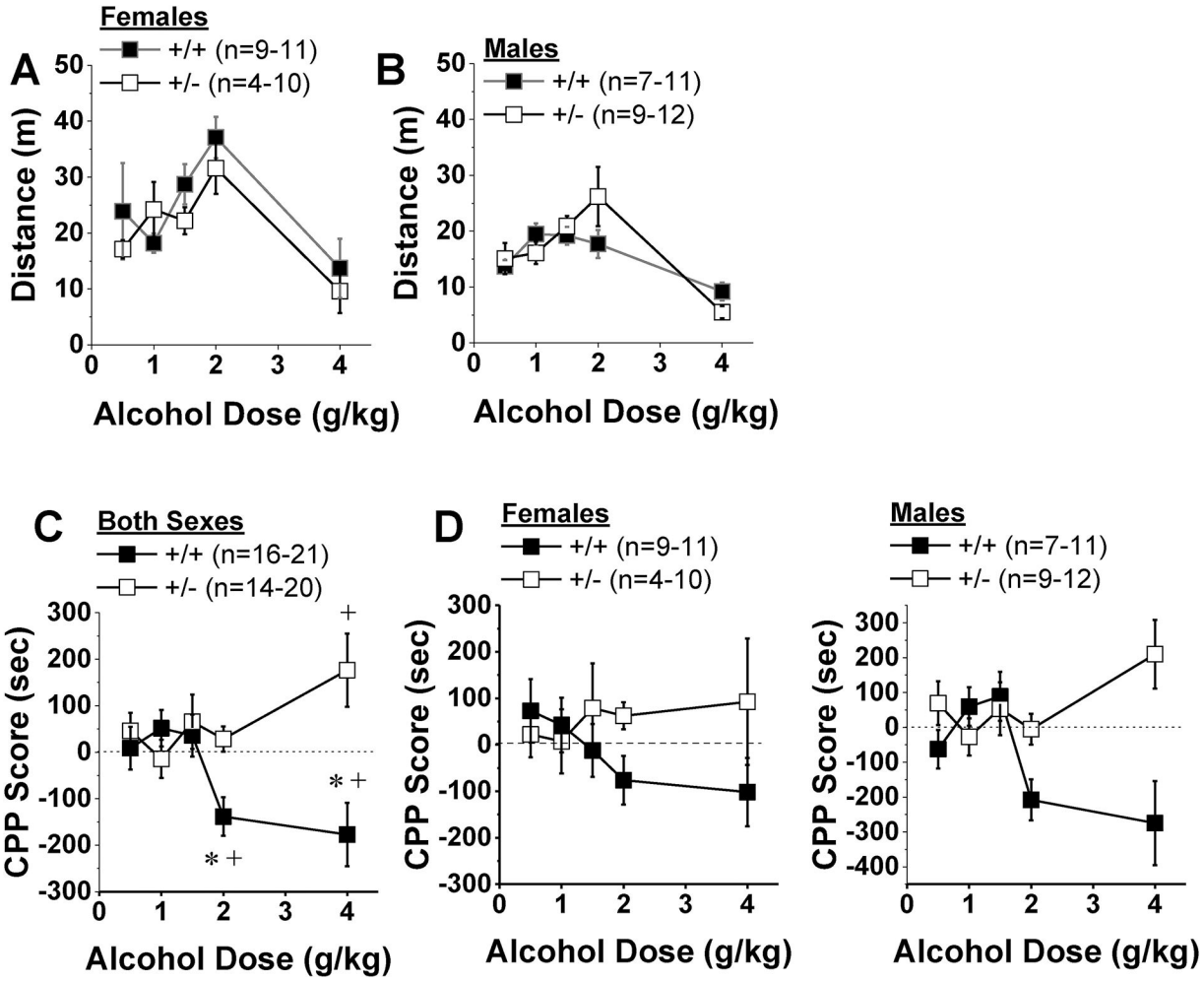

Figure 4:

Hnrnph1+/− mice do not exhibit alcohol-induced place-aversion. Wild-type mice (+/+) and their littermates with a heterozygous deletion of Hnrnph1 (+/−) underwent an alcohol-induced place-conditioning procedure involving 8 pairings of alcohol (0.5–4 g/kg) with a distinct compartment of a 2-compartment apparatus. While female mice (A) locomoted more than males (B) in response to alcohol injection during the first conditioning session, no genotypic differences were detected in the shape of the dose-response function for acute alcohol-induced locomotion. (C) When allowed free-access to both compartments following conditioning, we detected no sex difference in alcohol-induced place-conditioning. Thus, the data were collapsed across sexes to illustrate the large genotypic difference in the direction of the conditioned response between +/+ (aversion) and +/− mice (preference). The data represent the means ± SEMs of the number of mice indicated in Panels A and B. *$p < 0.05$ vs. +/+; +$p < 0.05$ vs. unpaired side (place-conditioning)

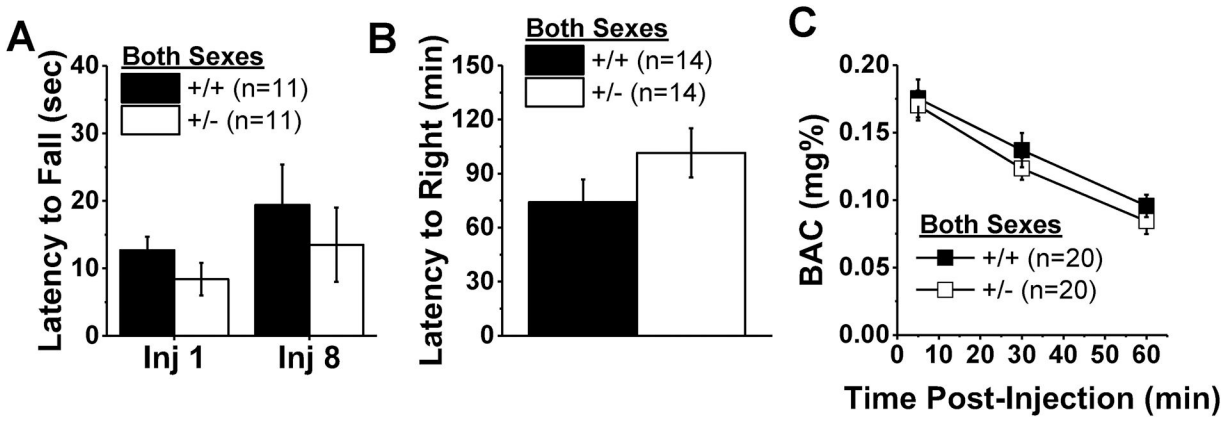

Figure 5:

Hnrnph1 deletion does not alter alcohol intoxication, sedation or metabolism. (A) Wild-type mice (+/+) and their littermates with a heterozygous deletion of Hnrnph1 (+/−) did not differ with regard to time spent on a fixed speed rotarod following their first and eighth injection of 3 g/kg alcohol. (B) Similarly, no genotypic difference was detected for the time taken to right themselves following an acute injection of 4 g/kg alcohol. (C) No genotypic difference in plasma alcohol levels were detect over the course of a 1-h period following injection with 3 g/kg alcohol. The data represent the means ± SEMs of the number of mice indicated in each panel. *$p < 0.05$ vs. +/+.

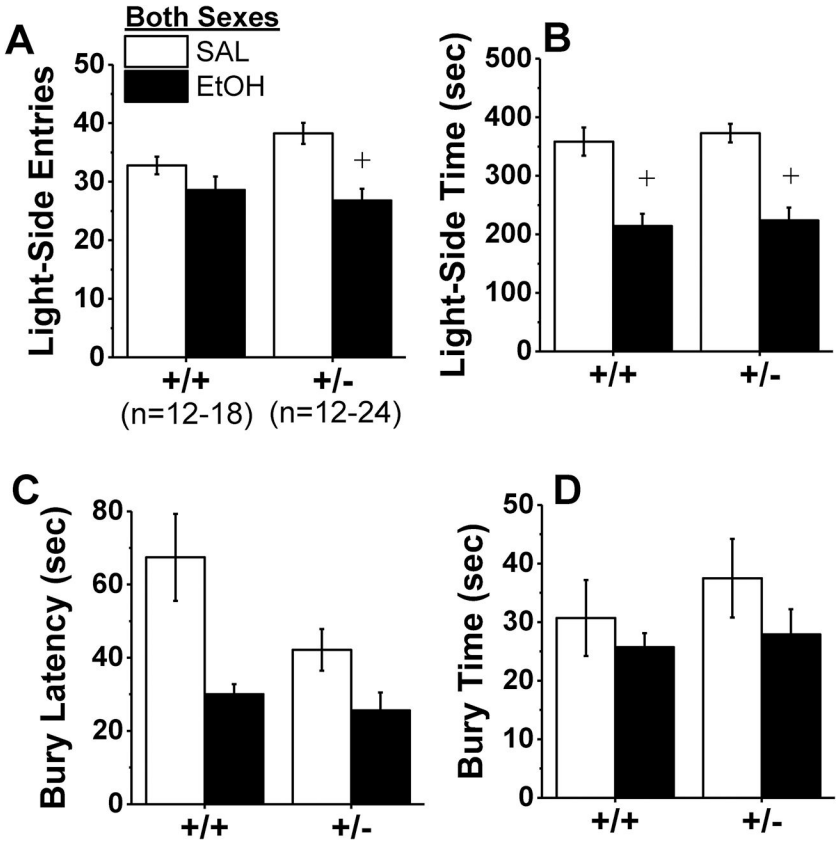

Figure 6:

Hnrnph1+/− deletion does not consistently alter baseline, or alcohol withdrawal-induced increases in anxiety-like behavior. Wild-type mice (+/+) and their littermates with a heterozygous deletion of Hnrnph1 (+/−) were injected repeatedly with 4 g/kg alcohol (EtOH) or saline (SAL) and then assayed for negative affect using a test battery including the light-dark shuttle-box and marble-burying tests. (A) +/− mice but not +/+ mice showed a significant withdrawal-induced decrease in the latency to enter the light side of a light-dark shuttle-box compared to their +/− control counterparts while (B) no genotypic difference was detected for the withdrawalinduced reduction in the time spent in the light side. In the marble-burying assay, alcohol withdrawal produced a nonsignificant reduction in the latency to begin burying marbles (C) and the time spent burying (D), but no genotypic differences were detected for either variable. The data represent the means ± SEMs of the number of mice indicated in Panel A. +$p < 0.05$ vs. SAL (alcohol withdrawal effect).

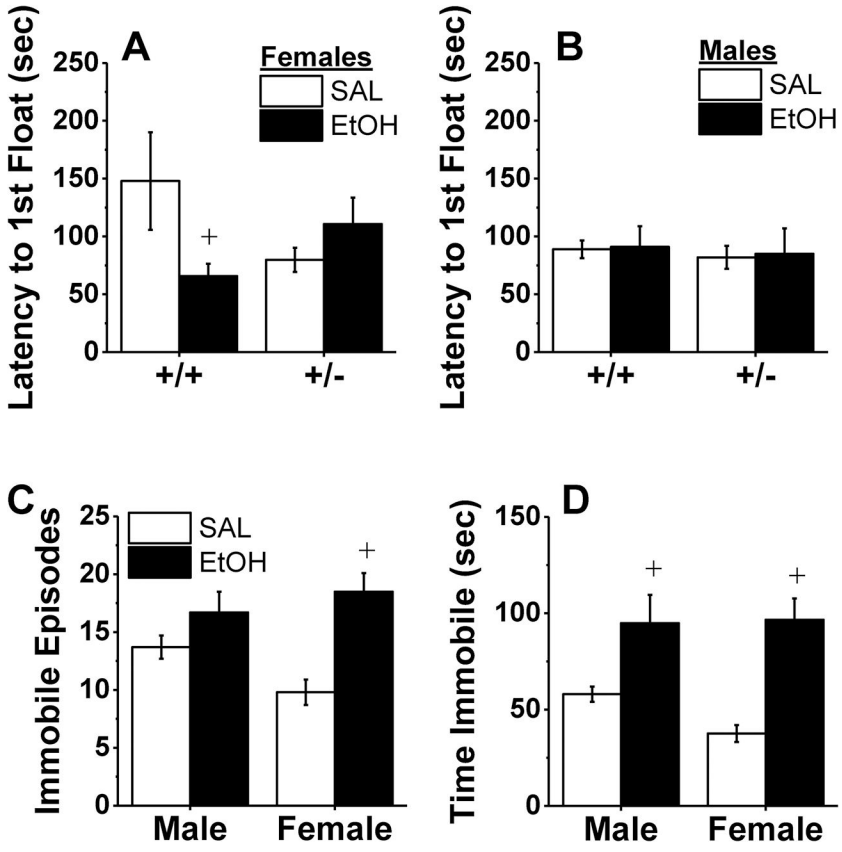

Figure 7.

Hnrnph1+/− deletion does not consistently alter baseline, or alcohol withdrawal-induced increases in depressivelike behavior. Wild-type mice (+/+) and their littermates with a heterozygous deletion of Hnrnph1 (+/−) were also assayed for genotypic differences in behavior in the forced swim test during early alcohol withdrawal. (A) A withdrawalinduced decrease in the latency to first float was detected in female +/+, but not female +/− mice, while no genotypic difference in float latency was observed in males (B). Alcohol withdrawal increased (C) the incidences of floating moreso in female versus male mice, while a withdrawal-induced increase in the time spent floating was comparable in male and female mice. No genotypic differences were detected for the number of floats or time spent floating, thus the data in Panels C and D are collapsed across genotype. The data represent the means ± SEMs of the number of mice indicated in Fig. 5, Panel A. +$p < 0.05$ vs. SAL (alcohol withdrawal effect).