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Chloride Intracellular Channels modulate acute ethanol behaviors in *Drosophila, C. elegans* and mice

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Abstract

Identifying genes that influence behavioral responses to alcohol is critical for understanding the molecular basis of alcoholism and ultimately developing therapeutic interventions for the disease. Using an integrated approach that combined the power of the *Drosophila*, *C. elegans* and mouse model systems with bioinformatics analyses, we established a novel, conserved role for Chloride Intracellular Channels (CLICs) in alcohol-related behavior. CLIC proteins might have several biochemical functions including intracellular chloride channel activity, modulation of TGF-β signaling, and regulation of ryanodine receptors and A-kinase anchoring proteins. We initially identified vertebrate *Clic4* as a candidate ethanol-responsive gene via bioinformatic analysis of data from published microarray studies of mouse and human ethanol-related genes. We confirmed that *Clic4* expression was increased by ethanol treatment in mouse prefrontal cortex and also uncovered a correlation between basal expression of *Clic4* in prefrontal cortex and the locomotor activating and sedating properties of ethanol across the BXD mouse genetic reference panel. Furthermore, we found that disruption of the sole *Clic Drosophila* orthologue significantly blunted sensitivity to alcohol in flies, that mutations in two *C. elegans Clic* orthologues, *exc-4* and *exl-1*, altered behavioral responses to acute ethanol in worms, and that viral-mediated overexpression of *Clic4* in mouse brain decreased the sedating properties of ethanol. Together, our studies demonstrate key roles for *Clic* genes in behavioral responses to acute alcohol in *Drosophila*, *C. elegans* and mice.

Keywords

alcohol; invertebrate; vertebrate; sensitivity; tolerance; genetics

Introduction

Alcohol abuse has broad negative effects on human health (Dhhs, 2000). Despite these health consequences, few effective treatments are available and currently there are few genes with established roles in human alcohol abuse (Bierut *et al.*, 2010, Edenberg *et al.*,

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2010, Johnson *et al.*, 2006, Kendler *et al.*, 2011, Kumar *et al.*, 2009, Lind *et al.*, 2010, Treutlein *et al.*, 2009). A more comprehensive understanding of the genes that influence behavioral responses to alcohol is critical for meaningfully predicting the potential of an individual to abuse the drug and for developing new therapeutic strategies aimed at novel molecular targets.

Short-term exposure to moderate doses of alcohol causes sedation in humans, mice, fruit flies (*Drosophila melanogaster*) and nematode worms (*Caenorhabditis elegans*), whereas repeated or longer-term exposure to ethanol leads to tolerance in these same species (Crabbe *et al.*, 2006, Davies *et al.*, 2004, Davies *et al.*, 2003, Heberlein, 2000, Kapfhamer *et al.*, 2008, Scholz *et al.*, 2000). Given the conservation in behavioral responses to ethanol, animal models have been used to investigate genetic pathways that influence behavioral responses to the drug. Forward genetic strategies have identified genes important for ethanol-related behaviors in flies (Scholz *et al.*, 2005), worms (Davies *et al.*, 2003) and, more recently, mice (Kapfhamer *et al.*, 2008). Reverse genetic approaches in flies and mice have also been used to examine the influence of genes predicted to play roles in behavioral responses to ethanol (Crabbe *et al.*, 2006, Rodan & Rothenfluh, 2010). Interestingly, flies harboring putative alleles of ethanol responsive genes (Kong *et al.*, 2009, Morozova *et al.*, 2006) or genes that are differentially expressed in flies artificially selected for ethanol sensitivity (Morozova *et al.*, 2007) exhibit altered ethanol-related behaviors. Thus, ethanol-responsive genes (genes that change expression in response to ethanol) or genes with expression levels that correlate with ethanol phenotypes are excellent candidate loci for influencing behavioral responses to the drug. Understandably, the vast majority of published studies have investigated the role of a gene or pathway of interest in a single species only, although there are several notable exceptions (Corl *et al.*, 2009, Kapfhamer *et al.*, 2008, Lasek *et al.*, 2011a, Lasek *et al.*, 2011b, Schumann *et al.*, 2011). The general lack of cross-species studies, however, leaves unresolved whether many genes that influence ethanol-related behavior in one species have effects in others.

We took a two-step approach to identify novel genes with roles in alcohol behavior. First, we developed a list of candidate genes by analyzing ethanol-responsive loci in several microarray studies in mice and humans (Kerns *et al.*, 2005, Liu *et al.*, 2006, Mayfield *et al.*, 2002, Mulligan *et al.*, 2006). Second, we investigated the consequences of genetic manipulation of several of these genes on acute behavioral responses to alcohol in *Drosophila, C. elegans* and mice. Here, we report our studies on multiple members of the *Chloride Intracellular Channel* (*Clic*) family of genes and their effects on alcohol related behavior. CLICs are typically small proteins with single GST-CLIC domains at the N- and C-termini. CLICs have several proposed biochemical functions including intracellular chloride channel activity (Ashley, 2003), modulation of TGF-β or bone morphogenic protein (BMP) signaling (Shukla *et al.*, 2009), and regulation of ryanodine receptors (Jalilian *et al.*, 2008), 14-3-3 proteins (Suginta *et al.*, 2001) and A-kinase anchoring proteins (Shanks *et al.*, 2002). The *Clic* gene family is represented in mammals, flies and worms by six (*Clic1–Clic6*), one (*Clic*) and two (*exc-4* and *exl-1*) loci, respectively. Our studies establish that members of the *Clic* gene family are key modulators of ethanol behavior in flies, worms and mice.

Materials and Methods

Mouse husbandry

Mice were maintained in a room under controlled temperature (23±1°C) with 12 h light/dark cycles and free access to standard chow (Harlan Teklad #7912, Madison, WI) and water. Cages (4 mice/cage until viral injections and then single housing thereafter) and bedding (Harlan Sani-chips, #7090A, Harlan, Teklad, Madison, WI) were changed weekly. All

experimental manipulations or behavioral testing were done between 0900 and 1200 h. Procedures were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University and followed the NIH Guide for the Care and Use of Laboratory Animals. All mice were DBA/2J males from Jackson Laboratories (Bar Harbor, Maine) purchased at 10–13 weeks of age. All animals were allowed to habituate to the animal facility for at least 2 weeks prior to experimental procedures.

Recombinant adeno-associated virus over-expression of *Clic4* in mice

Helper-free adeno-associated serotype 2 was used throughout this study. Mouse *Clic4* cDNA was generated by PCR and cloned into the plasmid pAAV-IRES-hrGFP (Stratagene, La Jolla, CA) in frame with the FLAG epitope tag. FLAG-tagged pAAV-CLIC4-GFP was verified through sequencing and by green fluorescent protein (GFP) expression in HEK-293 cells via Arrest-in transfection (Open Biosystems, Huntsville, AL). Additional details are provided as Supplementary Material.

Behavioral analyses in mice

Three weeks after viral stereotactic injections, AAV-CLIC4 (*Clic4* over-expressing) and AAV-IRES (control) injected mice were evaluated for a sequential battery of behavioral responses to acute ethanol administration. Mice were habituated to injections with saline in their home cage for 2 days prior to behavioral studies. All animals were allowed a 1-hour acclimation period to the behavioral room prior to testing. Locomotor activity was measured immediately following injection with either saline or ethanol (2.0 g/kg) by photobeam breaks during a 10-minute session in locomotor activity chambers (Med-Associates, model ENV-510; St. Albans, VT) in ventilated sound attenuating boxes (ENV-022MD-27). Two weeks later, anxiety testing was done using the light-dark transition model (Crawley & Goodwin, 1980). Mice were injected i.p. with saline or ethanol (1.8 g/kg) and placed in the light compartment of the light-dark box (Med-Associates ENV-510 with black plexiglass inserts to divide into two 13.5 cm × 27 cm × 20 cm compartments. Light compartment was illuminated with 100 mA incandescent bulbs. Timing was initiated when animals entered the dark compartment and locomotor activity/position monitored for 10 minutes. Results were expressed as percent of time or locomotor activity in the light vs. dark. One week after anxiety testing, loss-of-righting reflex (LORR) assays were done by injecting mice i.p. with 3.8 g/kg of ethanol and hypnotic effects of ethanol were observed by inverting mice onto their backs in V-shaped troughs (Linsenbardt *et al.*, 2009). For LORR testing, all animals were injected with ethanol (n=28 each for AAV-IRES and AAV-CLIC4). The duration of LORR was initiated when the animal lost the ability to right itself onto its paws and terminated when the animal recovered its righting reflex by righting itself three times in 30 seconds. Behavioral measurements were discarded when ethanol leaked from the injection site and/or the genital area or when the animal did not lose the righting reflex. Two animals each for AAV-IRES and AAV-CLIC4 groups was excluded for these reasons (final n=26 each). All animals were subjected to supervised randomization between each phase of the behavioral battery so that both groups being tested for each behavior had identical prior experimental exposure.

Detection of *in vivo Clic4* expression by immunohistochemistry and qRT-PCR

Two weeks following the conclusion of behavioral studies (~9 weeks after stereotactic injections) *Clic4* expression from AAV-CLIC4 injected animals was detected *in vivo* by immunohistochemistry using antisera against the FLAG epitope tag. Ethanol regulation of endogenous *Clic4* mRNA in mouse brain was determined through quantitative real-time PCR (qRT-PCR) to confirm prior microarray studies (Kerns *et al.*, 2005). Additional details are provided as Supplementary Material.

Bioinformatics analyses of *Clic4* gene expression in BXD mice

A *Clic4* co-expression network was initially generated using Pearson product-moment correlation coefficients with basal gene expression data from medial PFC across 27 BXD recombinant inbred mice, as well as C57BL/6J and DBA/2J progenitors (n = 29). This was done within the GeneNetwork resource for genetic analysis of genomic and phenotypic traits (www.genenetwork.org). To address possible false positives due to multiple comparisons, p-values were corrected using the q-value false-discovery rate method (Qian & Huang, 2005) within the R statistical framework (http://www.bioconductor.org/packages/2.4/bioc/html/qvalue.html), and resulting q-values were filtered for $q<0.05$, resulting in a total of 1015 probe sets. Ingenuity Pathway Analysis (www.ingenuity.com) was utilized to assess the biological significance and relationships between these genes, based on current scientific literature, using the Pearson correlation coefficients (positive and negative) as signals for individual genes (Figure S7).

Drosophila husbandry, genetics and qRT-PCR

Flies were grown at 20°C (*Clic* homozygous mutants) or 25°C (all other genotypes) and 55% relative humidity on a standard sugar:yeast:cornmeal:agar medium (10%:2%:3.3%:1% w/v) supplemented with 0.2% Tegosept (Sigma Chemical Co., St. Louis, MO, USA) and active dry yeast under a 12-h light-dark cycle. *P[EPgy2]Clic*^{EY04209} (i.e. *Clic*^{EY04209}) and *P[w[+mC]=lacW]Clic[G0472]* (i.e. *Clic*^{G0472}) were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). To assess *Clic* expression, qRT-PCR studies were performed using standard methods as described (Jones *et al.*, 2009). Additional details are provided as Supplementary Material.

Drosophila ethanol-related behaviors and internal ethanol measurements

For all fly behavioral studies, groups of 25 adult flies (2–5 days-old, grown in parallel) were collected under brief CO_2 anesthesia, transferred to fresh food vials, and housed overnight at 25°C and 55% relative humidity. Each group of 25 flies constituted n=1 (7,425 total flies were used for the reported behavioral studies). Sensitivity and rapid tolerance to ethanol (provided as a vapor) were determined in flies using ethanol Rapid Iterative Negative Geotaxis (eRING) assays at 25°C and 55–65% relative humidity as described (Bhandari *et al.*, 2009). Internal ethanol concentrations in flies exposed to ethanol in eRING assays were determined as described previously (Bhandari *et al.*, 2009). Additional details are provided as Supplementary Material.

C. elegans genetics and ethanol-related behaviors

C. elegans were cultured according to Brenner (Brenner, 1974). Strains used in this study (N2 control and *Clic* mutant strains *exc-4 (rh133)* and *exl-1(ok857))* were obtained from the *C. elegans* Genetics Center, which is funded by NIH-NCRR. The double mutant *exc-4(rh133);exl-1(ok857)* was generated by standard genetic crosses, using the recessive excretory canal phenotype that is associated with *exc-4(rh133)* to detect that mutation and a set of PCR primers to detect the *exl-1(ok857)* deletion mutation (5'-GTGCAATCTCGTCAGGACCAGGC-3', 5'-ATGCGTTACGATGCCCCGACAC-3'). Ethanol response assays were carried out as previously described (Davies *et al.*, 2004, Davies *et al.*, 2003) except additional time points were measured and ImagePro Plus was used for object tracking. Additional details are provided as Supplementary Materials.

Internal ethanol measurements in *C. elegans*

Young-adult age-matched worms, reared at 20°C, were placed on unseeded plates containing 0 mM or 400 mM ethanol (prepared as for ethanol response assays) for 5, 10 or 30 minutes. Ethanol-exposed worms were frozen at −80°C and homogenized in H_2O.

Internal ethanol was determined in the homogenates using a commercially available alcohol reagent (Pointe Scientific) and by calculating the volume of worms. Additional details are provided as Supplementary Materials.

Statistical Analyses

To determine statistical significance ($p \leq 0.05$) in studies comparing two or more groups, t tests (Prism, GraphPad, San Diego, CA, USA), one- and two-way ANOVA (JMP, SAS Institute, Cary, NC) followed by Bonferroni multiple comparison tests were performed as appropriate. Data from qRT-PCR studies in Figure 2B were analyzed by one-sample t tests (Prism) to individually compare *Clic* mRNA levels in mutants and revertants to control (100%).

Results

Expression of Mammalian *Clic4* and Acute Ethanol Behaviors

To identify novel candidate genes involved in alcohol responses, we used gene set overlap analysis within the Ontological Discovery Environment (Baker *et al.*, 2009) and the Ethanol-Related Gene Resource (Guo *et al.*, 2009) to rank data from multiple microarray studies of ethanol-related genes in mouse (Kerns *et al.*, 2005, Mulligan *et al.*, 2006) and human alcoholic brain (Liu *et al.*, 2006, Mayfield *et al.*, 2002). The most highly ranked gene from our analyses, *Chloride Intracellular Channel 4* (*Clic4*), was represented across six independent microarray, linkage and association studies. For example, our prior microarray studies showed that *Clic4* had lower basal expression but greater ethanol-responsive expression in prefrontal cortex (PFC) of DBA/2J mice compared to C57BL/6 mice (Supplemental Figure S1; two-way ANOVA; treatment, $F(1,12)=7.28$, $p=0.027$; genotype, $F(1,12)=8.082$, $p=0.022$; interaction, $F(1,12)=11.158$, $p=0.010$, $n=3$); also see (Kerns *et al.*, 2005)). These two mouse strains differ widely in terms of multiple behavioral responses to acute or chronic ethanol, as well as ethanol consumption (Metten *et al.*, 1998). We also found that *Clic4* was in other published datasets for genes associated with ethanol drinking preference in mice (Mulligan *et al.*, 2006) and genes with altered expression in frontal cortex of alcoholics (Mayfield *et al.*, 2002). Furthermore, *Clic4* is located within a confirmed quantitative trait locus (QTL) for ethanol drinking behavior on distal mouse chromosome 4 (Tarantino *et al.*, 1998) and we also found that ethanol treatment (4 g/kg × 4 h) increased expression of *Clic4* in PFC of DBA/2J mice (Figure 1A; *, two-sample t-test; $t(6)=3.091$, $p=0.027$, $n=4$ for EtOH and 3 for Saline), validating our prior microarray data (Kerns *et al.*, 2005).

As an initial characterization of the role for *Clic4* in ethanol responses in mammals, we used genomic studies across the BXD genetic panel of mice to determine whether basal expression of *Clic4* in PFC correlated with ethanol-related behaviors in the GeneNetwork web resource (www.genenetwork.org). Through this analysis we found that *Clic4* PFC basal expression correlated positively with low dose (2 g/kg) ethanol-induced locomotor activation (Figure 1B) and inversely with high dose (4 g/kg) ethanol-induced ataxia (Figure 1C) in mice. Our analysis of data from human and rodent studies thus suggests that *Clic4* or related genes might be important determinants of acute level of response to ethanol across a range of species.

Functional analysis of *Clic* in *Drosophila* ethanol behavior

We used *Drosophila* to directly test the hypothesis that *Clic* genes are important for acute ethanol behavior. We confirmed two independent transposon insertions (*G0472* and *EY04209*, Figure 2A) in the sole *Clic* locus in flies and subsequently backcrossed both elements into w^{1118} (our standard laboratory stock) to control for genetic background effects

on ethanol behavior. Both transposon insertions were homozygous female and hemizygous male lethal at 25°C. Female flies heterozygous for *G0472* and *EY04209*, however, were fertile, appeared morphologically normal and seemed generally healthy. qRT-PCR analysis showed that *Clic* mRNA in *G0472*/+ and *EY04209*/+ flies was reduced by approximately 40% compared to w^{1118} control (Figure 2B; *, one sample t tests; t(2)=27.01 and t(2)=4.95 respectively, p<0.05, n=3). *G0472* and *EY04209* are therefore partial loss of function mutations in *Drosophila Clic*.

To assess ethanol behavior in *Clic* mutant flies, we used an ethanol Rapid Iterative Negative Geotaxis (eRING) assay developed in one of our component laboratories (Bhandari *et al.*, 2009). Female $Clic^{G0472}$/+ and $Clic^{EY04209}$/+ mutants maintained their negative geotaxis behavior longer than did the w^{1118} female controls during exposure to ethanol (Supplemental Figure S2; two-way ANOVA; time, F(7,216)=249.75, p<0.0001; genotype, F(2,216)=40.36, p<0.0001; n=5), suggesting that *Clic* mutants have reduced sensitivity to the sedative effects of ethanol. Data from several experiments confirmed the blunted ethanol sensitivity in female $Clic^{G0472}$/+ and $Clic^{EY04209}$/+ flies (Figure 3A and B, respectively; *, t tests; $Clic^{G0472}$/+, t(26)=6.31, p<0.0001; $Clic^{EY04209}$/+, t(28)=6.87, p<0.0001, n=13–15). Additionally, $Clic^{G0472}$ and $Clic^{EY04209}$ hemizygous males and homozygous females (reared at 20°C to improve viability) had diminished ethanol sensitivity (Supplemental Figure S3; two-sample t tests; $Clic^{G0472}$ males, t(6)=3.55, p=0.012; $Clic^{G0472}$ females, t(6)=9.39, p<0.0001; $Clic^{EY04209}$ males, t(7)=5.62, p=0.0008; $Clic^{EY04209}$ females, t(8)=13.04, p<0.0001, n=3–5). (Note that data in Figure 3 and Supplemental Figure S3 are T50 values that reflect the time required for ethanol to inhibit negative geotaxis by 50%. Higher values indicate blunted ethanol sensitivity; i.e. higher doses of ethanol are required to produce the same level of response.) In contrast to the effect of *Clic* mutations on ethanol sensitivity, we did not observe a significant alteration in rapid ethanol tolerance in these animals (Supplemental Figure S4; two-sample t tests; $Clic^{G0472}$/+, t(16)=0.777, p=0.448; $Clic^{EY04209}$/+, t(17)=1.86, p=0.080; n=9–10). *Clic* might therefore be an important determinant of ethanol sensitivity in *Drosophila* without having major effects on rapid ethanol tolerance in this species.

Although the $Clic^{G0472}$ and $Clic^{EY04209}$ alleles were independently derived and backcrossed for several generations to normalize the genetic background, it was formally possible that the transposon insertions in *Clic* were not responsible for the blunted ethanol sensitivity in flies. To address this issue, we generated two revertants ($Clic^{G0472.R4}$ and $Clic^{G0472.R9}$) of the *G0472* chromosome through precise excision of the transposon. Expression of *Clic* returned to normal in the two revertants (Figure 2B; one sample t tests; $Clic^{G0472.4}$, t(2)=0.0, p=1.00, n.s.; $Clic^{G0472.R9}$, t(2)=1.22, p=0.348, n.s.; n=3) as did the ethanol sensitivity phenotype of $Clic^{G0472}$ flies (Figure 3C; one-way ANOVA; genotype, F(3,44)=69.93, p<0.0001; *Bonferroni multiple comparison, Control w^{1118} t(22)=9.40, p<0.05; $Clic^{G0472R.4}$ t(22)=7.68, p<0.05; $Clic^{G0472R.9}$ t(25)=14.29, p<0.05; n=10–15). These data confirm that the *G0472* transposon insertion disrupted *Clic* expression and blunted ethanol sensitivity.

To address the possibility that blunted ethanol sensitivity in *Drosophila Clic* mutants could be secondary to altered ethanol uptake or metabolism, we determined internal ethanol concentrations in flies exposed to ethanol in eRING assays (Bhandari *et al.*, 2009). Duration of ethanol exposure had a significant effect on internal ethanol concentrations as expected (individual two-way ANOVAs; Figure 4A, F(2,12)=115.69, p<0.0001; Figure 4B, F(2,12)=84.39, p<0.0001; n=3), but there was no effect of *Clic* genotype on internal ethanol concentrations (Figure 4A, F(1,12)=0.417, p=0.529; Figure 4B, F(1,12)=0.283, p=0.603) or an interaction between genotype and duration of ethanol exposure (Figure 4A, F(2,12)=0.340, p=0.569; Figure 4B, F(2,12)=0.888, p=0.362). *Clic* mutants, therefore, have

a blunted level of response to acute ethanol exposure that is not related to a significant change in tissue concentrations of the drug.

It seemed possible that partial loss of function in *Clic* could, in principle, lead to a global improvement in negative geotaxis behavior that manifested as blunted sensitivity to ethanol in eRING assays. We addressed this issue by carefully examining two key components of negative geotaxis, latency to initiate the behavior and climbing speed, in control and *Clic* mutants in the absence of ethanol. Climbing speed in negative geotaxis assays was indistinguishable in $Clic^{G04072}$/+, $Clic^{EY04209}$/+ and control w^{1118} flies (Supplemental Figure S5A and C; two-sample t tests; $Clic^{G04072}$/+, t(28)=1.66, p=0.107; $Clic^{EY04209}$/+, t(28)=0.891, p=0.381; n=14–15). Although climbing latency was decreased in $Clic^{G0472}$/+ (Supplemental Figure S5B; two-sample t test; t(26)=3.40, p=0.002, n=13–14), this effect was not seen in $Clic^{EY04209}$/+ (Supplemental Figure S5D; two-sample t test; t(27)=1.26, p=0.218; n=14). We conclude that partial loss of function in *Clic* does not consistently alter negative geotaxis in flies and, therefore, that the blunted ethanol sensitivity in *Clic* mutants must be independent of global improvements in negative geotaxis or climbing behavior.

Functional Analysis of *Clic* orthologues in *C. elegans*

To determine if *Clic* genes act exclusively in initial sensitivity or if they also influence the development of acute functional tolerance to ethanol, we extended our genetic analysis to another invertebrate model, the nematode *C. elegans*. Worms have two *Clic* genes, *exc-4* and *exl-1* (Berry *et al.*, 2003). We assessed the responses to acute ethanol using an established behavioral assay in which worms were exposed to the drug on agar plates and locomotor speed was assessed as a function of exposure time (Davies *et al.*, 2004, Davies *et al.*, 2003, Kapfhamer *et al.*, 2008). In this assay, locomotor speed in N2 control worms is increasingly depressed in the presence of ethanol over the first 15 minutes of drug exposure (indicating initial sensitivity). Thereafter, locomotor speed increases despite the continued presence of ethanol (indicating the development of acute functional tolerance to ethanol) (Davies *et al.*, 2004). Repeated measures two-way ANOVA indicated significant effects of time (F(10,120)=14.66, p=0.0244) and genotype (F(3,120)=11.06, p=0.0009) on locomotor behavior in the presence of ethanol with no interaction between the factors (F(10,120)=1.78, p=0.177). Worms harboring *exc-4(rh133)*, a null allele (Berry *et al.*, 2003), had diminished ethanol sensitivity during the first few minutes of drug exposure (Figure 5A, Supplemental Figure S6A; #, Bonferroni multiple comparison, t(7)=2.91–4.92, p<0.05) and a trend toward blunted acute tolerance to ethanol at later time-points (Figure 5A). In contrast, worms carrying the *exl-1(ok857)* mutation, also likely a null allele (Berry & Hobert, 2006), had wild-type initial sensitivity to ethanol followed by significantly enhanced acute functional ethanol tolerance (Figure 5A; †, Bonferroni multiple comparison; t(7)=2.91–4.92, p<0.05). Importantly, while internal ethanol concentrations increased with the duration of ethanol exposure as expected, neither mutation significantly altered internal tissue concentrations of ethanol determined at key time-points of behavioral testing as compared to N2 control animals (Figure 5B; two-way ANOVA; duration, (F(2,60)=29.19, p<0.0001); genotype (F(3,60)=3.07, p=0.0439; interaction between duration and genotype, (F(3,60)=0.1143, p=0.951, n.s.; Bonferroni multiple comparisons between N2 and other genotypes, t(11)=0.0824–1.94, p>0.05, n.s., n=6). The behavioral consequences of *exc-4* and *exl-1* mutations are therefore likely to be related to changes in the pharmacodynamic responses to ethanol as opposed to altered pharmacokinetics of the drug. These studies indicate that both *Clic* orthologues in *C. elegans* influence ethanol-related behavior. The effects of mutations in *exc-4* and *exl-1* on initial sensitivity and acute tolerance in worms, however, are wholly distinct and suggest that different *Clic* genes could play distinct roles in ethanol-related behavior.

To address the possibility that the *Clic* genes *exc-4* and *exl-1* might work in concert, we also assessed the behavioral response to ethanol in *exc-4;exl-1* double mutants. Interestingly, initial sensitivity in the double mutants was essentially normal (similar to *exl-1* mutants), whereas acute tolerance was significantly suppressed in these animals (similar to *exc-4* mutants) (Figure 5A). Since internal ethanol concentrations were not significantly altered in *exc-4(rh133);exl-1(ok857)* double mutants compared to wild-type N2 controls (Figure 5B), we tentatively conclude that the effect of loss of *exc-4* function on initial sensitivity requires normal expression of *exl-1* and conversely that the effect of loss of *exl-1* function on acute tolerance requires normal expression of *exc-4*.

Clic4 influences ethanol sensitivity in mice

The gene expression-correlation studies in Figure 1, combined with the functional genetic analysis of *Clic* orthologues in *Drosophila* (Figures 2–4) and *C. elegans* (Figure 5), suggested that *Clic4* might influence acute behavioral sensitivity to ethanol in mammals. To directly test this possibility, we constructed AAV2 viral vectors to over-express CLIC4 protein in specific regions of mouse brain. As expected, stereotactic injection of AAV2/*Clic4* virus in medial prefrontal cortex (including cingulate and secondary motor cortex) in DBA/2J mice increased *Clic4* transcript abundance (not shown) and increased expression of epitope-tagged CLIC4 protein (Figure 6A) compared to control animals injected with empty vector (Figure 6B). AAV2-mediated overexpression of *Clic4* in DBA/2J mice also decreased the sensitivity to high-dose ethanol (3.8 g/kg) loss-of-righting reflex (LORR) (Figure 6C; *, two-sample t-test, $t(49)=2.49$, $p=0.016$, $n=26$) without changing the anxiolytic or locomotor-activating responses to lower doses of ethanol (1.8 g/kg, not shown). The decreased LORR time with Clic4 overexpression is consistent with results of Fig. 1C showing that higher Clic4 expression correlates with higher blood ethanol levels being required for initiation of rotarod ataxia (decreased sensitivity). These results indicate that the expression level of *Clic4* can influence the level of response to high-doses of ethanol in DBA/2J mice, consistent with a role for CLIC proteins in behavioral responses to acute ethanol in mammals.

Bioinformatics analysis for *Clic4* functional mechanisms in mice

The *Clic* family of genes has been implicated in a number of different biological processes or molecular functions (Maeda *et al.*, 2008, Shukla *et al.*, 2009, Singh & Ashley, 2007, Suh *et al.*, 2004, Ulmasov *et al.*, 2009). Many of these functions, however, remain largely unconfirmed or poorly characterized. As a non-biased analysis of possible mechanisms for *Clic4* action on ethanol responses in mouse brain PFC, where we observed altered LORR after *Clic4* over-expression, we performed a genetic correlation analysis using the GeneNetwork web resource (www.genenetwork.org) to identify genes with expression tightly coupled with basal *Clic4* expression in PFC across the BXD genetic panel of mice. *Clic4* expression was strongly correlated with a large set of genes in PFC (Supplemental Table S1). This gene set was strikingly over-represented ($p< 10^{-15}$) for a network of genes involved in RNA processing as determined via Ingenuity Pathway Analysis (Supplementary Figure S7 and Supplemental Table S2). This result raises the possibility that *Clic4* might influence ethanol sensitivity in mice by functioning within a network of genes that impinge on RNA processing and trafficking.

Discussion

Using animal models to identify genes and genetic pathways that influence ethanol-related behavior holds tremendous promise for providing mechanistic insight into the basic biology of alcohol abuse. Studies in animal models could lay the foundation for developing novel drug targets for treating alcohol abuse and for estimating risk of abusing alcohol in human

populations. Starting with ethanol-responsive gene sets and other ethanol-related data from studies in mammals, we selected mammalian *Clic4* as a candidate gene in ethanol behavior. Subsequent studies in multiple animal models directly linked the *Clic* family of genes to acute behavioral responses to alcohol. Partial loss of function in the sole *Clic* orthologue in *Drosophila* blunted the locomotor sedating effects of ethanol. In *C. elegans*, loss of function in the *Clic* orthologue *exc-4* blunted ethanol sensitivity during the first few minutes of drug exposure, whereas loss of function in another *Clic* orthologue, *exl-1*, enhanced the development of acute functional tolerance to alcohol. Additionally, viral-mediated brain-specific overexpression of mammalian *Clic4* decreased the sedating effects of high dose ethanol in mice. The ethanol behavior phenotypes were unrelated to altered uptake/metabolism of the drug in worms or flies, indicating that CLIC proteins influence the pharmacodynamic properties of ethanol in these species. We did not perform pharmacokinetic studies on ethanol in the virally injected mice, but we have not observed altered ethanol metabolism in other AAV gene delivery studies on PFC (Miles and Meng, unpublished data). Although we cannot totally exclude the possibility that expression of *Clic4* in a relatively small population of PFC neurons in adult animals would lead to peripheral differences in ethanol metabolism (as might occur with a traditional gene knockout approach), we believe it highly unlikely. Collectively, our data establish that several members of the *Clic* gene family influence ethanol-related behaviors in multiple species. We are currently investigating whether variance in *Clic* genes might be associated with human responses to alcohol or alcohol abuse.

We focused our *Clic* genetic studies reported here on the level of response to acute ethanol exposure because there is an intriguing literature indicating that level of response correlates with alcohol abuse or intake. Men with low level of response to acute ethanol are more likely to develop alcohol dependence than those with high level of response (Schuckit, 1994, Schuckit & Smith, 1996). A similar inverse relationship between acute ethanol sensitivity and ethanol drinking behavior is also often seen in several studies with gene-targeted mice. For example, mice with knockout of the A_{2A} adenosine receptor (Naassila *et al.*, 2002), neuropeptide Y (Thiele *et al.*, 1998) or the protein kinase A regulatory subunit RIIβ (Thiele *et al.*, 2000) exhibit decreased sensitivity to ethanol and increased ethanol consumption. Conversely, protein kinase-C epsilon knockout mice have increased sensitivity to the effects of ethanol and decreased ethanol drinking (Hodge *et al.*, 1999). Not all gene-targeted mouse studies, however, show this inverse relationship. In an extensive review of the subject, Crabbe et al. found that 19 out of 48 (40%) genes studied showed opposite effects on ethanol consumption vs. acute sedation, but 12% showed increases in both traits and another 48% were equivocal (Crabbe *et al.*, 2006). These studies thus indicate that acute level of response to ethanol often, but not always, has predictive value for identifying molecular events relevant to ethanol consumption including long-term ethanol drinking behavior in humans. The studies on *Clic* in this report document a novel gene affecting the acute level of response to ethanol across multiple species, and thus could have important implications for understanding the mechanisms that affect ethanol consumption.

All genetic manipulations of *Clic* that we investigated led to altered ethanol-response behavior. The observed phenotypes between or even within a species, however, were not identical. Although we currently do not understand the range of phenotypes observed in animals with altered *Clic* expression, one possible explanation is that CLIC proteins could be direct targets of ethanol, but different *Clic* genes might be expressed in different cells (e.g. excitatory versus inhibitory neurons) leading to varying effects on ethanol responses. The *C. elegans* CLIC protein EXL-1 is expressed in PVD and CAN neurons in addition to neurons that have not been identified, while EXC-4, the other CLIC protein in worms, is expressed in a distinct but somewhat overlapping pattern in the nervous system (Berry *et al.*, 2003, Berry & Hobert, 2006). Additionally, while both EXL-1 and EXC-4 are expressed in

the intestine, EXL-1 is expressed in body muscle and coloemocytes whereas EXC-4 is localized to the excretory canal cell, hypodermis, sheath cells, rectal gland and vulval cells (Berry & Hobert, 2006). Although the connections between these various cells or tissues and ethanol behavior in worms are not immediately obvious, the distinct patterns of EXL-1 and EXC-4 expression could contribute to the different ethanol-related behaviors observed in *exl-1* and *exc-4* mutants. Similarly, it is possible that *Clic4* over-expression in regions of the mouse brain other than the PFC could produce varying effects on LORR or even other ethanol behavioral phenotypes.

Although there is evidence that *Clic* genes encode intracellular chloride channels (Ashley, 2003), this channel activity is somewhat controversial and several other functions have been suggested. Vertebrate CLIC4 or other CLIC proteins bind and likely regulate the function of ryanodine receptors (Jalilian *et al.*, 2008), 14-3-3 proteins (Suginta *et al.*, 2001) and A-kinase anchoring proteins (Shanks *et al.*, 2002) Additionally, CLIC4 interacts with Smad proteins and is consequently a mediator of TGF-β or bone morphogenic protein (BMP) signaling (Shukla *et al.*, 2009). The relevance of these biochemical functions for CLIC4 or other CLIC proteins in behavioral responses to ethanol is unknown currently. Our bioinformatics studies on gene networks associated with *Clic4* basal expression in mouse PFC (Supplementary Figure S7), however, provide correlative evidence that *Clic4* might modulate RNA processing and trafficking. This possibility is particularly interesting since CLIC4 is a mediator of TGF-β signaling and this pathway is known to modulate RNA processing via Smad protein interaction with RNA binding sites on miRNA (Davis *et al.*, 2008). Thus, CLIC4 might regulate TGF-β/Smad-mediated modulation of RNA processing and therefore protein expression, which could ultimately influence neuronal responses to ethanol. While such a role for CLIC proteins in modulating ethanol-related behavior is speculative at this time, our approach of using multiple model organisms will allow us to investigate this and other possible mechanisms of CLIC proteins in behavioral responses to ethanol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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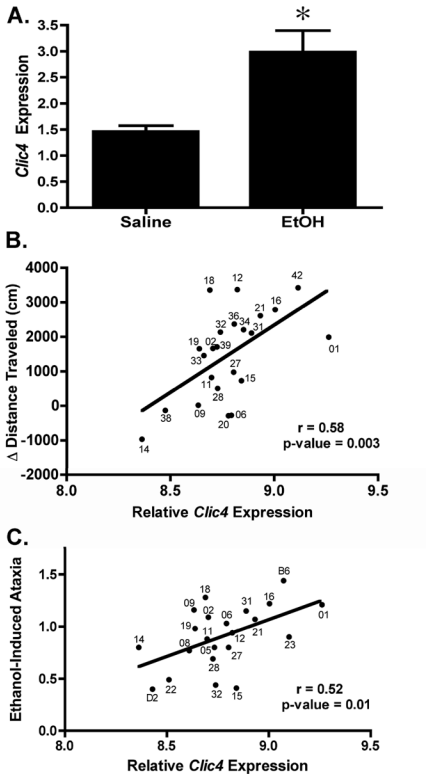

Figure 1. Ethanol-responsive and basal expression of *Clic4* in mouse PFC

(A) qRT-PCR analysis of basal (Saline) and ethanol-responsive (EtOH, 4 g/kg, 4 hr) *Clic4* expression in DBA2/2J mice. Expression of *Clic4* is elevated after ethanol treatment, validating prior microarray results (Kerns *et al.*, 2005) . (B and C) Pearson correlation of *Clic4* basal expression in PFC (x-axis) of BXD recombinant inbred lines (numbered points) with ethanol-induced locomotor activity (GeneNetwork trait ID 11962 (Philip *et al.*, 2010)) (B) and initial sensitivity to ethanol-induced rotarod ataxia following first of five injections (onset of ataxia brain ethanol threshold, mg ethanol/g brain – GeneNetwork ID 10144 (Gallaher *et al.*, 1996)) (C). Scattergrams were generated in GeneNetwork (www.genenetwork.org) using the VCU PFC saline database (Wolen and Miles, unpublished). B6 and D2 strains were not tested in experiments shown in panel B

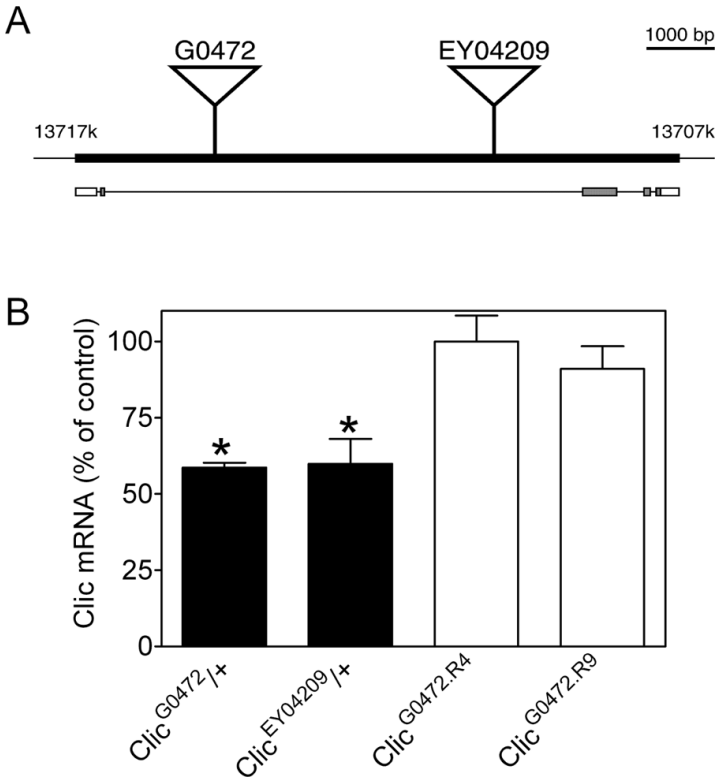

Figure 2. Transposon insertions cause partial loss of function in *Drosophila Clic*

(A) The *Clic* locus and transposon insertions. Transcription of *Clic* is from left to right. The *Clic* transcription unit is represented by the filled rectangle with nucleotide positions indicated above (coordinates from FlyBase annotation release 5.33). Exons are represented by rectangles below the transcription unit with protein coding sequences and untranslated regions depicted as grey and open rectangles, respectively. Introns are represented as a line and transposons as triangles. Scale bar (upper right) is 1000 bp. Schematic adapted from FlyBase. (B) Whole-body *Clic* mRNA expression in transposon lines. Expression of *Clic* mRNA in flies heterozygous for the *G0472* and the *EY04209* transposons was reduced relative to w^{1118} controls.

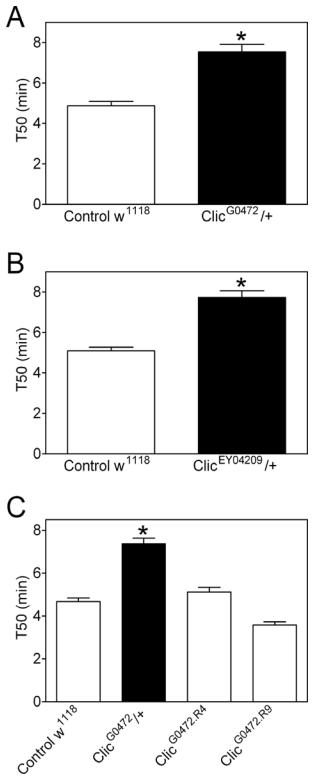

Figure 3. Ethanol sensitivity in *Drosophila Clic* transposon mutants and revertants

Ethanol sensitivity represented as T50 values in $Clic^{G0472}$/+ and $Clic^{EY04209}$/+ (black bars, A and B, respectively) and Control w^{1118} flies (open bars). *Clic* mutants had significantly higher T50 values than controls. (C) Ethanol sensitivity (T50 values) in $Clic^{G0472}$ heterozygous transposon mutants and revertants. $Clic^{G0472}$/+ flies (black bar) had higher T50 values than Control w^{1118} flies or revertants ($Clic^{G0472.R4}$ and $Clic^{G0472.R9}$) (white bars).

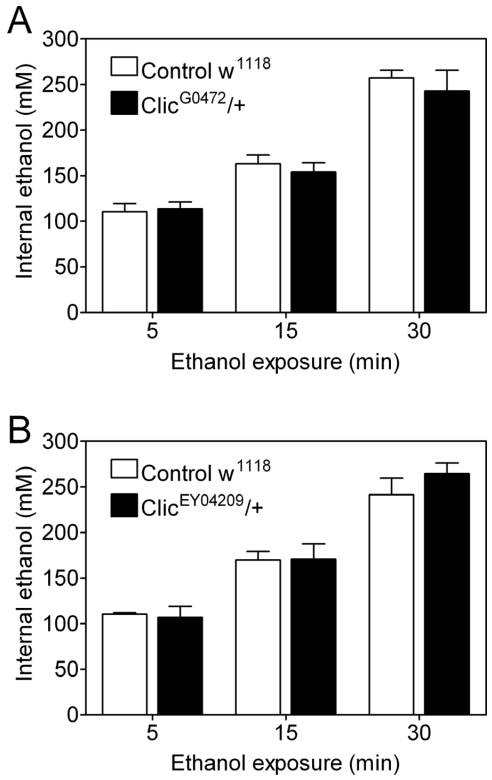

Figure 4. Internal ethanol concentrations in *Drosophila Clic* mutants

Clic^{G0472}/+ (A) and *Clic*^{EY04209}/+ (B) were exposed to ethanol vapor for the indicated durations in eRING assays in parallel with Control *w*^{1118} flies. Internal ethanol concentrations were determined as described in Materials and Methods

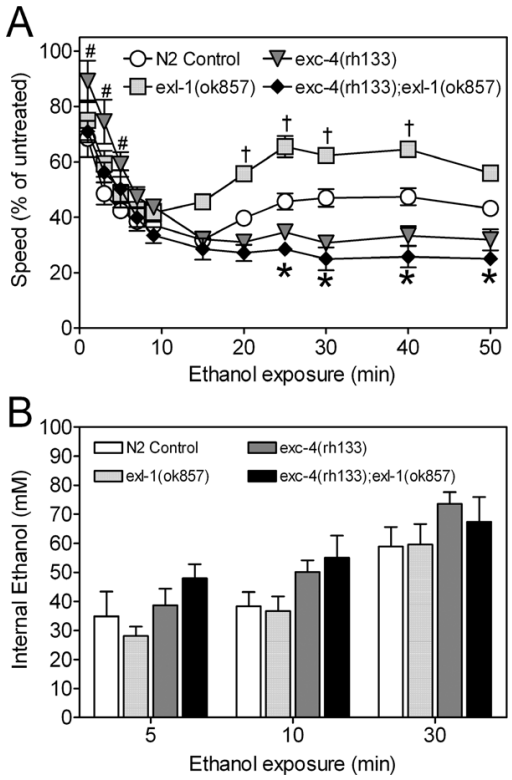

Figure 5. Ethanol sensitivity and acute functional tolerance in *C. elegans* with mutations in *Clic* orthologues

(A) Effect of ethanol exposure on relative locomotor speed (percent of untreated animals) in N2 control (open circles) and *Clic* mutants (*exl-1(ok857)*, light grey squares; *exc-4(rh133)*, dark grey triangles; *exc-4(rh133);exl-1(ok857)*, black diamonds). Data are from 4 independent experiments where 10 worms per genotype contributed to an average speed for a population. (B) Internal ethanol concentrations in N2 control and *Clic* mutants were determined as described in Materials and Methods

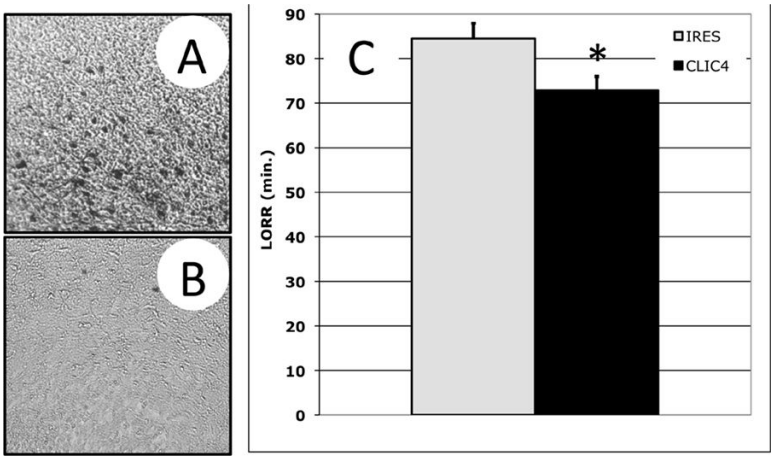

Figure 6. Altered loss of righting reflex (LORR) in mice with AAV2 viral vector-mediated expression of *Clic4* in brain

AAV2 vectors expressing a *Clic4*-FLAG fusion protein (AAV-CLIC4; panel A) or empty vector (AAV-IRES; panel B) were stereotactically injected into male DBA/2J mouse PFC. Panels A and B show immunohistochemistry results for FLAG epitope primary antibody staining. Immunohistochemistry was done 2 weeks after the last behavioral studies (~9 weeks after viral injections). Panel C shows that *Clic4* over-expressing animals (black) had a shorter duration of LORR following 3.8 g/kg IP of ethanol compared to control (grey). Behavioral testing for LORR was done ~7 weeks after viral injections.