Dpp6 Homozygous Knockout Mice Exhibit Increased Ethanol Conditioned Place Preference and Acute Ethanol-Induced Anxiolytic Behavior

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Ethics approval statement

The animal studies protocols were approved by the Institutional Animal Care and Use Committee and the Animal Resource Facility at the University of New Mexico Health Sciences Center

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest disclosure

The authors declare no conflicts of interest.

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Abstract

The gene *DPP6* has been associated with behavioral phenotypes of alcohol use disorder (AUD) in recent human genome wide association studies. To further assess the role of this gene in ethanol-related traits, we tested *Dpp6* knockout (KO) mice for ethanol conditioned place preference (CPP), locomotor activity, and ethanol-induced anxiolysis. Male homozygous KO mice (HOM) showed greater preference for the ethanol-paired context compared to wild type littermates (WT) and heterozygous KO mice (HET), while female mice showed no genotypic difference. HOM of both sexes exhibited greater novelty-induced hyperactivity in the CPP apparatus than HET and WT mice in the first two minutes. In a separate experiment, HOM mice showed enhanced locomotor activity following a 1.5 g/kg ethanol injection; however, they also displayed greater locomotor activity during habituation, suggesting basal locomotor differences. Following 1.5 and 2 g/kg injections, HOM mice exhibited EtOH-induced anxiolysis in the first 5 minutes, while the HET and WT mice did not. Lastly, HOM mice displayed a significant sedative response compared to WT animals following a 2 g/kg injection of ethanol. Ultimately, these findings validate a role for *Dpp6* in modulating ethanol's rewarding, anxiolytic, and sedative effects in a sex-dependent manner.

Keywords: Alcohol, Dpp6, knockouts, conditioned place preference, acute alcohol

Introduction

As of 2023, 10.2% of the United States population aged 12 and older met criteria for Alcohol Use Disorder (AUD) within the past year (1). AUD is a complex disorder with both genetic and environmental factors contributing to its etiology. In fact, genetics contribute roughly 50% (2), and emerging evidence from large human genome-wide association studies (GWAS) have identified several novel genes associated with AUD phenotypes. More recently, the *dipeptidyl peptidase-like protein 6 (DPP6)* gene has been associated with measures of alcohol consumption (e.g., number of drinks per week), problematic alcohol use, and alcohol use disorder (AUD) measurement (3–5), suggesting it may play a role in modulating alcohol-related behaviors.

DPP6 encodes an auxiliary subunit that modulates the biophysical properties and surface expression of A-type voltage-gated potassium channels, particularly Kv4.2. Notably, *Dpp6* is most densely expressed in the hippocampus (HPC) and prefrontal cortex (PFC), regions that are both implicated in reward processing and learning (6–11). Behavioral phenotyping of *Dpp6* knockout (KO) mice has revealed impairments in tasks related to recognition and spatial learning and memory, as demonstrated in paradigms such as the novel object recognition and Morris water maze tasks (12). Additionally, significant changes in locomotor behavior and weight have been reported in mice with a *Dpp6* deletion. More specifically, KO mice tend to show increased locomotor activity and exhibit lower body and brain weights than wild types (WT) (12,13). However, no preclinical studies have assessed the role of *Dpp6* in any alcohol-related phenotypes.

Ethanol (EtOH) conditioned place preference (CPP) is a well-established associative learning paradigm, whereby EtOH is paired with a specific context to elicit an unconditioned stimulus response. In this case, we can assess the rewarding/aversive properties of EtOH, which contributes to the development of AUD. Several brain regions have been implicated in CPP acquisition and expression, such as the bed nucleus of the stria terminalis (BNST), nucleus accumbens (NAc), PFC, and HPC (14–17). Beyond the behavioral differences, *Dpp6* KO mice show changes in dendritic morphology and excitatory neurotransmission in the HPC (18), which could result in changes in EtOH CPP expression.

Here, we sought to functionally validate the role of *Dpp6* in modulating sensitivity to ethanol's rewarding and locomotor effects through two complementary experiments. Experiment 1 used an ethanol (EtOH) conditioned place preference (CPP) protocol to determine whether *Dpp6* KO mice exhibit enhanced conditioned preference for an EtOH-paired context compared to WT controls. Experiment 2 assessed whether *Dpp6* KO alters the acute locomotor and anxiolytic response to a stimulatory (1.5 g/kg) or mildly sedative (2 g/kg) dose of EtOH in each of the genotypes. Together these experiments highlight that the global knockout of *Dpp6* alters behavioral responses to EtOH.

Methods

Animals

A total of 66 adult *Dpp6* homozygous KO (HOM), heterozygous KO (HET), and wild type (WT) littermate mice were used in Experiment 1 (both sexes; postnatal day [PND] 57–152; n=4-17/sex/genotype). Experiment 2 used 20 adult mice of all three genotypes (both sexes; PND 74–101; n=4-8/sex/genotype). All animals came from an in-house breeding colony established

from B6.Cg-Dpp6$^{tm1.1Dahn}$/J cryorecovered breeders (Jax stock # 017972; Su et al. 2011) and maintained with HET x HET breeder pairs. All mice were genotyped via TransnetYX (Memphis, TN) using validated protocols. Mice were group-housed (2–5 per cage) in large Plexiglas cages equipped with a paper house and plastic upper-level compartment for enrichment and maintained on a 12:12 h light/dark cycle (lights on at 07:00). Food (PicoLab 5L0D) and water were available *ad libitum*. All procedures complied with institutional and national ethical standards and were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Place conditioning and open field apparatus

CPP testing was conducted using a two-chambered apparatus (Med Associates, Inc., VT) fitted into a clear Plexiglas chamber (27.3 × 27.3 × 20.3 cm). The CPP insert consisted of two tactilely distinct compartments separated by a black wall and a removable guillotine door. One side featured a stainless-steel bar floor, and the other a grid floor; both were flanked by clear walls. The open field test used the same Plexiglas chamber without the CPP insert. Locomotor and positional data were recorded using the Med Associates Activity Monitor system. Chambers were cleaned with 10% isopropyl alcohol between animals to eliminate olfactory cues.

Drugs and solutions

0.9% sterile saline (UFC Bio, Amherst, NY, via Amazon) and 20% EtOH (v/v in saline) were administered intraperitoneally (i.p.) immediately prior to placement in the CPP chamber. Conditioning sessions alternated between 10 ml/kg saline or 2 g/kg EtOH injections. For the limited access drinking sessions, 200 proof EtOH (Decon Laboratories, King of Prussia, PA) was diluted in tap water to make a 20% (v/v) solution. During the open field experiment, mice received a 1.5 g/kg and 2 g/kg EtOH (in 20% v/v in saline) injection, and the saline volume equivalent of a 1.5 g/kg injection. All solutions were prepared daily prior to behavioral testing.

Experiment 1: Ethanol conditioned place preference

The CPP procedure consisted of a pretest phase, a conditioning phase, and a posttest phase. On the pretest day, mice were moved into the testing room, weighed, and allowed to acclimate for at least 60 min. They were then injected with saline and placed in the center of the apparatus and allowed to explore freely for 30 min to assess initial chamber preference. Due to an average baseline preference of 60.17% ± .012 for the grid floor chamber, a biased conditioning approach was used where each animal was assigned to receive ethanol in the initially non-preferred chamber. During conditioning days, the guillotine door was inserted, and animals were injected with the assigned drug and then placed in the corresponding chamber for five minutes. For example, on day 1, animals were all placed in the bar floor chamber following either 2 g/kg or 10 ml/kg saline injection depending on whether the bar floor type was their conditioned stimulus (CS+) or unconditioned stimulus (CS-), respectively. The following day animals were placed in the chamber with the grid floor type and received injections according to their conditioning group. Four days of conditioning trials were given each week, alternating between saline and ethanol trials. Finally, in the posttest phase (testing days 5, 12, and 19), all animals were injected with saline and placed in the center of the testing apparatus to assess preference (30-minute test). Time spent in each chamber and distance traveled were automatically recorded on each test day. We also computed a CPP change score for each test (% time in EtOH chamber on test day - % time in EtOH chamber on pretest). Two days following test 3, all mice underwent a two hour two-bottle choice homecage drinking procedure (see supplemental material).

Experiment 2: Open field activity

Locomotor activity was recorded on three consecutive days in 30-minute sessions. On days 1 and 2, mice received a saline injection prior to placement in the open field chamber. On day 3, mice were injected with 1.5 g/kg ethanol (20% v/v in saline). A follow-up test was conducted one week later following a 2 g/kg ethanol injection, during which activity was recorded over 60 minutes. Distance traveled (cm) and time spent in the center zone (14.45 cm x 14.45 cm) were automatically recorded.

Data Analysis

All data were analyzed using SPSS software (SPSS, Version 30, Chicago, IL) and graphed and analyzed with GraphPad Prism software (GraphPad Prism, v. 10.5.0, La Jolla, CA). Animals were excluded from analyses on certain days if they had a sipper leak or if the MedAssociates program did not track their behavior correctly. No animals were removed from the entire study as these issues were resolved for testing the following day. Repeated measure two-way or three-way analysis of variance (RM ANOVA) tests were used to assess test*genotype*sex or time*genotype*sex interactions. If there were no significant interactions with sex, data were collapsed. Significance values were set at $p < 0.05$. All data were assessed for normality using the Shapiro-Wilks Test, and if normality was violated, non-parametric tests were used. For repeated-measures data, violations of Mauchly's test for sphericity were corrected using Greenhouse–Geisser estimates.

Results

HOM males significantly increased their time spent on the CS+, but not HOM females.

Experiment 1. This experiment tested whether the global homozygous or heterozygous knockout of *Dpp6* would alter EtOH CPP. Figure 1 shows time spent on the ethanol-paired floor and CPP change score for each test. A three-way RM ANOVA of time spent on the EtOH-paired floor (test* sex* genotype; GGe $\varepsilon = .74$) revealed a significant main effect of test, [$F(2.21, 132.84) = 9.5$, $p < .001$], and significant test*sex, [$F(2.21, 132.84) = 4.28$, $p = .01$] and test*sex*genotype interactions, [$F(4.43, 132.84) = 3.11$, $p = .01$] (Figure 1A-B). To probe the three-way interaction, two-way RM ANOVAs of test*genotype for each sex were used. In females, no significant main effects or interactions emerged (p's > .05; Figure 1A). In males, a two-way RM ANOVA (GGe corrected, $\varepsilon = .75$) revealed a significant main effect of test, [$F(2.26, 81.23) = 14.68$, $p < .001$], and a significant interaction of test*genotype, [$F(6, 108) = 3.87$, $p = .01$] (Figure 1B). Tukey's multiple comparisons test indicated HOM males significantly increased their time spent on the EtOH-paired floor from pretest to tests 2 and 3, and from test 1 to tests 2 and 3 ($p \leq 0.03$ for all). At test 3, HOM males differed significantly from WT males ($p = 0.02$). Taken together, these data suggest that time spent on the CS+ was significantly increased in the male HOMs, but not in WT or HET males or females of any genotype.

HOM Males Show Greater EtOH CPP Scores Compared to WTs, HETs, and HOM Females

To account for variations in baseline preference, we also analyzed CPP change scores (% time in CS+ on test N – % time in CS+ on pretest) using three-way RM ANOVA (GGe corrected, $\varepsilon = .87$). We found a significant main effect of test, [$F(1.73, 103.91) = 11.2$, $p < .001$], and significant interactions of test*sex, [$F(2, 120) = 7.7$, $p = .001$], and test*sex*genotype, [$F(4, 120) = 5.47$, $p < .001$] (Figure 1C-D). To probe the three-way interaction, we ran separate two-way RM ANOVAs

of test and genotype for each sex. For females, there were no significant main effects or interactions (Figure 1C). In males, a two-way RM ANOVA (GGe corrected, $\varepsilon = .79$) revealed a significant main effect of test, [$F(1.58, 56.83) = 19.43$, $p < .001$], and a significant interaction of test*genotype, [$F(4, 72) = 5.79$, $p < .001$] (Figure 1D). Tukey's multiple comparisons tests confirmed significant increases in CPP score in HOM males from test 1 to tests 2 and 3 ($p \leq 0.02$), as well as between WT and HOM males on tests 2 and 3 ($p \leq 0.02$). These data suggest that CPP change score was significantly increased in HOM males only, consistent with the raw CS+ preference scores.

Only Males Show Significant EtOH CPP Expression

One-sample t tests of CPP change scores (vs. zero) were used to assess the development of significant EtOH CPP expression. In females (Figure 1C), no group showed significant effects, though HETs showed trends at test 2 ($p = .051$) and test 3 ($p = .077$). In the males (Figure 1D), HETs showed significance at test 2 (M = 12.98%, SD = 24.199%), [$t(16) = 2.2$, $p = .042$], and a trend at test 3 ($p = .068$). HOM males showed significant effects at test 2 (M = 22.47%, SD = 12.87%), [$t(7) = 4.94$, $p = .002$], and test 3 (M = 25.84%, SD = 14.85%), [$t(7) = 4.92$, $p = .002$]. Taken together, we can confirm that the HOM males and the HET males showed significant changes in preference for the CS+ context after two and three rounds of conditioning.

HOM Mice Display Novelty-Induced Hyperactivity

Next, we assessed locomotor activity during the pretest (Figure 2A). A three-way RM ANOVA of time (30 minutes), sex, and genotype (GGe corrected, $\varepsilon = .04$) revealed significant main effects of time, [$F(1.14, 68.40) = 931.95$, $p < .001$], and sex, [$F(1, 60) = 10.09$, $p = .002$], and a significant interaction of time*sex, [$F(29, 1740) = 9.5$, $p < .001$]. When collapsed by genotype, a two-way RM ANOVA of time and sex revealed a significant main effect of time, [$F(16.66, 1067) = 58.20$, $p < .001$], and sex, [$F(1, 64) = 11.97$, $p < .001$], and no significant interactions.

A focused analysis of the first five minutes revealed significant main effects of time, [$F(4, 240) = 20.13$, $p < .001$], and sex, [$F(1, 60) = 10.03$, $p = .002$], and a significant interaction of time*genotype, [$F(8, 240) = 4.01$, $p < .001$] (Figure 2B). There was a trend towards a time*sex*genotype interaction ($p = .06$). Collapsing across sex, a two-way RM ANOVA, confirmed significant main effects of time [$F(4, 252) = 122.8$, $p < .001$] and genotype, [$F(2, 63) = 5.264$, $p < .001$], and a time*genotype interaction, [$F(8, 252) = 11.35$, $p < .001$]. Tukey's multiple comparisons tests indicated the HOMs were significantly more active than WTs and HETs at minute 1 (both p's < .001), and minute 2 (both p's < .001). However, total locomotor activity showed only a main effect of sex, [$F(1, 60) = 10.05$, $p = .002$], suggesting that the locomotor differences are due to the initial novelty of the context (Figure 2C).

HOMs Show Greater Locomotor Activity on Saline Injection Days in Open Field Test

Figure 3 shows locomotor activity on each day of the open field test. On habituation day 1, a three-way RM ANOA of time (30 minutes), sex, and genotype (GGe corrected, $\varepsilon = .34$) revealed main effects of time, [$F(9.99, 289.63) = 30.06$, $p < .001$, $\eta p^2 = .51$], sex, [$F(1, 29) = 8.14$, $p = .01$], and genotype, [$F(2, 29) = 13.68$, $p < .001$], and significant interactions of time*sex, [$F(9.99, 289.63) = 2.46$], and time*genotype, [$F(19.97, 289.63) = 2.08$, $p = .01$] (Figure 3B). To probe the interactions, we collapsed on either sex or genotype. A two-way RM ANOVA of time and sex (GGe corrected, $\varepsilon = .29$) revealed a significant main effect of time, [$F(8.5, 280.6) = 29.69$, $p <$

.001], sex, [F(1, 33) = 4.25, p = .047] and a time*sex interaction, [F(8.50, 280.6), p = .017]. Tukey's multiple comparisons tests indicated that females were more active than males at minute 1, 3, 5, 13, 23, 24, and 30 (p's < .05). Next, a two-way RM ANOVA of time and genotype (GGe corrected, ε = .32) revealed a significant main effect of time, [F(9.33, 298.47) = 28.94, p < .001], genotype, [F(2, 32) = 9.84, p < .001], and an interaction of time*genotype, [F(18.66, 298.47) = 1.86, p = .017]. Tukey's multiple comparisons tests revealed elevated activity in HOMs vs. WTs at multiple time points: 1-3, 10, 11, 14, 18, 19, and 22 (p's < .05) and HETs at minute 28 (p = .02). Additionally, a two-way ANOVA of sex and genotype on total locomotor activity revealed a main effect of sex, [F (1, 29) = 8.144, p < .001], and genotype, [F (2, 29) = 13.68, p < .001] (Figure 3C), suggesting that females show greater locomotor activity than males and that HOMs show greater locomotor activity in a 30-minute test in a novel context.

On habituation day 2, a three-way RM ANOVA revealed significant main effects of time, [F(29, 812) = 31.42, p < .001], sex, [F(1, 28) = 7.39, p = .01], and genotype, [F(2, 28) = 5.35, p = .01], with a trend toward a time*genotype interaction (p = .06). These data suggest that on the second day of the saline injection, females moved more than males and, overall, there were genotype differences across time (Figure 3D). Total distance travelled showed a genotype effect, [F (2, 28) = 5.48, p = .01], and a trend toward a main effect of sex (p = .06; Figure 3E). A post hoc Tukey's multiple comparisons test revealed that HOM mice had significantly greater total distance traveled than WT mice (p = .007).

HOMs Show Greater Locomotor Activity Following a 1.5 g/kg EtOH Injection, but not a 2 g/kg EtOH Injection

On the first acute ethanol treatment day, a three-way RM ANOVA (GGe corrected, ε = .23) revealed significant main effects of time, [F(6.69, 194.09) = 31.98, p < .001], sex, [F(1, 29) = 8.17, p = .01], and genotype, [F(2, 29) = 5.58, p = .01]. These data suggest that an acute injection of 1.5 g/kg ethanol shows a significantly more stimulatory response in females compared to males and that there are significant differences between genotypes (Figure 3F). A two-way ANOVA of total locomotor activity showed similar effects of sex, [F (1, 29) = 8.17, p = .01], and genotype, [F (2, 29) = 5.58, p = .01] (Figure 3G), indicating enhanced EtOH-induced stimulation in HOMs compared to WTs and females compared to males.

Following a 2 g/kg injection of EtOH, a three-way RM ANOVA (GGe corrected, ε = .21) revealed significant main effects of time, [F(6.07, 176.06) = 50.95, p < .001], and sex, [F(1, 29) = 10.74, p < .01] (Figure 3H). In the first 30 minutes post-injection (Figure 3I), a two-way ANOVA revealed a main effect of sex, [F(1, 29) = 10.74, p < .01], and no other significant effects, suggesting that at a higher dose locomotor activity was similar across all genotypes.

HOMs Show Sedative Response to 2 g/kg EtOH Injection

Change scores were calculated for each EtOH dose using day 2 (saline) as baseline. At the 1.5 g/kg EtOH dose, a two-way ANOVA of sex and genotype did not yield any significant effects, suggesting that the 1.5 g/kg dose did not induce a greater stimulatory response in the KOs (Figure 5A). At the 2 g/kg EtOH dose, a two-way ANOVA of sex and genotype revealed a significant main effect of genotype, [F (1, 28) = 0.2368, p = .02]. When collapsed by sex, a one-way ANOVA confirmed this genotype effect, [F (2, 31) = 4.702, p = .017]. Specifically, there was a significant difference between WTs and HOMs (p = .018), suggesting that HOMs display a greater sedative response in the first 30 minutes compared to WTs (Figure 5B).

Only HOMs Show EtOH-Induced Anxiolytic Behavior

Percent of time spent in the center of the Open Field Activity chambers across injection days can be seen in Figure 4. When collapsed by sex, a mixed-effects analysis (GGe corrected, $\varepsilon = .64$) revealed main effects of day, [$F(1.917, 60.71) = 27.64$, $p < .001$], genotype, [$F(2, 32) = 8.868$, $p < .001$], and a day*genotype interaction, [$F(6, 95) = 3.942$, $p = .002$] (Figure 4A). Tukey's multiple comparisons test revealed HOMs spent significantly greater time in the center than WTs and HETs on day 3 (1.5 g/kg; p's = .01 – .02) and day 10 (2 g/kg; p's = .02 – .03), suggesting a genotypic-specific increase in anxiolytic response to ethanol. Within-genotype comparisons showed significant changes across days. In WTs, time spent in the center was lower on days 2, 3, and 10 compared to day 1 (p's < .05). In the HETs, time spent in the center was lower on days 3 and 10 compared to both day 1 and day 2(p's < .05). Lastly, HOMs spent significantly more time in the center on day 1 vs. day 2 and 3 (p's < .05). These data indicate that the HOMs did not significantly differ from their second saline injection day on days where they received EtOH, when analyzing the entire 30-minute session (Figure 4A).

Because of the genotypic differences in locomotor activity during the initial minutes of testing, we decided to look at the change in percent time spent in the center in the first five minutes (Figure 4B). A mixed effects analysis (GGe corrected, $\varepsilon = .80$) revealed significant main effects of day, [$F(2.398, 75.95) = 12.54$, $p < .001$], and genotype, [$F(2, 32) = 15.96$, $p < .001$], and a significant interaction of day*genotype, [$F(6, 95) = 5.561$, $p < .001$]. Tukey's multiple comparisons test indicated significant findings between WTs and HOMs ($p < .01$; $p < .001$) and HETs and HOMs ($p < .01$; $p < .001$) at the 1.5 g/kg and 2 g/kg dose, respectively, with HOMs having greater center time than the other genotypes. There were also significant differences between days in each group. In WTs, there were differences between day 1 vs. day 2 and day 3 (p's < .05). In HETs, there were differences between day 1 vs. day 2 and 3 (p's < .05). Finally, in HOMs, there were differences between day 1 vs. day 10 ($p < .05$), day 2 vs. day 3 and 10 (p's < .05), suggesting that in the first 5 minutes of the session HOMs exhibited an EtOH-induced anxiolytic response. Representative images of locomotor activity in the chamber on day 3 (1.5 g/kg EtOH injection) can be seen in Figure 4C.

Discussion

To our knowledge, this study is the first to validate the involvement of *Dpp6* in alcohol-related phenotypes and reveals several key differences between WT and KO mice. In experiment 1, HOM males exhibited a greater CPP response than the other genotypes and HOM females. Additionally, HOM males and females exhibited significant increases in their initial locomotor activity during the pretest, suggesting novelty-induced hyperactivity. In experiment 2, we replicated novelty-induced hyperactivity in the HOMs, and we saw greater total locomotor activity across days. Interestingly, in HOM males and females, both a 1.5 g/kg and a 2 g/kg injection of EtOH significantly increased the percentage of time spent in the center of the open field test compared to the other genotypes, suggesting a greater anxiolytic response to EtOH in the first 5 minutes of the locomotor test, but not when analyzing the entire 30-minute session. However, when we compared the difference between each EtOH injection day and habituation day 2, we found that the higher dose produced a significant sedative response in the HOMs as compared to the WTs. Beyond behavioral differences, we assessed weights in these mice because a loss of *Dpp6* is associated with lower body weight (12,13). Specifically, Lin et al. (2018) found that *Dpp6* loss resulted in significantly lower body weights and brain weights at

311 different developmental times. Here, we did not find significant differences between our WT and
312 KO littermates (Figure S1 & S4); however, we did see a trend in the same direction.

313 First, we found a significant CPP response on test 2 and 3 in the male HOMs, and on test 2 in
314 the male HETs (Figure 1D), suggesting that the male KOs are more sensitive to the rewarding
315 properties of EtOH. A limitation of this study is that the WTs did not show preference for the
316 EtOH-paired chamber, which could be explained by their C57BL6/J (B6) background. Several
317 studies have highlighted that B6 mice are not as sensitive to the rewarding properties of EtOH
318 as compared to DBA mice, and only modestly show EtOH CPP (19). Thus, regardless of the WT
319 indifference, the male KOs were able to form stronger context-drug associations. Interestingly,
320 we found that female KOs did not condition to the EtOH-paired chamber, which suggests that
321 *Dpp6* might be involved in a sex-dependent manner on associative or context-dependent
322 learning; however, this remains a gap in the field.

323 There are several brain regions implicated in CPP, including the NAc, HPC, BNST, and the
324 prelimbic cortex (20–22). *Dpp6* is highly expressed in the HPC and is one of two auxiliary β-
325 subunits that modulates the expression and function of A-type voltage-gated Kv4.2 channels,
326 which are important for neuronal excitability (23,24). Thus, increased dendritic excitability in the
327 HPC and, therefore, increased excitatory projections to the NAc could be driving the differences
328 in the expression of EtOH CPP (25); however, other brain regions co-expressing *Dpp6* and
329 Kv4.2 channels, such as the striatum or cerebellar cortex should be considered (23).
330 Additionally, in *Dpp6* KO mice, Lin et al. (2018) found that KO mice displayed greater locomotor
331 activity and deficits in several learning and memory paradigms. Similarly, Kiselycznyk, Hoffman,
332 and Holmes (2012) found that Kv4.2 KO mice displayed greater locomotor activity and greater
333 anxiolytic behavior, which is consistent with our results. Together, these findings support the
334 notion that both *Dpp6* and Kv4.2 channels are co-expressed (23), and suggests that both are
335 also important for changes in locomotor activity.

336 Next, we found that HOM KO mice displayed greater novelty-induced hyperactivity in the CPP
337 chamber and the open field chamber. This behavioral response involves the HPC, VTA, and
338 locus coeruleus among other brain regions (26,27). Hippocampal circuits play an important role
339 in novelty recognition (28), which further suggests that deletion of this gene disrupts
340 hippocampal neurotransmission that may be important for this behavior. This is in line with
341 Procaccini et al. (2011) who found more c-Fos expression in the hippocampus of GluA1-KO
342 mice following novelty-induced hyperactivity.

343 Additionally, studies of both *Dpp6* KO and Kv4.2 KO models have found that anxiety-like
344 behavior is reduced measured by time spent in the open arms of the elevated plus maze(12,29).
345 We did not significantly detect differences in anxiety-like behavior as measured by percent of
346 the time spent in the center on habituation day 1 when mice received an injection of saline,
347 which is in line with unpublished data from Lin et al. (2018). However, this study demonstrated
348 that a 1.5 and 2 g/kg dose of EtOH significantly decreased anxiety-like behavior in male and
349 female HOM KO mice compared to other genotypes, as measured by the percent of time they
350 spent in the center of the open field chamber. The ventral HPC (vHPC) is implicated in anxiety-
351 related behaviors (28). In fact, lesions and chemogenetic inhibition of the vHPC have shown to
352 decrease anxiety-like behavior (30,31), suggesting that the reduction in anxiety-like behavior
353 following EtOH seen in our experiment might be due to disruption of the ventral, but not dorsal,
354 HPC. While EtOH has been shown to produce anxiolytic behavior independent of locomotor
355 increases (32), we did not find this in our WT or HET animals, suggesting that full deletion of

Dpp6 exacerbates the anxiolytic effect potentially produced by EtOH, and that this effect is present following both a 1.5 and 2 g/kg injection in the first 5 minutes.

Lastly, we found greater total locomotor activity in HOMs on all test days except following a 2 g/kg EtOH dose, suggesting that this dose was sufficient to blunt their hyperlocomotor activity. This is unexpected, given our findings in experiment 1; however, mice were only in the CPP chamber for 5 minutes, whereas they were in the open field chamber for 30 minutes to one hour. The sedative response found in the HOMs in the first 30 minutes in experiment 2 suggests that they may be more sensitive to the sedative properties of EtOH than WT or HET mice. While we did not see a stimulatory response at the 1.5 g/kg dose, it remains to be seen if the HOMs would have displayed an increased stimulatory response at a lower dose, given that CPP expression was greater at 2 g/kg. Additionally, these effects seem to be specific to systemic administration of EtOH. When mice self-administered EtOH, there were no significant differences in intake, nor did these intakes correlate with their CPP expression on test 3 (Figure S2). One limitation, though, is that these mice were given a two-hour drinking session in the regular light cycle to match the CPP experiment. Thus, future work should focus on intake patterns during their active cycle to see if *Dpp6* has a role in EtOH consumption.

Conclusion

DPP6 is involved in several alcohol-related phenotypes, which warrants further investigation. This was the first study looking at the role of *Dpp6* in the rewarding and sedative properties of alcohol. Overall, we found that male, but not female, HOM KOs are more sensitive to both the rewarding properties and sedative effects of 2.0 g/kg injection dose. Anxiolytic behavior following EtOH injections of both 1.5 and 2.0 g/kg was seen in both male and female HOM KO mice compared to HET KO and WT, and both sexes seemed to show a greater novelty-induced locomotor response than the other genotypes. Future work focusing on the underlying mechanisms are necessary to understand the behavioral differences seen here and their potential relationship to risk for AUD.

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Figures

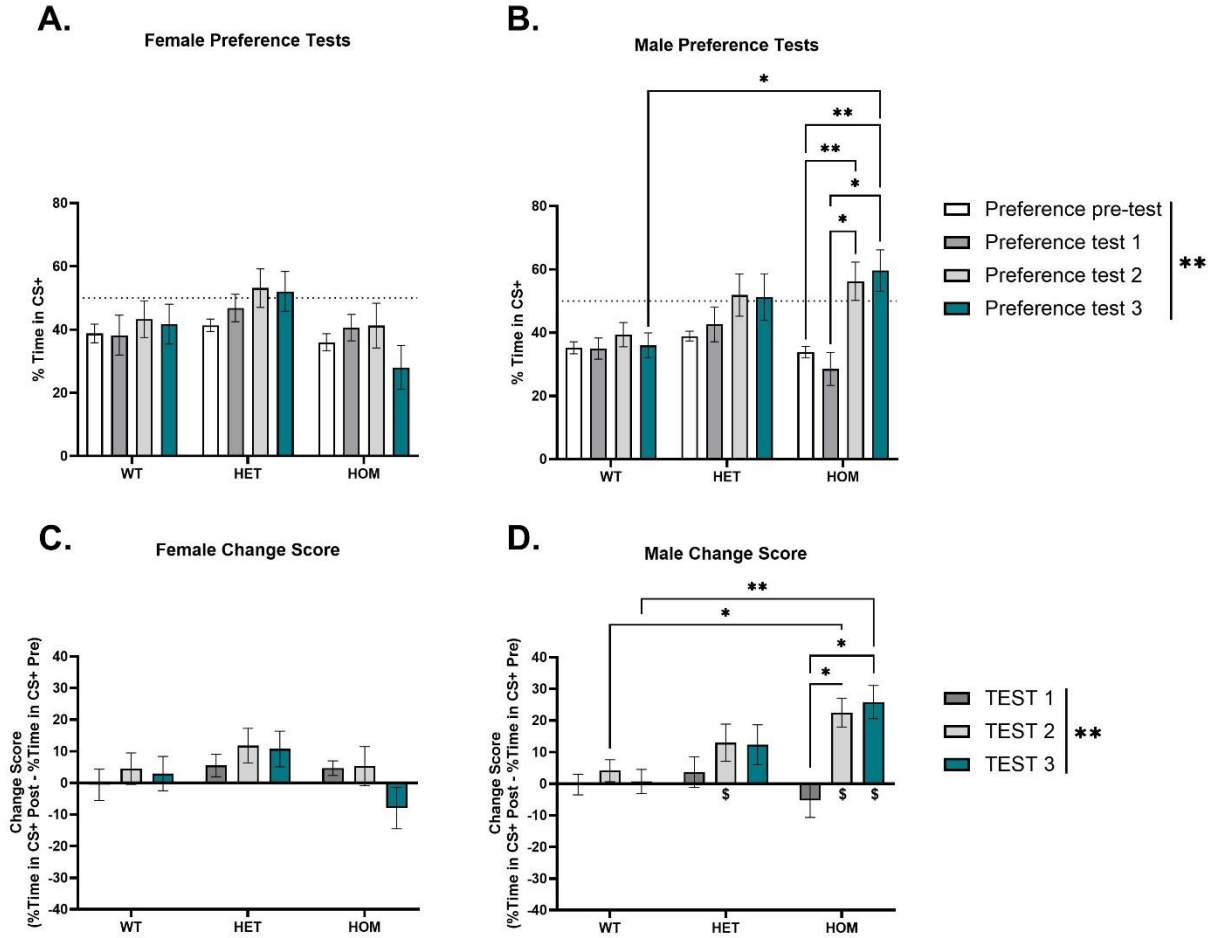

Figure 1. CS+ preference and change scores for *DPP6* WT, HET, and HOM mice. A) Female mice did not show any significant changes in their preference for the CS+ after conditioning. B) Male HOM KO mice show significant increases in preference for CS+ on preference test 2 and 3, and on test 3 they show a significant increase as compared to WT males. C) Similar to preference scores, females did not show changes in their change score or in their ability to express CPP. D) Male KOs show significantly increased CPP change scores after conditioning on test 2 and test 3, both of which are significantly higher as compared to WTs. When assessing their CPP expression, only HET males on test 2 and HOM males on test 2 and 3 showed significant change from zero. WT Female (n = 8); HET Female (n=15); HOM Female (n= 4); WT Male (n= 14); HET Male (n= 17); HOM Male (n= 8) (*p < .05) (**p < .01) ($p < .05, change from zero) ($$p < .01, change from zero)

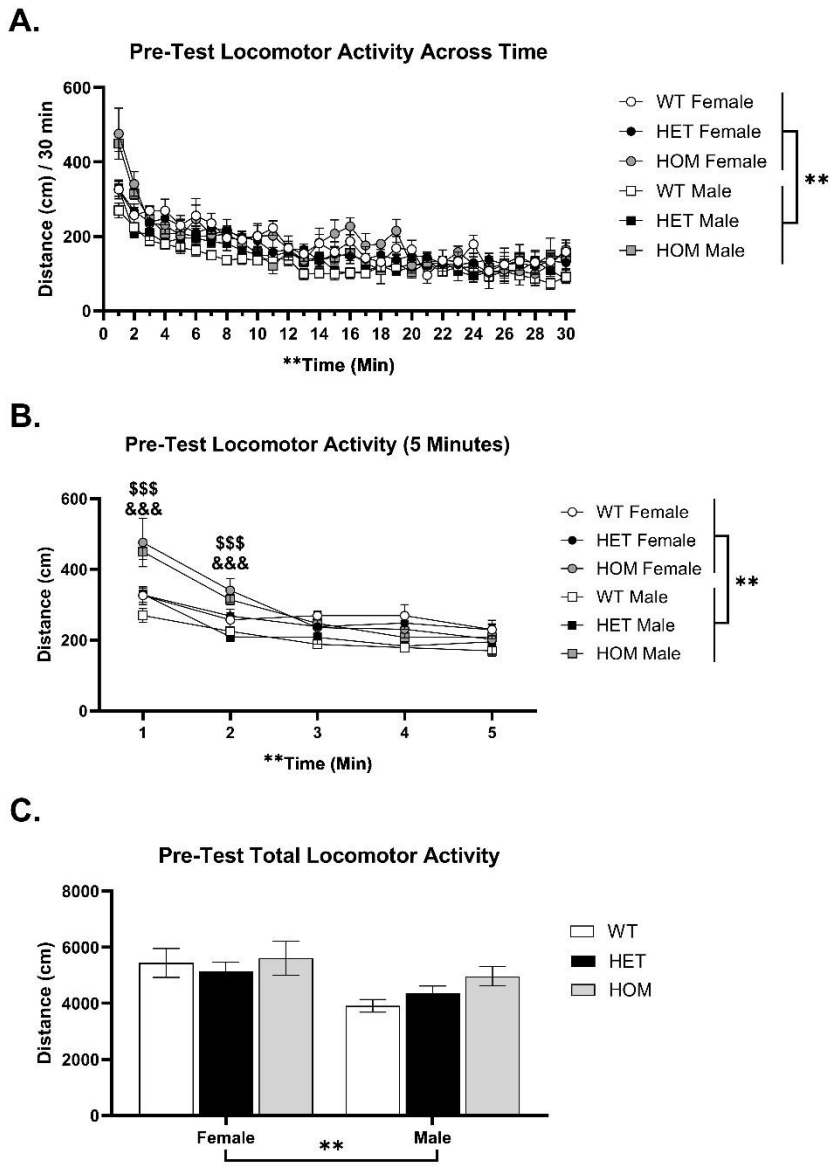

Figure 2. Pretest locomotor activity in Dpp6 WT, HET, and HOM females and males. A) pretest locomotor activity changed across the 30-minute session time and females moved around more than males. B) In the first 5 minutes of the session, both female and male HOM KOs exhibited greater locomotor activity in the first two minutes compared to WT and HET animals. Females also moved more than males in general. C) Total locomotor activity only showed a significant difference between females and males, suggesting that the genotypes move similarly. (*$p < .05$) (**$p < .01$) ($^{\#\#}p < .01$, ME sex) ($^{\$\$\$}p < .001$, HOM vs WT) ($^{\&\&\&}p < .001$, HOM vs HET)

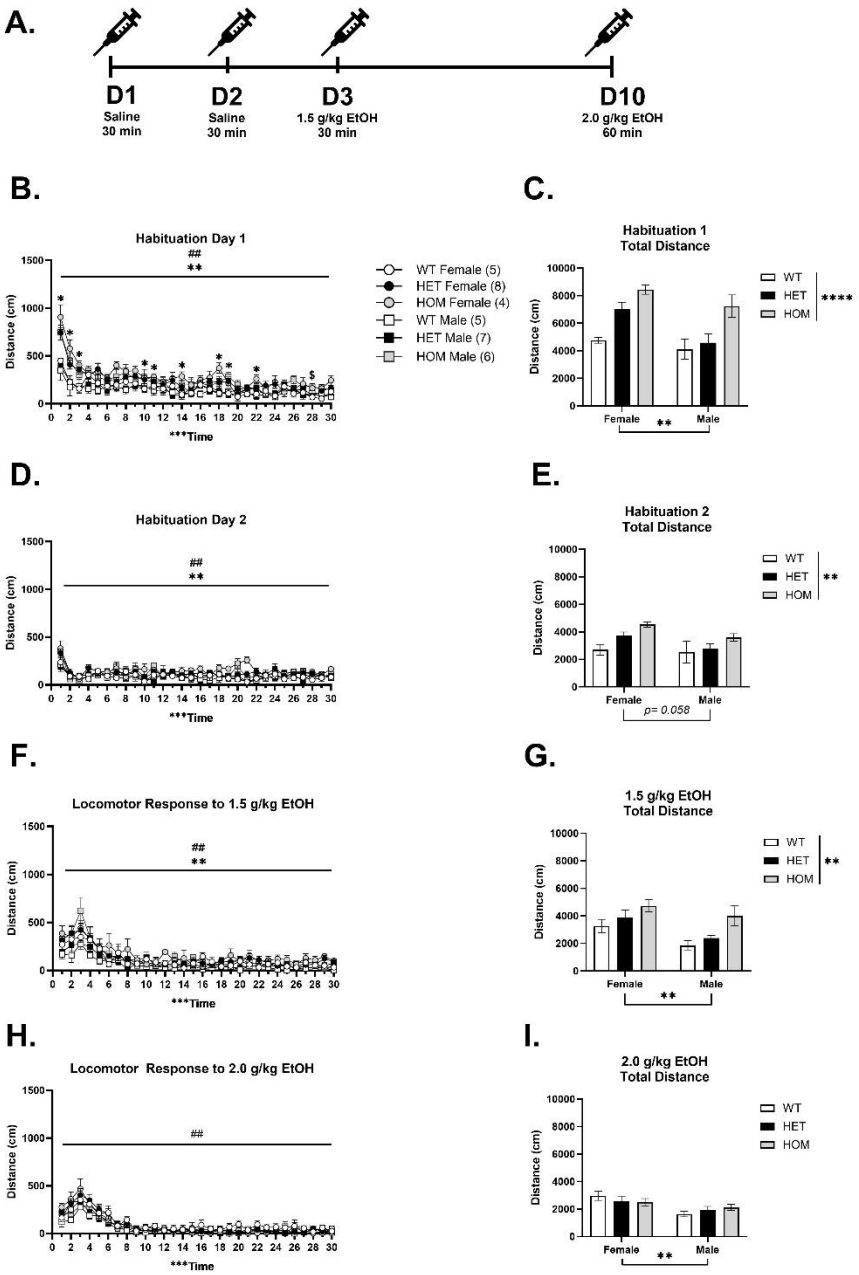

Figure 3. Assessment of 30-minute locomotor activity each day. A) A timeline of experiment 2. B) Habituation day 1 showed significant effects of sex, genotype, and time. C) Total locomotor activity showed main effects of sex and genotype. D) Habituation day 2 also showed significant effects of sex, genotype, and time. E) Total locomotor activity on day 2 shows a significant effect of genotype. F) An acute injection of 1.5 g/kg EtOH showed significant effects of sex, genotype, and time. G) Total locomotor on day 3 showed significant effects of sex and genotype. Lastly, H) an injection of 2 g/kg on day 10 showed significant effects of sex and time but not genotype. Similarly, I) shows a main effect of sex only. WT Female (n = 5); HET Female (n=8); HOM Female (n= 4); WT Male (n= 5); HET Male (n= 7); HOM Male (n= 6); (***$p < .001$) ($^{\#\#}p < .01$, ME of sex) (**$p < .01$ and ME genotype across time)

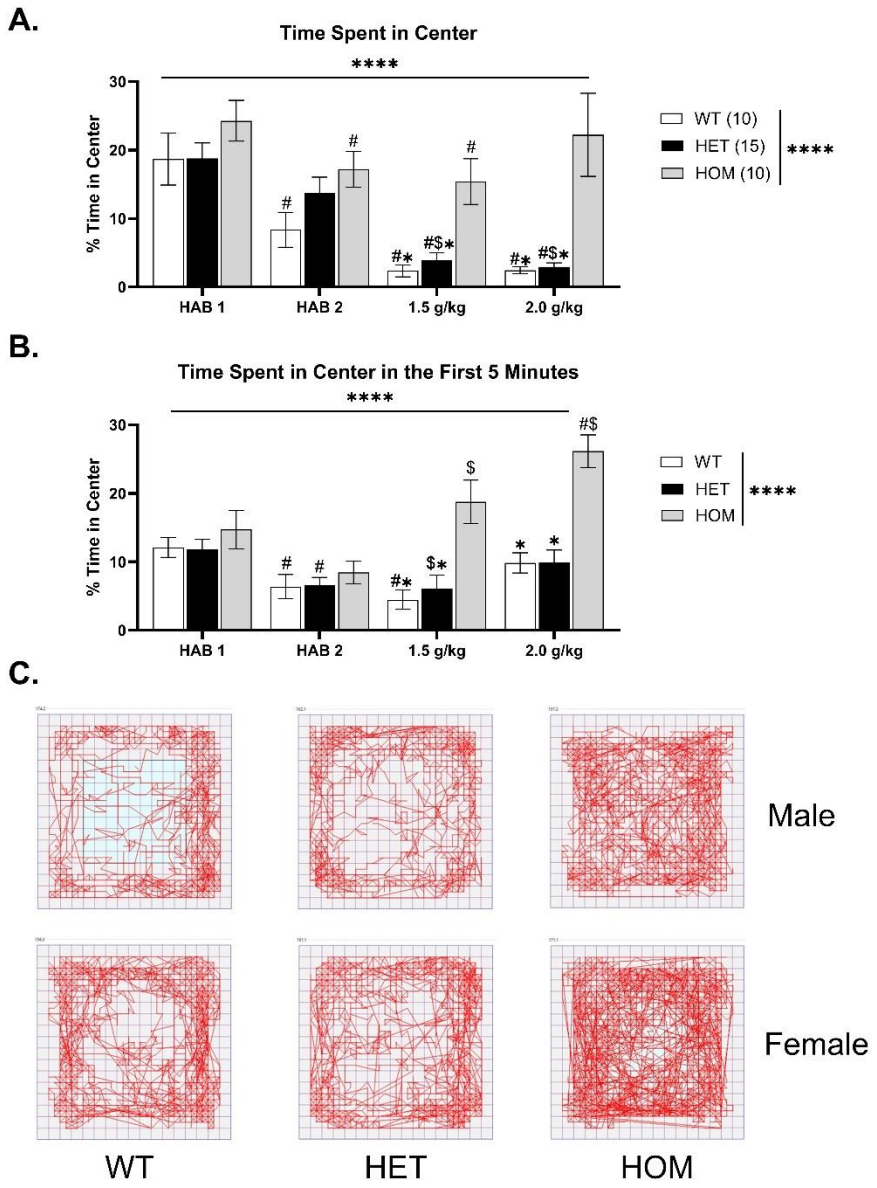

Figure 4. Anxiety-like behavior across test days. A) The percent time spent in the center was significantly difference between genotypes across days. HOMs WTs and HETs showed decreased anxiolytic behavior compared to HOMs on the EtOH injection days. B) When we looked at the first five minutes, we found that genotypes were different across testing days. Similarly, WTs and HETs were significantly different from HOMs on days where EtOH was administered. C) Representative images of the locomotor activity seen in each genotype on day 3 (1.5 g/kg injection; $^{\#}p < .05$, different from Hab 1) ($^{\$}p < .05$, different from Hab 2) ($^{*}p < .05$, different from HOMs in the same group).

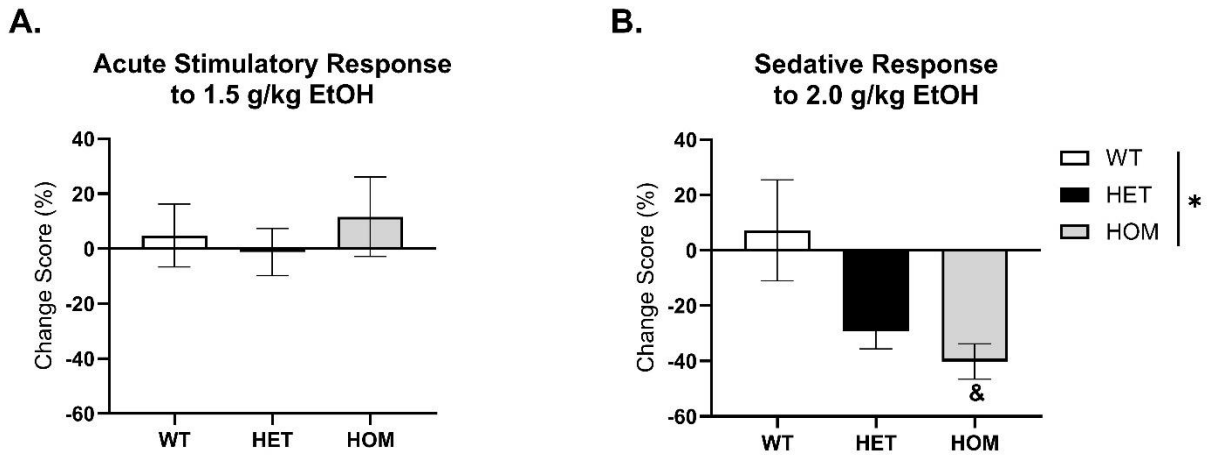

Figure 5. Change scores of EtOH injection days from habituation day 2. There were no main effects of sex, so groups were collapsed. A) 1.5 g/kg injection did not show any significant difference between sex or genotype. B) A 2 g/kg EtOH injection resulted in a main effect of genotype. Specifically, the HOMs showed a greater sedative response compared to WTs. (*$p < .05$) ($^{\&}p < .05$, different from WT)