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Cytoplasmic FMR1-interacting protein 2 allelic variation influences free-choice ethanol drinking, but not binge-like drinking or wheel-running activity, in C57BL/6J and C57BL/6NJ mice

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Abstract

Background: Since the origination of the C57BL/6 (B6) mouse strain, several phenotypically and genetically distinct B6 substrains have emerged. For example, C57BL/6J substrain (B6J) mice display greater voluntary ethanol consumption, increased locomotor response to psychostimulants, and differences in nucleus accumbens synaptic physiology relative to mice of the C57BL/6N (B6N) substrain. Furthermore, a non-synonymous point mutation of serine to phenylalanine (S968F) in the cytoplasmic FMR1-interacting protein 2 (*Cyfip2*) gene, has been shown to underlie both the differential locomotor response to cocaine and accumbal physiology exhibited by B6J and B6N substrains. Thus, the present study was designed to determine whether *Cyfip2* allelic variation underlies B6 substrain differences in other reward-related phenotypes, such as ethanol intake and wheel-running activity.

Methods: Voluntary ethanol consumption, wheel-running, and binge-like ethanol drinking were compared in male and female B6J and B6NJ substrains (both obtained from The Jackson Laboratory). When substrain differences were observed, additional experiments were performed in two novel mouse models in which the previously identified B6N *Cyfip2* mutation was either introduced (S968F) into the B6J background or corrected (F968S) in the B6N background via CRISPR/Cas9 technology.

Results: B6J consumed significantly more ethanol than B6NJ, while allelic variation in *Cyfip2* contributed substantially to this substrain difference. In contrast, B6NJ displayed significantly more daily wheel-running than B6J, but *Cyfip2* allelic variation played only a minor role in this substrain difference. Lastly, no substrain differences were observed in binge-like ethanol drinking.

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Conclusions: These results contribute to the characterization of behavior-genetic differences between B6 substrains, support previous work indicating that free-choice and binge-like ethanol drinking are dependent on partially distinct genetic networks, and identify a novel phenotypic difference between B6 substrains in wheel-running activity.

Keywords

ethanol intake; *Cyfip2* ; DID; wheel-running; B6 substrain

1. Introduction

C57BL/6J (B6J) mice are the preferred inbred mouse model among alcohol researchers due to their high levels of voluntary ethanol intake and preference relative to other inbred strains (Belknap et al., 1993; Yoneyama et al., 2008). While B6J mice originated at The Jackson Laboratory (JAX), a population of B6J were sent to the National Institutes of Health (NIH) in 1951 to form a new colony. However, whenever a new colony is established and maintained separately from an existing colony for 20 or more generations, it can become a genotypically distinct substrain via effects of genetic drift, residual heterozygosity in the founder population, and/or genetic contamination (Bailey, 1982). Over time, these B6J mice (maintained at NIH) became recognized as C57BL/6N (B6N) mice, formally distinguishing B6J and B6N as distinct B6 substrains (Bryant, 2011; Morse, 1978). Moreover, separate colonies of B6N have since been established (and are currently being maintained) at several other locations, including Charles River Laboratories in 1974 (C57BL/6NCrl; B6NCrl), Taconic Biosciences in 1991 (C57BL/6NTac; B6NTac), and JAX in 2005 (C57BL/6NJ; B6NJ).

As a result of genetic drift (Kumar et al., 2013), significant genotypic and phenotypic differences have emerged among various B6 substrains (Ahlgren & Voikar, 2019; Bryant, 2011; Keane et al., 2011; Mortazavi et al., 2022; Simon et al., 2013), with most work specifically comparing B6J (the parental substrain) to B6N and/or its derivatives (B6NCrl, B6NTac, B6NJ) (Bryant et al., 2008; Matsuo et al., 2010; Mekada et al., 2009). Unfortunately, within the broader scientific research community, substrain differences are often unacknowledged, generally due to lack of awareness. However, subtle genotypic and phenotypic differences can be meaningful, especially given that different B6 substrains have been utilized in several large-scale genomics initiatives. For example, B6J mice were selected for the original Mouse Genome Sequencing Consortium (Waterston et al., 2002) whereas B6N are currently used in various projects associated with the International Knockout Mouse Consortium (Austin et al., 2004; Pettitt et al., 2009). It is critical, therefore, that researchers take specific notice of genotypic and phenotypic differences between B6 substrains when planning experiments and especially when comparing data across studies. On the other hand, the emergence of various B6 substrains provides a potentially valuable tool for experimental elucidation of previously unknown genes and alleles contributing to complex traits (Kumar et al., 2013; Miura et al., 2022).

B6 substrains differ significantly in several affective-behavioral phenotypes, including contextual fear conditioning (Bryant et al., 2008), nociception (Bryant et al., 2008; Matsuo

et al., 2010), acute and sensitized locomotor response to psychostimulants (i.e., cocaine, methamphetamine, and nicotine) (Akinola et al., 2019; Kumar et al., 2013), naloxone-induced conditioned place aversion (Kirkpatrick & Bryant, 2014), binge eating (Kirkpatrick et al., 2017), and corticosterone sensitivity (Sturm et al., 2015). Of particular interest to the current work, B6 substrains exhibit differences in several ethanol consumption paradigms. B6J mice reliably demonstrate greater ethanol consumption and preference in the two-bottle free-choice model of voluntary ethanol intake compared to several B6N derivatives (Blum et al., 1982; Mulligan et al., 2008; Ramachandra et al., 2007; Warden et al., 2020; Yoshimoto & Komura, 1987), while B6NJ mice show more robust expression of the alcohol deprivation effect (ADE) than B6J (Khisti et al., 2006). However, B6J and B6N substrains have not yet been directly compared on many other relevant ethanol-related phenotypes. Such studies would be highly valuable, however, given the known genetic correlations among inbred mouse strains between two-bottle free-choice ethanol drinking and other distinct ethanol-related phenotypes, such as handling-induced convulsions (HIC) severity (Hitzemann et al., 2009; Metten et al., 1998) and binge-like drinking (Crabbe et al., 2012). Our laboratory has begun to assess these potential correlations in B6J and B6NJ mice, though we recently found little evidence for substrain differences in either ethanol acute withdrawal severity or abstinence-induced affective disruption (Hartmann et al., 2020).

Initial progress has been made in revealing the genetic bases of addiction-relevant phenotypic differences among B6 substrains. For example, a single-nucleotide polymorphism, a non-synonymous point mutation of serine to phenylalanine (S968F) in the cytoplasmic FMR1-interacting protein 2 (*Cyfip2*) gene has been shown to underlie, at least in part, differences in psychostimulant-induced locomotor response and in multiple aspects of nucleus accumbens synaptic physiology between B6J and B6N mice (Kumar et al., 2013) as well as differences in binge eating between these substrains (Kirkpatrick et al., 2017). Further, heterozygous *Cyfip2* knockout mice (*Cyfip2*$^{N/-}$) show partial reversal of decreased psychostimulant-induced locomotor response, dendritic spine density and glutamatergic activity in the nucleus accumbens shell (Kumar et al., 2013), and increased binge eating (Kirkpatrick et al., 2017). Together, these results suggest that *Cyfip2* may mediate a wide range of reward- and addiction-related differences between B6 substrains. Therefore, the present study compared voluntary ethanol consumption, wheel-running, and binge-like ethanol drinking in C57BL/6J (B6J) and C57BL/6NJ (B6NJ) substrains, and when substrain differences were present, in two novel CRISPR/Cas9 engineered mouse models in which either the previously identified B6N *Cyfip2* mutation was introduced (S968F) into the B6J background (B6J-*Cyfip2*$^{N/N}$) or corrected (F968S) in the B6N background (B6NJ-*Cyfip2*$^{J/J}$).

In Experiment 1, two-bottle free-choice ethanol consumption and preference, as well as daily wheel-running activity, were examined in male and female B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$ and B6NJ-*Cyfip2*$^{J/J}$ mice. Since B6J mice have been shown to exhibit higher ethanol consumption and preference than B6N mice from multiple sources (Blum et al., 1982; Mulligan et al., 2008; Ramachandra et al., 2007; Yoshimoto & Komura, 1987), we expected B6J mice to exhibit significantly greater ethanol consumption and preference than B6NJ. Further, we hypothesized that insertion of the B6N *Cyfip2* mutation (S968F) into the B6J background (B6J-*Cyfip2*$^{N/N}$) would significantly reduce ethanol consumption and

preference, while correcting the mutation (F968S) in the B6NJ background (B6NJ-*Cyfip2*$^{J/J}$) would significantly increase ethanol consumption and preference. Wheel-running was also examined since previous work suggests wheel-running is a rewarding and reinforcing behavior that relies on overlapping neural circuitry that mediates drug reward (Brené et al., 2007). Prior work indicates that B6N mice display higher daily wheel-turns than B6J in both light-dark (LD) and constant darkness (DD) (Banks et al., 2015). In contrast, however, B6J and B6NCrl mice do not significantly differ in daily wheel-turns under constant light (LL) (Capri et al., 2019). Therefore, we also sought to further characterize substrain differences in this phenotype and to assess the potential contribution of the substrain-specific *Cyfip2* mutation.

In Experiment 2, male and female B6J and B6NJ mice were tested for non-dependent binge-like ethanol drinking using the well-validated Drinking-in-the-Dark (DID) protocol (Crabbe et al., 2012; Rhodes et al., 2007). Because previous studies involving inbred mice show a positive correlation between binge-like ethanol drinking in the DID test and voluntary ethanol consumption and preference under two-bottle free-choice conditions (Crabbe et al., 2012; Rhodes et al., 2007), we hypothesized that B6J mice would demonstrate higher levels of DID drinking than B6NJ. Such a result would imply that overlapping sets of genes contribute to both voluntary free-choice ethanol intake (as measured in Experiment 1) and non-dependent binge-like ethanol drinking (as measured in Experiment 2).

2. Materials and Methods

2.1. Animals

Male and female C57BL/6J (JAX Stock No. 000664; B6J; M, $n = 14$; F, $n = 16$), C57BL/6NJ (JAX Stock No. 005304; B6NJ; M, $n = 14$; F, $n = 16$), B6J-*Cyfip2*$^{N/N}$ (JAX Stock No. 028895; M, $n = 13$; F, $n = 12$), and B6NJ-*Cyfip2*$^{J/J}$ (JAX Stock No. 028897; M, $n = 11$; F, $n = 13$) mice were shipped to the University of Maine from The Jackson Laboratory (Bar Harbor, ME). Specifically, *Cyfip2* knock-in mice (B6J-*Cyfip2*$^{N/N}$, B6NJ-*Cyfip2*$^{J/J}$) were generated by the Kumar Laboratory through utilization of CRISPR/Cas9 endonuclease mediated genome editing to introduce the previously identified *Cyfip2* mutation (S968F) into the B6J background (B6J-*Cyfip2*$^{N/N}$) and correct this mutation (F968S) in the B6N background (B6NJ-*Cyfip2*$^{J/J}$). Mice arrived in the laboratory at approximately 6 weeks of age and were immediately individually housed in running-wheel cages (32 × 20 × 14 cm; see below) under a LD 12:12 lighting regimen (lights off at 1400) for the duration of the experiment. Running-wheel cages were placed within light-shielded and sound-attenuating metal cabinets equipped with standard fluorescent bulbs on each shelf. Food (Prolab RMH 3000; LabDiet, St. Louis, MO) and tap water were available *ad libitum* throughout the experiment. During the two-bottle free-choice ethanol drinking protocol, ethanol solutions of various concentrations were available via a second drinking bottle, as described below. All experimental procedures were approved by the University of Maine Institutional Animal Care and Use Committee (IACUC).

Male and female C57BL/6J (B6J; M, $n = 10$; F, $n = 10$) and C57BL/6NJ (B6NJ; M, $n = 9$; F, $n = 9$) mice were shipped to the University of Maine from The Jackson Laboratory

(Bar Harbor, ME). Mice arrived in the laboratory at approximately 6 weeks of age and were immediately individually housed in standard mouse cages (30 × 18 × 12 cm) under a reverse LD 12:12 lighting regimen (lights off at 1200). Cages were placed in a light-shielded and sound-attenuating metal cabinet equipped with a standard fluorescent bulb on each shelf. Food (Prolab RMH 3000; LabDiet, St. Louis, MO) was available *ad libitum* throughout the experiment, whereas tap water was available *ad libitum* except during single-bottle ethanol access, as described below. All experimental procedures were approved by the University of Maine Institutional Animal Care and Use Committee (IACUC).

2.2. Procedures

24 hours following arrival in the laboratory, mice were placed individually in running-wheel cages and wheel-turns were recorded for a 15-day period with *ad libitum* access to food and water (but not ethanol). Afterwards, all mice underwent an extended two-bottle free-choice ethanol drinking protocol, while running wheels remained available throughout ethanol access. There were 10–16 mice for each sex/substrain combination; exact *n* for each group is available for ethanol analyses and wheel-turn per day analyses in Fig. 2 and Fig. 6, respectively. Due to running-wheel equipment malfunction, data were unavailable for a small subset of mice (B6J-*Cyfip2*$^{N/N}$, $n = 2$; B6NJ-*Cyfip2*$^{J/J}$, $n = 4$) that were thus excluded from analyses of daily activity.

After one week of acclimation to the reversed LD cycle, all mice underwent the DID protocol, a widely accepted mouse model of binge-like ethanol drinking originally developed by Rhodes et al. (2005) and described fully below. Immediately following the final ethanol access period, blood samples were obtained from all mice for analysis of blood ethanol concentration (BEC) (see below).

Despite the use of a one-week acclimation to the reverse LD schedule, our first attempt at the DID protocol yielded uncharacteristically low ethanol intake on Day 4 (data not shown). Since the success of the DID protocol is strongly dependent on the time within the animal's circadian rhythm at which ethanol access occurs (Thiele & Navarro, 2014), we suspect that mice were not fully entrained to the reverse light-dark schedule by the start of testing. Therefore, mice were subsequently given 10 days of additional exposure to the reversed LD cycle, with no ethanol access, after which the entire DID protocol was repeated. We believe that this approach is justified by previous work showing stable levels of binge-like ethanol drinking even after up to 10 successive 4-day DID episodes (Cox et al., 2013), and thus only data from the second DID test is shown. There were 9–10 mice for each sex/substrain combination; exact *n* for each group is available in Table 1.

2.2.1. Wheel-running

Mice were given continuous access to an in-cage running-wheel (wheel diameter: 11.5 cm; model ACT-551; Coulbourn Instruments, Whitehall, PA) for the duration of the experiment. Wheel-turns were monitored via microswitch and stored in 1-minute bins using the ClockLab interface system (Coulbourn Instruments, Whitehall, PA). Daily wheel-turns during the 15-day period prior to ethanol access were used for data analysis.

2.2.2. Two-Bottle Free-Choice Ethanol Consumption

Mice were given continuous free-choice access to bottles containing either an ethanol solution or plain water for a total of 35 days. Ethanol concentration was initially set at 3% (v/v) and increased in 3% increments, every 5 days, through a final concentration of 21%. The physical location (right or left) of the water and ethanol solutions was switched every 5 days, in a counterbalanced manner, to reduce the effects of potential side preference. Pre- and post-measurements of bottle weight, along with respective body weights following each 5-day period of ethanol access (data not shown), were used to obtain body weight-adjusted ethanol intake (g/kg) over the course of the experiment. Ethanol preference was determined by dividing the volume of ethanol solution consumed by total fluid intake.

2.2.3. Drinking-in-the-Dark

For 3 consecutive days, beginning 3 hours into the dark cycle, water bottles were removed from all cages and replaced with bottles containing 20% (v/v) ethanol solution. Mice were given 2 hours of access to ethanol, after which the ethanol bottles were removed from cages and water bottles replaced. This same procedure was followed on Day 4 except that ethanol access was extended from 2 to 4 hours. Pre- and post-measurements of bottle weight on each day, along with initial respective body weights (data not shown), were used to obtain body weight-adjusted ethanol intake (g/kg). Experimenters who weighed the bottles each day were blinded to the sex and genotype of the mice. Separate empty cages ($n = 3$) were set up and handled identically to account for bottle leakage potentially caused by mice tampering with the drinking spout, cage handling, and/or bottle weighing. Obtained leakage values (g) were subtracted from individual mouse raw intake data (g) to calculate corrected ethanol intake (g/kg). The 4-day DID procedure has been previously shown to generate high levels of voluntary ethanol intake and to reliably yield binge-like BEC consistent with intoxication (i.e., greater than 80 mg/dL; Rhodes et al., 2005; 2007).

2.2.4. Measurement of Ethanol Concentrations in Tail Blood—In Experiment 2, BEC were measured immediately following cessation of the ethanol access period on Day 4. A small (approximately 20 μL) blood sample was collected from each mouse via tail snip and centrifuged for 10 minutes at 1000 g to separate plasma from serum. BEC were determined from 5 μL plasma samples using an AM-1 alcohol analyzer (Analox Instruments, Lunenburg, MA).

2.2.5. Statistics—Data are presented as means ±SEM and effects were considered statistically significant when $p < .05$. Only statistically significant main effects and interactions are described in the text. Full analysis of variance (ANOVA) results (i.e., F, df, p, partial η^2) are provided for the omnibus analyses, but only p-values are indicated for follow-up tests. Data analyses were performed using SPSS 25.0 (IBM Inc., Armonk, NY) and figures were generated using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA).

Ethanol intake and preference (via two-bottle free-choice) were analyzed using 3-factor (genotype, sex, concentration) mixed-design ANOVA followed where appropriate by

separate 2-factor ANOVAs. Wheel-turns per day was analyzed using 3-factor (genotype, sex, day) mixed-design ANOVA, followed where appropriate by separate 2-factor ANOVAs.

Ethanol intake (via Drinking-in-the-Dark) was analyzed using 3-factor (substrain, sex, day) mixed-design ANOVA and BEC data were analyzed using 2-factor (substrain, sex) ANOVA. Additionally, correlations among ethanol intake and BEC measurement were analyzed for each substrain.

3. Results

3.1. Experiment 1.

3.1.1. *Cyfip2* allelic variation has a substantial, but asymmetric, influence on the greater two-bottle free-choice ethanol consumption observed in both males and female B6J versus B6NJ.—

Here, we evaluated potential effects of genotype and sex across ethanol concentrations in male and female B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$ and B6NJ-*Cyfip2*$^{J/J}$ mice two-bottle free-choice ethanol consumption. ANOVA revealed significant main effects of genotype ($F_{3,101} = 11.58$, $p < .001$, partial $\eta^2 = .256$), sex ($F_{1,101} = 41.44$, $p < .001$, partial $\eta^2 = .291$), and ethanol concentration ($F_{6,606} = 76.268$, $p < .001$, partial $\eta^2 = .430$), as well as significant genotype x sex ($F_{3,101} = 3.37$, $p = .021$, partial $\eta^2 = .091$), genotype x concentration ($F_{6,606} = 2.40$, $p = .001$, partial $\eta^2 = .066$), and sex x concentration ($F_{6,606} = 7.71$, $p < .001$, partial $\eta^2 = .071$) interactions (Fig. 1). Ethanol intake generally increased as a function of concentration, while females displayed significantly greater intake than males, both overall and at each concentration except 3% and 9% (Fig. 1). Regarding genotype, ethanol intake was generally highest in B6J, lowest in B6NJ, and intermediate in the two *Cyfip2* knock-in mouse models (Fig. 1). While B6J displayed significantly greater overall intake than any other genotype, pairwise comparisons among genotypes varied as a function of concentration. Specifically, B6J displayed significantly greater intake than B6NJ at all concentrations of 9% and higher; B6J displayed significantly greater intake than B6J-*Cyfip2*$^{N/N}$ at concentrations of 12%, 18%, and 21%; and B6J displayed significantly greater intake than B6NJ-*Cyfip2*$^{J/J}$ at concentrations of 12% and greater (Fig. 1). While B6J-*Cyfip2*$^{N/N}$ exhibited greater intake than B6NJ overall, this effect was significant only at the 15% concentration (Fig. 1). Finally, while B6NJ-*Cyfip2*$^{J/J}$ showed significantly greater overall intake than B6NJ, this effect was not significant at any specific concentration (Fig. 1). Separate analyses for females and males (Fig. 2) showed that female B6J displayed significantly greater intake than females of any other genotype (Fig. 2A, whereas among males, B6J displayed significantly greater intake than B6NJ and B6J-*Cyfip2*$^{N/N}$ but did not differ from B6NJ-*Cyfip2*$^{J/J}$ (Fig. 2B). Finally, male B6NJ exhibited significantly lower intake than any other genotype (Fig. 2B).

3.1.2. *Cyfip2* allelic variation has a minor contribution on greater two-bottle free-choice ethanol preference observed in both males and female B6J versus B6NJ.—

Next, we assessed potential effects of genotype and sex across ethanol concentrations in male and female B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$ and B6NJ-*Cyfip2*$^{J/J}$ mice two-bottle free-choice ethanol preference. ANOVA revealed significant main effects of genotype ($F_{3,101} = 8.41$, $p < .001$, partial $\eta^2 = .200$), sex ($F_{1,101} = 8.43$, $p = .005$, partial

η^2 = .077], and ethanol concentration ($F_{6,606}$ = 7.59, p < .001, partial η^2 = .070), as well as significant genotype x sex ($F_{3,101}$ = 4.45, p = .005, partial η^2 = .119) and sex x concentration ($F_{6,606}$ = 3.39, p = .003, partial η^2 = .032) interactions (Fig. 3). Ethanol preference was generally stable across concentrations, though somewhat lower preference was observed at the higher concentrations (Fig. 3). Females displayed significantly higher preference than males overall, and at concentrations of 15% and greater (Fig. 3). Across concentrations, B6NJ exhibited significantly lower preference than all other genotypes. Separate analyses for females and males showed that female B6J displayed significantly greater preference than both B6NJ and B6NJ-*Cyfip2*$^{J/J}$, but not B6J-*Cyfip2*$^{N/N}$ (Fig. 4A), whereas male B6NJ showed significantly lower preference than all other genotypes (Fig. 4B).

3.1.3. Substrain differences in wheel-running were also observed, but in the direction opposite to two-bottle free-choice ethanol drinking differences.—

Here, we examined potential effects of genotype and sex across a 15-day period of wheel-running activity (prior to ethanol access) in male and female B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$ and B6NJ-*Cyfip2*$^{J/J}$ mice. As expected from prior research (Kandasamy et al., 2016), wheel-turns per day generally increased over successive days but stabilized by about Day 8 of wheel access (Fig. 5). ANOVA revealed significant main effects of genotype ($F_{3,95}$ = 35.02, p < .001, partial η^2 = .525), sex ($F_{1,95}$ = 8.32, p = .005, partial η^2 = .081), and day ($F_{14,1330}$ = 74.16, p = .002, partial η^2 = .438), as well as significant genotype x sex ($F_{3,95}$ = 3.09, p = .031, partial η^2 = .089) and genotype x day ($F_{42,665}$ = 2.09, p = .013, partial η^2 = .062) interactions (Fig. 5). Overall, females exhibited significantly higher wheel-turns per day than males, B6NJ displayed significantly higher wheel-turns per day than all other genotypes, and B6NJ-*Cyfip2*$^{J/J}$ showed significantly higher wheel-turns per day than B6J and B6J-*Cyfip2*$^{N/N}$ (who did not differ from each other; Fig. 5). Separate analyses for females and males showed that female B6NJ and B6NJ-*Cyfip2*$^{J/J}$ displayed higher wheel-turns per day than both female B6J and B6J-*Cyfip2*$^{N/N}$ (Fig. 6A). In contrast, male B6NJ displayed significantly higher wheel-turns per day than all other genotypes, while male B6NJ-*Cyfip2*$^{J/J}$ showed significantly higher wheel-turns per day than male B6J-*Cyfip2*$^{N/N}$ (Fig. 6B).

3.2. Experiment 2.

3.2.1. No substrain or sex differences in binge-like drinking.—

Here, we tested potential effects of genotype and sex on non-dependent binge-like ethanol drinking in male and female B6J and B6NJ mice undergoing the standard DID protocol. ANOVA revealed a significant main effect of day ($F_{3,102}$ = 48.78, p < .001, partial η^2 = .589), but no significant effects of sex or substrain (Fig. 7). *Post hoc* pairwise comparisons showed that ethanol intake was significantly lowest on Day 1 and highest on Day 4 (Fig. 7). In addition, exploratory *post hoc* analyses conducted on each individual day revealed that B6J displayed significantly higher ethanol intake than B6NJ ($F_{3,34}$ = 6.77, p = .014, partial η^2 = .166) only on Day 1 (Fig. 7).

3.2.2. No substrain or sex differences in BEC.—

Following conclusion of ethanol access on Day 4, all groups showed mean BEC above the National Institute on Alcohol Abuse and Alcoholism defined criterion for a “binge” episode, 80 mg/dL. While there were

no significant effects of substrain or sex, females showed numerically higher BEC than males (Table 1). Lastly, both B6J (r = .589, n = 20, p = .006; Fig. 8A) and B6NJ (r = .581, n = 18, p = .011; Fig. 8B) demonstrated moderate, positive correlations between Day 4 ethanol intake and subsequent BEC.

4. Discussion

Overall, the present set of experiments detected substantial substrain differences between B6J and B6NJ mice of both sexes in two-bottle free-choice ethanol drinking and daily wheel-running, but not in binge-like ethanol drinking. Moreover, the use of two novel CRISPR/Cas9-engineered mouse models revealed that allelic variation in *Cyfip2* substantially modulates substrain differences in ethanol intake, and to a lesser extent in wheel-running, though observed genotypic effects often interacted with other factors such as sex, ethanol concentration, and/or day of running-wheel access.

Consistent with prior two-bottle free-choice studies which utilized a different B6N derivative (i.e., B6NCrl; Mulligan et al., 2008; Ramachandra et al., 2007), B6J mice of both sexes showed greater overall ethanol intake and higher levels of ethanol preference compared to B6NJ. As expected, females generally consumed more ethanol than males in both B6J and B6NJ, but the substrain difference in ethanol consumption was substantially larger in males than in females. While introduction of the previously identified B6N *Cyfip2* mutation (S968F) into the B6J background (B6J-*Cyfip2*$^{N/N}$) significantly reduced ethanol intake in both sexes, correcting this mutation (F968S) in the B6N background (B6NJ-*Cyfip2*$^{J/J}$) significantly increased ethanol intake only in males. Further, while male B6J-*Cyfip2*$^{N/N}$ showed significantly higher ethanol intake than male B6NJ, female B6J-*Cyfip2*$^{N/N}$ exhibited similar levels of ethanol intake as did female B6NJ. Overall, males were generally more affected by background genotype and *Cyfip2* allele variation, suggesting an interaction between sex-dependent genetic background effects and the *Cyfip2* allele underlies the observed differences in ethanol intake between B6J and B6NJ. In fact, sex-dependent genetic background effects have been recently shown to interact with allelic variation in cytoplasmic FMR1-interacting protein 1 (*Cyfip1*), a homolog of *Cyfip2*, to influence binge eating susceptibility in mice (Babbs et al., 2019). Such preclinical findings provide further justification to incorporate sex as a biological variable in experimental design, especially as human epidemiological data has already established that males generally have a greater susceptibility to developing alcohol use disorder (Grant et al., 2015).

Although there was no *a priori* hypothesis predicting an asymmetric effect of the *Cyfip2* mutation manipulation, the current data reflects a greater propensity of the *Cyfip2* mutation (S968F) insertion into a high-drinking B6J background to reduce ethanol intake than its correction (F968S) in a comparatively low-drinking B6NJ background to increase ethanol intake. It is possible that the corrected *Cyfip2* allele requires sex-specific gene-gene interactions to generate the high ethanol intake customarily seen in B6J mice, while the *Cyfip2* mutation is sufficient to yield low ethanol intake regardless of sex. Sex-specific mapping studies could help identify potential gene-gene interactions modulating the effects of *Cyfip2* on two-bottle free-choice ethanol drinking.

Genotypic differences in ethanol intake also varied as a function of ethanol concentration, as B6J displayed significantly greater intake than B6NJ only at concentrations of 9% and higher. This finding differs somewhat from previous work (comparing B6J and B6NCrl) which utilized the identical series of ethanol concentrations as employed here yet observed higher voluntary ethanol intake (B6J > B6NCrl) at all concentrations, including at 3% and 6% (Mulligan et al., 2008). It should be noted that the current experimental design, like that of Mulligan et al. (2008), involved an increasing ethanol concentration series, thus confounding time with increasing concentration. That is, we may very well have observed a different outcome at any specific concentration if we had employed a descending concentration series or even tested independent groups at each concentration. However, five days was allotted to consume each respective ethanol solution and previous work indicates that approximately four days is the optimal duration of two-bottle free-choice access for detecting murine strain differences (Tordoff & Bachmanov, 2002).

Similar to ethanol intake, B6J mice of both sexes showed greater overall ethanol preference compared to B6NJ, consistent with prior work comparing B6J to B6NCrl (Mulligan et al., 2008; Ramachandra et al., 2007). In contrast to our ethanol intake data, B6J-*Cyfip2*$^{N/N}$ did not display significantly reduced ethanol preference relative to B6J in either sex, whereas B6NJ-*Cyfip2*$^{J/J}$ exhibited significantly increased ethanol preference relative to B6NJ, but only in males. These results suggest a stronger effect of the *Cyfip2* SNP on ethanol intake than on ethanol preference, which was not anticipated.

Naturally, ethanol preference depends on both ethanol intake and water intake, and important differences between ethanol intake and preference have historically resulted in a greater emphasis on reporting consumption in g/kg rather than preference ratios (Crabbe et al., 2014). The amount of ethanol consumed by an animal typically increases progressively across increasing concentrations, until eventually plateauing at high concentrations that may become more aversive than pleasurable. In contrast, as increasing concentrations are offered, ethanol preference ratios often follow an inverted “U-shaped” pattern, with the highest ethanol preference ratio occurring at a strain-specific intermediate concentration (Crabbe et al., 2014). Some strains may simply show patterns of increasing or decreasing preference for ethanol over many days, as ethanol-avoiding animals generally exhibit decreased preference across time, whereas ethanol-preferring animals typically show increased preference (Blizard et al., 2008). Moreover, unlike previous work, our mice had access to in-cage running-wheels throughout the two-bottle free-choice protocol, which our lab (Rosenwasser et al., 2013; Rosenwasser et al., 2015) and others (Crews et al., 2004; Ozburn et al., 2008) have shown to typically reduce ethanol preference, due to increased water intake, while having little effect on overall ethanol intake.

Substrain differences in wheel-running were also observed, but in the direction opposite to two-bottle free-choice ethanol drinking differences. B6NJ mice of both sexes showed higher levels of wheel-running than B6J, even though B6J are themselves known as a “high-running” substrain. Intriguingly, B6J-*Cyfip2*$^{N/N}$ did not display significantly increased wheel-running relative to B6J in either sex, yet B6NJ-*Cyfip2*$^{J/J}$ exhibited significantly decreased wheel-running relative to B6NJ, but only in males. The asymmetric and sex-

dependent nature of these effects reflect a more modest influence of *Cyfip2* allelic variation on wheel-running compared to two-bottle free-choice ethanol drinking.

Despite considerable differences in two-bottle free-choice drinking (Experiment 1), no substrain differences were found in either binge-like ethanol drinking or resultant BEC levels during a standard 4-day DID protocol (Experiment 2). Both substrains displayed the expected elevated consumption and BEC within the range of intoxication on Day 4, while results were quantitatively similar to that seen in previous work with B6J mice (Rhodes et al., 2005; Rhodes et al., 2007). Additionally, both substrains demonstrated moderate, positive correlations between Day 4 ethanol intake and BEC levels, confirming that intake readings were indeed due to actual consumption and not accidental leakage from mice tampering with the drinking spout. In contrast to prior work (Rhodes et al., 2005; Rhodes et al., 2007), we did not observe effects of sex on either ethanol consumption or BEC levels, though females showed numerically higher ethanol consumption and resultant BEC. Interestingly, a recent study (Jimenez Chavez et al., 2021) compared B6J and B6NJ mice in a modified DID protocol that, unlike ours, did not employ an extended 4-hour access test day. They also reported no substrain difference but found that B6J consumed more ethanol than B6NJ during a 3-bottle version of the test in which multiple concentrations were offered concurrently. Further work is needed to clarify the exact experimental conditions of binge-like drinking paradigms where substrain differences emerge, as even seemingly insignificant factors, such as position and number of available solutions have been shown to notably influence results. (Bachmanov, Reed, Beauchamp, & Tordoff, 2002; Tordoff & Bachmanov, 2003).

Though the substrain x day interaction was not significant, exploratory analyses of potential substrain differences on individual test days indicated that B6J consumed significantly more ethanol than B6NJ on Day 1 only. This disparity on the first access day likely reflects the substrain difference in free-choice ethanol consumption observed in Experiment 1. These findings provide evidence that certain genes, such as *Cyfip2*, can selectively contribute to a distinct ethanol-related phenotype.

Previous data from inbred strain panels has suggested common genetic influences on limited-access and continuous two-bottle free-choice drinking (Crabbe et al., 2012; Rhodes et al., 2007). Correlations from 23 inbred strains indicate that the DID protocol shares about 50–70% of genetic variance in common with the standard two-bottle free-choice test (Crabbe et al., 2012). However, contrasting evidence from selected lines has emerged. High Drinking in the Dark (HDID) mice, selectively bred for high resultant BEC via the DID protocol, do not significantly differ in voluntary ethanol consumption under two-bottle free-choice conditions compared to non-selected control mice from the genetically heterogenous progenitor line (Crabbe et al., 2011; Rosenwasser et al., 2013), implying that genes underlying two-bottle free-choice drinking are at least partially distinct from those promoting binge-like drinking during the DID protocol.

Though these lines of evidence may appear contradictory, the different genetic animal models employed to evaluate the strength of the genetic association between continuous two-bottle free-choice and DID drinking must be acknowledged. For instance, since the

process of inbreeding intrinsically eliminates heterozygosity, relevant dominant alleles are theoretically absent within inbred strains (Crabbe et al., 2011). In turn, genetic dominance has been shown to influence both continuous two-bottle free-choice (Blednov et al., 2005; Blednov et al., 2010) and DID drinking (Phillips et al., 2010), which perhaps explains, in part, the disparity between data derived from inbred strain panels and selected lines. Additionally, there are differences in underlying mechanisms between the behaviors elicited by these different paradigms. Unlike continuous two-bottle free-choice drinking, the DID protocol results in intoxicating BEC levels and significantly impaired motor coordination (Rhodes et al., 2007). The presence or absence of intoxication is possibly a reason why selection for high voluntary ethanol consumption under two-bottle free-choice conditions and for high BEC via the DID protocol is not entirely symmetrical (Crabbe et al., 2011). Indeed, estimated heritability of the DID trait (h^2 = 0.096; Crabbe et al., 2009) is markedly less than that of high two-bottle free-choice drinking (h^2 = 0.46 – 0.74; Belknap et al., 1993; Wahlsten et al., 2006; Yoneyama et al., 2008). Also, since correlations between the DID protocol and the two-bottle free-choice test among inbred strains are substantial, but not absolute, a lack of differential ethanol preference drinking between HDID mice and a genetically heterogenous stock is plausible. Specific alleles that promote high resultant BEC through the DID protocol are likely distinct in ethanol-preferring inbred strains and HDID mice. In fact, ethanol-related phenotypic correlations are typically seen much more reliably among inbred strains than in selectively-bred lines (Hitzemann et al., 2009; Metten et al., 1998). Thus, while partially overlapping gene sets contribute to both two-bottle free-choice and binge-like DID drinking among inbred strains, evidence from selectively-bred HDID mice and B6 substrains indicate that the genes underlying two-bottle free-choice drinking are at least partially distinct from those promoting binge-like drinking in the DID protocol.

Although not evaluated in the current set of experiments, it we would interesting to examine whether B6J and B6NJ mice differ in dependence-induced escalation of voluntary ethanol intake. As expected, such work almost exclusively utilizes B6J mice (Becker & Lopez, 2004; Griffin, Lopez, & Becker, 2009a; Griffin, Lopez, Yanke, et al., 2009b; Lopez & Becker, 2005). Intriguingly, previous data from selectively-bred lines do not suggest substantial common genetic influence on DID and dependence-induced escalation of voluntary ethanol intake (Crabbe et al., 2012). Therefore, since we found that B6J and B6NJ did not differ in DID drinking (Experiment 2), it is plausible that significant substrain differences would be observed in dependence-induced escalation of voluntary ethanol intake.

In sum, despite the evidence for considerable overlap between the genetic influences on two-bottle free-choice and DID drinking, there are likely also genetic factors involved that contribute to one, but not the other, trait. Utilization of B6 substrains allowed discovery of an uncommon instance where two genetically distinct populations (albeit more genetically similar compared to two distinct inbred strains) substantially differed in two-bottle free-choice ethanol drinking but not binge-like ethanol drinking. B6 substrains allow a surprisingly robust balance between genetic similarity and diversity which can produce considerable phenotypic differences. Since genetic variants that underlie B6 substrain differences are likely to frequently be different from variants identified in inbred

strain panels, we suggest use of such experimental framework as a powerful approach for uncovering novel genetic and allelic contributions of various ethanol-related phenotypes.

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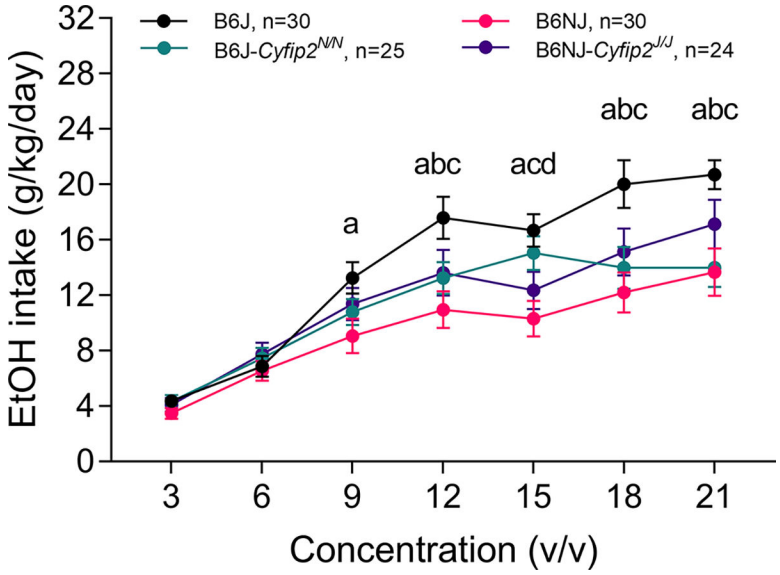

Figure 1.

Ethanol intake across concentrations. Mean (±SEM) ethanol intake (g/kg/day) during two-bottle free-choice in B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$, and B6NJ-*Cyfip2*$^{J/J}$ mice. Letters denote the following significant comparisons ($p < .05$): a = B6J > B6NJ, b = B6J > B6J-*Cyfip2*$^{N/N}$, c = B6J > B6NJ-*Cyfip2*$^{J/J}$, d = B6J-*Cyfip2*$^{N/N}$ > B6NJ.

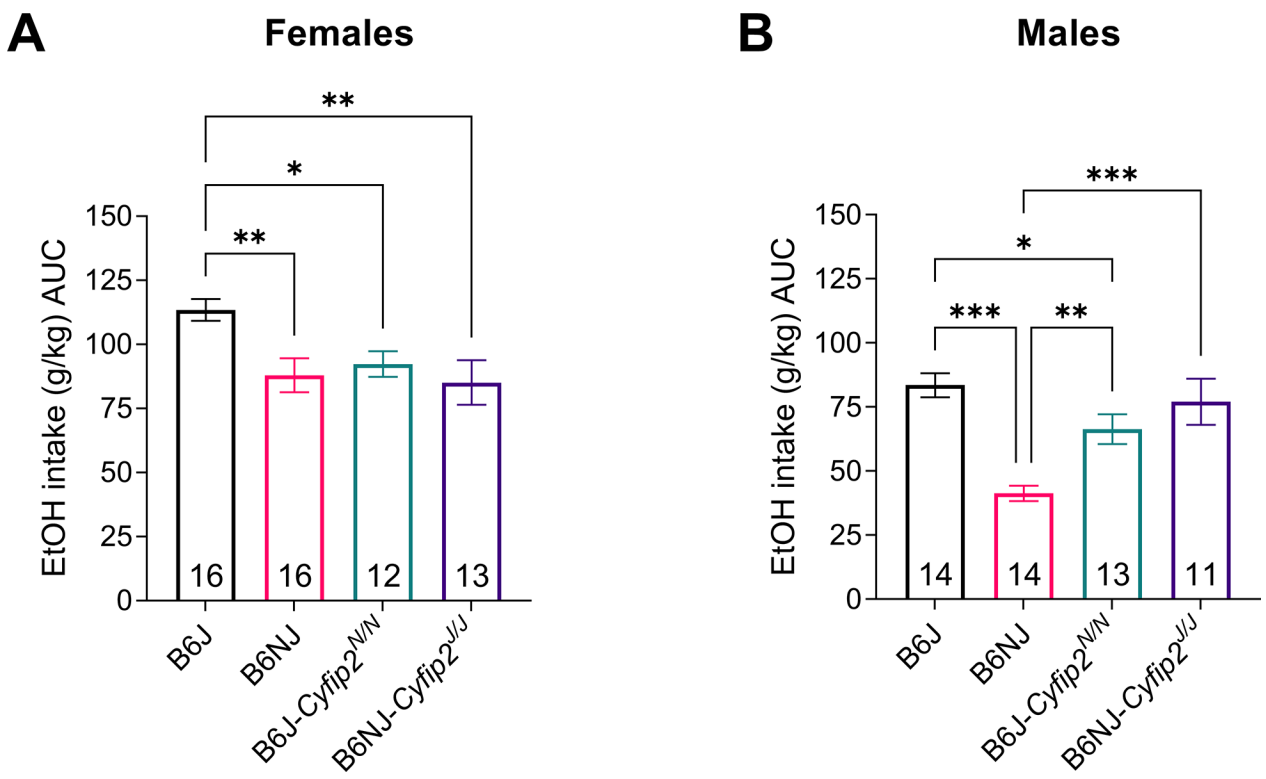

Figure 2.

Total ethanol intake across sex. Mean (±SEM) total ethanol intake (g/kg), calculated by area under the curve (AUC) approximation, during two-bottle free-choice in female (**A**) and male (**B**) B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$, and B6NJ-*Cyfip2*$^{J/J}$ mice. Asterisk symbols indicate the following: *** = $p < .001$, ** = $p < .01$, * = $p < .05$.

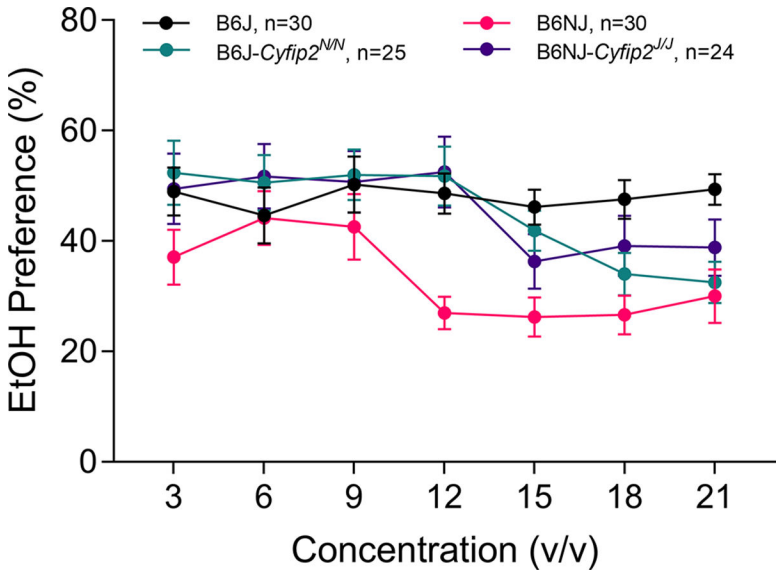

Figure 3.

Ethanol preference across concentrations. Mean (±SEM) ethanol preference during two-bottle free-choice in B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$, and B6NJ-*Cyfip2*$^{J/J}$ mice.

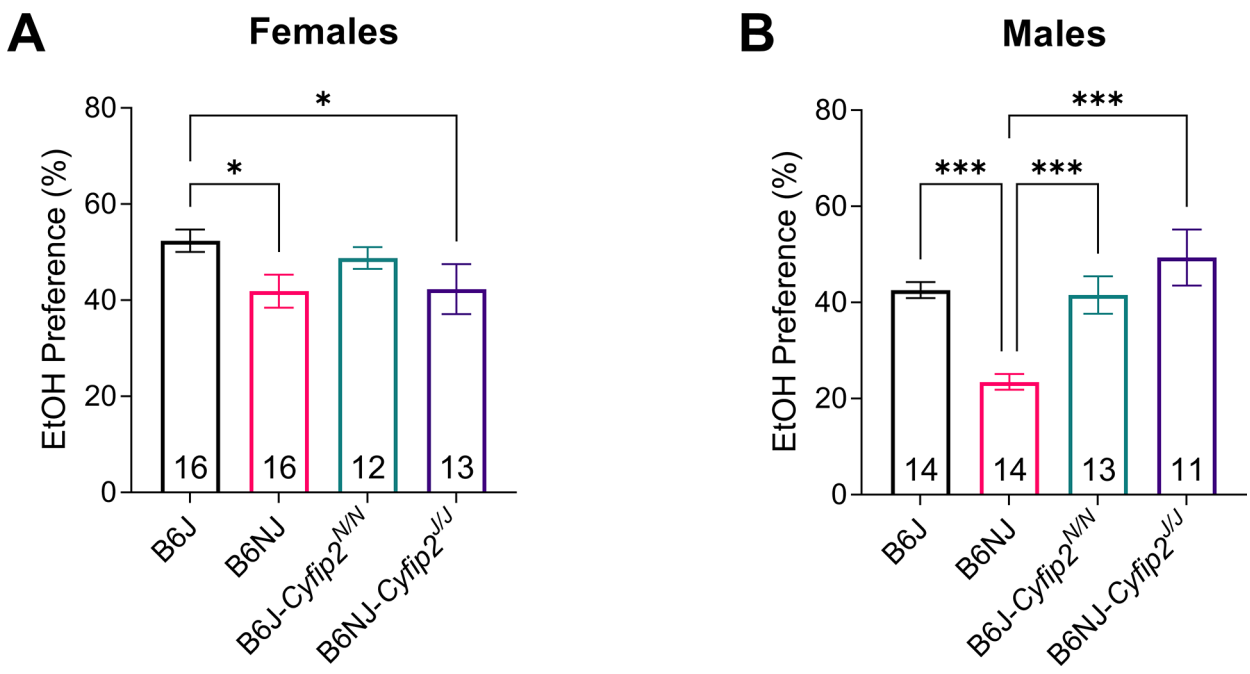

Figure 4.

Ethanol preference across sex. Mean (±SEM) ethanol preference during two-bottle free-choice, collapsed across concentration, in female (**A**) and male (**B**) B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$, and B6NJ-*Cyfip2*$^{J/J}$ mice. Asterisk symbols indicate the following: *** = $p < .001$, ** = $p < .01$, * = $p < .05$.

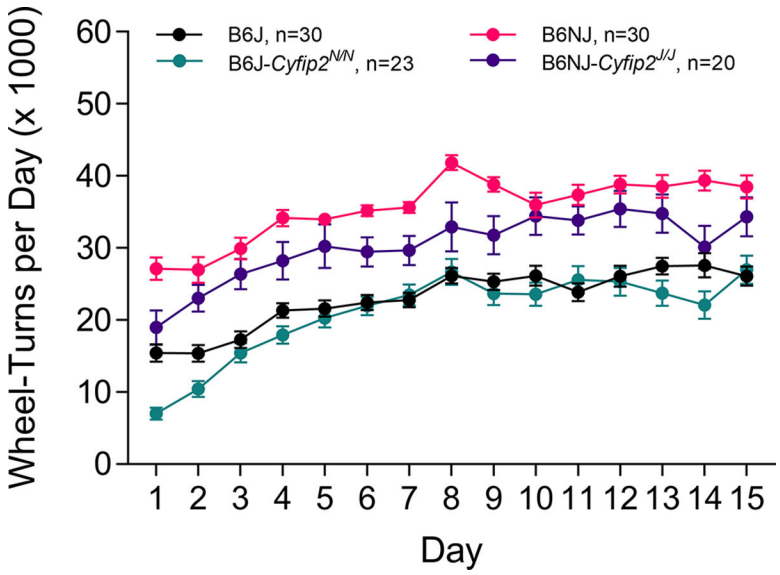

Figure 5.

Daily wheel-turns across 15-day period prior to ethanol access. Mean (±SEM) wheel-turns per day in B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$, and B6NJ-*Cyfip2*$^{J/J}$ mice.

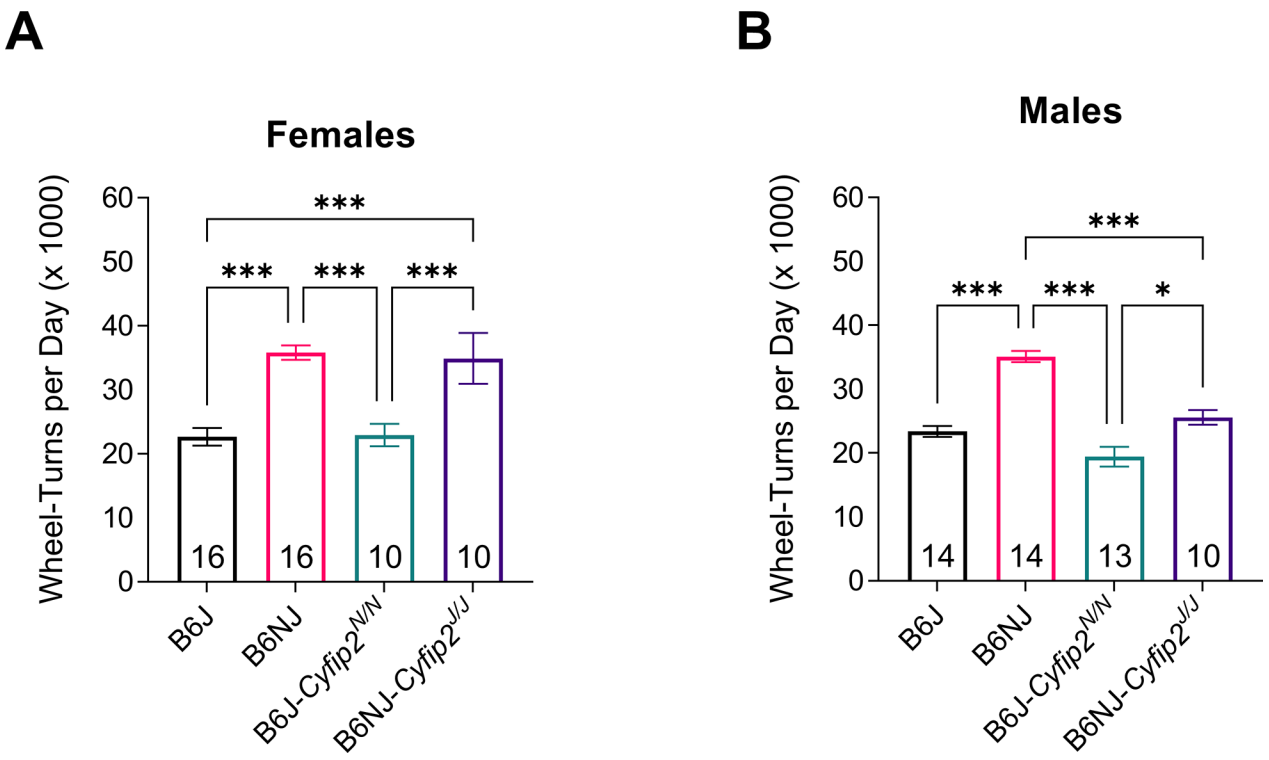

Figure 6.

Daily wheel-turns across sex. Mean (±SEM) wheel-turns per day (Days 8–15), collapsed across day, in female (**A**) and male (**B**) B6J, B6NJ, B6J-*Cyfip2*$^{N/N}$, and B6NJ-*Cyfip2*$^{J/J}$ mice. Asterisk symbols indicate the following: *** = $p < .001$, ** = $p < .01$, * = $p < .05$.

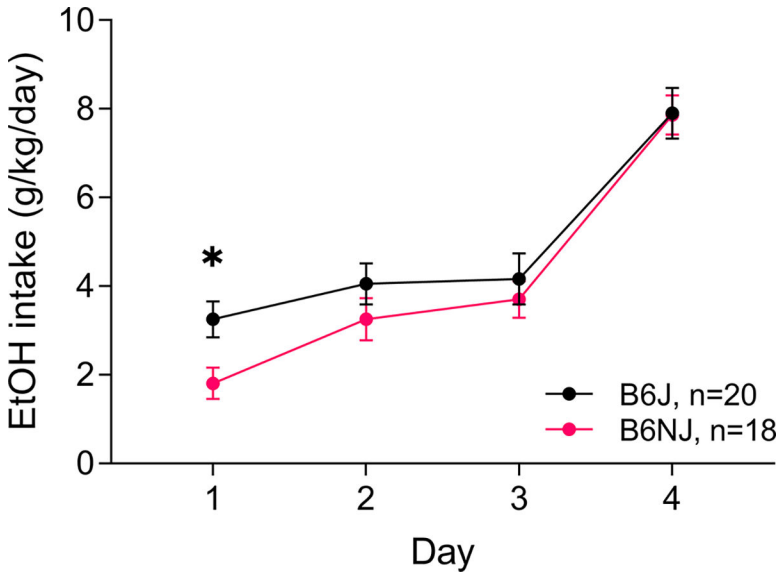

Figure 7.

Ethanol intake across days. Mean (±SEM) ethanol intake (g/kg) during 4-day Drinking-in-the-Dark (DID) protocol in B6J and B6NJ mice. Asterisk symbols indicate $p < .05$ for substrain comparisons within individual access days.

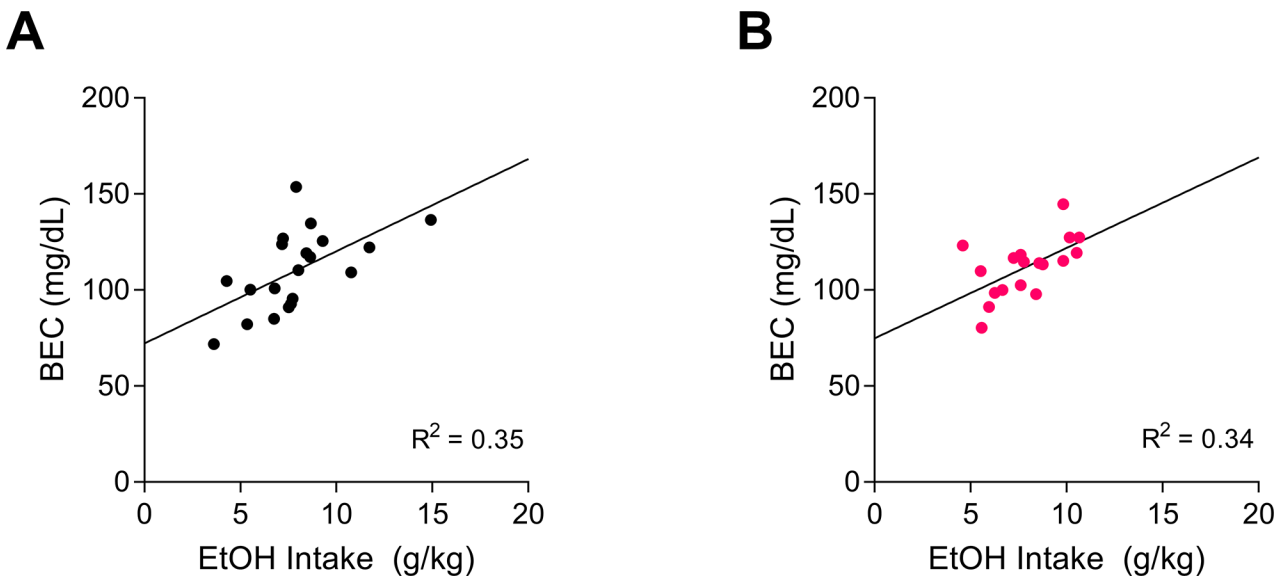

Figure 8.

BEC vs. Day 4 ethanol intake. Means for blood ethanol concentration (BEC; mg/dL) plotted against means for ethanol intake (g/kg) on Day 4 of the Drinking-in-the-Dark (DID) protocol in B6J (**A**) and B6NJ (**B**) mice. Correlation estimates (R^2 values) are shown for each respective substrain.

Table 1.

Experiment 2: BEC produced by DID protocol. Mean (±SEM) for blood ethanol concentration (BEC) for male (M) and female (F) B6J (J) and B6NJ (NJ) mice immediately following the ethanol access period on Day 4.

B6 Strain	Sex	Treatment	*n*	BEC (mg/dL)
J	F	DID	10	118.4 ± 6.7
J	M	DID	10	101.7 ± 5.5
NJ	F	DID	9	113.9 ± 5.3
NJ	M	DID	9	109.7 ± 4.9