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RESEARCH ARTICLE

Natural allelic variation modifies acute ethanol response phenotypes in wild strains of *C. elegans*

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Abstract

Background: Genetic variation contributes to the likelihood that an individual will develop an alcohol use disorder (AUD). Traditional laboratory studies in animal models have elucidated the molecular pharmacology of ethanol, but laboratory-derived genetic manipulations rarely model the naturally occurring genetic variation observed in wild populations. Rather, these manipulations are biased toward identifying genes of central importance in the phenotypes. Because changes in such genes can confer selective disadvantages, they are not ideal candidates for carrying AUD risk alleles in humans. We sought to exploit *Caenorhabditis elegans* to identify allelic variation existing in the wild that modulates ethanol response behaviors.

Methods: We tested the acute ethanol responses of four strains recently isolated from the wild (JU1511, JU1926, JU1931, and JU1941) and 41 multiparental recombinant inbred lines (mpRILs) derived from them. We assessed locomotion at 10, 30, and 50 min on low and high ethanol concentrations. We performed principal component analyses (PCA) on the different phenotypes, tested for transgressive behavior, calculated heritability, and determined the correlations between behavioral responses.

Results: We observed a range of responses to ethanol across the strains. We detected a low-concentration locomotor activation effect in some of the mpRILs not seen in the laboratory wild-type strain. PCA showed different ethanol response behaviors to be independent. We observed transgressive behavior for many of the measured phenotypes and found that multiple behaviors were uncorrelated. The average broad-sense heritability for all phenotypes was 23.2%.

Conclusions: Genetic variation significantly affects multiple acute ethanol response behaviors, many of which are independent of one another. This suggests that the genetic variation captured by these strains likely affects multiple biological mechanisms through which ethanol acts. Further study of these strains may allow these distinct mechanisms to be identified.

KEYWORDS
acute functional tolerance, alcohol sensitivity, behavior, *Caenorhabditis elegans*, natural variation

INTRODUCTION

Alcohol misuse is a major public health problem; it is estimated that more than 5% of people over 15 years old suffer from alcohol use disorder (AUD) worldwide (World Health Organization, 2018). Genetics plays a major role in AUD; approximately 50% of an individual's lifetime risk for developing AUD is heritable (Prescott & Kendler, 1999; Verhulst et al., 2015). Many studies have worked to identify candidate genetic liability loci in human populations, but only a small number of verified genes have been found (Jorgenson et al., 2017; Kranzler et al., 2019; Sanchez-Roige et al., 2019; Zhou et al., 2020). One difficulty with identifying natural genetic variants associated with AUD is the complex polygenic genetic architecture of this disease (Clarke et al., 2017).

An individual's acute physiological response to alcohol is strongly predictive of their lifetime liability to develop an alcohol use problem (Schuckit, 1994; Schuckit et al., 2007), and this phenotype is heritable (Edwards et al., 2018; Schuckit, 2018). Understanding the genetic contributors to alcohol responses has been the focus of much work. One important approach has been to use experimental animal models to first identify genes that affect responses to EtOH and then to ask whether allelic variation in human homologs of those genes is associated with problematic alcohol use. This work has been essential in shaping our understanding of the molecular physiology of alcohol's effects, and these studies have had successes in identifying liability loci in human populations (Grotewiel & Bettinger, 2015). However, this approach is unlikely to be able to fully examine relevant human natural genetic variation. One characteristic of most gene manipulations that are commonly used in animal models is that they have very large effects on gene function; they are usually strong loss- or gain-of-function alleles. Animals carrying these mutations can often be maintained in artificial laboratory conditions where selective pressures are minimized, but such mutations are likely to confer significant selective disadvantages in the wild, so are unlikely to persist in natural populations. The allelic variation in humans that impacts alcohol response phenotypes is very likely to primarily consist of changes that subtly modify gene function; such changes are less likely to confer selective disadvantages and therefore can be tolerated in the population. Identification of alleles that affect EtOH responses and can be maintained in the wild can be achieved by examining natural allelic variation that affects EtOH responses in wild isolates of model organisms.

The nematode *Caenorhabditis elegans* is a powerful model in which to study natural variation in EtOH phenotypes. The *C. elegans* machinery of nervous system function is strongly conserved from *C. elegans* to humans (Bargmann, 1998; Chase & Koelle, 2007; Hobert, 2013), and the molecular effects of EtOH are also conserved (Davies et al., 2003; Grotewiel & Bettinger, 2015). *C. elegans* are found worldwide (Frezal & Felix, 2015), and many genetically distinct natural isolates have been collected and their genetic diversity has been extensively characterized (Cook et al., 2017). Here, we examine acute EtOH responses of four strains isolated from the wild (JU1511, JU1926, JU1931, and JU1941) and 41 multiparental recombinant inbred lines (mpRILs) derived from them (Snoek et al., 2019; Volkers et al., 2013). The four wild strains were collected in two locations in France; JU1511 and JU1941 were found on decaying apples in Orsay, and JU1926 and JU1941 were found in Santeuil on decaying hogweed stems (Volkers et al., 2013). There is wide genetic variation between these strains, including approximately 9000 coding SNPs between them (Snoek et al., 2019). The mpRIL panel harbors genetic variation that explains natural variation in a number of life-history traits, and under different stresses and environments (Snoek et al., 2019). A major advantage of using this wild-derived mpRIL panel is that it minimizes the confounding effects of lab-derived alleles; we have previously found that the lab-derived *npr-1* gain-of-function allele present in the canonical laboratory N2 strain has a profound impact on EtOH response behaviors (Davies et al., 2004).

We find that there is genetic variation in these strains that significantly affects several acute EtOH response behaviors, strongly supporting the further characterization of these strains to identify naturally occurring variants that modify the physiological response to EtOH.

MATERIALS AND METHODS

C. elegans strains and husbandry

Caenorhabditis elegans strains were maintained at 20°C on nematode growth medium (NGM) plates containing 2% agar with lawns of *Escherichia coli* strain OP50 as their food source. We use 2% agar plates to discourage burrowing by the wild and wild-derived strains. Strains used in this study were: N2, JU1511, JU1926, JU1931, and JU1941 and 41 multiparental recombinant inbred lines (mpRILs) that were derived from crosses between JU1511, JU1926, JU1931, and JU1941: WN1001 (V), WN1002 (B), WN1004 (Y), WN1005 (G), WN1006 (HH), WN1007 (II), WN1008 (U), WN1011 (FF), WN1012 (BB), WN1013 (I), WN1015 (W), WN1016 (KK), WN1017 (L), WN1018 (H), WN1019 (P), WN1020 (Z), WN1021 (NN), WN1022 (JJ), WN1023 (T), WN1024 (DD), WN1025 (MM), WN1026 (S), WN1027 (O), WN1029 (X), WN1030 (GG), WN1031 (CC), WN1032 (D), WN1033 (K), WN1035 (EE), WN1036 (C), WN1038 (N), WN1040 (J), WN1041 (Q), WN1043 (AA), WN1044 (A), WN1045 (E), WN1046 (R), WN1049 (LL), WN1051 (M), WN1052 (F), and WN1073 (OO) (Snoek et al., 2019, Volkers et al., 2013). Letters in parentheses represent the shorthand designations for these mpRIL strains in the graphs shown in Figures 1–3, and Figure S2; they have been ordered based on their rankings in Figure 1A.

All animals used in behavioral assays were first-day adults that had been reared for at least two generations in well-fed, uncrowded conditions.

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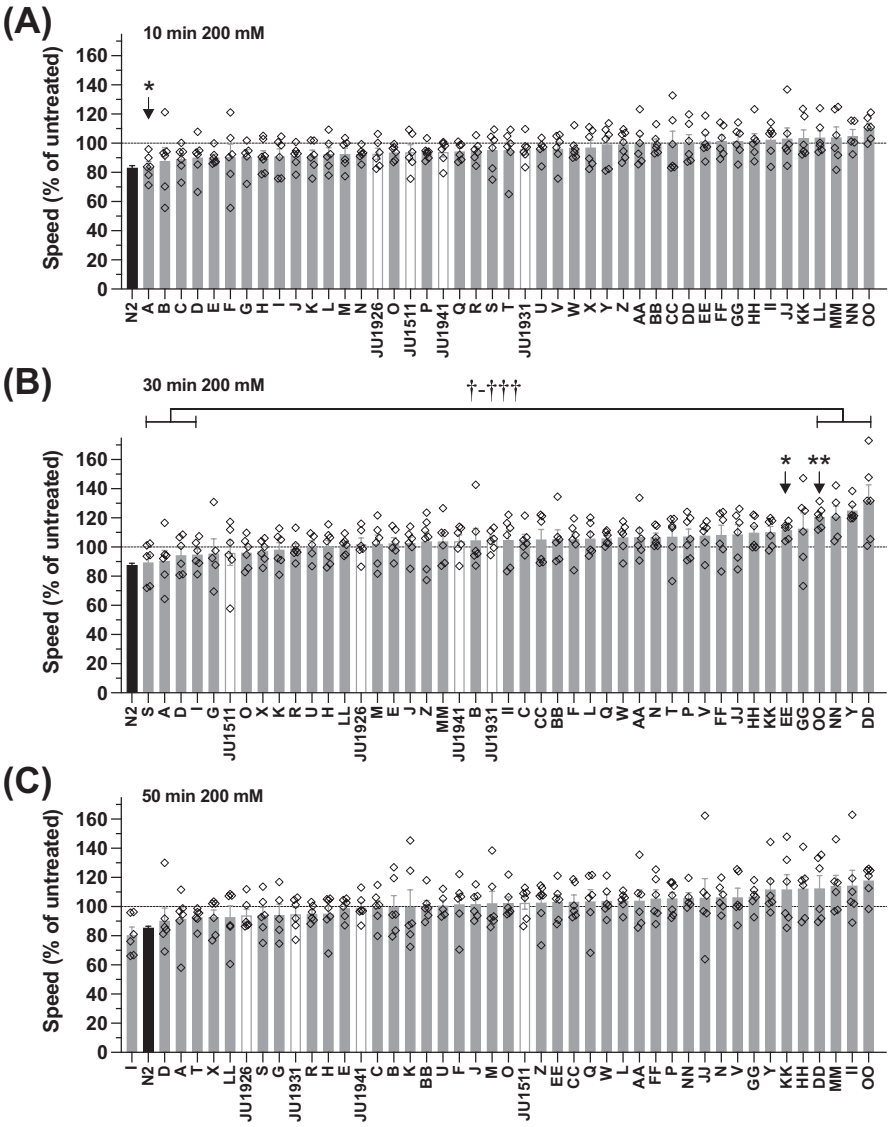

FIGURE 1 Individual strain responses to low-concentration EtOH during an acute exposure. Graphs illustrate the average relative speeds (treated speed/untreated speed of the same strain at the same time point × 100) (±SEM) after (A) 10-, (B) 30-, and (C) 50-min of continuous exposure to 200 mM EtOH in the absence of food ($n = 5–7$). Untreated speed is indicated by the dotted line at 100%. Pooled average data across all laboratory wild-type (N2) trials ($n = 97$) are indicated by solid black bars for reference (N2 was not included in any statistical comparisons with the mpRIL strains). The wild parent strains are indicated by open white bars. The mpRIL strains (WN10##) are named using letters for ease of comparison across this figure and Figures 2 and 3 (see Materials and methods for key). (A) 10 min of 200 mM EtOH exposure has minimal effect on the speeds of parent wild strains and the mpRIL strains. (B) After 30 min of 200 mM EtOH exposure, several mpRIL strains show a behavioral trend of speed activation, such that their average speeds on EtOH are faster than their untreated speeds. We have named this behavioral response low-concentration locomotor activation (LCLA). (C) After 50 min of exposure to 200 mM EtOH, most mpRIL strains display speeds that are similar to their untreated speeds at the same time point. Significant differences between untreated and treated speeds at the same time point for an individual strain are shown (Multiple unpaired *t*-tests: *, adj. $q < 0.05$; **, adj. $q < 0.01$). Each of the highest performing (top 10%) strains was significantly different from the lowest performing (bottom 10%) strains when relative speeds after 30 min of EtOH exposure were compared across these two groups (1-way ANOVA, $F_{(44,225)} = 2.10$, $p < 0.001$, Šídák's multiple comparison tests, † – †††, adj. $p < 0.05$ – adj. $p < 0.0001$).

Behavioral assays

Locomotion tracking and analysis were performed as described previously (Davies et al., 2004, 2015). Briefly, acclimatization and assay plates (NGM, 2% agar) were dried without lids at 37°C for 1 h. Each assay plate was weighed to determine the volume of NGM. Four copper rings were melted into the surface of each of the plates; the rings act as corrals, and we can fit four rings in a single field of view, allowing us to test four different strains simultaneously. Cold 100% EtOH was added to the assay plates to a final concentration of 0,

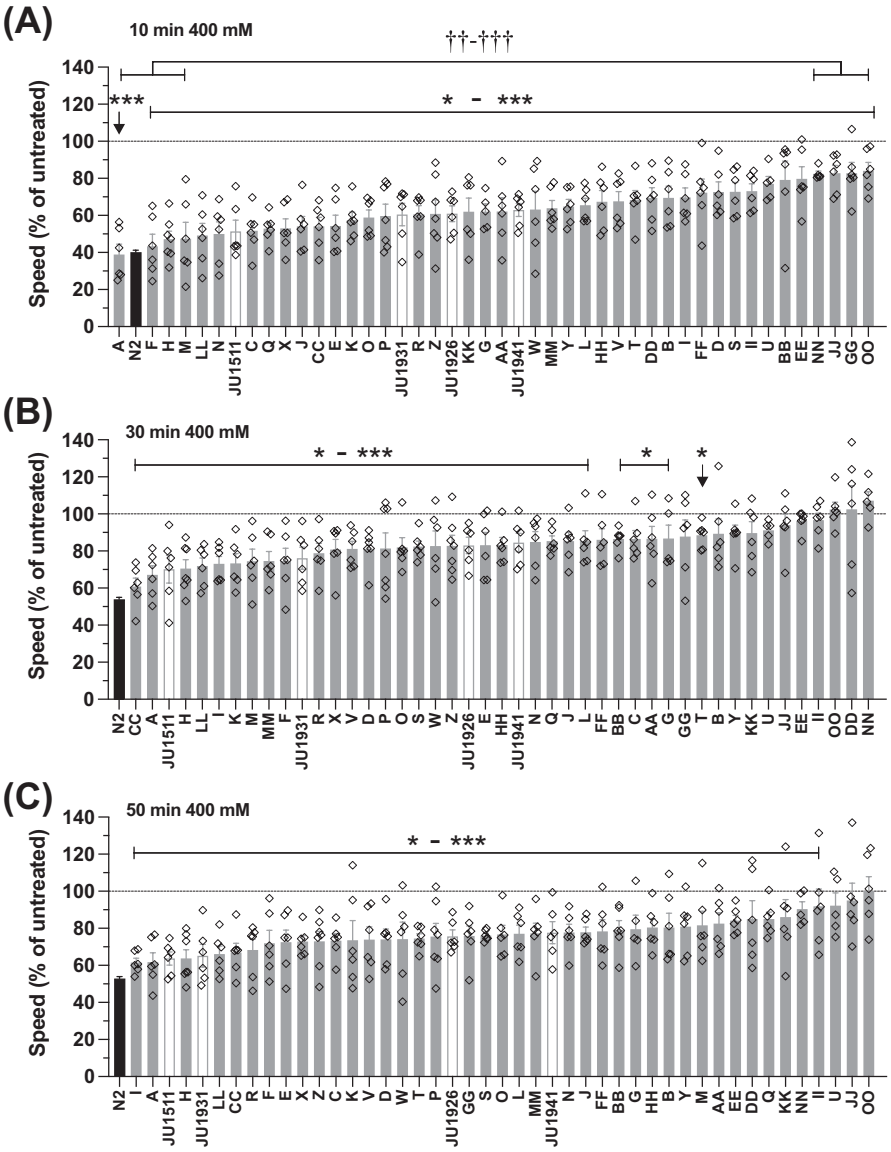

FIGURE 2 Individual strain responses to high-concentration EtOH during an acute exposure. Graphs illustrate the average relative speeds (treated speed/untreated speed of the same strain at the same time point × 100) (±SEM) after (A) 10-, (B) 30-, and (C) 50-min of continuous exposure to 400 mM EtOH in the absence of food ($n = 5–7$). Untreated speed is indicated by the dotted line at 100%. Pooled average data across all laboratory wild-type (N2) trials ($n = 97$) are indicated by solid black bars for reference (N2 was not included in any statistical comparisons with the mpRIL strains). The wild parent strains are indicated by open white bars. mpRIL strains (WN10##) are named using letters for ease of comparison across this figure and Figures 1 and 3 (see Materials and methods for key). (A) 10 min of 400 mM EtOH exposure significantly reduces the speed of all wild parent and mpRIL strains, although the degree of effect is different, as there are significant differences between the lowest performing (bottom 10%) and highest performing (top 10%) mpRIL strains. (B) After 30 min of 400 mM EtOH exposure, due to the development of significant levels of acute functional tolerance (AFT) (see Figure 3), some mpRIL strains are approaching their untreated speeds at the equivalent time point, while others remain significantly affected. (C) After 50 min of exposure to 400 mM EtOH, most mpRIL strains display speeds that are significantly lower than their untreated speeds at the same time point. Significant differences between untreated and treated speeds at the same time point for an individual strain are shown (Multiple unpaired *t*-tests: *, adj. $q < 0.05$; **, adj. $q < 0.01$; ***, adj. $q < 0.001$). Each of the highest performing (top 10%) strains was significantly different from the lowest performing (bottom 10%) strains when relative speeds after 10-min of EtOH exposure were compared across these two groups (1-way ANOVA, $F_{(44,225)} = 3.67$, $p < 0.0001$, Šídák's multiple comparison tests, † – †††, adj. $p < 0.001$ – adj. $p < 0.0001$).

200, or 400 mM, and the EtOH was allowed to absorb into the plates for 2 h.

Worms were allowed to become accustomed to the lack of food by being placed into copper rings on dried acclimatization plates. After 30 min, 10 first-day adult worms were moved from acclimatization plates and placed in each copper ring on assay plates, one strain per ring, and allowed to crawl freely. The N2 laboratory wild strain was tested on each plate. Assays were performed in the

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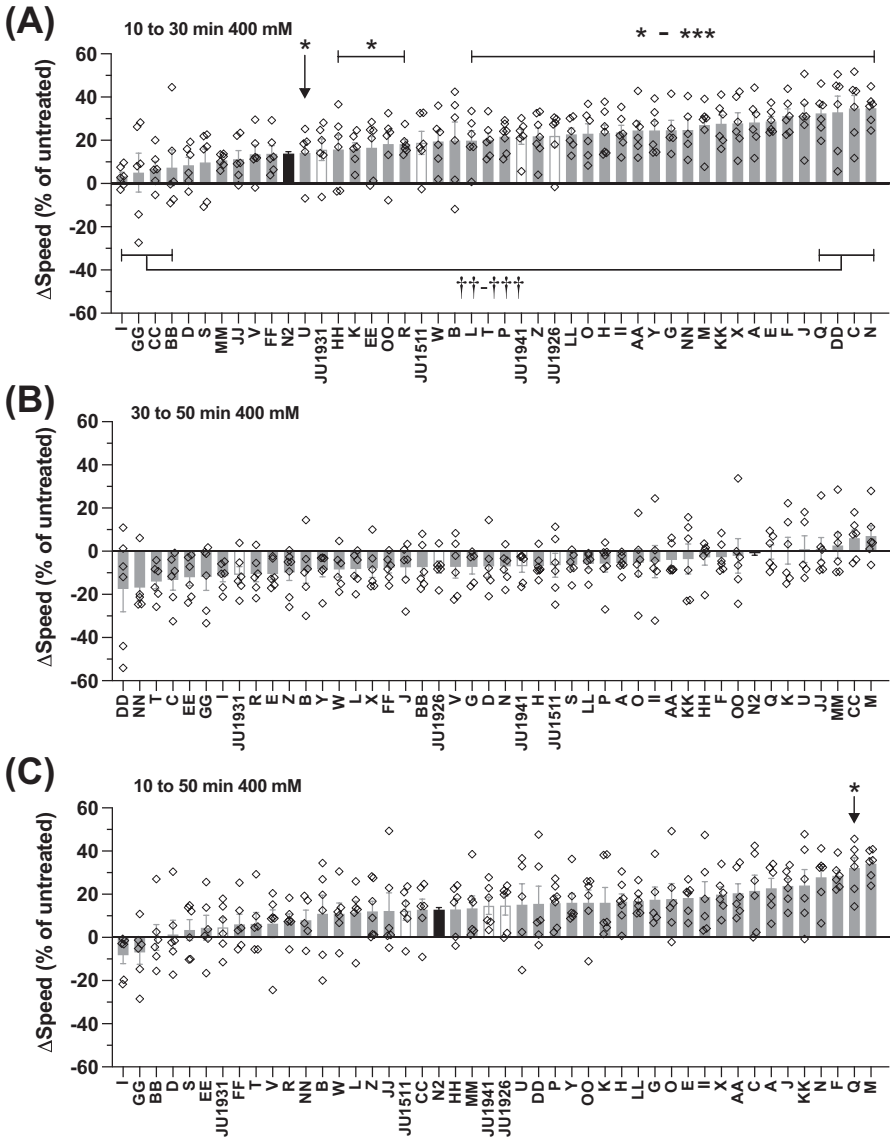

FIGURE 3 The development of acute functional tolerance (AFT) to the effects of high-concentration EtOH by individual strains over time. Graphs illustrate the average difference in relative speed (treated speed/untreated speed of the same strain at the same time point × 100) (±SEM) between (A) 10–30 min, (B) 30–50 min, and (C) 10–50 min of continuous exposure to 400 mM EtOH in the absence of food ($n = 5$–7). Positive and negative ΔSpeed values represent increases and decreases in relative speed during the specific time period, respectively. Pooled average data across all laboratory wild-type (N2) trials ($n = 97$) are indicated by solid black bars for reference (not visible in B as it is close to 0) (N2 was not included in any statistical comparisons with the mpRIL strains). The wild parent strains are indicated by open white bars. mpRIL strains (WN10##) are named using letters for ease of comparison across this figure and Figures 1 and 2 (see Materials and methods for key). (A) Many mpRIL strains show significant development of AFT during the 10–30 min period of EtOH exposure and there are significant differences in AFT between the upper and lower performing mpRIL strains. (B) During the late phase of exposure (30–50 min) there is a general trend toward decreases in speed, although no strain showed a statistically significant decrease in speed when multiple testing was considered. (C) After 50 min of exposure to 400 mM EtOH, there is an overall increase in average speed for the majority of mpRIL strains compared with the speeds after 10 min, although only one strain showed a significant increase when multiple testing was taken into account. Relative speeds for each strain at the relevant time points (10 & 30, 30 & 50, or 10 & 50) were compared with determine whether statistically significant AFT (positive or negative) had occurred (Multiple unpaired *t*-tests: *, adj. $q < 0.05$; **, adj. $q < 0.01$; ***, adj. $q < 0.001$). Each of the highest performing (top 10%) strains was significantly different from the lowest performing (bottom 10%) strains when average AFT values for the 10–30 min time period were compared across these two groups (1-way ANOVA, $F_{(44,225)} = 2.88$, $p < 0.0001$, Šídák's multiple comparison tests, †† – †††, adj. $p < 0.01$ – adj. $p < 0.0001$).

absence of bacterial food to encourage the worms to crawl consistently at high speeds. Two-minute video recordings were made at 10–12, 30–32, and 50–52 min of exposure, and object tracking software (ImagePro Plus; Media Cybernetics, Inc.) was used to determine the average speed (μm/s) of each animal and to calculate an average speed for the 10 worms in each trial ($n = 1$). Assays on EtOH were always performed in parallel with assays in the absence of EtOH.

Ambient humidity and temperature were recorded for each assay. All locomotion data are included in Data S1.

Phenotypes

We examined 7 phenotypes.

- *Raw speed* at each time point is the speed of the animals measured in μm/s. The basal speed at each time point is the speed of the animals on 0 mM EtOH.
- All phenotypes except raw speed use *Relative speed* in their calculations = the speed of animals on EtOH (200 or 400 mM)/speed of animals on 0 mM EtOH at a time point.
- *Low-concentration initial sensitivity to EtOH* is the relative speed of animals at 10 min of exposure to 200 mM EtOH. A number below 100% reflects depression of speed by EtOH.
- *Low-concentration locomotor activation (LCLA)* is observed using the relative speed of animals at 30 min of exposure to 200 mM EtOH. In this study, some strains moved faster when exposed to 200 mM EtOH, which is reflected in a number above 100%; this is a phenomenon that we do not observe in the N2 strain.
- *High-concentration initial sensitivity to EtOH* is the relative speed of animals at 10 min of exposure to 400 mM EtOH.
- *High-concentration (400 mM) change in speed over time* was measured for three intervals: (10–30) = relative speed at 30 min—relative speed at 10 min; (30–50) = relative speed at 50 min—relative speed at 30 min; (10–50) = relative speed at 50 min—relative speed at 10 min. We have previously observed that wild-type animals demonstrate a decrease in the depressive effects of EtOH (an increase in speed despite an increase in tissue EtOH concentration) over time. This is *Acute Functional Tolerance (AFT)* (Davies et al., 2004; Mathies et al., 2015).

Data analysis

Data analyses were performed either using Prism (version 9.5.1) (GraphPad Software) or the statistical software R (version 4.0.2) using RStudio (version 1.3.1093). For general data loading, visualization and manipulation, the packages tidyverse (version 1.3.1), openxlsx (version 4.1.5), and ggpubr (version 0.4.0) were used.

Outliers

Prism (version 9.5.1) (GraphPad Software) was used to identify and remove statistical outlier data using the ROUT (robust regression and outlier removal) method ($Q = 1\%$). When we observed an outlier data point, we removed the entire biological replicate (including 0, 200, and 400 mM EtOH) from our analysis. We removed 7 outliers (of 3330 data points) affecting 3 trials (of 370 trials) from the behavioral data; these are highlighted in Data S1.

Individual strain analyses

To compare the raw treated and untreated speeds at any given time point or relative speeds across two time points for each of the individual wild parent or mpRIL strains, we used the recommended parameters (Prism) to perform multiple unpaired *t*-tests using a false discovery rate (FDR) approach and the Benjamini, Krieger, and Yekutieli two-stage step-up method with a desired FDR of 5%. To compare high and low performing strains with each other for relative speeds at specific time points or rates of development of AFT we used recommended parameters (Prism) to perform 1-way ANOVA analyses across all strains followed by selected post-hoc comparisons between the top and bottom four strains (upper and lower 10%) using Šídák's multiple comparisons tests. To compare the untreated and treated (200 mM) raw speeds at three time points for two strains that showed significant LCLA, we used recommended parameters (Prism) to perform repeated measure 2-way ANOVA, using Šídák's multiple comparisons tests to examine the significance at each time point. The unpaired *t*-test comparison of average treated and untreated speeds for the N2 strain used data for the N2 strain that was pooled from all assays ($n = 97$).

Genetic variation analyses

Principal component analysis

We used the function *prcomp()* with *scale. = TRUE*. Strains with missing values were omitted from the analysis.

ANOVA

An analysis of variance was conducted using the function *aov()*. Each phenotype was analyzed separately.

Correlation analysis

We examined the degree of correlation between different EtOH phenotypes with the R function *cor()* using Pearson correlation for underived and derived phenotypes separately. *p* Values were adjusted using Bonferroni correction for multiple testing.

Transgression

The most extreme parents were determined for each phenotype. Any mpRIL that had a mean that was more extreme than the mean of the most extreme parent was filtered. Two Dunnett's tests were performed using the function *DunnettTest()* with *alternative = less or greater* and *p value adjustment method = single-step* to determine whether there were animals that showed significant transgressive

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behavior. If only one mpRIL had a more extreme phenotype compared with the most extreme parent, a one-sided *t*-test was performed.

Heritability

Per phenotype, the broad-sense heritability was calculated using the *repeatability()* function of the heritability package (version 1.3). This function determines broad-sense heritability (H^2) based on ANOVA using the general formula:

$$H^2 = \frac{V_g}{V_g + V_e},$$

where H^2 is the broad-sense heritability; V_g is the genotypic variance; and V_e is the residual variance. Humidity and temperature were used as covariates.

To calculate the narrow-sense heritability (h^2) per phenotype, a kinship matrix was generated using the function *popkin()* from the package popkin (version 1.3.1). The input for the kinship matrix was the previously made SNP map of the mpRILs (Snoek et al., 2019). Using the *marker_h2()* function from the heritability package (version 1.3) the REML estimates of the genetic and residual variance and their standard deviations were calculated per phenotype using temperature and humidity as covariates. Based on these values, narrow-sense heritability estimates were given.

RESULTS

To explore the natural phenotypic variation in EtOH responses in *C. elegans*, we behaviorally characterized four strains that are derived from wild isolates (JU1511, JU1926, JU1931, and JU1941) (Volkers et al., 2013), 41 mpRILs that were the result of crosses between the four wild-derived strains (Snoek et al., 2019), and the standard laboratory wild-type strain Bristol N2. We measured the speed of locomotion in the absence of food, and the effect of acute exposure to two concentrations of EtOH on locomotion speed using our well-established EtOH response assay (Davies et al., 2004, 2015). We exposed animals to 0, 200, or 400 mM EtOH and measured their locomotion speeds at 10, 30, and 50 min of continuous exogenous EtOH exposure (Data S1).

Low-concentration (200 mM) EtOH exposure phenotypes

We examined the speed of each strain exposed to 200 mM EtOH at each time point compared with the untreated speed of each strain at the same time point (Figure 1). While this low concentration at 10 min of exposure depressed locomotion of the N2 strain (unpaired *t*-test, $t_{192} = 9.77$, $p < 0.0001$), it had only a mild, nonsignificant, effect on locomotion speed in all of the wild and wild-derived mpRILs, with one statistically significant exception (WN1044 (A)) (Figure 1A). Each mpRIL is labeled with a letter code based on their order in Figure 1A (key is in Materials and methods); this code is maintained in Figures 1–3. Intriguingly, when we looked at locomotion after 30 min of exposure to 200 mM EtOH, we observed a new phenotype in two of the wild-derived mpRILs (WN1035 (EE) and WN1073 (OO)): exposure to 200 mM EtOH enhanced locomotion such that the animals moved significantly faster than their untreated speeds, a phenotype that we call low-concentration locomotor activation (LCLA; Figure 1B). We also observed evidence of a trend toward the expression of this phenotype in several other mpRIL strains that did not remain statistically significant when adjusted for repeated testing. This is a phenotype that we did not observe in N2, which remains significantly depressed relative to its untreated speed after 30 min of 200 mM EtOH exposure ($t_{192} = 8.47$, $p < 0.0001$) (Figure S1). Because we had not previously observed LCLA in the N2 strain, we examined the time course for this phenotype more carefully in the two strains that showed significant LCLA (EE and OO) (Figure S2). We found that the two strains behaved quite differently; EE only displayed activation at 30 min of exposure and was not activated at 10 and 50 min of exposure, by contrast, OO, when examined in isolation, was activated at all three time points (Figure S2; Data S2). For both strains, however, the maximal activation was at 30 min of exposure. Although the LCLA of only two strains achieved statistical significance, we did observe a strong suggestion that other mpRILs also demonstrated LCLA; to further examine the range of phenotypes at 30 min, we compared the highest and lowest performing strains (top and bottom 10%) to each other and found that each of these groups was significantly different from the other (Figure 1B; Data S2). After 50 min of 200 mM exposure, no mpRIL strain showed a statistically significant LCLA phenotype (Figure 1C), suggesting that this EtOH-induced locomotor activation is time-dependent.

High-concentration (400 mM) EtOH exposure phenotypes

Exposure to 400 mM EtOH for 10, 30, and 50 min causes strong locomotor depression in the N2 strain (Davies et al., 2003, 2004). We previously characterized the time course of EtOH intoxication in N2 and N2-derived strains and found that the maximal acute effect of 400 mM EtOH on locomotion speed is observable at 10 min of exposure (Alaimo et al., 2012). We refer to the response to 400 mM EtOH at 10 min as *initial sensitivity*. We examined the relative speeds of the wild and mpRIL strains and found that there was an extremely wide range in initial sensitivity, from approximately 60% depression of speed to approximately 15% depression of speed across the strains (Figure 2A). These differences are borne out in the comparison of the highest and lowest performing strains (top and bottom 10%), which found that each of these strains was significantly different from the strains in the opposite group (Figure 2A; Data S2). We examined all strains for evidence of acute functional tolerance. At 30 min of exposure, the relative speed (treated speed/untreated speed) of most

strains was significantly faster than at 10 min, clear evidence of the development of AFT (Figure 3A). For some strains, this recovery was such that they approached or exceeded their untreated speeds at 30 min (Figure 2B). We asked whether there was a significant difference between individual strains at the lower and higher ends of the range of rates of AFT. Each of the lowest performing strains (bottom 10%) was significantly different from each of the highest performing strains (top 10%) for the rate at which they developed AFT during the 10–30-min time interval (Figure 3A; Data S2). For most strains, AFT did not persist in the 30–50-min interval, such that after 50 min of exposure to 400 mM EtOH, only one strain (WN1041 (Q)) maintained a statistically significant difference (with accounting for repeated testing) from its relative speed at 10 min (Figure 3B,C; Multiple unpaired *t*-tests, *p*.adj < 0.001).

Genetic variation in EtOH response phenotypes

We explored the phenotypic ranges in the EtOH responses in the wild strains and the mpRILs derived from them. We found that the three wild isolates JU1926, JU1931, and JU1941 often approximated the average value for most phenotypes (Figures 1–3), particularly at the early time points. The wild parent strain JU1511 and the laboratory strain N2 were among the strains most strongly affected in all of the EtOH response phenotypes, and often were significantly slower than JU1926, JU1931, and/or JU1941 (ANOVA, Tukey *p*.adj < 0.05) (Figures 1–3, Figure S1), although for every measure, at least one mpRIL was more affected by EtOH than JU1511. Interestingly, we observed that particular mpRILs resided in the left tail of the phenotypic distribution for some phenotypes and in the right tail of the phenotypic distribution in other phenotypes (Figures 1–3).

To characterize the factors that contribute to these behaviors, we conducted principal component analyses (PCA). We first examined the speed of animals on each concentration of EtOH at each time point. We found that the first two principal components (PC), PC1 and PC2, explained 71.3% and 17.4% of the variance, respectively (Figure 4A). As expected, in these PCs, the loadings clustered by EtOH concentration, indicating that EtOH concentration has a larger influence on speed than the time of EtOH exposure. When we examined the variables of temperature and humidity, we observed humidity- and temperature-dependent patterns in the first two principal components, suggesting that these environmental conditions had an influence on EtOH response behaviors (Figure 4B,C). Next, we performed PCA on the time-dependent phenotypes of acute functional tolerance and the relative speeds. For these phenotypes, PC1 and PC2 explained 52.6% and 33.5%, respectively, of the variance in the data (Figure 4D). The loadings were widely spaced, indicating that the different EtOH response behaviors are likely to be independent of each other, which is consistent with previous observations (Bettinger et al., 2012; Mathies et al., 2015; Raabe et al., 2014). As we expected their effects on speed, temperature, and humidity also influenced these derived phenotypes (Figure 4E,F).

We asked how much the covariates of temperature and humidity influenced our observed EtOH responses by performing an ANOVA on each phenotype separately and calculating the amount of variance that temperature and humidity explained. Both temperature and humidity explained less than 2% of the variance in each phenotype (Data S3). Humidity and temperature significantly influenced the EtOH responses of 4 of 14 phenotypes (ANOVA, $p < 0.05$). Thus, both temperature and humidity influence EtOH response behaviors in *C. elegans*, but their associated effects are small.

To ask whether we could find evidence that alleles combine in the mpRILs in new ways that could confer phenotypes outside of the phenotypic range of the parents, we tested whether a phenotype contained transgressive strains. To test for transgressive behavior, we determined the two most extreme parents, on each end of the phenotypic spectrum, and performed a Dunnett's test (or a one-sided *t*-test if only one mpRIL transcended the most extreme parent) with all the mpRILs that were more extreme than the most extreme parent (Data S4). We found statistical evidence for transgressive behavior for nine traits: basal locomotion speed at 10 and 30 min; the effects of 200 mM EtOH on locomotion speed at 10, 30, and 50 min; the effect of 200 mM EtOH on the relative speed at 30 min (LCLA); and the effects of 400 mM EtOH on locomotion speed at 10, 30 and 50 min (one-sided Dunnett's test, *p*.adjust < 0.05 or one-sided *t*-test, $p < 0.05$). However, although we observed that there were strains that appeared to have extreme AFT phenotypes when compared to the wild parent strains, there were no statistically significant cases of transgression for this phenotype.

Next, we investigated the genetic basis of these different phenotypes by calculating broad-sense heritability (H^2). We calculated the H^2 of 14 phenotypes: Basal speed (0 mM) at 10, 30, and 50 min; low-concentration speed (200 mM), at 10, 30, and 50 min; high-concentration speed at 10, 30, and 50 min; low-concentration initial sensitivity, LCLA, initial sensitivity, AFT (30 min), AFT (50 min). The average H^2 of all combined phenotypes was 23.2% and ranged from 2.0% to 35.5% (Figure 5A). This indicates that a proportion of the observed phenotypic variance in EtOH responses is due to genetic factors. Additive genetic factors seem to play only a small role since the narrow-sense heritability h^2 only explained 5.6% of the phenotypic

FIGURE 4 Principal component analysis (PCA) reveals that the phenotypes are uncorrelated and are influenced by humidity and temperature. (A–C) PCA on the raw speeds. The first principal component explained 71.3% of the variance and the second principal component explained 17.4% of the variance in the dataset. (A) The loadings cluster together by EtOH concentration. (B, C) The first two PCs are influenced by humidity (B) and temperature (C). (D–F) PCA on the relative speeds and acute functional tolerance phenotypes. The first principal component explained 52.6% of the variance and the second principal component explained 33.5% of the variance in the dataset. (E, F) The first two PCs are influenced by humidity (E) and temperature (F); the influence of humidity and temperature explains less than 2% of the variance in the affected phenotypes.

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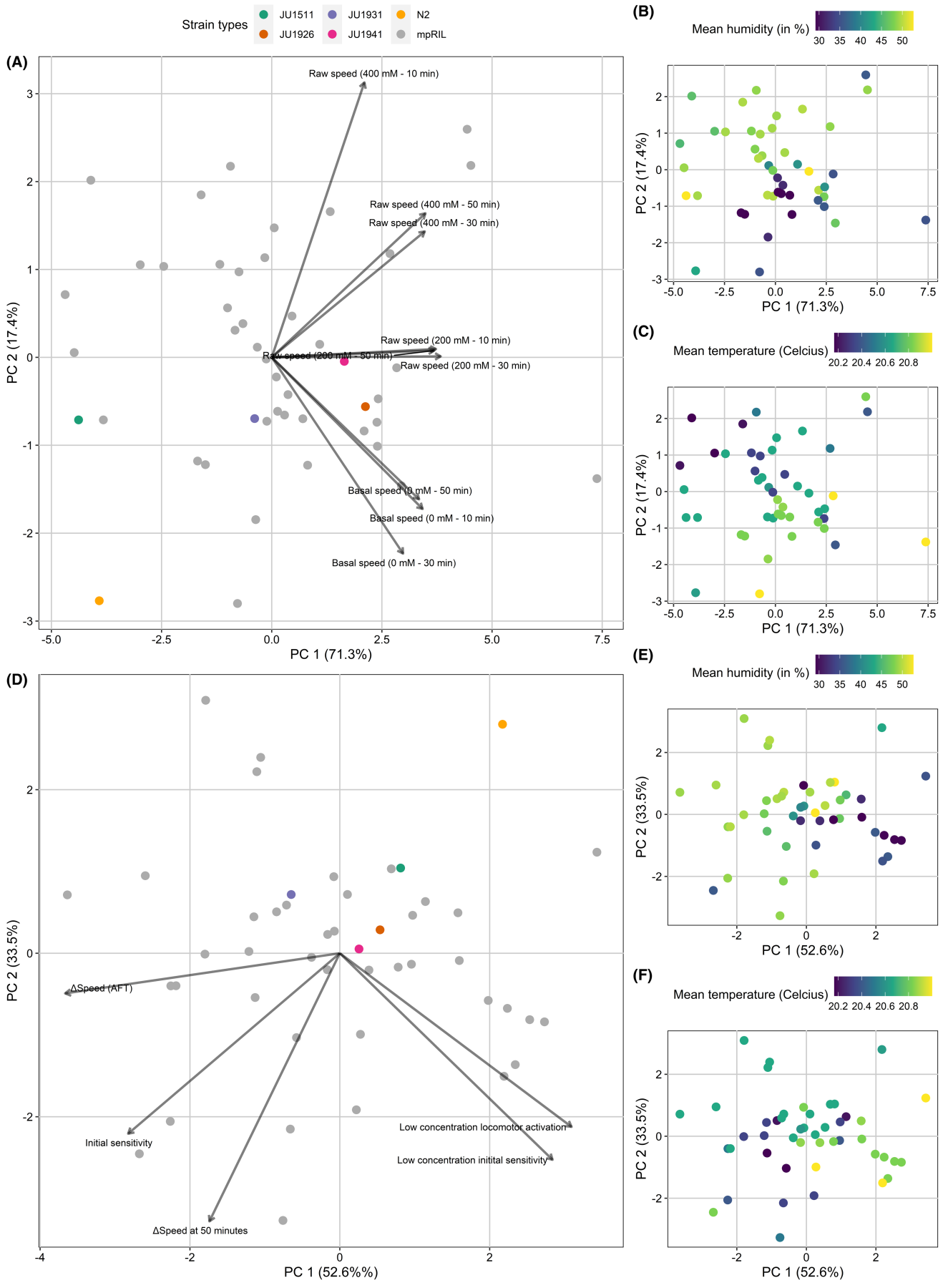

(A)

	H2	h2
Basal speed (0 mM - 10 min)	0.35	0.04
Basal speed (0 mM - 30 min)	0.27	0.01
Basal speed (0 mM - 50 min)	0.19	0.04
Raw speed (200 mM - 10 min)	0.17	0.02
Raw speed (200 mM - 30 min)	0.16	0.04
Raw speed (200 mM - 50 min)	0.02	0.06
Raw speed (400 mM - 10 min)	0.22	0.09
Raw speed (400 mM - 30 min)	0.16	0.15
Raw speed (400 mM - 50 min)	0.32	0.08
Low concentration initital sensitivity	0.31	0.06
Low concentration locomotor activation	0.22	0.07
Initial sensitivity	0.22	0.09
ΔSpeed (AFT)	0.34	0.04
ΔSpeed at 50 minutes	0.30	0.00

(B)

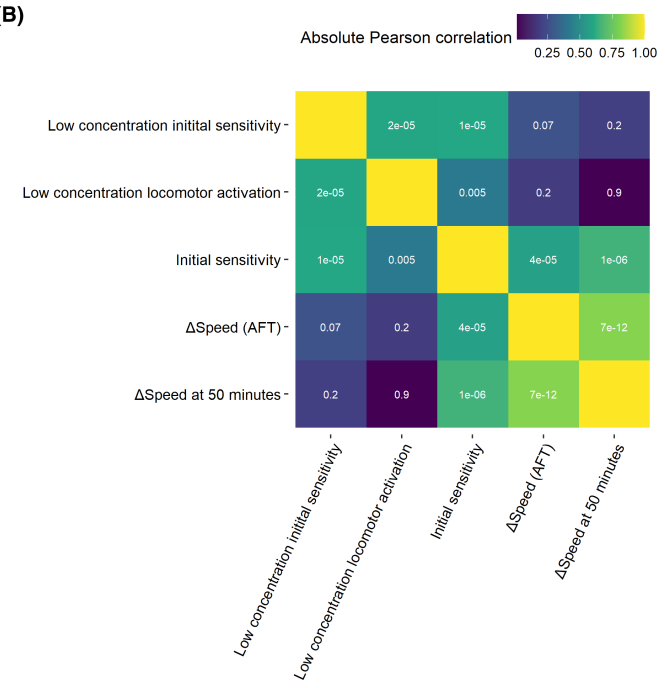

FIGURE 5 The broad-sense heritability of EtOH responses is moderate. Some of the EtOH response phenotypes are correlated, while some are uncorrelated, indicating that there are both shared and separate genetic mechanisms underlying these phenotypes. (A) Broad- and narrow-sense heritability per phenotype. (B) Correlations of phenotypes using the absolute Pearson correlation. Bonferroni-adjusted *p* values are depicted in white.

variance on average, with SD = 3.8%, ranging from 0.0% to 15.0% (Figure 5A). Because the mpRILs have been inbred for multiple generations, their genomes are nearly homozygous. Dominance and recessiveness are thus unlikely to have effects on the phenotypes, suggesting that closely linked genes and/or gene–gene interactions may be important in the genetic architecture of EtOH responses in *C. elegans*.

Finally, we wondered to what extent the EtOH response phenotypes were correlated with each other. We observed that 4 of 10 EtOH response phenotypes did not correlate significantly (Pearson correlation, Bonferroni corrected p value < 0.05) (Figure 5B). Notably, the low-concentration phenotypes of sensitivity (10 min at 200 mM EtOH and LCLA) did not correlate with the high-concentration AFT phenotypes (10–30 and 10–50 min). We have previously shown that AFT and initial sensitivity (10 min at 400 mM EtOH) have shared and distinct genetic influences (Bettinger et al., 2012; Grotewiel & Bettinger, 2015; Mathies et al., 2015), and, consistent with those observations, here we found that these phenotypes are correlated but not entirely overlapping. Together, these results indicate that the genetic mechanisms underlying these different EtOH response behaviors are partially distinct.

DISCUSSION

Many gene-finding experiments in model organisms using phenotypic screens are biased toward detecting mutations that cause large phenotypic effects. Such genes are likely to be central to the function being studied and often are key players in core biological processes. While many studies have successfully used these approaches to identify molecular machinery that is affected by relevant concentrations of EtOH, these findings have not always led to the identification of genetic variants that are associated with alcohol use. We speculate that there are at least two reasons for this: First, laboratory-induced mutations tend to either completely eliminate or strongly enhance gene function, and this does not accurately reflect the majority of mutations that exist in human populations, which cause subtle effects on function. Second, mutation of many of the genes of large effect identified in these studies may not be selected for in the wild. We anticipate that, in many cases, the selective cost of genetic variation in core molecular elements would be too high for such variants to have long-term viability across generations in the population. Therefore, many primary EtOH targets may not be good candidates for human liability loci. For example, we have previously identified the SLO-1 BK channel as a major molecular target of EtOH in worms (Davies et al., 2003), and many studies have demonstrated the conserved importance of the BK channel in EtOH responses across species including humans (Bettinger & Davies, 2014; Dopico et al., 2016). Despite the importance of the BK channel in EtOH responses, however, there have been only subtle, nonsignificant, signals identified in the human BK channel gene (*KCNMA1*) in GWAS for alcohol use disorder or other relevant alcohol phenotypes (Edenberg et al., 2010; Han et al., 2013; Kendler et al., 2011; Schuckit et al., 2005). A possible explanation is that variation in *KCNMA1* is not widely spread in the human population because making major

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changes in BK function can cause substantially deleterious phenotypes (Cui, 2021; Du et al., 2020).

One approach that may circumvent this problem is to identify variation affecting EtOH responses that exist in wild populations of model organisms. Such variation may point to aspects of biological pathways in which changes can be tolerated in the wild. Here, we have taken this approach to begin to identify natural allelic variation that impacts EtOH responses in wild strains of *C. elegans*. We characterized the acute behavioral response to low and high concentrations of EtOH of four wild strains relatively recently isolated in France (Volkers et al., 2013), and a set of 41 mpRILs that were derived from an intercross between these four wild strains (Snoek et al., 2019).

We and others have extensively characterized behavioral responses to EtOH intoxication of the laboratory standard wild-type strain N2 (Alaimo et al., 2012; Chen et al., 2018; Davies et al., 2003, 2004; Graham et al., 2009; Grotewiel & Bettinger, 2015; Mitchell et al., 2010; Morgan & Sedensky, 1995; Oh & Kim, 2019; Scott et al., 2017; Topper et al., 2014). Worms respond to high concentrations of EtOH by decreasing their locomotion speed in a concentration-dependent manner (Davies et al., 2003). The maximal depression of locomotion (initial sensitivity) is observable at 10 min of exposure and over the course of the next 20 min, speed increases in a process that reflects the development of acute functional tolerance (AFT) to EtOH (Davies et al., 2004). Low-concentration EtOH causes a much more modest depression of locomotion in N2 (Davies et al., 2003).

When we examined the wild and wild-derived mpRIL strains, we found that there was a wide array of behavioral responses to EtOH, demonstrating that, in these strains, there is genetic variation that can strongly affect EtOH response behaviors. We found that the parent strains had phenotypes in the middle of the distribution of all of the strains. We found that among the tested mpRILs, there were transgressive strains with more extreme phenotypes than the parents, indicating that, in the mpRILs, there has been a reshuffling of alleles that has caused the phenotypic range to expand. These data demonstrate that there are multiple alleles that vary in these strains that can interact to modulate EtOH response phenotypes.

Low-dose EtOH causes behavioral activation; genetic variation influences this

Low doses of EtOH are known to cause behavioral disinhibition in mammals; this effect is strongly influenced by genetics (Dudek et al., 1991; Rose et al., 2013; Wolstenholme et al., 2018) but is poorly understood. We characterized the effects of low-concentration EtOH on the wild and wild-derived strains and found that, like mice, *C. elegans* can be activated by EtOH. At 30 min of exposure to 200 mM EtOH, a subset of the mpRILs moved faster than their untreated speeds at the same time point. This is a phenotype that we do not observe in the laboratory wild-type N2 strain. This suggests that there are alleles in the wild populations that control this phenotype and highlights the importance of phenotyping a variety of different genetic backgrounds.

Variation in the regulation of acute functional tolerance that overcomes *npr-1* effects

We have previously shown that the neuropeptide Y-like receptor NPR-1 has an extremely strong effect on AFT to EtOH. NPR-1 function antagonizes AFT: more function causes a decrease in the development of AFT, and less or no NPR-1 function allows very fast development of AFT (Davies et al., 2004). In the early history of the domestication of the canonical laboratory N2 strain, *npr-1* was subject to selection that yielded a strong gain-of-function (*gf*) allele, this allele was fixed in the population and all N2-derived strains share this allele (McGrath et al., 2009). This *gf* allele of NPR-1 depresses the rate of AFT in N2 and N2-derived strains (Davies et al., 2004). Wild strains have the ancestral wild-type allele, and this is true of the four wild progenitor strains studied here. We expected then that the rate of AFT would be faster than N2 in each of our parent strains and mpRILs. To our surprise, while N2 was in the lower half of the phenotypic distribution, it was not the strain with the least AFT. These observations strongly support the hypothesis that there are additional genes that vary across the wild strains that act in the same direction and in the opposite direction from *npr-1* in AFT. Alternatively, it may be that some or all of this effect on AFT is due to genetic variation in the wild strains that alter the regulation of *npr-1*.

We have previously observed that the rate of development of AFT is not constant over time: after the initial depression of locomotion at 10 min, the worms move faster at 30 min than they did at 10 min, but from 30–50 min, there is a marked decrease in the speed of the animals, and most move more slowly at 50 min relative to 30 min (Davies et al., 2004). Here, we find that this dynamic response to EtOH is consistent across the parents and RILs. We can envision a number of not mutually exclusive explanations for the differing effects of EtOH during the 10–30 and 30–50 min intervals. First, we have previously shown that EtOH continues to accumulate in the tissue over the course of exposure (Alaimo et al., 2012). The depressing effects of EtOH are likely to increase in strength with increases in EtOH concentration. It may be that the AFT mechanisms that can act immediately between 10 and 30 min to counter the depressing effects of EtOH become progressively overwhelmed by the increasing concentration-dependent depression, so that the depressing effects of EtOH become predominant in the 30–50 interval. Another possibility is that the mechanism of AFT is limited such that it becomes exhausted after 30 min and is therefore less effective after that time.

We considered the possibility that AFT may reflect low-concentration activating effects of EtOH because the time courses of LCLA and AFT appear to overlap. In this model, LCLA effects would be predominant at the beginning of exposure when the concentration of EtOH is lowest but would be progressively overwhelmed by the concentration-dependent depressing effects of

EtOH as higher concentrations of EtOH accumulate over time. This hypothesis predicts that if AFT is the same thing as LCLA, the strains that have the highest AFT would also show the most LCLA. When we compared these two phenotypes, we did not find a correlation between them.

One striking observation is that for the different phenotypes we assessed, the distributions of the strains differ such that the strain with the most extreme of any one phenotype did not have the most extreme of another phenotype. This suggested that there is some but not complete overlap in the biological underpinnings of the various EtOH responses, so we wondered about the degree of correlation between the phenotypes tested in these strains. We found that the 200 mM phenotypes of the sensitivity to 200 mM at 10 min and the degree of LCLA at 30 min were only moderately correlated, suggesting that they share some but not all underlying genetic variation. We have previously found for initial sensitivity and AFT at 400 mM EtOH that genes may affect one or both of these phenotypes (Bettinger et al., 2012; Grotewiel & Bettinger, 2015; Mathies et al., 2015), so we were not surprised to find that in the wild and wild-derived strains, 400 mM initial sensitivity at 10 min and AFT at 30 min were only moderately correlated. The 200 and 400 mM responses were poorly correlated with each other, suggesting that different mechanisms underlie the effects of EtOH at these different concentrations.

Together, these data demonstrate that there is wild genetic variation in *C. elegans* strains that affect EtOH response behaviors. These findings justify further use of this approach in future in-depth analyses of the genetic architecture of EtOH responses.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

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