A Novel Cholinergic Action of Alcohol and the Development of Tolerance to That Effect in *Caenorhabditis elegans*

Edward G. Hawkins,* Ian Martin,* Lindsay M. Kondo,* Meredith E. Judy,† Victoria E. Brings,*
Chung-Lung Chan,* GinaMari G. Blackwell,* Jill C. Bettinger,* and Andrew G. Davies*,†,1

*Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia 23298, and †Ernest Gallo Clinic and Research Center, University of California, San Francisco, California 94608

ABSTRACT Understanding the genes and mechanisms involved in acute alcohol responses has the potential to allow us to predict an individual's predisposition to developing an alcohol use disorder. To better understand the molecular pathways involved in the activating effects of alcohol and the acute functional tolerance that can develop to such effects, we characterized a novel ethanol-induced hypercontraction response displayed by *Caenorhabditis elegans*. We compared body size of animals prior to and during ethanol treatment and showed that acute exposure to ethanol produced a concentration-dependent decrease in size followed by recovery to their untreated size by 40 min despite continuous treatment. An increase in cholinergic signaling, leading to muscle hypercontraction, is implicated in this effect because pretreatment with mecamylamine, a nicotinic acetylcholine receptor (nAChR) antagonist, blocked ethanol-induced hypercontraction, as did mutations causing defects in cholinergic signaling (*cha-1* and *unc-17*). Analysis of mutations affecting specific subunits of nAChRs excluded a role for the ACR-2R, the ACR-16R, and the levamisole-sensitive AChR and indicated that this excitation effect is dependent on an uncharacterized nAChR that contains the UNC-63 α-subunit. We performed a forward genetic screen and identified *eg200*, a mutation that affects a conserved glycine in EAT-6, the α-subunit of the Na+/K+ ATPase. The *eat-6(eg200)* mutant fails to develop tolerance to ethanol-induced hypercontraction and remains contracted for at least 3 hr of continuous ethanol exposure. These data suggest that cholinergic signaling through a specific α-subunit-containing nAChR is involved in ethanol-induced excitation and that tolerance to this ethanol effect is modulated by Na+/K+ ATPase function.

THE abuse of alcohol is a cause of significant societal and health-related problems. Despite the common usage of this drug, the molecular underpinnings of alcohol's acute actions on the brain are poorly understood. Alcohol (ethanol) has biphasic behavioral effects in humans and other animals, acting as a stimulant at lower concentrations and as a depressant at higher concentrations. Understanding the molecular nature of these biphasic effects is made difficult by the fact that ethanol interacts with, and alters the function of, many proteins, including neurotransmitter receptors and ion channels (Harris

et al. 2008; Spanagel 2009). A commonly suggested class of ethanol targets is ligand-gated ion channels (LGICs), which include NMDA glutamate receptors, and members of a subclass of LGICs, the Cys-loop superfamily, including GABAA, glycine, and nicotinic acetylcholine receptors (nAChRs) (Olsen *et al.* 2014). Effects on the GABAA and glutamate receptors are hypothesized to play a significant role in the depressant effects of ethanol (Harris *et al.* 2008). Locomotor activation by low to moderate concentrations of ethanol in rodents is thought to model the euphoric effects of ethanol in humans (Phillips and Shen 1996). Locomotor activation by ethanol has been linked to increased dopamine release in neural reward pathways, possibly via mechanisms that involve inhibition of the inhibitory GABAB metabotropic receptors by ethanol (Holstein *et al.* 2009; Kruse *et al.* 2012) and/or by ethanol activation of neuronal nAChRs (Soderpalm *et al.* 2000; Larsson *et al.* 2002; Kamens and Phillips 2008; Kamens *et al.* 2009).

Individual variation that alters the mechanisms responsible for the acute behavioral effects of ethanol is likely to contribute to variation in predisposition to develop alcohol use disorders (AUDs). An individual's level of response (LR) to acute ethanol intoxication is determined by two major opposing biological forces, the degree of sensitivity to intoxication and the rate at which tolerance develops to that acute intoxication during the intoxication session (called acute functional tolerance) (Newlin and Thomson 1990). LR has genetic determinants (Kalu *et al.* 2012) and LR in naive drinkers is a predictor of the predisposition to develop AUDs later in life (Schuckit 1994; Volavka *et al.* 1996; Heath *et al.* 1999). Therefore, it is important that we better understand the pathways responsible for the behavioral actions of ethanol and the mechanisms by which acute functional tolerance can develop to those behavioral effects. Studies in selectively bred mouse strains suggest that ethanol-induced locomotor activation and the rate of development of acute functional tolerance may share common mechanisms (Ponomarev and Crabbe 2002).

Caenorhabditis elegans represents a powerful model for identifying drug targets, using genetic approaches (Van Swinderen *et al.* 1999; Davies *et al.* 2003; Artal-Sanz *et al.* 2006; Kaletta and Hengartner 2006; Kwok *et al.* 2006). *C. elegans* are sensitive to acute ethanol exposure, demonstrating an increased speed of locomotion at low ethanol concentrations (Graham *et al.* 2009) and decreased amplitude of body bends and decreased rates of crawling, swimming, egg laying, and pharyngeal pumping at higher concentrations of ethanol (Davies *et al.* 2003; Mitchell *et al.* 2007; Speca *et al.* 2010). Ethanol also produces disinhibition of certain crawling behaviors that would normally be inhibited in a liquid environment (Topper *et al.* 2014). Acute functional tolerance, which is unrelated to metabolism of the drug, develops rapidly to the effects of ethanol on the speed of locomotion (Davies *et al.* 2004; Alaimo *et al.* 2012; Bettinger *et al.* 2012; Raabe *et al.* 2014). Longer ethanol exposures followed by removal from the drug can lead to withdrawal-related phenotypes (Davies *et al.* 2004; Mitchell *et al.* 2010). Therefore, these drug-induced behavioral effects are indicative of ethanol having activating and depressing actions in *C. elegans* as it does in mammals and demonstrate that tolerance can develop to acute and long-term ethanol exposures.

In this study, we describe a novel excitation effect of acute ethanol treatment of *C. elegans*, which we have termed **e**thanol-induced **h**yper**c**ontraction (EHC). EHC is time dependent; treated animals show a rapid onset of body hypercontraction and a relatively quick recovery while in the presence of the drug. This latter observation is indicative of the development of acute functional tolerance. We test the role of candidate neurotransmitter signaling systems in EHC and identify a specific nAChR subunit that mediates the excitation effect. We describe the identification of a mutant that displays a lack of development of tolerance to EHC as an allele of *eat-6*, the Na^+/K^+-ATPase α-subunit-encoding gene, suggesting the possibility that Na^+/K^+-ATPase function is required for the development of tolerance to EHC.

Materials and Methods

Nematode maintenance and strains

Nematodes were maintained at 20° on nematode growth medium (NGM) plates in the presence of *Escherichia coli* strain OP50. The strains used in this study were N2 (wild type), CB4856 (wild type), *eri-1(mg366)*, *cha-1(p1152)*, *unc-17(e245)*, *unc-17(e113)*, *acr-2(ok1887)*, *acr-16(ok789)*, *unc-38(e264)*, *unc-29(e193)*, *lev-1(e211)*, *unc-63(x13)*, *unc-63(gk234)*, *gbb-1(tm1406)*, *gbb-2(tm1165)*, *unc-25(e156)*, *unc-25(sa94)*, *unc-25(n2569)*, *unc-49(e407)*, *unc-30(e191)*, *unc-30(e318)*, *unc-47(gk192)*, *unc-47(e307)*, *slo-1(eg142)*, *npr-1(ky13)*, *eat-6(eg200)*, *eat-6(ad601)*, *eat-6(ad467)*, *eat-6(ad997)*, *egl-3(n150)*, *unc-31(e928)*, *nlp-12(ok335)*, *trp-4(sy695)*, *lon-3(e2175)*, *unc-61(e228)*, *unc-42(e270) yDf12 V/nT1[unc-?(n754) let-?](IV;V)*; *dpy-6(e14)X, unc-42(e270) arDf1 V/nT1[unc-?(n754) let-?](IV;V)*, and *nDf42 V/nT1[unc-?(n754) let-?](IV;V)*. Strains were provided by the *Caenorhabditis* Genetics Center (CGC) and the National Bioresource Project for the nematode (Japan).

Hypercontraction assay

Plates were prepared as previously described (Davies *et al.* 2003). Briefly, NGM plates (6 cm) were dried for 1.5 hr at 37° 18–20 hr before experiments were performed. Plates were weighed (to determine media volume) and four copper rings were melted into the surface of the agar of each plate to corral the worms. Plates were divided into the following groups: (1) acclimation, (2) 0 mM ethanol treatment, and (3) *X* mM ethanol treatment, where *X* represents the final exogenous concentration of ethanol (from 100 to 500 mM). Ethanol was added to the ethanol treatment plates (vol/vol) to the desired concentration and allowed to equilibrate for 2 hr before the experiment was performed. Ten age-matched first-day adults were assayed in each of the four rings. The acclimation plates were used to acclimate the worms to the absence of bacteria (food) for 30 min prior to the experiment being performed. The worms were then transferred to their respective treatment plates for the assay (0 mM or *X* mM ethanol). We have previously shown that a 400-mM exogenous dose generates a tissue concentration of ethanol in *C. elegans* of ∼44 mM (Alaimo *et al.* 2012).

To measure worm perimeter, images were captured with a Retiga 4000R (QImaging, BC, Canada) camera attached to an Olympus SZX7 microscope set to a magnification of 2.0× and fitted with a 0.5× objective. Images were taken using ImagePro Plus Version 6 (Media Cybernetic, Bethesda, MD) at the following time points: −1 min (on the acclimation plate, immediately prior to movement to the treatment plate) and 1, 5, 10, 20, 30, and 40 min. Worm size was determined using a macro written in the ImagePro Plus program (available on request). The macro converted the captured images to 8-bit gray scale and filtered the images to enhance the worm objects. First, the image was flattened (feature width 20) and then a large spectral filter was applied (Edge Minus, W10, H10, S1, P1). The resulting image

was inverted and the count/size feature was applied to detect the worm objects based on a gray-scale threshold. Most nonworm objects were excluded based on a size filter, using parameters that include area, feret size, and perimeter. Finally, a manual visual check was used to ensure that only worms were included in the measurements and that no worms were included that had generated a ring shape, which can form during an omega bend movement. The average perimeter size for the group of 10 animals ($n = 1$) in each copper ring was determined at each time point. To control for differences in the baseline perimeter for strains of different genotypes (Supporting Information, Table S2), data are presented as perimeter (% of untreated) ± SEM and were calculated using the following equation:

$$\frac{\text{Average size at time } X}{\text{Average size at time } (-1 \text{ or } -5)} \times 100$$
$$= \text{Perimeter } (\% \text{ of untreated}).$$

With the exception of Figure 1B, the data for worms assayed in the absence of ethanol are not shown. In the absence of ethanol, we found no significant differences for perimeter size measured over time for any of the strains we tested.

Locomotion

Basal speeds were determined as previously described (Alaimo *et al.* 2012). The plates and first-day adult animals were prepared as described for the hypercontraction assay. A 2-min movie was recorded at 30 min and the average speed was measured using ImagePro Plus Version 6 (Media Cybernetics, Bethesda, MD). The average speed and SEM of six trials were plotted and analyzed using a one-way ANOVA with Prism v 5.0 (GraphPad Software).

Identification of the eg200 mutation

The *eg200* mutant was isolated in a small forward genetic screen (1800 haploid genomes tested on 150–200 mM ethanol) on the basis of slower than wild-type speeds in the presence of ethanol. The prolonged ethanol-induced hypercontraction phenotype associated with *eg200* was used thereafter in the mapping and identification of the causative mutation. Using SNP mapping methods (Wicks *et al.* 2001; Davis *et al.* 2005) with the Hawaiian wild-type strain CB4856, the *eg200* mutation was initially mapped to the right arm of chromosome V between two SNPs ~1.8 map units apart (*pkP5068* and *pkP5087*). The SNPs assessed in fine-mapping *eg200* on chromosome V were *haw78359*, *pkP5067*, *pkP5068*, *pkP5069*, *pkP5125*, *pkP5087*, *pkP5086*, *pkP5084*, and *pkP5126* (http://wormbase.org). To facilitate the recovery of additional recombinants in the genetic interval containing *eg200*, a triple-mutant strain, *lon-3(e2175) eg200 unc-61(e228)*, was constructed using standard genetic crosses. The triple mutant was crossed to CB4856, F$_1$ progeny were allowed to self-fertilize, and F$_2$ progeny were isolated that were Lon non-Unc or Unc non-Lon. The
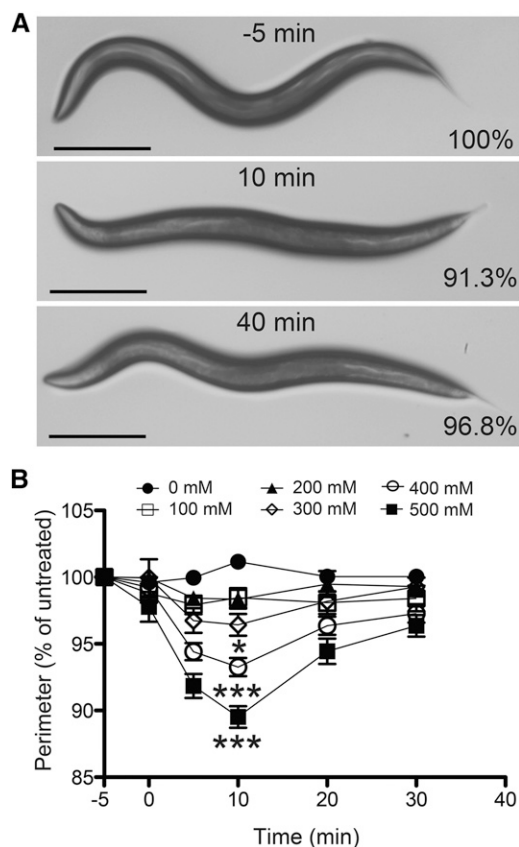
Figure 1 Ethanol dose-dependently induces hypercontraction. (A) Images of the same N2 (wild-type) worm before treatment (−5 min) and during treatment with 400 mM ethanol (10 and 40 min of exposure). Percentage indicates percentage of untreated perimeter at −5 min. Bar, 250 μm. (B) N2 (wild-type) worms treated with 0, 100, 200, 300, 400, and 500 mM exogenous ethanol. A significant decrease in size, indicating contraction, relative to 0 mM was obtained at the 10-min time point at 300, 400, and 500 mM ethanol. Recovery from the contraction was significant at 30 min at concentrations of 400 and 500 mM ethanol (*$P < 0.05$; ***$P < 0.001$; $n = 12$).

recombinant chromosome was made homozygous by selecting self-progeny individuals that were Lon non-Unc or Unc non-Lon and did not have self-progeny that were Lon Unc. The presence or absence of the *eg200* mutation in these recombinant strains was determined based on the EHC phenotype. The strains were then examined for N2/CB4856 SNPs in the interval. This mapping further narrowed the *eg200*-containing interval to between *pkP5068* and *snp_F57B1[3]* (http://wormbase.org).

The *eg200* mutation failed to complement the deficiencies *yDf12* and *nDf42*, while complementing *arDf1*. To do this, *eg200* homozygous males were crossed to the deficiency strains and non-Unc (*eg200/Df*) young adult progeny were tested for prolonged contraction in the presence of ethanol. Both *yDf12* and *arDf1* are known to delete the *lin-25* gene (Hsu and Meyer 1994; Tuck and Greenwald 1995), while the *max-1* gene is deleted by *yDf12* but not by *arDf1* (Huang *et al.* 2002), which suggested that *eg200* was in the vicinity of *max-1* to the right of the *arDf1* right breakpoint. To determine the extent of the right breakpoints

of *arDf1* and *yDf12*, the deficiency-bearing strains were crossed to CB4856 males and DNA was prepared from non-Unc (*+/Df*) progeny. The primers used to amplify DNA fragments (i–viii, shown in Figure 6D) in the interval of interest were previously described (Swan *et al.* 2002) (File S1, Table M1). Each of these primer pairs flanks a known dimorphic SNP present in the N2 and CB4856 strains. Sequencing of the amplified fragment was carried out to determine the genotype (only the CB4856 allele would be detected if the region is deleted by the deficiency or both the N2 and CB4856 alleles would be detected if the interval is not deleted by the deficiency). These experiments placed the right breakpoint of *arDf1* between *lin-25* and the PCR fragment labeled i in Figure 6D, whereas the right breakpoint of *yDf12* was between PCR fragments vii and viii.

We chose *eat-6* as a candidate gene within the *eg200*-containing genetic interval to test. To confirm that *eat-6* was affected by the *eg200* mutation, the genomic region spanning *eat-6* coding sequences was amplified by PCR, using genomic DNA purified from the *eg200* mutant as the template for the reaction (File S1, Table M2).

Aldicarb and levamisole treatment

The aldicarb assay was adapted from previously described methods (Mahoney *et al.* 2006). Aldicarb (Sigma-Aldrich, St. Louis) plates were prepared at a concentration of 0.5 or 1 mM 2–3 days before the assay. For assays determining the sensitivity to aldicarb-induced paralysis, 10 first-day adults were moved to the aldicarb treatment plates (1.0 mM) and animals were scored for paralysis every 30 min for 3 hr. Worms were counted as paralyzed if they did not move when prodded with a platinum pick on the head three times. For assays determining the degree of hypercontraction induced by aldicarb treatment, first-day adults were moved to the aldicarb plates (0.5 or 1.0 mM) after their body size was determined on an aldicarb-free plate, and then perimeters were measured as described above for hypercontraction assays. Levamisole paralysis assays were carried out as previously described (Nurrish *et al.* 1999). Age-matched young adult animals were transferred to plates with agar containing 100 μM levamisole (Sigma-Aldrich) and paralysis induced by the drug was determined (as above) at 0, 10, 30, 60, 90, and 120 min of exposure.

Mecamylamine treatment

First-day adult worms were pretreated with mecamylamine (Sigma-Aldrich) by constructing small-volume NGM pads containing 5 mM of the compound. Small-volume pads were used to reduce the quantity of drug required for treatment. Copper rings were set in the lid of 24-well plates. Agar from NGM plates dried as explained in the hypercontraction assay section was melted in a water bath at 85°. Mecamylamine was added to the melted NGM to 5 mM and 200 μl of this solution was pipetted into each copper ring and allowed to harden at room temperature. Equivalent 200-μl pads made without mecamylamine were used for acclimation, preexposure measurements, untreated controls, and ethanol treatment. Ethanol added to the ethanol-treatment pads was allowed to equilibrate for 2 hr prior to their use.

Statistical analysis

Treated mutant strains were compared to treated wild-type controls to determine whether the mutation caused a significant effect on the EHC phenotype. Respective time points were compared against one another, using a repeated-measures two-way ANOVA with Bonferroni *post hoc* tests unless otherwise indicated (GraphPad Prism).

Results

The ethanol-induced hypercontraction phenotype is dose dependent

In performing assays of the behavioral effects of ethanol on *C. elegans* locomotion, we observed that acutely exposed worms demonstrate a transient decrease in overall body length, which we have termed EHC (Figure 1A). We performed a dose-response analysis to quantify the degree of hypercontraction and to determine the duration of this effect of ethanol on wild-type (WT) N2 young adult animals. Worms were exposed to 0-, 100-, 200-, 300-, 400-, and 500-mM exogenous concentrations of ethanol over a 30-min period and their lengths were measured and compared with their preexposure untreated lengths (Figure 1B). Higher concentrations (600 and 700 mM ethanol) were also tested but ethanol-induced coiling of the treated worms limited collection of the length measurements (Table S1), which may bias the data to overrepresent less-affected animals. Over the time course of exposure, there were two apparent phases in the EHC phenotype: (1) an initial contraction phase (0–10 min) and (2) a recovery phase (10–30 min). Both phases of the EHC phenotype are shown in Figure 1A, which contains images of the same N2 worm untreated (−5 min) and treated with 400 mM ethanol at 10 min and 40 min. Also apparent in Figure 1A, in the second image, is the decrease in body bend amplitude induced by ethanol (Davies *et al.* 2003). This flattening of the worm obscures the length decrease visually, but computer-assisted length measurements of midline or perimeter reveal the effect of ethanol on overall body length. The worms demonstrated a maximum contraction at 10 min with significant differences from the 0-mM untreated controls at 300, 400, and 500 mM ethanol (Figure 1B; Table S1). Significant recovery from EHC was achieved within 20 min following the point of maximum contraction (10 min) at both 400 and 500 mM ethanol. We chose to investigate EHC further using 400 mM ethanol, which was the lowest exogenous concentration at which we saw both statistically significant hypercontraction and a significant recovery from that hypercontraction.

Hypertonic-sensitive worms are not hypersensitive to ethanol

Hypertonic environments have previously been shown to produce a transient shrinkage in *C. elegans* (Choe and Strange

2007). We observed that hypertonic-induced hypercontraction differs substantially from EHC; hypertonic conditions (*e.g.*, 349 mM NaCl) produce a greater decrease in body size and require a more prolonged recovery (4–5 hr) than hypercontraction of wild-type animals treated with 400 mM ethanol. We tested whether the mechanisms of EHC overlap with those responsible for the hypertonic hypercontraction response. Recovery from hypertonic shrinkage involves the activation of the **w**ith-**n**o-**k**ysine (WNK) WNK-1 serine–threonine kinase (Choe and Strange 2007). In agreement with this report, we found that RNA interference (RNAi) knockdown of WNK-1 inhibited recovery from hypertonic shock in *eri-1(lf)* worms (Figure S1A) [*eri-1(mg363)* animals are hypersensitive to RNAi]. If EHC occurred as a result of hypertonic stress, we hypothesized that knockdown of the *wnk-1* gene should increase sensitivity to ethanol or prolong the ethanol effect. When we examined the sensitivity to EHC of *wnk-1* RNAi-treated animals, we found that there was no difference in the degree of EHC compared to that in RNAi control-treated animals (Figure S1B), suggesting that the mechanisms of EHC do not overlap with the mediators of the response to hypertonic stress.

Other physical environmental properties of ethanol do not produce hypercontraction

We considered the possibility that other changes in the physical environment produced by ethanol might be the cause of EHC. At the concentration of ethanol we used, neither acidity nor viscosity are expected to be different from that in NGM media alone (Bates *et al.* 1963; Khattab *et al.* 2012). In the sealed petri plate, we might expect the presence of ethanol to alter vapor pressure. We tested vapor pressure, using a closed chamber system that we have used previously in the analysis of volatile inhalant drugs (Davies *et al.* 2012). Vapor pressure produced by 400 mM ethanol did not change the contraction state of wild-type animals (Figure S1C).

Hypercontraction to ethanol requires cholinergic signaling

Cholinergic signaling promotes contractile responses while GABAergic signaling induces relaxation responses in the longitudinal body wall muscle of the animal (Rand 2007). Pharmacological treatment of wild-type worms that leads to prolonged activation of cholinergic signaling has been shown to cause hypercontraction and paralyzed phenotypes (Lewis *et al.* 1980). While the worms displaying EHC are not paralyzed, we suspected that increased cholinergic signaling might be the mechanism responsible for EHC.

Mecamylamine is a nonselective noncompetitive antagonist of nAChRs. Preincubation of N2 worms for 1 hr with 5 mM mecamylamine did not significantly affect the body size of the worms during the pretreatment period (Figure 2A). When we pretreated animals with 5 mM mecamylamine and then exposed those worms to ethanol, we found that mecamylamine treatment significantly blocked EHC (Figure 2A). This result suggests that nAChR activity is required for eth-
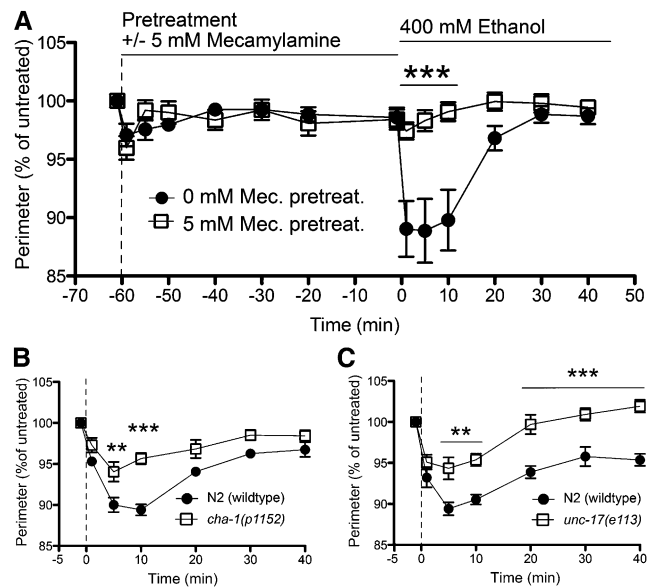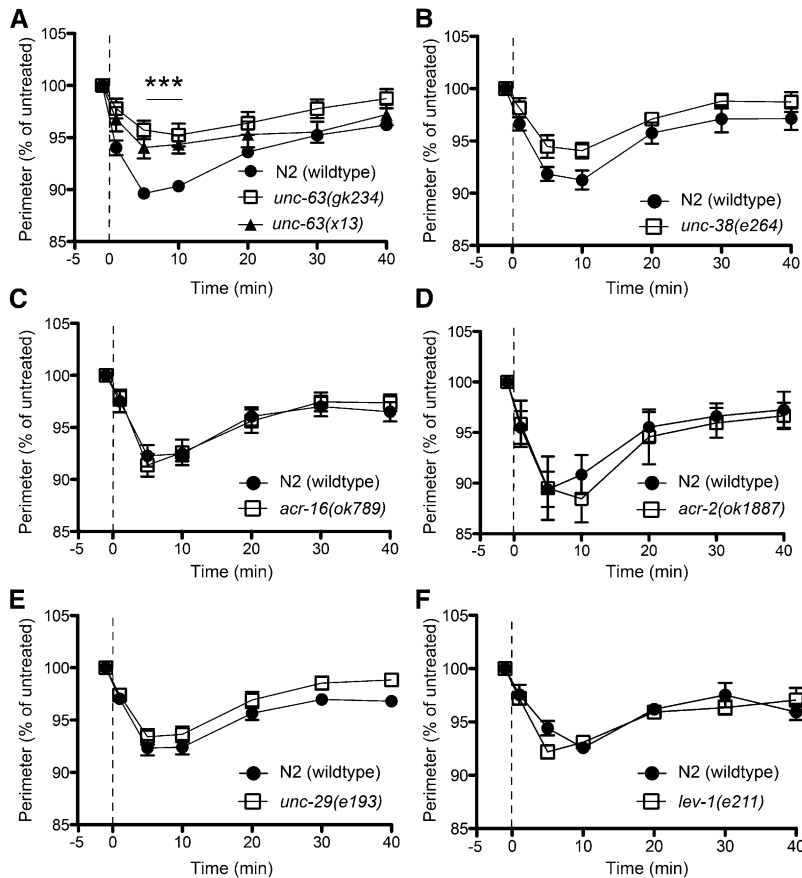

Figure 2 Hypercontraction requires cholinergic signaling. (A) Preincubation (−60 to 0 min) with mecamylamine (Mec.) significantly blocked the EHC phenotype induced by 400 mM ethanol on N2 (wild-type) worms at 10 min (***$P < 0.001$; $n = 11$). (B and C) Mutant worms deficient in presynaptic acetylcholine release, *cha-1* and *unc-17*, were significantly resistant to the hypercontraction induced by ethanol compared with N2 worms (**$P < 0.01$; ***$P < 0.001$; $n = 6$).

anol to induce hypercontraction. We tested this hypothesis further, using mutants in genes that are involved in cholinergic signaling.

The synthesis of acetylcholine (ACh) requires the choline acetyltransferase (ChAT) enzyme that is encoded by the *cha-1* gene (Rand and Russell 1984). Packaging into vesicles in presynaptic neurons requires the vesicular acetylcholine transporter (VAChT) that is encoded by the *unc-17* gene (Alfonso *et al.* 1993). Strains carrying partial loss-of-function mutations in *cha-1* or *unc-17* have decreased levels of ACh released into the synaptic cleft (Rand 2007). When *cha-1* or *unc-17* mutant worms were tested on ethanol, we observed significant resistance to EHC compared to that in wild-type worms (Figure 2, B and C). The *unc-17(e113)* mutant showed an unusual tolerance response; by 40 min of exposure, the mutant worms recovered to a size greater than their preethanol treatment size (Figure 2C). A second allele, *unc-17(e245)*, displayed the same resistance and tolerance phenotype (Table S2), confirming that this observation was not allele specific or a product of genetic background. The *unc-17* and *cha-1* data are in agreement with the mecamylamine data; together these results suggest a cholinergic mechanism of action for EHC.

Specific nAChRs play a role in EHC

The *C. elegans* genome contains a large family of nAChRs with 29 identified subunits compared to the human genome, which contains 17 (Brown *et al.* 2006; Jones *et al.* 2007; Rand 2007). The most well-characterized subunits in worms are those expressed in motor neurons and at the neuromuscular
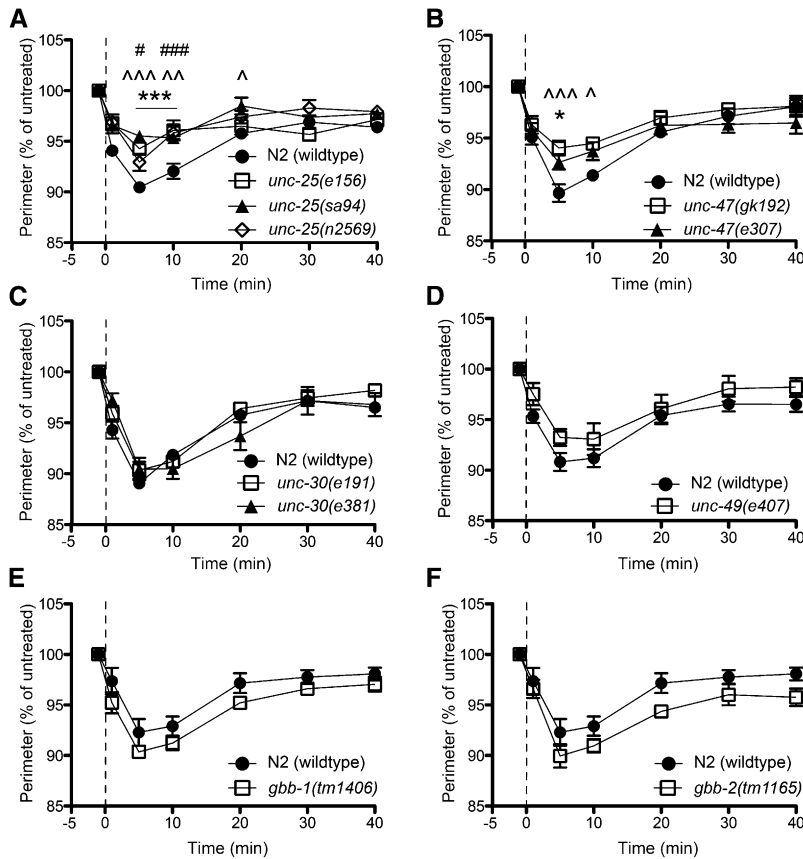
Figure 3 Ethanol-induced hypercontraction requires UNC-63. (A) Two different alleles of *unc-63(gk234)* and *unc-63(x13)* significantly reduced the hypercontraction response to 400 mM ethanol (***$P < 0.001$; $n = 7$). (B–F) *unc-38* ($n = 6$), *acr-16* ($n = 6$), *acr-2* ($n = 5$), *unc-29* ($n = 10$), and *lev-1* ($n = 5$) mutants did not demonstrate a significant difference from N2 (wild type) ($P > 0.05$). Given the conserved homology between *unc-63* and *unc-38*, the trend toward resistance for *unc-38* may be relevant.

junction (Richmond and Jorgensen 1999; Touroutine *et al.* 2005) and we tested many of these for a role in EHC. We focused first on the neuromuscular junction (NMJ) as EHC appears to involve muscle hyperactivation. Two receptor classes have been identified at the NMJ, those that are sensitive to the AChR agonist levamisole and those that are insensitive to levamisole. The levamisole-sensitive receptor requires the UNC-29, UNC-38, UNC-63, and LEV-1 subunits (Fleming *et al.* 1997; Culetto *et al.* 2004; Towers *et al.* 2005). The homomeric ACR-16R nAChR is the only defined levamisole-insensitive receptor at the NMJ (Touroutine *et al.* 2005). We tested members of each receptor class (levamisole sensitive, *unc-29*, *unc-38*, *unc-63*, and *lev-1*; levamisole insensitive, *acr-16*) for the effect of loss of function on EHC responses (Figure 3). We found that, of the genes tested, only mutations causing a loss of function in the *unc-63* gene (*x13* and *gk234*) significantly reduced the EHC response compared with that in wild-type animals (Figure 3A). The *unc-38(e264)* mutation produced a nonsignificant trend toward resistance to EHC (Figure 3B). That the *unc-29* and *lev-1* mutants displayed wild-type sensitivity to EHC (Figure 3, E and F) suggests that it is not the levamisole-sensitive receptor mediating the EHC effect because UNC-29 and LEV-1 are required subunits of that receptor. Instead, these data suggest the involvement of the UNC-63 subunit in a different receptor(s). One likely candidate was the neuronal ACR-2 receptor (ACR-2R), which has been shown to require UNC-63 and UNC-38 subunits (Jospin *et al.* 2009) and is expressed in cholinergic

motorneurons (Petrash *et al.* 2013). Loss of function of the *acr-2* gene eliminates the ACR-2R, so we tested *acr-2(ok1887)* for EHC, with the idea that if UNC-63 were acting in the ACR-2R to mediate EHC, then loss of ACR-2R should mimic the *unc-63(lf)* for this phenotype. *acr-2(ok1887)* mutants showed wild-type sensitivity to EHC (Figure 3D), which suggests that UNC-63 may belong to yet another nACh receptor class that is independent of the levamisole-sensitive receptor and the ACR-2R. These data indicate that the UNC-63 α-nAChR subunit plays a significant role in mediating EHC, and we have eliminated the levamisole-sensitive receptor and the ACR-2R as being required for the EHC effect.

GABA signaling in EHC

While ACh induces contraction of body wall muscle, GABA causes relaxation of the contralateral body wall muscle. We predicted that loss of GABA signaling might increase the degree of EHC due to a loss of inhibition at the NMJ. In contrast, we found that loss-of-function mutations in the *unc-25* gene, which encodes the GABA synthetic enzyme, glutamic acid decarboxylase (GAD), decrease the degree of EHC (Figure 4A). We also found this to be the case for mutations affecting the GABA vesicular transporter-encoding gene *unc-47* (Figure 4B), which is required for uptake of GABA into synaptic vesicles prior to neurotransmitter release. Mutations in *unc-25* and *unc-47* should reduce the release of GABA from GABAergic neurons (McIntire *et al.*

Figure 4 Loss of UNC-25 or UNC-47 function results in resistance to ethanol-induced hypercontraction. (A) GABA synthesis-defective *unc-25* mutants displayed a significant reduction in the hypercontraction phenotype compared to wild-type animals (*e156*, ***$P < 0.001$; *sa94*, ^^$P < 0.01$, ^^^$P < 0.001$; *n2569*, #$P < 0.05$, ###$P < 0.001$; $n = 6$). (B) Loss of function of the vesicular GABA transporter, UNC-47, resulted in a significant decrease in the EHC phenotype (*e307*, *$P < 0.05$; $n = 6$; *gk192*, ^$P < 0.05$, ^^^$P < 0.001$; $n = 6$). (C) Mutations affecting *unc-30*, which encodes a transcription factor responsible for development of type D GABAergic motor neurons, did not alter EHC ($P > 0.05$; $n = 6$). (D–F). Mutants with a loss of signaling through GABA_A (*unc-49*) or GABA_B (*gbb-1*, *gbb-2*) were not significantly different from wild-type animals for EHC ($P > 0.05$; $n = 5$).

1993a). Interestingly, mutations in the GABA_A receptor-encoding gene, *unc-49*, or in either of the GABA_B metabotropic receptor subunits, *gbb-1* and *gbb-2*, do not alter the sensitivity to ethanol for EHC (Figure 4, D–F). Finally, we examined the effect of mutations in the *unc-30* gene, which alters the developmental fate of the type D GABAergic motorneurons, such that they no longer express *unc-25* and *unc-47* (Eastman *et al.* 1999). Unexpectedly, the *unc-30* mutants showed a wild-type response to EHC (Figure 4C). We tested the possibility that the *unc-25* and *unc-47* mutants were already hypercontracted due to the lack of inhibition caused by a decrease in GABA signaling. In such a case, it may be that the GABA signaling mutants would not be able to contract further or to the same degree as WT animals, resulting in a floor effect in our measurements of EHC. To test this, we exposed *unc-25*, *unc-47*, and *unc-49* mutant animals to aldicarb (0.5 or 1.0 mM), an acetylcholinesterase inhibitor that causes progressive hypercontraction and paralysis of *C. elegans* (Mahoney *et al.* 2006). All of the mutants, when exposed to aldicarb, contracted to a greater degree than they did in the presence of ethanol (Figure S2), confirming that our measurements were not confounded by a floor effect. The resistance to EHC shown by the *unc-25* and *unc-47* mutants suggests a role for GABA in the action of ethanol that generates hypercontraction, while the lack of effect of the *unc-30* mutants suggests a role outside of the type D neurons.

Neuropeptide signaling is not required for hypercontraction

It has been reported that aldicarb treatment increases the release of the neuropeptide NLP-12 from the DVA neuron, which then potentiates cholinergic signaling at the NMJ of *C. elegans* (Hu *et al.* 2011). The DVA neuron is a stretch-sensitive sensory interneuron that requires the TRP-4 ion channel for its function. Worms exposed to ethanol exhibit a dose-dependent flattening of body bends (See Figure 1B) (Davies *et al.* 2003). We hypothesized that the EHC phenotype might be due to increased cholinergic signaling via NLP-12 release that is the result of the ethanol-induced reduction in body bend amplitude. We tested this hypothesis, using worms that are deficient in neuropeptide release and defective in DVA neuron function. Approximately 250 neuropeptides are released from dense core vesicles at the synaptic cleft in *C. elegans* (Li and Kim 2008), a process that requires UNC-31 and EGL-3. The *unc-31* gene encodes the calcium-dependent activator protein for secretion (CAPS); loss of this protein inhibits the calcium-mediated fusion of dense core vesicles to the membrane, which prevents peptide release (Speese *et al.* 2007). *egl-3* encodes a proprotein convertase type 2, which is required for the conversion of propeptides into active peptides (Kass *et al.* 2001). To test the role of neuropeptides in EHC, loss-of-function mutants for *unc-31* and *egl-3* were examined for sensitivity to EHC. Neither of these mutants exhibited a significantly different

EHC phenotype compared with wild-type animals (Figure 5, A and B). To examine directly the role of the stretch pathway in EHC, we tested loss-of-function mutants of *nlp-12* and *trp-4* (Figure S3, A and B), which eliminate the NLP-12 response (Hu *et al.* 2011). Both of these mutants were not different from wild-type animals for the EHC response, indicating that the hypercontraction phenotype is not the result of increased cholinergic transmission through DVA activation. Taken in conjunction with the *unc-31* and *egl-3* data, this shows that neuropeptide signaling is unlikely to be involved in EHC.

eat-6(eg200) is defective in the development of acute functional tolerance to EHC

In a forward genetic screen, using ethyl methanesulfonate mutagenesis to generate random mutations, we isolated mutants that are hypersensitive to the effects of ethanol on the speed of locomotion. One of these mutants, *eg200*, was isolated on the basis of having significantly slower speeds on ethanol than wild-type siblings (Figure 6, A and B). However, the most striking aspect of the response to ethanol of the *eg200* mutant animals was their continued hypercontraction for as long as they remained exposed to ethanol (Figure 7A), as opposed to the relatively rapid recovery demonstrated by wild-type animals (Figure 7A). We pursued identification of the gene affected by the *eg200* mutation to better understand the second phase of EHC, the recovery (or acute functional tolerance) phase.

We used genetics to map the *eg200* mutation to a region between *lin-25* and the dimorphic SNP, *snp_FF57B1[3]* on chromosome V (Figure 6D) (see *Materials and Methods*). We examined the proposed functions, where known, of the 228 predicted genes in the ~560-kb *eg200*-containing interval for candidate genes whose function might account for the hypersensitivity to and prolonged recovery from EHC. We investigated the *shw-3* Shaw-class potassium channel as an *eg200* candidate gene. Sequencing of the *shw-3* gene amplified from *eg200* genomic DNA did not reveal any mutations in the *shw-3* coding sequence (data not shown). We next sequenced the *eat-6* gene, which encodes the α-subunit of the Na⁺/K⁺-ATPase (Davis *et al.* 1995), which is an important regulator of excitable cell activity, and found a nucleotide substitution mutation that alters the codon for a highly conserved amino acid (glycine 77 in the EAT-6 protein) (Figure 6E), suggesting that the *eg200* mutation affects the *eat-6* gene.

The *eg200* mutant animals do not have the starved appearance found with some other mutant alleles of the *eat-6* gene (Avery 1993), but the *eg200* animals are smaller and slower growing than wild-type animals, which is consistent with mutants that have feeding defects (Avery 1993). Some *eat-6* mutants have been reported to be hypersensitive to the AChR agonist levamisole (Doi and Iwasaki 2008; Govorunova *et al.* 2010). We found that *eg200* mutant animals are hypersensitive to the paralyzing effects of levamisole (Figure 6C), consistent with the *eg200* mutant having a defect in the *eat-6* gene. Although *eg200* mutants do not have a starved appear-
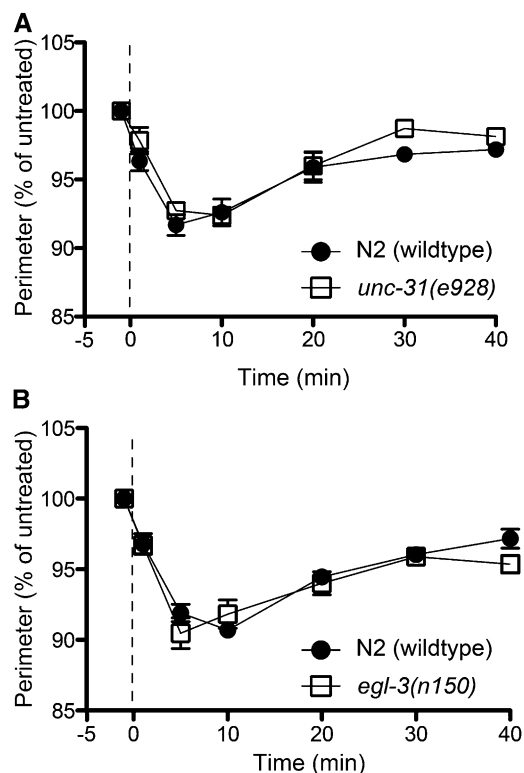
Figure 5 Neuropeptide signaling is not involved in ethanol-induced hypercontraction. (A and B) Mutations affecting two genes involved in neuropeptide release and activation, *unc-31* and *egl-3*, respectively, exhibited similar contraction phenotypes to N2 (wild type) to 400 mM ethanol ($P > 0.05$; $n = 6$).

ance, they do have a reduction in pharyngeal pumping rate (Table S3), similar in severity to another *eat-6* mutant, *ad601*, considered to be a weak allele for both starvation and pharyngeal pumping phenotypes (Davis *et al.* 1995; Govorunova *et al.* 2010). Two additional *eat-6* alleles, *ad467* and *ad997*, are reported to be stronger alleles with respect to mutant pharyngeal pumping and starvation phenotypes (Davis *et al.* 1995). We examined all three *eat-6* alleles for altered recovery from EHC (Figure 7 and Figure S4B).

The *eat-6(ad601)* mutants contracted to the same extent as the *eg200* mutant animals; however, unlike *eg200* animals, the *ad601* mutants initially recovered at the same rate as wild-type animals (Figure 7A). Interestingly, over the course of the next 2 hr of ethanol exposure, the *ad601* mutant animals slowly contracted again to the same level as *eg200* mutants, while wild-type animals did not undergo a second contraction over this time course (Figure 7A). We confirmed that *eg200* is an allele of the *eat-6* gene based on a failure to complement the *ad601* for the EHC phenotype (Figure S4A). The *trans*-heterozygote *eg200/ad601* displayed an EHC phenotype more similar to *ad601* homozygotes; the *eg200/ad601* animals initially recovered from the contracted state only to contract again at late time points of ethanol exposure.
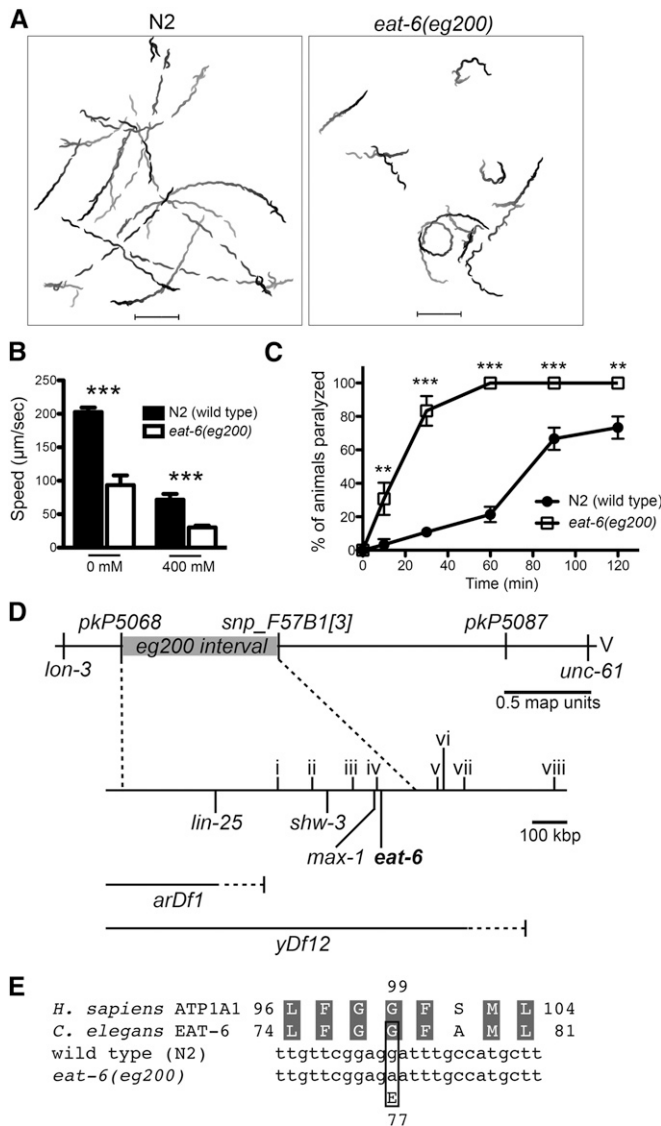
Figure 6 Mutations in the *eat-6* gene alter tolerance to ethanol-induced hypercontraction. (A) The *eg200* mutant animals have decreased movement compared with wild-type animals on ethanol. In these representative illustrations, images of wild-type and *eg200* worms were captured every 10 sec over a 2-min period after 30 min of ethanol exposure (400 mM). The progression of time is indicated by darkening worm silhouettes. Bars, 2 mm. (B) Average speeds of wild-type and *eg200* mutant animals in the absence and presence of ethanol (400 mM) after 30 min of exposure (***$P < 0.001$; $n = 5$). (C) The *eat-6(eg200)* mutation increases the sensitivity to paralysis caused by prolonged exposure to the AChR agonist, levamisole (100 μM) (**$P < 0.01$; ***$P < 0.001$; $n = 3$). (D) The *eg200* mutation was mapped between SNPs on chromosome V and between endpoints of the deletions *arDf1* and *yDf12*. The approximate endpoints of the deletions were determined by placing CB4856-specific SNPs (i–viii) in *trans* to the deletion-bearing chromosomes and sequencing to determine genotype at each SNP (hemizygous CB4856 or heterozygous N2/CB4856). (E) The *eg200* mutation is predicted to cause a substitution of a glutamic acid for a conserved glycine (Gly77) in the EAT-6 protein. The mutation is a G to A substitution in exon 2 of *eat-6* (nucleotide 230 of the mRNA sequence; position 13,129,568 of the chromosome V nucleotide sequence).
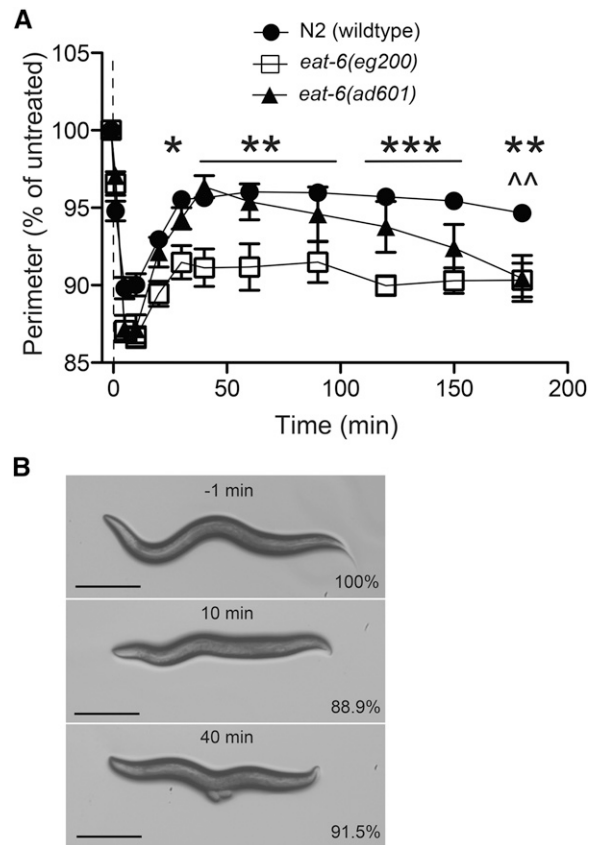
Figure 7 Two *eat-6* mutants show defects in the recovery from ethanol-induced hypercontraction. (A) N2 (wild type), *eat-6(eg200)*, and *eat-6(ad601)* contract to a maximum degree in the first 10 min. N2 (wild type) and *eat-6(ad601)* recover while *eat-6(eg200)* remains significantly smaller after 40 min on 400 mM ethanol. *eat-6(ad601)* fails to maintain the recovery of contraction state; at 180 min both *eat-6* alleles were significantly contracted compared to N2 (*eg200*, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; *ad601*, ^^$P < 0.01$; $n = 6$). (B) Images of the same *eat-6(eg200)* worm treated with 400 mM ethanol at −1, 10, and 40 min. Percentages indicate percentage of the untreated perimeter at −1 min. Bar, 250 μm.

Surprisingly, the two strong *eat-6* mutants, *ad467* and *ad997*, showed wild-type responses to EHC (Figure S4B). This result suggests that the strengths of the starvation and pharyngeal pumping mutant phenotypes are inversely related to the strength of the EHC phenotype. The same inverse relationship has been reported for sensitivity to the acetylcholinesterase inhibitor, aldicarb; *ad601* has been reported to be more sensitive to the paralytic effects of aldicarb than the stronger alleles that cause visible starvation (Doi and Iwasaki 2008; Govorunova *et al.* 2010). We examined each of the *eat-6* mutants for aldicarb sensitivity and found that *eg200* was significantly more sensitive than any of the three remaining *eat-6* alleles (Figure S4C). Under the conditions we used (1 mM aldicarb, no food), *ad601* did not appear more sensitive to aldicarb than *ad467* as has been reported (Doi and Iwasaki 2008; Govorunova *et al.* 2010), although in our assay all three mutations were more sensitive to aldicarb than wild-type animals (Figure S4C).

While the two mutants, *eg200* and *ad601*, do not share identical EHC phenotypes, their ethanol responses are consistent with the EAT-6 Na$^+$/K$^+$-ATPase playing a role in the development and maintenance of tolerance to EHC.

EHC is distinct from previously identified ethanol responses

Mutations in multiple genes have previously been shown to generate altered phenotypes compared to wild-type animals upon treatment with ethanol (Davies *et al.* 2003, 2004; Kayser *et al.* 2003; Kapfhamer *et al.* 2008; Graham *et al.* 2009; Speca *et al.* 2010; Bettinger *et al.* 2012; Bhandari *et al.* 2012; Jee *et al.* 2013; Raabe *et al.* 2014). We examined two of these genes that have robust mutant ethanol response phenotypes, *slo-1* and *npr-1*. Loss-of-function mutations in the BK potassium channel-encoding gene, *slo-1*, produce a significant level of resistance to the acute depressing effects of ethanol on locomotion speed and egg-laying frequency (Davies *et al.* 2003). We tested the null mutant *slo-1(eg142)* for its EHC response and found that *slo-1(eg142)* mutant animals have a wild-type level of EHC (Figure S3C). This suggests that the nAChR-mediated EHC effect is independent of the actions of ethanol that are mediated by SLO-1. Loss-of-function mutations in the neuropeptide Y receptor-like-encoding gene, *npr-1*, produce a more rapid development of acute functional tolerance to the depressing effects of ethanol on locomotion within an ethanol exposure session compared with wild-type animals (Davies *et al.* 2004). We tested whether the null mutant *npr-1(ky13)* would also alter the rate of development of tolerance to the EHC effect. As with the *slo-1* mutant, the *npr-1(ky13)* mutant animals displayed wild-type sensitivity and rate of recovery to the EHC effect (Figure S3D), suggesting that the tolerance to the effects of ethanol on locomotion is independent of the tolerance that develops to EHC.

Discussion

The excitatory effects of ethanol have been phenotypically characterized in several different species, yet the molecular mechanism by which ethanol acts as an excitatory drug remains uncharacterized (Gingras and Cools 1996; Addicott *et al.* 2007; Arias *et al.* 2009; Beckstead and Phillips 2009; Kong *et al.* 2010; Rose *et al.* 2013). Using the model organism *C. elegans*, we developed a hypercontraction assay to study both the excitatory effects of ethanol and the tolerance that develops to these effects. This assay describes an effect of ethanol that is distinct from previously identified ethanol effects in the worm. Using an *in vivo* genetic approach, we were able to identify cholinergic and GABAergic signaling as being involved in mediating this excitatory effect. We identified an unusual mutation in the *eat-6* Na$^+$/K$^+$-ATPase-encoding gene that prevented the development of acute functional tolerance to this effect.

Cholinergic signaling

Our data support a model in which exogenous administration of ethanol augments cholinergic signaling, causing a decrease in body length, presumably via an increase in the acetylcholine-mediated contraction of the body wall muscle. The nonselective and noncompetitive nAChR antagonist mecamylamine significantly blocked the EHC phenotype (Figure 2A), identifying a requirement for normal AChR function in this effect. Furthermore, animals carrying loss-of-function mutations in the genes encoding choline acetyltransferase *cha-1* and the vesicular acetylcholine transporter *unc-17*, which result in low levels of ACh release into the synaptic cleft (Rand 2007), showed significant resistance to the EHC effect (Figure 2, B and C). These data support the hypothesis that ethanol increases cholinergic signaling to bring about EHC and that the availability of ACh for synaptic release is important for the degree of EHC.

We favored a hypothesis in which ethanol acts through specific AChRs because previous research has linked specific mammalian AChR subunits with electrophysiological and behavioral effects of ethanol. *In vitro* studies have shown direct effects of ethanol on nAChR currents, including potentiating effects (Cardoso *et al.* 1999; Godden *et al.* 2001). Our data indicate that there is a requirement for wild-type ACh availability for EHC and are consistent with the hypothesis that ethanol potentiates normal ACh signaling to generate EHC. Consistent with our mecamylamine result, it has been shown that mecamylamine can inhibit the locomotor-stimulation effects of ethanol in mice (Kamens and Phillips 2008). Our data indicate that *unc-63*, which encodes a nAChR α-subunit, is necessary for wild-type levels of EHC (Figure 3A). A mutation in *unc-38*, a paralog of *unc-63*, trended toward resistance of the EHC phenotype (Figure 3B), indicating that these related α-subunits may both play a role in EHC. The genes identified as orthologs of *unc-63* and *unc-38* in the *Homo sapiens* genome are *CHRNA2* (α2), *CHRNA3* (α3), *CHRNA4* (α4), and *CHRNA6* (α6) (Shaye and Greenwald 2011). Electrophysiological activities of specific subunits (α2β2, α2β4, α4β2, and α4β4) are enhanced in the presence of ACh and ethanol when measured in *Xenopus* oocytes (Cardoso *et al.* 1999; Borghese *et al.* 2003). The use of receptor-specific antagonists suggests that α3β2- or β3-containing receptors are involved in ethanol-induced locomotor stimulation in mice (Jerlhag *et al.* 2006), whereas α4β2- and α6-containing receptors are not (Larsson *et al.* 2002; Jerlhag *et al.* 2006). The involvement of α3 in locomotor stimulation agrees with genetic mapping and expression studies that suggest a role for the nAChR α3-subunit-encoding gene *CHRNA3* in ethanol-induced locomotor stimulation in mice (Kamens *et al.* 2009). Behaviorally, heterozygotes containing an α3-knockout mutation were more sensitive to the depressant effects of ethanol than wild-type mice (Kamens *et al.* 2009), which suggests a possible loss of excitatory effects. Human genetics studies have found association with a cluster of three AChR-encoding genes that includes the α3-encoding gene and symptoms of alcohol abuse/dependence and with an acute level of response to alcohol (Joslyn *et al.* 2008; Chen *et al.* 2009; Sherva *et al.* 2010). That UNC-63 is considered to be the *C. elegans* ortholog of α3 suggests that

there may be significant conservation between the ethanol-induced stimulation effect in mice, alcohol abuse/dependence in humans, and the EHC effect in worms that we have described here. Therefore, our data provide further support for the mammalian studies implicating specific α-subunits of the nAChRs in ethanol-induced stimulatory effects and strongly support this *C. elegans* phenotype as a relevant model of ethanol-induced excitatory effects.

Our finding that other subunits required for the levamisole-sensitive nAChR (Fleming *et al.* 1997; Culetto *et al.* 2004) are not required for EHC suggests that UNC-63 is acting in a different receptor to mediate EHC. We tested the only additional receptor currently known to include the UNC-63 subunit, the neuronal ACR-2 receptor (Jospin *et al.* 2009; Petrash *et al.* 2013), and found that loss of that receptor, through loss of function in the *acr-2* gene, did not alter sensitivity to EHC. These results suggest that UNC-63 is likely to act in an as yet unidentified receptor(s) to mediate EHC.

GABA signaling in EHC

GABA signaling acts to oppose cholinergic signaling in coordinating body muscle contractions in *C. elegans*. We reasoned that if ethanol is acting through AChRs to mediate increased body muscle contraction, then loss of GABA signaling should enhance the effect of ethanol. Instead, we found the opposite result; a reduction in GABA synthesis and vesicular packaging produced via mutations in *unc-25* and *unc-47*, respectively, reduced the degree of contraction induced by ethanol. We confirmed that this result was not due to a preexisting level of hypercontraction in the *unc-25* and *unc-47* mutants that limited the degree to which they could contract further (Figure S2A). One hypothesis to explain this outcome is that reduced GABA production and release may lead to a compensatory response that down-regulates cholinergic signaling, which may then attenuate the degree to which ethanol can act through AChRs. If this is true, then the compensation must be specific in nature because it has been shown previously (Vashlishan *et al.* 2008) that GABAergic mutants, including *unc-25*, are actually more sensitive to aldicarb-induced paralysis, suggesting that with a reduction in GABA signaling, the body wall muscle is more sensitive to excitation rather than less sensitive as a compensatory model might predict. This suggests that there may be a more specific reduction in levels or activity of the UNC-63-containing receptor(s) in the *unc-25* and *unc-47* mutant backgrounds; such a reduction might not affect aldicarb sensitivity for muscle paralysis but would alter ethanol sensitivity. Also of interest are the differential effects on EHC of reduced GABA production and release in the *unc-25* and *unc-47* mutants compared with loss of either the major GABA$_A$ receptor UNC-49 or the two GABA$_B$ receptors GBB-1 and GBB-2. These results suggest the possibility that loss of both receptor types is required to mimic the reduction of GABA synthesis or, alternatively, that the impact of GABA on EHC is mediated by a third receptor type. The lack of effect of the loss of UNC-49, the major inhibitory receptor on the body muscle, suggests that the site of action for ethanol in producing EHC may not be directly on body muscle; instead an action on upstream neurons would better explain our observed data. We also found that *unc-30* mutants, which lack GABA synthesis and vesicular packaging in the type D neurons (McIntire *et al.* 1993a), show a wild-type EHC phenotype. This result implicates the remaining GABAergic neurons (AVL, DVB, RIS, and RMEs) that are affected by *unc-25* and *unc-47* mutations but are unaffected in the *unc-30* mutant animals. Of those neurons, AVL, DVB, and the four RMEs are primarily involved in defecation and in head foraging movement (McIntire *et al.* 1993b), and a GABAergic function associated with the RIS interneuron is yet to be identified. The mechanism by which decreased GABA synthesis and release reduces sensitivity to EHC remains to be identified.

eat-6 mutations affect tolerance to EHC

The *eat-6(eg200)* mutant demonstrates a prolonged response to EHC. The continued hypercontraction in the presence of ethanol by *eat-6(eg200)* and the return to hypercontraction following an initial recovery that is shown by the *eat-6(ad601)* mutant highlight the requirement for EAT-6 function in the acute functional tolerance that is occurring in wild-type animals to this effect of ethanol during the period of ethanol exposure.

The *eat-6* gene encodes the α-subunit of the Na$^+$/K$^+$-ATPase (Davis *et al.* 1995), which pumps three sodium ions out of and two potassium ions into the cell. As a result, maintenance of a negative membrane potential is achieved as dictated by the Goldman equation (Hille 2001). Loss of function of this protein would be predicted to result in a depolarized and hyperexcitable cell (Davis *et al.* 1995). One possibility to explain the prolonged EHC phenotype in the *eat-6(eg200)* mutant animals is that *eg200* is a loss-of-function allele that leads to a hyperexcitable state in cells activated by ethanol and, in combination with potentiation of cholinergic signaling by ethanol, the affected cells cannot compensate for the strong hyperactivation to achieve a homeostatic recovery. The strong levamisole and aldicarb hypersensitivity phenotypes associated with *eg200* support the model that *eg200* mutants are particularly sensitive to cholinergic activation. However, the idea of *eg200* as a straightforward loss-of-function mutation is not supported by the EHC or aldicarb phenotypes displayed by other alleles of *eat-6*, some of which have stronger effects on pharyngeal pumping (Davis *et al.* 1995).

eat-6 mutants have been described as having several phenotypes, including defects in pharyngeal pumping, and altered sensitivities to aldicarb, levamisole, nicotine, and serotonin (Avery 1993; Davis *et al.* 1995; Doi and Iwasaki 2008; Govorunova *et al.* 2010). Different alleles of *eat-6* have different constellations of phenotypes, suggesting that they do not fall into a simple allelic series representing an increasing degree of loss of function; instead different alleles

have different strengths of effect on a variety of different neuromuscular phenotypes, to which we have added the development of tolerance to EHC (Table S3). The *eg200* and *ad601* mutations are both weak alleles for effects on starvation, and both produce hypersensitivity to levamisole, but they differ in the time course for their effect on the development of tolerance to EHC. The *ad467* and *ad997* mutations have strong effects on pharyngeal pumping and feeding, but have no detectable effect on EHC. All alleles show hypersensitivity to aldicarb-induced paralysis in our assay but *eg200* shows the greatest level of hypersensitivity. These observations combine to suggest that the *eat-6* mutation is unusual in comparison with other isolated *eat-6* mutations.

An alternative model to explain the effect of the *eg200* mutation is that tolerance to the EHC effect may require regulation of Na^+/K^+-ATPase function, and the *eg200* mutation specifically affects the ability of the Na^+/K^+-ATPase to make such changes. The *eg200* mutation is predicted to result in glycine to glutamic acid substitution at residue 77. The role of the equivalent conserved amino acid, Gly94, in the rat Na^+/K^+-ATPase has been investigated (Einholm *et al.* 2005). Substitution of Gly94 by Ala94 in the M1 transmembrane region interferes with binding of both Na^+ and K^+ by the enzyme. The results were particularly striking for the E_2P conformational state of the enzyme, which had a ninefold reduced affinity for the K^+ cation, resulting in a dysfunctional transporter (Einholm *et al.* 2005). The importance of that specific amino acid in enzyme function, particularly in certain conformations, may be significant in the development of tolerance to EHC.

One other possibility for the role of EAT-6 in altering cholinergic signaling and the sensitivity to, or recovery from, EHC is that EAT-6 has been found to act in an allele-specific and pump-independent manner to regulate nAChR localization at the postsynaptic membrane of the NMJ (Doi and Iwasaki 2008). The *ad601* mutation was found to have the strongest effect on nAChR localization (Doi and Iwasaki 2008); we found that when exposed to ethanol, *ad601* had a delayed return to a hypercontracted state following an initial wild-type-like recovery. The possibility that the number of UNC-63-containing receptors could be increased postsynaptically in *eat-6(eg200)* mutant animals might also account for a prolonged EHC effect, particularly if the regulation of the localization of those receptors was abnormal. The observation of increased levamisole and aldicarb sensitivity in the *eat-6(eg200)* mutant would also be consistent with *eg200* acting in a similar manner to *ad601* for its effects on the levamisole-sensitive receptor at the NMJ. The impact of *eat-6(eg200)* on the unknown ethanol-sensitive receptor(s) containing UNC-63 is worthy of future examination.

Conclusion

We identified an excitatory effect of ethanol that we have termed EHC in the model organism *C. elegans* and developed a novel software-assisted assay method to aid in data analysis. We used both genetic and pharmacological approaches to determine the molecular mechanisms involved in this ethanol-induced effect. With this *in vivo* model we found that the excitatory phenotype is cholinergic and functions through the UNC-63 α-nAChR subunit, which is orthologous to the mammalian α2, α3, α4, and α6 nAChR subunits. This excitation effect of ethanol characterizes a property of an unidentified receptor type because known receptors that include the UNC-63 subunit were not required for this effect. Furthermore, we show that levels of GABA release alter the degree of EHC; however, the mechanism involved does not appear to involve the well-characterized *C. elegans* $GABA_A$ or $GABA_B$ receptors. In addition to the acute effect of ethanol on excitation, we show that acute functional tolerance develops rapidly to the effect. We identify a missense mutation of *eat-6* that alters a conserved amino acid in the Na^+/K^+-ATPase as having the effect of eliminating the development of tolerance to the ethanol effect, causing a prolonged hypercontracted phenotype. This suggests that the Na^+/K^+-ATPase plays a role in the development of tolerance to this ethanol effect. Genes previously shown to affect acute sensitivity and the development of acute functional tolerance to the depressive effect of ethanol on locomotion speed, *slo-1* and *npr-1*, respectively, are not involved in the excitatory mechanism. Our data indicate that the mechanisms of ethanol-induced excitation and depression and the mechanisms of tolerance to these effects are different from each other.

Literature Cited

Addicott, M. A., D. M. Marsh-Richard, C. W. Mathias, and D. M. Dougherty, 2007 The biphasic effects of alcohol: comparisons of subjective and objective measures of stimulation, sedation, and physical activity. Alcohol. Clin. Exp. Res. 31: 1883–1890.

Alaimo, J. T., S. J. Davis, S. S. Song, C. R. Burnette, M. Grotewiel *et al.*, 2012 Ethanol metabolism and osmolarity modify behavioral responses to ethanol in *C. elegans*. Alcohol. Clin. Exp. Res. 36: 1840–1850.

Alfonso, A., K. Grundahl, J. S. Duerr, H. P. Han, and J. B. Rand, 1993 The *Caenorhabditis elegans unc-17* gene: a putative vesicular acetylcholine transporter. Science 261: 617–619.

Arias, C., E. C. Mlewski, J. C. Molina, and N. E. Spear, 2009 Ethanol induces locomotor activating effects in preweanling Sprague-Dawley rats. Alcohol 43: 13–23.

Artal-Sanz, M., and L. de Jong, and N. Tavernarakis, 2006 *Caenorhabditis elegans*: a versatile platform for drug discovery. Biotechnol. J. 1: 1405–1418.

Avery, L., 1993 The genetics of feeding in *Caenorhabditis elegans*. Genetics 133: 897–917.

Bates, R. G., M. Paabo, and R. A. Robinson, 1963 Interpretation of pH measurements in alcohol-water solvents. J. Phys. Chem. 67: 1833–1838.

Beckstead, M. J., and T. J. Phillips, 2009 Mice selectively bred for high- or low-alcohol-induced locomotion exhibit differences in dopamine neuron function. J. Pharmacol. Exp. Ther. 329: 342–349.

Bettinger, J. C., K. Leung, M. H. Bolling, A. D. Goldsmith, and A. G. Davies, 2012 Lipid environment modulates the development of acute tolerance to ethanol in *Caenorhabditis elegans*. PLoS ONE 7: e35192.

Bhandari, P., J. S. Hill, S. P. Farris, B. Costin, I. Martin *et al.*, 2012 Chloride intracellular channels modulate acute ethanol behaviors in *Drosophila*, *Caenorhabditis elegans* and mice. Genes Brain Behav. 11: 387–397.

Borghese, C. M., L. A. Henderson, V. Bleck, J. R. Trudell, and R. A. Harris, 2003 Sites of excitatory and inhibitory actions of alcohols on neuronal alpha2beta4 nicotinic acetylcholine receptors. J. Pharmacol. Exp. Ther. 307: 42–52.

Brown, L. A., A. K. Jones, S. D. Buckingham, C. J. Mee, and D. B. Sattelle, 2006 Contributions from *Caenorhabditis elegans* functional genetics to antiparasitic drug target identification and validation: nicotinic acetylcholine receptors, a case study. Int. J. Parasitol. 36: 617–624.

Cardoso, R. A., S. J. Brozowski, L. E. Chavez-Noriega, M. Harpold, C. F. Valenzuela *et al.*, 1999 Effects of ethanol on recombinant human neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. J. Pharmacol. Exp. Ther. 289: 774–780.

Chen, X., J. Chen, V. S. Williamson, S. S. An, J. M. Hettema *et al.*, 2009 Variants in nicotinic acetylcholine receptors alpha5 and alpha3 increase risks to nicotine dependence. Am. J. Med. Genet. B Neuropsychiatr. Genet. 150B: 926–933.

Choe, K. P., and K. Strange, 2007 Evolutionarily conserved WNK and Ste20 kinases are essential for acute volume recovery and survival after hypertonic shrinkage in *Caenorhabditis elegans*. Am. J. Physiol. Cell Physiol. 293: C915–C927.

Culetto, E., H. A. Baylis, J. E. Richmond, A. K. Jones, J. T. Fleming *et al.*, 2004 The *Caenorhabditis elegans* unc-63 gene encodes a levamisole-sensitive nicotinic acetylcholine receptor alpha subunit. J. Biol. Chem. 279: 42476–42483.

Davies, A. G., J. T. Pierce-Shimomura, H. Kim, M. K. Vanhoven, T. R. Thiele *et al.*, 2003 A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. Cell 115: 655–666.

Davies, A. G., J. C. Bettinger, T. R. Thiele, M. E. Judy, and S. L. McIntire, 2004 Natural variation in the *npr-1* gene modifies ethanol responses of wild strains of *C. elegans*. Neuron 42: 731–743.

Davies, A. G., R. I. Friedberg, H. Gupta, C.-L. Chan, K. L. Shelton *et al.*, 2012 Different genes influence toluene- and ethanol-induced locomotor impairment in *C. elegans*. Drug Alcohol Depend. 122: 47–54.

Davis, M. W., D. Somerville, R. Y. Lee, S. Lockery, L. Avery *et al.*, 1995 Mutations in the *Caenorhabditis elegans* Na,K-ATPase alpha-subunit gene, *eat-6*, disrupt excitable cell function. J. Neurosci. 15: 8408–8418.

Davis, M. W., M. Hammarlund, T. Harrach, P. Hullett, S. Olsen *et al.*, 2005 Rapid single nucleotide polymorphism mapping in *C. elegans*. BMC Genomics 6: 118.

Doi, M., and K. Iwasaki, 2008 Na^+/K^+ ATPase regulates the expression and localization of acetylcholine receptors in a pump activity-independent manner. Mol. Cell. Neurosci. 38: 548–558.

Eastman, C., H. R. Horvitz, and Y. Jin, 1999 Coordinated transcriptional regulation of the *unc-25* glutamic acid decarboxylase and the *unc-47* GABA vesicular transporter by the *Caenorhabditis elegans* UNC-30 homeodomain protein. J. Neurosci. 19: 6225–6234.

Einholm, A. P., M. Toustrup-Jensen, J. P. Andersen, and B. Vilsen, 2005 Mutation of Gly-94 in transmembrane segment M1 of Na^+,K^+-ATPase interferes with Na^+ and K^+ binding in E_2P conformation. Proc. Natl. Acad. Sci. USA 102: 11254–11259.

Fleming, J. T., M. D. Squire, T. M. Barnes, C. Tornoe, K. Matsuda *et al.*, 1997 *Caenorhabditis elegans* levamisole resistance genes *lev-1*, *unc-29*, and *unc-38* encode functional nicotinic acetylcholine receptor subunits. J. Neurosci. 17: 5843–5857.

Gingras, M. A., and A. R. Cools, 1996 Analysis of the biphasic locomotor response to ethanol in high and low responders to novelty: a study in Nijmegen Wistar rats. Psychopharmacology 125: 258–264.

Godden, E. L., R. A. Harris, and T. V. Dunwiddie, 2001 Correlation between molecular volume and effects of *n*-alcohols on human neuronal nicotinic acetylcholine receptors expressed in *Xenopus* oocytes. J. Pharmacol. Exp. Ther. 296: 716–722.

Govorunova, E. G., M. Moussaif, A. Kullyev, K. C. Nguyen, T. V. Mcdonald *et al.*, 2010 A homolog of FHM2 is involved in modulation of excitatory neurotransmission by serotonin in *C. elegans*. PLoS ONE 5: e10368.

Graham, M. E., M. R. Edwards, L. Holden-Dye, A. Morgan, R. D. Burgoyne *et al.*, 2009 UNC-18 modulates ethanol sensitivity in *Caenorhabditis elegans*. Mol. Biol. Cell 20: 43–55.

Harris, R. A., J. R. Trudell, and S. J. Mihic, 2008 Ethanol's molecular targets. Sci. Signal. 1: re7.

Heath, A., P. Madden, K. Bucholz, S. Dinwiddie, W. Slutske *et al.*, 1999 Genetic differences in alcohol sensitivity and the inheritance of alcoholism risk. Psychol. Med. 29: 1069–1081.

Hille, B., 2001 *Ion Channels of Excitable Membranes*. Sinauer Associates, Sunderland, MA.

Holstein, S. E., L. Dobbs, and T. J. Phillips, 2009 Attenuation of the stimulant response to ethanol is associated with enhanced ataxia for a $GABA_A$, but not a $GABA_B$, receptor agonist. Alcohol. Clin. Exp. Res. 33: 108–120.

Hsu, D. R., and B. J. Meyer, 1994 The *dpy-30* gene encodes an essential component of the *Caenorhabditis elegans* dosage compensation machinery. Genetics 137: 999–1018.

Hu, Z., E. C. Pym, K. Babu, A. B. Vashlishan Murray, and J. M. Kaplan, 2011 A neuropeptide-mediated stretch response links muscle contraction to changes in neurotransmitter release. Neuron 71: 92–102.

Huang, X., H. J. Cheng, M. Tessier-Lavigne, and Y. Jin, 2002 MAX-1, a novel PH/MyTH4/FERM domain cytoplasmic protein implicated in netrin-mediated axon repulsion. Neuron 34: 563–576.

Jee, C., J. Lee, J. P. Lim, D. Parry, R. O. Messing *et al.*, 2013 SEB-3, a CRF receptor-like GPCR, regulates locomotor activity states, stress responses and ethanol tolerance in *Caenorhabditis elegans*. Genes Brain Behav. 12: 250–262.

Jerlhag, E., M. Grotli, K. Luthman, L. Svensson, and J. A. Engel, 2006 Role of the subunit composition of central nicotinic acetylcholine receptors for the stimulatory and dopamine-enhancing effects of ethanol. Alcohol Alcohol. 41: 486–493.

Jones, A. K., P. Davis, J. Hodgkin, and D. B. Sattelle, 2007 The nicotinic acetylcholine receptor gene family of the nematode *Caenorhabditis elegans*: an update on nomenclature. Invert. Neurosci. 7: 129–131.

Joslyn, G., G. Brush, M. Robertson, T. L. Smith, J. Kalmijn *et al.*, 2008 Chromosome 15q25.1 genetic markers associated with level of response to alcohol in humans. Proc. Natl. Acad. Sci. USA 105: 20368–20373.

Jospin, M., Y. B. Qi, T. M. Stawicki, T. Boulin, K. R. Schuske et al., 2009   A neuronal acetylcholine receptor regulates the balance of muscle excitation and inhibition in Caenorhabditis elegans. PLoS Biol. 7: e1000265.

Kaletta, T., and M. O. Hengartner, 2006   Finding function in novel targets: C. elegans as a model organism. Nat. Rev. Drug Discov. 5: 387–398.

Kalu, N., and V. M. Ramchandani, V. Marshall, D. Scott, C. Ferguson et al., 2012   Heritability of level of response and association with recent drinking history in nonalcohol-dependent drinkers. Alcohol. Clin. Exp. Res. 36: 522–529.

Kamens, H. M., and T. J. Phillips, 2008   A role for neuronal nicotinic acetylcholine receptors in ethanol-induced stimulation, but not cocaine- or methamphetamine-induced stimulation. Psychopharmacology 196: 377–387.

Kamens, H. M., C. S. Mckinnon, N. Li, M. L. Helms, J. K. Belknap et al., 2009   The alpha 3 subunit gene of the nicotinic acetylcholine receptor is a candidate gene for ethanol stimulation. Genes Brain Behav. 8: 600–609.

Kapfhamer, D., J. C. Bettinger, A. G. Davies, C. L. Eastman, E. A. Smail et al., 2008   Loss of RAB-3/A in Caenorhabditis elegans and the mouse affects behavioral response to ethanol. Genes Brain Behav. 7: 669–676.

Kass, J., T. C. Jacob, P. Kim, and J. M. Kaplan, 2001   The EGL-3 proprotein convertase regulates mechanosensory responses of Caenorhabditis elegans. J. Neurosci. 21: 9265–9272.

Kayser, E. B., C. L. Hoppel, P. G. Morgan, and M. M. Sedensky, 2003   A mutation in mitochondrial complex I increases ethanol sensitivity in Caenorhabditis elegans. Alcohol. Clin. Exp. Res. 27: 584–592.

Khattab, I. S., F. Bandarkar, M. A. A. Fakhree, and A. Jouyban, 2012   Density, viscosity, and surface tension of water+ethanol mixtures from 293 to 323K. Korean J. Chem. Eng. 29: 812–817.

Kong, E. C., K. Woo, H. Li, T. Lebestky, N. Mayer et al., 2010   A pair of dopamine neurons target the D1-like dopamine receptor DopR in the central complex to promote ethanol-stimulated locomotion in Drosophila. PLoS ONE 5: e9954.

Kruse, L. C., D. N. Linsenbardt, and S. L. Boehm, 2nd, 2012   Positive allosteric modulation of the GABA$_B$ receptor by GS39783 attenuates the locomotor stimulant actions of ethanol and potentiates the induction of locomotor sensitization. Alcohol 46: 455–462.

Kwok, T. C., N. Ricker, R. Fraser, A. W. Chan, A. Burns et al., 2006   A small-molecule screen in C. elegans yields a new calcium channel antagonist. Nature 441: 91–95.

Larsson, A., L. Svensson, B. Soderpalm, and J. A. Engel, 2002   Role of different nicotinic acetylcholine receptors in mediating behavioral and neurochemical effects of ethanol in mice. Alcohol 28: 157–167.

Lewis, J. A., C. H. Wu, H. Berg, and J. H. Levine, 1980   The genetics of levamisole resistance in the nematode Caenorhabditis elegans. Genetics 95: 905–928.

Li, C., and K. Kim, 2008   Neuropeptides (April 7, 2008), WormBook, ed. The C. elegans Research Community, WormBook, doi:10.1895/wormbook.1.142.1, http://www.wormbook.org.

Mahoney, T. R., S. Luo, and M. L. Nonet, 2006   Analysis of synaptic transmission in Caenorhabditis elegans using an aldicarb-sensitivity assay. Nat. Protoc. 1: 1772–1777.

McIntire, S. L., E. M. Jorgensen, and H. R. Horvitz, 1993a   Genes required for GABA function in Caenorhabditis elegans. Nature 364: 334–337.

McIntire, S. L., E. M. Jorgensen, J. M. Kaplan, and H. R. Horvitz, 1993b   The GABAergic nervous system of Caenorhabditis elegans. Nature 364: 337–341.

Mitchell, P., R. Mould, J. Dillon, S. Glautier, I. Andrianakis et al., 2010   A differential role for neuropeptides in acute and chronic adaptive responses to alcohol: behavioural and genetic analysis in Caenorhabditis elegans. PLoS ONE 5: e10422.

Mitchell, P. H., K. Bull, S. Glautier, N. A. Hopper, L. Holden-Dye et al., 2007   The concentration-dependent effects of ethanol on Caenorhabditis elegans behaviour. Pharmacogenomics J. 7: 411–417.

Newlin, D. B., and J. B. Thomson, 1990   Alcohol challenge with sons of alcoholics: a critical review and analysis. Psychol. Bull. 108: 383–402.

Nurrish, S., L. Segalat, and J. M. Kaplan, 1999   Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. Neuron 24: 231–242.

Olsen, R., G. Li, M. Wallner, J. Trudell, E. Bertaccini et al., 2014   Structural models of ligand-gated ion channels: sites of action for anesthetics and ethanol. Alcohol. Clin. Exp. Res. 38: 595–603.

Petrash, H. A., A. Philbrook, M. Haburcak, B. Barbagallo, and M. M. Francis, 2013   ACR-12 ionotropic acetylcholine receptor complexes regulate inhibitory motor neuron activity in Caenorhabditis elegans. J. Neurosci. 33: 5524–5532.

Phillips, T. J., and E. H. Shen, 1996   Neurochemical bases of locomotion and ethanol stimulant effects. Int. Rev. Neurobiol. 39: 243–282.

Ponomarev, I., and J. C. Crabbe, 2002   Ethanol-induced activation and rapid development of tolerance may have some underlying genes in common. Genes Brain Behav. 1: 82–87.

Raabe, R., L. Mathies, A. Davies, and J. Bettinger, 2014   The omega-3 fatty acid eicosapentaenoic acid is required for normal alcohol response behaviors in C. elegans. PLoS ONE 9: e105999.

Rand, J. B., 2007   Acetylcholine (January 30, 2007), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.131.1, http://www.wormbook.org.

Rand, J. B., and R. L. Russell, 1984   Choline acetyltransferase-deficient mutants of the nematode Caenorhabditis elegans. Genetics 106: 227–248.

Richmond, J. E., and E. M. Jorgensen, 1999   One GABA and two acetylcholine receptors function at the C. elegans neuromuscular junction. Nat. Neurosci. 2: 791–797.

Rose, J. H., E. S. Calipari, T. A. Mathews, and S. R. Jones, 2013   Greater ethanol-induced locomotor activation in DBA/2J vs. C57BL/6J mice is not predicted by presynaptic striatal dopamine dynamics. PLoS ONE 8: e83852.

Schuckit, M. A., 1994   Low level of response to alcohol as a predictor of future alcoholism. Am. J. Psychiatry 151: 184–189.

Shaye, D., and I. Greenwald, 2011   OrthoList: a compendium of C. elegans genes with human orthologs. PLoS ONE 6: e20085.

Sherva, R., H. R. Kranzler, Y. Yu, M. W. Logue, J. Poling et al., 2010   Variation in nicotinic acetylcholine receptor genes is associated with multiple substance dependence phenotypes. Neuropsychopharmacology 35: 1921–1931.

Soderpalm, B., M. Ericson, P. Olausson, O. Blomqvist, and J. A. Engel, 2000   Nicotinic mechanisms involved in the dopamine activating and reinforcing properties of ethanol. Behav. Brain Res. 113: 85–96.

Spanagel, R., 2009   Alcoholism: a systems approach from molecular physiology to addictive behavior. Physiol. Rev. 89: 649–705.

Speca, D. J., D. Chihara, A. M. Ashique, M. S. Bowers, J. T. Pierce-Shimomura et al., 2010   Conserved role of unc-79 in ethanol responses in lightweight mutant mice. PLoS Genet. 6: e1001057.

Speese, S., M. Petrie, K. Schuske, M. Ailion, K. Ann et al., 2007   UNC-31 (CAPS) is required for dense-core vesicle but not synaptic vesicle exocytosis in Caenorhabditis elegans. J. Neurosci. 27: 6150–6162.

Swan, K. A., D. E. Curtis, K. B. Mckusick, A. V. Voinov, F. A. Mapa et al., 2002   High-throughput gene mapping in Caenorhabditis elegans. Genome Res. 12: 1100–1105.

Topper, S., S. Aguilar, V. Topper, E. Elbel, and J. T. Pierce-Shimomura, 2014   Alcohol disinhibition of behaviors in *C. elegans*. PLoS ONE 9: e92965.

Touroutine, D., R. M. Fox, and S. E. Von Stetina, A. Burdina, D. M. Miller, 3rd *et al.*, 2005   *acr-16* encodes an essential subunit of the levamisole-resistant nicotinic receptor at the *Caenorhabditis elegans* neuromuscular junction. J. Biol. Chem. 280: 27013–27021.

Towers, P. R., B. Edwards, J. E. Richmond, and D. B. Sattelle, 2005   The *Caenorhabditis elegans lev-8* gene encodes a novel type of nicotinic acetylcholine receptor alpha subunit. J. Neurochem. 93: 1–9.

Tuck, S., and I. Greenwald, 1995   *lin-25*, a gene required for vulval induction in *Caenorhabditis elegans*. Genes Dev. 9: 341–357.

Van Swinderen, B., O. Saifee, L. Shebester, R. Roberson, M. L. Nonet *et al.*, 1999   A neomorphic syntaxin mutation blocks volatile-anesthetic action in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 96: 2479–2484.

Vashlishan, A. B., J. M. Madison, M. Dybbs, J. Bai, D. Sieburth *et al.*, 2008   An RNAi screen identifies genes that regulate GABA synapses. Neuron 58: 346–361.

Volavka, J., P. Czobor, D. W. Goodwin, W. F. Gabrielli, Jr., E. C. Penick *et al.*, 1996   The electroencephalogram after alcohol administration in high-risk men and the development of alcohol use disorders 10 years later. Arch. Gen. Psychiatry 53: 258–263.

Wicks, S. R., R. T. Yeh, W. R. Gish, R. H. Waterston, and R. H. Plasterk, 2001   Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. Nat. Genet. 28: 160–164.

Communicating editor: D. I. Greenstein

GENETICS

A Novel Cholinergic Action of Alcohol and the Development of Tolerance to That Effect in *Caenorhabditis elegans*

Edward G. Hawkins, Ian Martin, Lindsay M. Kondo, Meredith E. Judy, Victoria E. Brings, Chung-Lung Chan, GinaMari G. Blackwell, Jill C. Bettinger, and Andrew G. Davies

SUPPLEMENTAL MATERIALS AND METHODS

RNAi induction: Knock-down of function using RNAi was performed as previously described (Ahringer, 2006; Choe and Strange, 2007; Alaimo *et al.*, 2012). Worms were fed *E. coli* that transcribe and generate double stranded RNA (dsRNA). *E. coli* strains containing the control empty vector plasmid (L4440) and the *wnk-1* dsRNA-expressing plasmid were obtained from the Ahringer library developed at the University of Cambridge (Geneservice, Cambridge, UK). NGM plates made with 1mM isopropyl β–D–1–thiogalactopyranoside (IPTG) and 25µg/mL ampicillin were seeded with the bacteria, which was allowed to grow at room temperature for 24 hrs. Six adult *eri-1(mg363)* were allowed to lay eggs on the plates for 36–40 hours and adults from this first generation were moved to a second RNAi bacteria-containing plate and allowed to lay eggs for 2 hours. Adult worms from the second generation were used for experiments.

Hypertonic Stress: The assay was performed as previously described (Choe and Strange, 2007). Hypertonic stress was achieved by adding NaCl (349 mM) to RNAi plates. RNAi plates without added NaCl were used as control plates. Hypertonic stress-induced hypercontraction was assessed at the following time points -1, 1, 30, 60, 120, 180, 240 and 300 minutes as described for ethanol-induced hypercontraction.

Vapor pressure: The nematodes and plates were prepared as described for the hypercontraction assay. The assay was performed in a sealed container with a volume of 1.6 L as previously described (Davies *et al.*, 2012). A picture at the -1 untreated time point was taken before the worms were moved to the testing plate. A 100 ml beaker was placed into the chamber containing no solution, 60 ml of deionized water, or 60 ml of 400 mM ethanol in water. For the positive control, a 400 mM ethanol plate was prepared as described for the hypercontraction assay. The testing plates were placed in the chamber for each testing condition, without a plate lid, and the chamber was sealed for the duration of the experiment. Pictures were taken and analyzed as described for the hypercontraction assay.

Pharyngeal pumping: Pharyngeal pumping was measured as previously described (Raizen *et al.*, 1995). Ten young adult hermaphrodites were transferred to a plate containing OP50 (grown for 16 hours at room temperature). For all strains, videos were taken using a Leica MZFLIII dissecting microscope (Leica Microsystems, Buffalo Grove, IL, USA) with a 10X objective and 10X magnification and a QImaging Retiga 4000R camera (QImaging, Surrey, BC, Canada) and analyzed using ImagePro Plus Version 6 (Media Cybernetics, Bethesda, MD, USA). Movies were played at 1 / 5 speed in order to clearly count a backward movement of the grinder. Data represented as mean ± SEM.

LITERATURE CITED

Ahringer, J., 2006 Reverse genetics, doi/10.1895/wormbook.1.47.1, http://www.wormbook.org in *WormBook*, edited by The *C. elegans* Research Community.

Alaimo, J. T., S. J. Davis, S. S. Song, C. R. Burnette, M. Grotewiel *et al.*, 2012 Ethanol metabolism and osmolarity modify behavioral responses to ethanol in *C. elegans*. Alcohol. Clin. Exp. Res. 36: 1840-1850.

Choe, K. P., and K. Strange, 2007 Evolutionarily conserved WNK and Ste20 kinases are essential for acute volume recovery and survival after hypertonic shrinkage in *Caenorhabditis elegans*. Am. J. Physiol. Cell. Physiol. 293: C915-27.

Davies, A. G., R. I. Friedberg, H. Gupta, C. -L. Chan, K. L. Shelton *et al.*, 2012 Different genes influence toluene- and ethanol-induced locomotor impairment in *C. elegans*. Drug Alcohol Depend. 122: 47-54.

Raizen, D. M., R. Y. Lee, and L. Avery, 1995 Interacting genes required for pharyngeal excitation by motor neuron MC in *Caenorhabditis elegans*. Genetics 141: 1365–1382.

Table M1 Deficiency mapping primers

PCR product	Matching cosmid	Nucleotide positions covered	Forward (F) and reverse (R) primers
i	C44H9	12,835,742– 12,836,025	F: 5'-TTTTTCCACCATCAGAACCC-3' R: 5'-TTTTCGGTTGTAGGTGTGCT-3'
ii	T16G1	12,935,045– 12,935,280	F: 5'-ATCTGAAAGCGACAAATGGC-3' R: 5'-CAGGGAAAACTAATCCTTTATACCC-3'
iii	F42E8	13,051,106– 13,051,268	F: 5'-TGCAAGTGCAGAGGCAATAC-3' R: 5'-CCATCAGGTTGTAAGCCGTT-3'
iv	B0365	13,120,346– 13,120,584	F: 5'-GGATTTTTGACGGAATCGTG-3' R: 5'-GGAGATGTGAACGAAGGGAA-3'
v	C26E1	13,293,639– 13,293,889	F: 5'-TTTGGGCTGATGTTAAACCC-3' R: 5'-AATGGCTTCGAGGCTAGTGA-3'
vi	C50B6	13,311,219– 13,311,453	F: 5'-TCTTCGACACCCTCCATCTC-3' R: 5'-AAGACGAGCGAGCTGAAATC-3'
vii	M04G12	13,370,458– 13,370,703	F: 5'-AGTGCAGCTTTGAACGGTTT-3' R: 5'-GTTGCATGCCCCTAACATCT-3'
viii	F53F4	13,628,573– 13,628,781	F: 5'-GAAGCTGGTTTCGCATTGA-3'T R: 5'-TCGCTTTCATCATTGATTCG-3'

Table M2 *eat-6* sequencing primers

Forward (F) and Reverse (R)	
*eat-6-F1	5'-GTATAGGACGGGACTTTCTGTCGACTTG-3'
eat-6-F2	5'-CGACGACAGAAAGTAGCATCAC-3'
eat-6-F3	5'-GGTCTTGGAGATGATCAAATC-3'
eat-6-F4	5'-GTTCCGAGATCGATACAAAG-3'
eat-6-F5	5'-GATGATCTCGGCAAGCTGATC-3'
eat-6-F6	5'-CTTCGCGAAGTTTGTCACTAAG-3'
eat-6-F7	5'-GGTTTGAGTTTCGGTGGTATC-3'
eat-6-F8	5'-CGGTGTTATCTCCAGTGTAAATG-3'
eat-6-R1	5'-CCAACAGAAAGATCCTCGAATAC-3'
eat-6-R2	5'-TCTCTCCATTCTTGACCTAC-3'
eat-6-R3	5'-TTCCAGTCGAAGAAGTCAAC-3'
eat-6-R4	5'-ATGAAGGGTGCTCCAGAAAG-3'
eat-6-R5	5'-GGAACTCTCACCCAAAACAG-3'
eat-6-R6	5'-CCCGTAACATCGCTTTCTTC-3'
eat-6-R7	5'-GGATTTGCCATGCTTCTTTG-3'
eat-6-R8	5'-CCGCTGTGCATGTATCTTTAC-3'
*eat-6-R9	5'-GGCAGGCAACACGAACAATCTTTTG-3'

* Primers used to generate fragment for sequencing

Table S1 Ethanol dose response for effects on perimeter length

Ethanol (mM)	Untreated perimeter length (μm)	% of untreated 10 min	% of untreated 30 min
0	2308.6 ± 25.6	101.2 ± 0.5	100.0 ± 0.6
100	2368.2 ± 13.4	98.5 ± 0.7	98.4 ± 1.0
200	2339.1 ± 42.3	98.3 ± 0.7	99.3 ± 0.7
300	2274.4 ± 32.0	96.4 ± 0.8*	99.3 ± 0.7
400	2282.1 ± 19.1	93.3 ± 0.7***	97.3 ± 0.7
500	2386.0 ± 38.0	89.5 ± 0.9***	96.4 ± 0.8
600[a]	2361.2 ± 20.2	88.6 ± 0.7	95.5 ± 0.7
700[b]	2361.1 ± 26.2	86.6 ± 0.3	92.5 ± 0.2

*, $p < 0.05$, ***, $p < 0.001$ compared with 0 mM

[a, b] Statistics were not performed because worms coiled at these ethanol concentrations resulting in only 20–50% of worms being measured.

Table S2 Mutant alleles tested for ethanol-induced hypercontraction

| Gene (allele)	Untreated perimeter (µm)		Perimeter (% of untreated)			
	N2 (WT)	Mutant	WT 10'	Mutant 10'	WT 40'	Mutant 40'
cha-1(p1152)	2352.5 ± 19.7	1477.6 ± 10.5	89.4 ± 0.7	95.7 ± 0.7***	96.8 ± 0.9	98.4 ± 0.7
unc-17(e245)	2366.5 ± 9.2	1616.4 ± 0.8	91.7 ± 0.8	98.8 ± 0.7***	97.0 ± 1.2	105.3 ± 0.8***
unc-17(e113)	2370.4 ± 9.0	1949.1 ± 14.3	90.5 ± 0.6	95.4 ± 0.7**	95.4 ± 0.7	101.9 ± 0.8***
acr-16(ok789)	2273.4 ± 18.5	2191.3 ± 23.5	92.5 ± 0.7	92.6 ± 1.2	96.5 ± 0.9	97.4 ± 0.8
acr-2(ok1887)	2283.2 ± 46.3	2293.4 ± 15.2	90.9 ± 0.8	88.5 ± 1.0	97.2 ± 0.7	96.7 ± 0.5
unc-38(e264)	2260.4 ± 0.9	1920.0 ± 42.9	91.3 ± 0.9	94.1 ± 0.7	97.1 ± 1.1	98.7 ± 1.0
unc-29 (e193)	2312.2 ± 20.5	2018.8 ± 17.5	92.4 ± 0.7	93.7 ± 0.7	96.8 ± 0.6	98.8 ± 0.6
lev-1(e211)	2196.7 ± 21.9	2106.0 ± 23.8	92.6 ± 0.6	93.1 ± 0.6	95.9 ± 0.8	97.1 ± 0.7
unc-63(x13)[a]	2273.1 ± 17.0	2133.7 ± 43.3	89.4 ± 0.6	94.3 ± 0.9***	95.9 ± 0.8	97.2 ± 1.0
unc-63(gk234)[a]	2273.1 ± 17.0	2049.9 ± 22.9	89.4 ± 0.6	95.2 ± 0.8***	95.9 ± 0.8	98.7 ± 0.9
unc-25(e156)[b]	2209.8 ± 20.3	1716.3 ± 16.0	92.0 ± 0.8	96.1 ± 1.0***	96.3 ± 0.5	97.2 ± 0.6
unc-25(sa94)[b]	2209.8 ± 20.3	2012.5 ± 20.0	92.0 ± 0.8	95.3 ± 0.6**	96.3 ± 0.5	97.7 ± 0.3
unc-25(n2569)[b]	2209.8 ± 20.3	1894.1 ± 25.0	92.0 ± 0.8	95.8 ± 0.6***	96.3 ± 0.5	97.9 ± 0.5
unc-49(e407)	2289.0 ± 27.8	2081.0 ± 34.3	91.4 ± 0.6	92.4 ± 1.0	96.9 ± 0.5	97.8 ± 0.7
unc-30(e191)[c]	2152.3 ± 23.3	1853.1 ± 12.6	91.9 ± 0.5	91.2 ± 0.5	96.5 ± 0.3	98.1 ± 0.5
unc-30(e381)[c]	2152.3 ± 23.3	1883.3 ± 11.9	91.9 ± 0.5	90.5 ± 1.0	96.5 ± 0.3	96.8 ± 1.1
unc-47(gk192)[d]	2182.2 ± 21.1	1804.3 ± 12.2	91.4 ± 0.5	94.5 ± 0.4*	98.1 ± 0.8	98.1 ± 1.0
unc-47(e307)[d]	2182.2 ± 21.1	1843.8 ± 4.0	91.4 ± 0.5	93.7 ± 0.9	98.1 ± 0.8	96.5 ± 1.0
gbb-1(tm1406)[e]	2160.5 ± 16.7	2113.7 ± 30.0	92.9 ± 1.0	91.2 ± 0.7	98.0 ± 0.6	97.1 ± 0.7
gbb-2(tm1165)[e]	2160.5 ± 16.7	2155.6 ± 27.1	92.9 ± 1.0	91.0 ± 0.7	98.0 ± 0.6	95.8 ± 0.9
egl-3(n150)	2276.7 ± 14.09	2330.7 ± 19.1	90.7 ± 0.3	91.8 ± 1.0	97.2 ± 0.7	95.4 ± 0.6
unc-31(e928)	2268.3 ± 49.2	2268.3 ± 49.2	92.6 ± 1.0	92.4 ± 0.6	97.2 ± 0.6	98.1 ± 0.3
nlp-12(ok335)	2291.1 ± 44.1	2106.3 ± 50.0	90.7 ± 0.6	91.2 ± 1.0	95. 8 ± 0.9	97.3 ± 0.5
trp-4(sy695)	2295.4 ± 23.7	2254.4 ± 43.0	89.0 ± 1.5	90.5 ± 0.4	94.4 ± 1.1	95.9 ± 0.4
eat-6(eg200)[f]	2326.3 ± 16.4	2087.0 ± 18.7	90.0 ± 0.7	86.7 ± 0.7	95.6 ± 0.2	91.1 ± 1.2**
eat-6(ad601)[f]	2326.3 ± 16.4	2155.9 ± 24.5	90.0 ± 0.7	87.2 ± 0.9	95.6 ± 0.2	96.4 ± 0.7
eat-6(eg200)[g]	2203.3 ± 29.1	2087.0 ± 18.7	92.2 ± 0.7	88.8 ± 0.6	96.9 ± 0.6	91.2 ± 0.7***
eat-6(ad467)[g]	2203.3 ± 29.1	1634.7 ± 58.3	92.15 ± 0.7	94.0 ± 1.69	96.9 ± 0.6	98.6 ± 0.4
eat-6(ad997)[g]	2203.3 ± 29.1	1582.1 ± 28.8	92.2 ± 0.7	93.71 ± 1.08	96.9 ± 0.6	99.1 ± 1.4
eat-6(eg200)[h]	2210.7 ± 10.0	1987.0 ± 32.1	92.2 ± 1.0	89.41 ± 0.51	97.3 ± 0.5	90.6 ± 0.6***
eat-6(ad601)[h]	2210.7 ± 10.0	2155.9 ± 24.5	92.2 ± 1.0	88.69 ± 0.78*	97.3 ± 0.5	98.2 ± 0.9
eat-6(eg200/ad601)[h]	2210.7 ± 10.0	1989.2 ± 26.1	92.2 ± 1.0	88.19 ± 0.83**	97.3 ± 0.5	95.4 ± 1.8
slo-1(eg142)	2282.9 ± 28.7	1970.5 ± 16.5	90.8 ± 0.7	89.05 ± 0.37	96.7 ± 1.1	94.2 ± 0.9
npr-1(ky13)	2266.4 ± 15.1	2079.8 ± 7.4	92.5 ± 0.7	92.59 ± 0.5	96.1 ± 0.4	97.5 ± 0.4

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with N2 (WT).

[a, b, c, d, e, f, g, h] Indicates these strains were tested on the same plates

**Table S3** Strength of *eat-6* mutant phenotypes

Genotype	Starved appearance	Pharangeal function (pumps/10s)	Adult perimeter (µm)	EHC sensitivity	Speed off food (µm/s)	Aldicarb sensitivity (1 mM)
N2	No	40.2 ± 1.3	2203.3 ± 29.1	wild-type	190.2 ± 6.9	wild-type
eat-6(eg200)	No	30.7 ± 1.3	2087.0 ± 18.7	↑↑↑	100.1 ± 4.0	↑↑↑
eat-6(ad601)	No	32.7 ± 0.5	2155.9 ± 24.5	↑	149.5 ± 3.4	↑
eat-6(ad467)	Yes	21.8 ± 0.9	1634.7 ± 58.3	wild-type	162.5 ± 4.1	↑
eat-6(ad997)	Yes	22.8 ± 1.5	1582.1 ± 28.8	wild-type	189.6 ± 7.0	↑

↑ – ↑↑↑   Indicates level of increased sensitivity
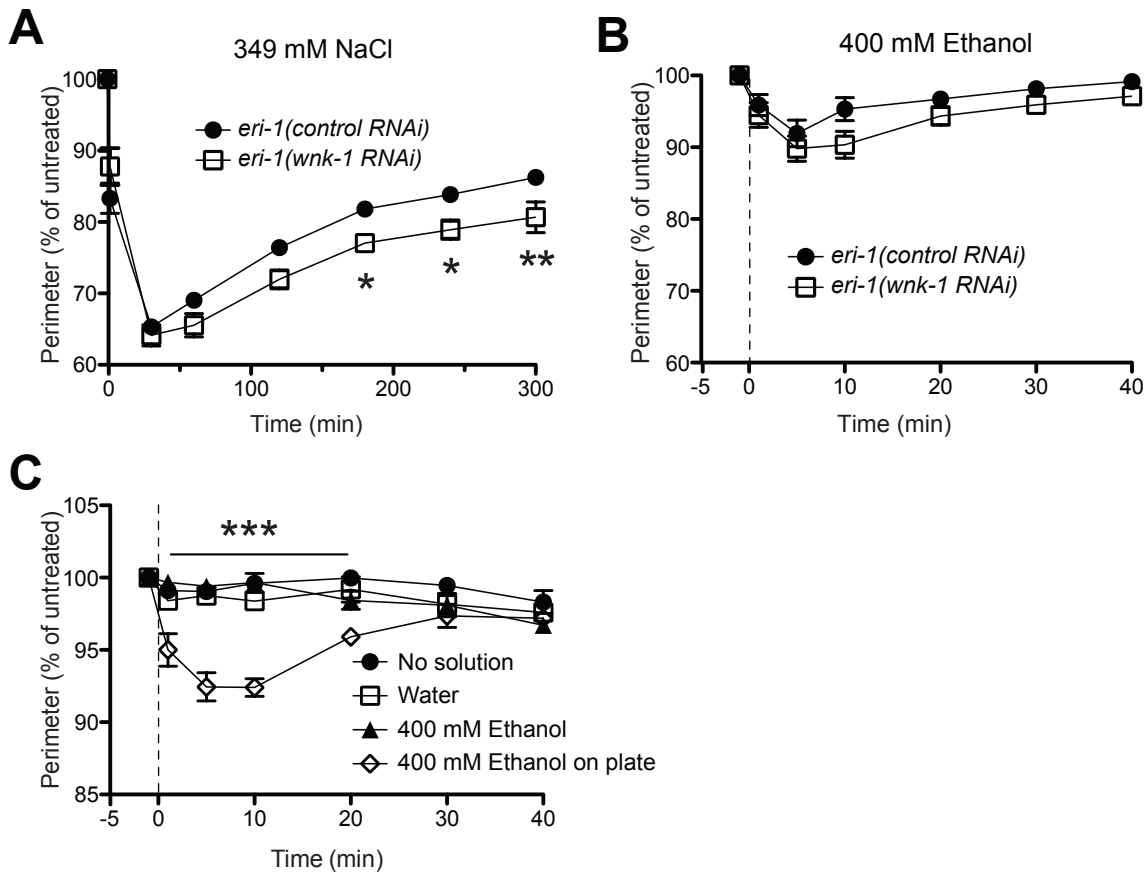
Figure S1 Hypersensitivity to hypertonic environments does not affect EHC responses and increased vapor pressure does not alter contraction state. (A) Maximum shrinkage to a hypertonic challenge (349 mM NaCl) occurred in 30 minutes in *eri-1(control RNAi)* with recovery over four hours. RNAi knockdown of *wnk-1* in the *eri-1* background decreased the rate of recovery significantly compared to the control animals (*, $p < 0.05$; **, $p < 0.01$; $n = 6$). (B) The EHC response to 400 mM ethanol in *eri-1(control RNAi)* and *eri-1(wnk-1 RNAi)* animals were not significantly different ($p > 0.05$; $n = 6$). (C) Increasing vapor pressure with water or 400 mM ethanol did not elicit a hypercontraction phenotype ($p > 0.05$, $n = 6$), in contrast to worms directly exposed to 400 mM ethanol in the NGM agar (***, $p < 0.001$; $n = 6$).
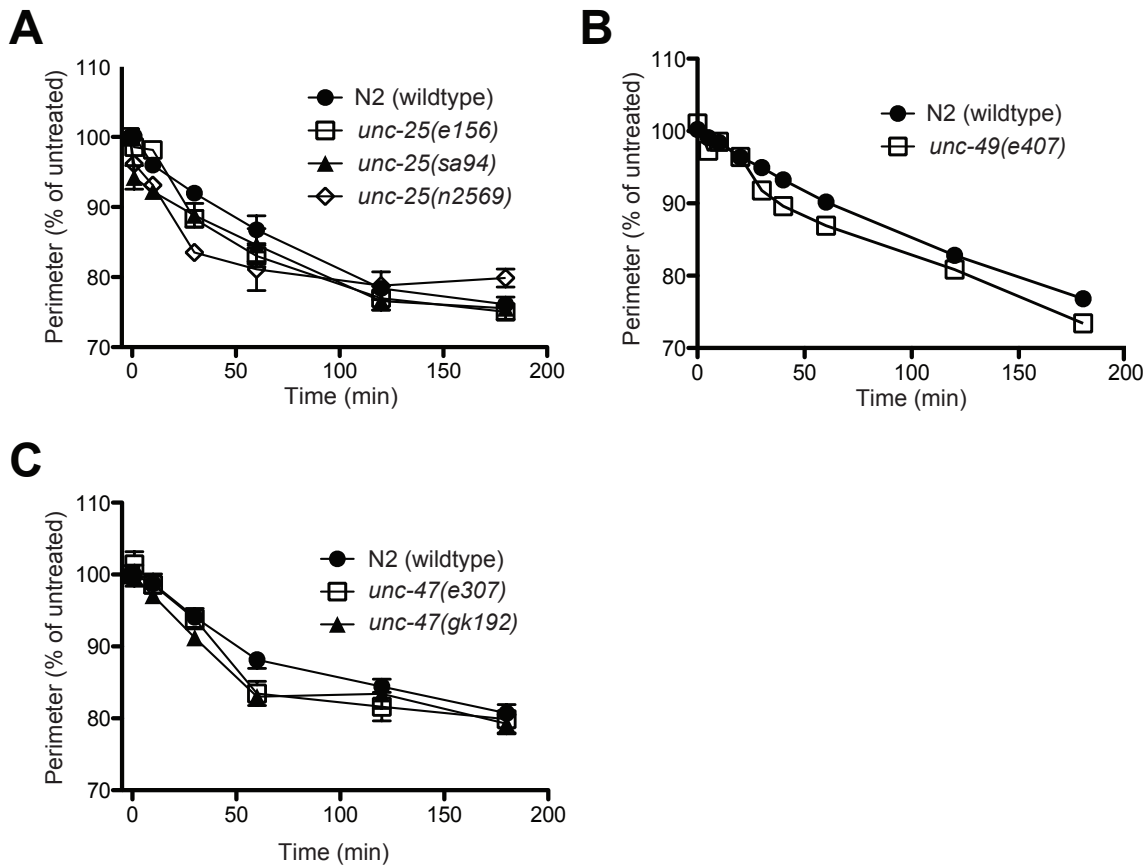
Figure S2 Mutants of *unc-25*, *unc-49, and unc-47* can contract beyond that induced by ethanol. (A) Three alleles of *unc-25*, *e156*, *sa94*, and *n2569,* contracted to 80% of their untreated size after three hours on 1.0 mM aldicarb. (B) *unc-49(e407)* contracted to 75% of their untreated size after three hours on 0.5 mM aldicarb. (C) Two alleles of *unc-47, e307* and *gk192,* contracted to 80% of their untreated size after 3 hours on 1 mM aldicarb.
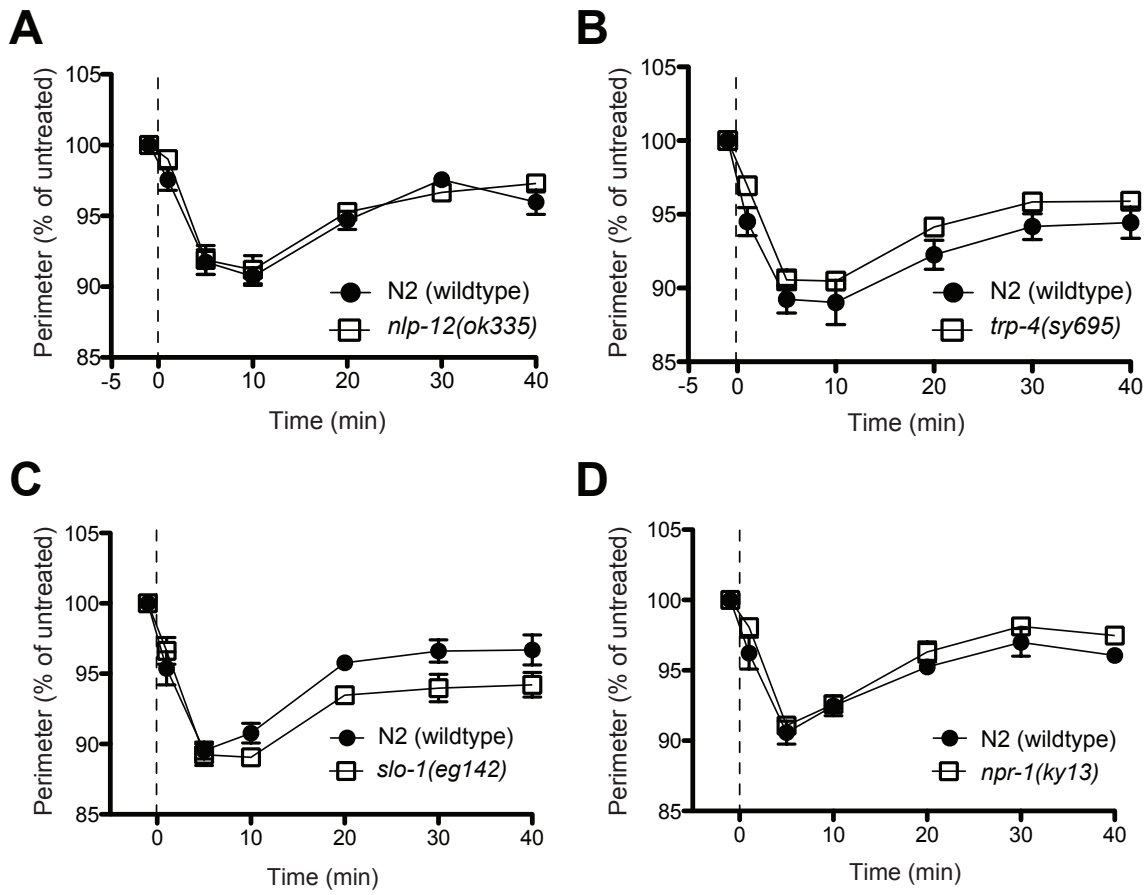
Figure S3 *nlp-12*, *trp-4*, *slo-1* and *npr-1* are not involved in EHC (A) *nlp-12(ok335)* mutant animals have wild-type sensitivity to EHC ($p > 0.05$; $n = 6$). (B) *trp-4(sy695)* mutant animals are not significantly different from N2 (wild type) ($p > 0.05$; $n = 6$). (C) The *slo-1(eg142)* mutant, which is resistant to the depressive effects of ethanol on rates of locomotion, is not significantly different from wild-type animals for EHC ($p > 0.05$, $n = 6$). (D) The *npr-1(ky13)* mutant, which has increased rates of development of acute functional tolerance to the effects of ethanol on rates of locomotion, was not significantly different from N2 (wild type) for EHC ($p > 0.05$; $n = 6$).
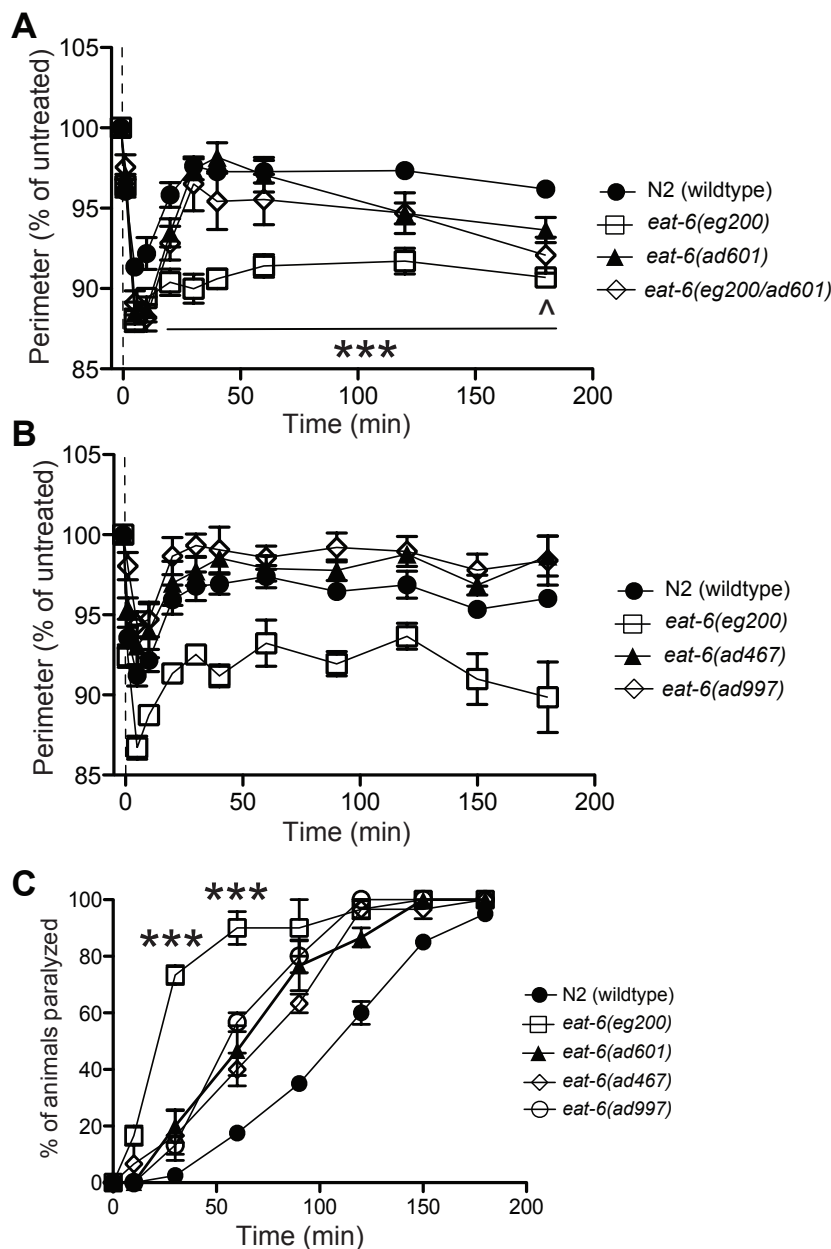
Figure S4 The *eg200* mutation is an unusual *eat-6* allele. (A) *eat-6(ad601)* fails to complement *eat-6(eg200)* for the ethanol-induced hypercontraction phenotype (*eg200/ad601*: ^, $p < 0.05$; *eg200*: ***, $p < 0.001$; $n = 9$). (B) Strong loss-of-function alleles of *eat-6*, *ad467* and *ad997*, are not different from wild-type animals for the EHC phenotype. Significant differences between *eat-6(eg200)* and N2 are described here (time = 5*, 20**, 30*, 40***, 60*, 90*, 150*, 180***; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, $n = 6$). (C) *eat-6(eg200)* was significantly more sensitive to paralysis induced by 1 mM aldicarb compared with each of the other tested *eat-6* alleles at the 30- and 60-minute time points (***, $p < 0.001$; $n = 3$). All *eat-6* mutants were more sensitive to the paralytic effects of aldicarb compared with the wild-type animals (*eg200*: *, 10, 150 min.; ***, 30, 60, 90, 120, 150 min.; *ad601*: *, 30, 150 min.; ***, 60, 90, 120 min.; *ad467*: ***, 60, 90, 120 min.; *ad997*: *, 150 min.; ***, 60, 90, 120 min.; * $p < 0.05$; *** $p < 0.001$; $n = 3$).