BK channel clustering is required for normal behavioral alcohol sensitivity in *C. elegans*

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The large conductance, calcium- and voltage-activated potassium channel, known as the BK channel, is one of the central proteins that mediate alcohol intoxication and tolerance across species. Although ethanol targets BK channels through direct interaction, how ethanol-mediated BK channel activation causes behavioral intoxication is poorly understood. In *C. elegans*, loss of function in SLO-1, the BK channel ortholog, confers profound ethanol resistance in movement and egg-laying behaviors. Here, we show that depletion of SLO-1 channels clustered at the active zones with no change in the overall channel expression level results in locomotory resistance to the intoxicating effect of ethanol, equivalent to that of slo-1 loss-of-function mutants. Likewise, depletion of clustered SLO-1 channels in the sarcolemma and neurons leads to ethanol-resistant egg-laying behavior. By contrast, reduction in the overall SLO-1 channel level by over 70% causes only moderate ethanol resistance in movement, and minimal, if any, resistance in egg laying. Our findings strongly suggest that behavioral ethanol sensitivity is conferred by local, but not global, depression of excitability via clustered BK channels. Given that clustered BK channels are functionally coupled to, and localize near, calcium channels, ethanol may mediate its behavioral effects by targeting BK channels and their coupled calcium channels.

BK channels have emerged as a major mediator of alcohol response across species from *C. elegans* to humans. In *C. elegans* genetic screens designed to identify genes that mediate the intoxicating effects of ethanol, slo-1 mutants were most frequently identified and showed profound resistance. Moreover, ethanol-mediated BK channel activation is commonly observed in mice, rats, and humans, indicating that the effect of ethanol on BK channels is an evolutionarily conserved phenomenon. Recent studies of *C. elegans*, mouse, and human BK channels have demonstrated that single amino acid mutations in the cytoplasmic C-terminal domain abolish ethanol-mediated BK channel activation without altering overall channel properties, suggesting that ethanol activates BK channels through direct interaction with these amino acids. Importantly, the fact that these potential ethanol-binding sites are positioned away from known calcium binding sites suggests that ethanol may act on BK channels independently of calcium binding, although, it is difficult to completely exclude the possibility that they may affect calcium binding indirectly through an allosteric gating mechanism. However, it is worthwhile to note that the consequence of the interaction between BK channels and ethanol is not straightforward in mammals. While ethanol activates BK channels at calcium ion concentration below 20 μM, ethanol inhibits BK channels as calcium ion concentrations further increase. Furthermore, β subunits of mammalian BK channels influence the calcium sensitivity of the channels and thus shift the range of calcium concentrations at which ethanol results in potentiation. Given that the range of intracellular calcium concentrations is highly variable among different subcellular compartments, it is an open question whether ethanol uniformly targets all of the BK channels present in the plasma membrane or prefers certain types of BK channels that mediate the behavioral effects of ethanol.

Studies on SLO-1 in *C. elegans* provided insights into its localization and function in muscles and neurons. CTN-1, an α-catenin ortholog that shares homology with α-catenin and vinculin, clusters SLO-1 channels at either muscle excitation sites or presynaptic terminals by direct physical interaction, and ERG-28 (a homolog of ergosterol biosynthetic protein 28), an endoplasmic reticulum (ER) membrane protein, promotes the trafficking of SLO-1 channels from the ER. Consistent with these cell biological data, cit-1 and erg-28 mutants

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were initially isolated as suppressors of slo-1 (gf, gain-of-function) phenotypes and were found to have deficits in evoked synaptic responses in electrophysiological recordings at the neuromuscular junction\(^{13,15}\). Despite their common involvement in the SLO-1-mediated synaptic response, ERG-28, but not CTN-1, is required for the inhibitory function of SLO-1 channels in calcium signaling during the establishment of the left-right asymmetry of AWC (Amphid WIng Cell) olfactory neurons\(^{13,15,18}\). This raised the question of how these two different genes distinctively contribute to SLO-1 function.

In this study, we used C. elegans to investigate how alcohol intoxication at the behavioral level is mediated via SLO-1 channels. Using different mutants that affect either SLO-1 channel clustering or density, we discovered that clustered, but not diffuse, SLO-1 channels at presynaptic terminals and presumed muscle excitation sites mediate SLO-1 function. Using different mutants that affect either SLO-1 channel clustering or density, we discovered that slo-1\(^{-}\) (slo-1) distinctively contribute to SLO-1 function.

### Results

**cnl-1 mutants exhibit more prominent ethanol-resistant locomotory behavior than erg-28 mutants.** The cnl-1 and erg-28 genes were previously identified as regulators of SLO-1 in a genetic screen to identify suppressors of sluggish movement of the slo-1(ky399) gain-of-function mutant\(^{13,17}\). While mutations in either gene suppress the sluggish movement of the slo-1(ky399gf) mutant, only an erg-28, but not a cnl-1, mutation suppresses the defective asymmetric differentiation of the AWC neuron pair in a slo-1(ky399gf) mutant\(^{13,17,18}\). This prompted us to determine whether mutations in these two genes have differential effects on ethanol intoxication, another well-known slo-1 function. Although we have recently reported that mutations in erg-28 confer resistance to the intoxicating effect of ethanol\(^{17}\), the effect of cnl-1 mutation on ethanol-mediated behavior has not been investigated. As previously reported, at an exogenous concentration that causes complete intoxication of the wild-type animals, slo-1(lf, loss-of-function) mutants exhibited a strongly ethanol-resistant locomotory phenotype\(^4\), and erg-28(gk697770) mutants exhibited a moderately ethanol-resistant locomotory phenotype (Fig. 1, Supplementary Video 1). We found that cnl-1(eg1167) mutants showed as much resistance as slo-1(lf) mutants (Fig. 1, Supplementary Video 1). The average speeds of these tested animals in the absence of ethanol did not differ by genotype (wild-type: 191 ± 12.3, erg-28: 197 ± 11.8, slo-1: 187 ± 8.8, slo-1: 197 ± 7.6 μm sec\(^{-1}\), mean ± sem). Thus, whereas erg-28 mutation, but not cnl-1 mutation, suppresses defective asymmetric differentiation of the AWC neuron pair in the slo-1(ky399gf) mutant\(^{15}\), cnl-1 mutation is more effective than erg-28 mutation in abolishing the function of SLO-1 in ethanol intoxication.

**CTN-1 is essential for SLO-1 channel clustering, while ERG-28 is necessary to maintain the levels of SLO-1 channels.** What could be the mechanism for these differential effects of erg-28 and cnl-1 mutation? ERG-28 is required for efficient trafficking of SLO-1 to the plasma membrane and erg-28 mutation leads to a drastic reduction in the number of SLO-1 channels, but SLO-1 puncta are still present at presynaptic terminals. CTN-1 is necessary for SLO-1 to cluster at presynaptic terminals. This led us to hypothesize that the function of SLO-1 in ethanol intoxication is critically dependent on its clustering at presynaptic terminals, while its function in AWC neuron differentiation is not. In a previous study on CTN-1\(^{15}\), it was not conclusive whether cnl-1 mutation affect the SLO-1 protein level in addition to clustering because a transgenic line overexpressing SLO-1 in a select group of motor neurons was used. Hence, we used a genome-edited line expressing GFP-tagged SLO-1 to determine SLO-1 expression levels and localization in the cnl-1 mutant and compared them with the erg-28 mutant. This genome-edited line, cim105[slo-1::GFP], is indistinguishable from wild-type animals in locomotive

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**Figure 1.** cnl-1 and erg-28 mutations confer differential resistance to the intoxicating effect of ethanol. (A) The locomotory speeds of wild-type, slo-1(eg142), erg-28(gk697770), or cnl-1(eg1167) mutant animals were measured in the presence of ethanol and their values were divided by the average speed of untreated animals. Error bars represent S.E.M. One-way ANOVA with Tukey’s post-hoc analysis (wild-type vs. erg-28: *p* = 0.0473, wild-type vs. cnl-1: *p* < 0.0001, wild-type vs. slo-1: *p* < 0.0001, slo-1 vs. cnl-1: *p* = 0.8136). Data points represent three independent trials of 10 animals. See Supplementary Video 1. (B) Snapshots of single animals in the wild-type vs. ctn-1 mutant and erg-28 mutant. This genome-edited line, cim105[slo-1::GFP], is indistinguishable from wild-type animals in locomotive.
Figure 2. Mutation of the ctn-1 gene depletes clustered SLO-1 channels in neurons, whereas mutation of the erg-28 gene reduces the number of clustered SLO-1 channels. (A) SLO-1 localization pattern in the dorsal cord of wild-type, erg-28(gk697770), and ctn-1(eg1167) mutant animals. The slo-1(cim105[slo-1::GFP]) strain, which was generated using genome-editing17, was used in the analysis. Scale bar, 5 μm. (B) The number of SLO-1 puncta representing clustered SLO-1 channels in the dorsal cord. n = 10. Sample numbers represent independent biological replicates.; F(9, 9) = 4.173, p = 0.0447, ANOVA, t(10.23) = 18, p < 0.0001, unpaired two-tailed t-test. ctn-1 mutants were excluded from the test, since we could not detect stable puncta.

SLO-1 channels clustered at the presynaptic terminals confer ethanol sensitivity in locomotion. A previous study showed that neuronal SLO-1 function is critical for the intoxicating effect of ethanol on locomotory behavior4. Expression of the slo-1 gene in neurons converts the ethanol-resistant locomotory phenotype of slo-1(ff) mutants to a wild-type-like sensitive phenotype. Therefore, we asked whether the clustering of SLO-1 channels at presynaptic terminals of neurons is essential for mediating alcohol-induced sedation. To answer this question, we first expressed a wild-type copy of ctn-1 in either muscles or neurons of ctn-1(eg1167) mutant animals and determined whether SLO-1 channel clusters were restored. Muscular and neuronal expression restored SLO-1 channel clusters in each transgenic line (Fig. 4A–D). Next, we determined whether we could reverse ethanol-resistant locomotory behavior of ctn-1 mutants in neuronal and muscular transgenic lines (Fig. 4E). We found that neuronal expression, but not muscular expression, of the ctn-1 gene reversed the ethanol-resistant locomotory behavior of the ctn-1 mutants (Fig. 4E). Taken together, these results indicate that clustered SLO-1 channels in neurons are responsible for mediating the intoxicating effects of ethanol.
Ethanol-mediated egg-laying suppression is primarily mediated by clustered SLO-1 channels. In addition to locomotory behavior, slo-1 mutants exhibit robust ethanol-resistant egg-laying behavior. The neural circuits for egg-laying behavior are different from the circuits for locomotory behavior. While locomotory behavior is mediated by striated body wall muscle that receives cholinergic and GABAergic inputs from motor neurons, egg laying is mediated by smooth muscle cells that receive serotonergic and cholinergic inputs from the HSN (Hermaphrodite Specific Neuron) and VC (Ventral Cord) neurons. We examined whether erg-28(gk697770) and ctn-1(eg1167) mutants also exhibit ethanol-resistant egg-laying behavior (Fig. 5). In the presence of an ethanol dose that suppresses the egg laying of wild-type animals almost completely, slo-1 and ctn-1 mutants laid significantly more eggs over 60 min than wild-type animals (Fig. 5). However, erg-28 mutants show minimal, if any, egg laying under the same conditions. These results indicate that clustered SLO-1 channels are responsible for ethanol-mediated suppression of egg laying. Next, we asked in which tissue the clustering of SLO-1 channels is responsible for ethanol-mediated suppression of egg laying. Specifically, we investigated whether the ethanol-mediated change in egg laying behavior is mediated through egg-laying muscles or upstream neurons (Fig. 6).
Figure 4. CTN-1-mediated SLO-1 clustering in neurons is critical for alcohol intoxication. (A,B) Muscle expression of a wild-type *ctn-1* in *ctn-1*(eg1167) mutant animals restores SLO-1 clustering in the sarcolemma, but not the neurons in the dorsal cord. The white arrows indicate the dorsal cord. + muscle: *myo-3* promoter-driven expression of *ctn-1*. Scale bar, 10 μm. Comparison of muscle SLO-1 channel clusters between wild-type and muscle-rescued *ctn-1* mutant animals: F(29, 29) = 1.393, p = 0.3770, ANOVA, t(1.296) = 58, p = 0.2002, unpaired two-tailed t-test. (C,D) Neuronal expression of a wild-type *ctn-1* in *ctn-1*(eg1167) mutant animals restores SLO-1 clustering at presynaptic terminals, but not sarcolemma. + neuron: *rgef-1* promoter-driven expression of *ctn-1*. Scale bar, 10 μm. Comparison of neuronal SLO-1 channel clusters between wild-type and neuron-rescued *ctn-1* mutant animals: F(9, 9) = 1.406, p = 0.6197, ANOVA, t(5.405) = 18, p < 0.0001, unpaired two-tailed t-test. (E) Neuronal, but not muscular, *ctn-1* expression restores normal ethanol-sensitive locomotory behavior. Locomotory speed was measured as in Fig. 1. + muscle: *myo-3* promoter-driven expression of *ctn-1*. + neuron: *H20* pan-neuronal promoter-driven expression of *ctn-1*. Error bars represent S.E.M. One-way ANOVA with Tukey's post-hoc analysis (*ctn-1* ethanol treated vs. *ctn-1* (+neuron) ethanol treated: p = 0.0145, *ctn-1* ethanol treated vs. *ctn-1* (+muscle) ethanol treated: p = 0.8477). Data points represent three independent trials of 10 animals.
The plasma membrane. By contrast, mutation, which disrupts normal SLO-1 channel trafficking in the ER, 
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ctn-1 terminals and presumed muscle excitation sites. Contrary to this belief, our data indicate that the density of 
ion channels is often regulated by the retrieval from the plasma membrane and subsequent degradation by 
proteasomes or endocytosis/lysosomes. It is conceivable that SLO-1 channels similarly undergo degradation 
via proteasomal- or endocytosis/lysosome-mediated degradation when they cannot form clusters at presynaptic 
terminals and presumed muscle excitation sites. Contrary to this belief, our data indicate that the density of SLO-1 
channels is not regulated by the retrieval from the plasma membrane. This steady level of SLO-1 is in contrast with the 
dramatically reduced SLO-1 channel level observed in the ctn-1 mutant is in contrast with the dramatically reduced SLO-1 channel level observed in the egg-laying muscles (Fig. 6A). This restored SLO-1 channel clustering reduced egg laying in the presence of ethanol within a 60-min time period. These results indicate that SLO-1 channel clusters in egg-laying muscles significantly contribute to ethanol sensitivity (Fig. 6B). Likewise, animals expressing a wild-type copy of the ctn-1 gene under the control of a pan-neuronal promoter in ctn-1 mutants, which restores SLO-1 clustering in the neuron (Fig. 2), laid moderately fewer eggs than ctn-1(eg1167) mutants within a 60-min time period in response to the same ethanol dose. Taken together, these results indicate that, unlike locomotory behavior, ethanol-mediated suppression of egg laying is mediated by clustered SLO-1 channels present in both egg-laying muscles and neurons.

**Discussions**

BK channels are known to mediate ethanol sensitivity across a wide variety of species, including C. elegans. Here, we demonstrate in a C. elegans genetic study that the behavioral effects of ethanol are mediated by clustered, but not dispersed, SLO-1 channels. We show that ctn-1 mutation causes a drastic elimination of clustered SLO-1 channels and, as a consequence, an increase in diffuse SLO-1 channels without affecting their overall level. We found that this ctn-1 mutation confers ethanol-resistant locomotory and egg-laying behaviors equivalent to slo-1 null mutations, indicating that the behavioral effect of ethanol is mediated by clustered SLO-1 channels.

Our study provides evidence that some of the functions of SLO-1, such as alcohol intoxication, are mediated uniquely by clustered SLO-1 channels, while other functions, such as asymmetry of olfactory neurons, are mediated by diffuse, scattered SLO-1 channels. Our previous study showed that ctn-1 mutation abolishes SLO-1 puncta in neurons and muscles, although we were not able to definitively conclude whether ctn-1 mutation causes any change in diffuse, scattered SLO-1 channels. Using a SLO-1::GFP genome-edited strain we now show that ctn-1 mutation disperses SLO-1 channels without affecting their overall abundance. This steady level of SLO-1 channels in the ctn-1 mutant is in contrast with the dramatically reduced SLO-1 channel level observed in the erg-28 mutant. Mutation in the erg-28 gene causes ineffective exit of SLO-1 channels from the ER, thus resulting in the degradation of a majority of SLO-1 channels and a consequent reduction in SLO-1 channel density. Mutation in the ctn-1 gene does not suppress the defect in the left-right asymmetry of AWC olfactory neurons observed in slo-1 gain-of-function mutants, while erg-28 mutation does. These results indicate that the left-right asymmetry of AWC olfactory neurons is associated with the total number of SLO-1 channels, but not the clustering of SLO-1 channels. On the other hand, ctn-1 mutants exhibit prominent ethanol-resistant locomotory and egg-laying behaviors, whereas erg-28 mutants show moderate ethanol-resistant locomotory and weak, if any, egg-laying behaviors.

Another interesting result obtained in our study is how the density of BK channels is regulated. The density of ion channels is often regulated by the retrieval from the plasma membrane and subsequent degradation by proteasomes or endocytosis/lysosomes. It is conceivable that SLO-1 channels similarly undergo degradation via proteasomal- or endocytosis/lysosome-mediated degradation when they cannot form clusters at presynaptic terminals and presumed muscle excitation sites. Contrary to this belief, our data indicate that the density of SLO-1 channels is not regulated by the retrieval from the plasma membrane. ctn-1 mutation abolishes SLO-1 channel clustering and causes SLO-1 channels to disperse through neurons and muscles but does not influence the overall levels of SLO-1 channels. Furthermore, because ctn-1 mutation does not suppress the defect in the left-right asymmetry of slo-1(gf) AWC neurons, diffuse SLO-1 channels are functional and are likely present at the plasma membrane. By contrast, erg-28 mutation, which disrupts normal SLO-1 channel trafficking in the ER,
Figure 6. CTN-1-mediated SLO-1 clustering in neurons and egg-laying muscles contributes to ethanol-mediated suppression of egg laying behavior. (A) Muscle expression of a wild-type copy of ctn-1 in ctn-1(eg1167) mutant animals restores SLO-1 clustering at egg-laying muscles. The images represent a maximum projection of Z-stack image sections. The asterisks, arrows, and arrowheads denote the ventral nerve cord, the vulval slit, and SLO-1 clusters present in egg-laying muscle cells, respectively. The number of SLO-1 channel clusters were quantified from a region of a single muscle cell, which was not overlapped with body wall muscle or the ventral cord. Comparison of vulval muscle SLO-1 channel clusters between wild-type and muscle-rescued ctn-1 mutant animals: F(6, 6) = 1.039, p = 0.96, ANOVA, t(1.482) = 12, p < 0.1642, unpaired two-tailed t-test. WT: wild-type, +muscle: myo-3 promoter-driven expression of ctn-1. (B) Neuronal and muscular SLO-1 channel clustering contributes to ethanol-mediated suppression of egg laying. Egg laying was measured as in Fig. 5. Egg laying was measured in 10 age-matched animals (30 h post L4) and repeated three times (biological replicates). +muscle: myo-3 promoter-driven expression of ctn-1, +neuron: H20 pan-neuronal promoter-driven expression of ctn-1. Error bars represent S.E.M. One-way ANOVA with Tukey’s post-hoc analysis (ctn-1 ethanol treated vs. ctn-1(+neuron) ethanol treated: p = 0.0086, ctn-1 ethanol treated vs. ctn-1(+muscle) ethanol treated: p < 0.0001).
reduce the abundance of SLO-1 channels by over 70%. Therefore, the major regulatory point of the overall SLO-1 channel level is channel trafficking in the ER.

Why does SLO-1 channel-mediated alcohol intoxication require clustering at presynaptic terminals and the sarcolemma? One possibility is that ethanol can activate both clustered and diffuse SLO-1 channels but only clustered SLO-1 channels are relevant to ethanol sensitivity due to their coupling with calcium channels that mediate behavioral sensitivity to ethanol. It has been reported that BK channels are functionally coupled with a wide variety of calcium channels23–27, which localize to distinct subcellular compartments, including pre- and post-synaptic sites, the ER, and muscle excitation sites30–32. Although clustered and diffuse SLO-1 channels both increase potassium efflux in response to ethanol, only clustered SLO-1 channels lead to an inhibition of behaviorally-relevant calcium channels, thereby causing sedation at the behavioral level. Alternatively, it is possible that, due to their unique locations, SLO-1 channels localized in the clusters bind to ethanol more readily than diffuse channels do. These clustered SLO-1 channels are found at the major entry sites for calcium ions, such as presynaptic terminals and muscle excitation sites, and thus could be exposed to a specific level of calcium ions that makes channel conformation most favorable for ethanol binding. BK channels have indeed been described to adopt multiple complex conformations depending on calcium and voltage8,30–32.

Our study demonstrates that the clustering of SLO-1 at presynaptic terminals or presumed muscle excitation sites plays a critical role in mediating ethanol sensitivity. It is generally thought that ethanol-mediated SLO-1 activation reduces overall neural excitability by hyperpolarizing the entire neuron. However, our study indicates that the depressive behavioral effects of ethanol on both locomotory and egg-laying behavior are mediated by clustered SLO-1 channels, suggesting that ethanol inhibits specific molecular steps through a local hyperpolarization mediated by SLO-1 channels. Given that BK channels are functionally coupled with calcium channels in muscles and neurons, these molecular steps likely involve the functions of calcium channels. While our recent study showed that neuron-specific P/Q-type voltage-gated UNC-2 calcium channels, which localize to presynaptic terminals33, indirectly contribute to SLO-1 localization in select cholinergic neurons34, whether alcohol-mediated SLO-1 activation suppresses UNC-2 function is not clear. Furthermore, it is not clear which calcium channel is the target of alcohol-mediated SLO-1 activation in egg-laying muscles. In the future, it will be necessary to identify calcium channels that couple with clustered SLO-1 channels and mediate the behavioral effects of ethanol.

Drug targeting of BK channels has been suggested as a tool to control alcohol dependence or alcohol withdrawal34–37. Given that both diffuse and clustered BK channels serve overlapping and distinct functions in C. elegans and mammals38,39, targeting clustered BK channels may offer a better therapeutic strategy than indiscriminate drug targeting of BK channels. Blocking BK channel clustering will be an effective way of de-coupling BK channels from calcium channels that mediate behavioral alcohol response while sparing the functions dependent on diffuse, disperse BK channels.

Methods
Worm strains and maintenance. All C. elegans strains were cultured at 20 °C on NGM (nematode growth medium) plates seeded with E. coli OP50. Table 1 lists all strains along with their genotypes and origins. Transgenic strains were constructed using standard DNA microinjection methods with either the coelomocyte unc-122p::GFP (or mCherry) or AIY (Amphid Interneuron Y) neuron ttx-3p::RFP co-injection marker40.

Ethanol-resistant locomotory behavior assay. All animals used for locomotory assays were day 1 adults (20–24 hours post L4). The overall method for the assay has been previously described41. The NGM agar plates used for all of the experiments were dried without lids for 25 min in a 60 °C incubator. This drying process

Table 1. List of C. elegans strains used in this study.

| Strain   | Genotype                                      | Source              | Notes                                      |
|----------|-----------------------------------------------|---------------------|--------------------------------------------|
| N2       | +                                             | CGC                 |                                            |
| HKK94    | ctn-1(eg167)I                                 | Abraham et al.13    |                                            |
| HKK538   | erg-28(gk697770)                              | Oh et al.17         |                                            |
| BZ142    | slo-1(eg1420)                                 | Davies et al.1      | slo-1 loss-of-function                     |
| HKK796   | slo-1(cim105[slo-1::GFP])                     | Oh et al.17         | CRISPR/Cas9, C-terminal GFP fusion to SLO-1|
| HKK123   | ctn-1(eg167)I; cimEx6[myo-3p::GFP, myo-3p::ctn-1, unc-122p::GFP] | Abraham et al.13    | ctn-1 muscle rescue                        |
| HKK124   | ctn-1(eg167)I; cimEx7[H20p::GFP, H20p::ctn-1, unc-122p::GFP] | Abraham et al.13    | ctn-1 neuronal rescue                      |
| HKK838   | erg-28(gk697770) slo-1(cim105)Y                | Oh et al.17         |                                            |
| HKK839   | ctn-1(eg167)I; slo-1(cim105)Y                 | This study          |                                            |
| HKK1039  | ctn-1(eg167)I; cimEx100[odr-1p::slo-1::gfp, unc-122p::mCherry] | This study          | SLO-1::GFP expression in AWC and AWB (Amphid Wing B cell) neurons |
| HKK1037  | cimEx98[odr-1p::slo-1::gfp, unc-122p::mCherry] | This study          | SLO-1::GFP expression in AWC and AWB neurons |
| HKK823   | ctn-1(eg167)I; slo-1(cim105)Y; cim14[unc-129p::mCherry::rab-3, odr-1p::GFP]; cimEx70[ttx-3p::ctn-1, ttx-3p::RFP] | This study          | neuronal expression of ctn-1               |
| HKK821   | ctn-1(eg167)I; slo-1(cim105)Y; cim1s14, cimEx69[myo-3p::ctn-1, ttx-3p::RFP] | This study          | muscle expression of ctn-1                |
was necessary to prevent the animals from exhibiting a swimming-like movement on wet plates, and this process usually reduces the agar to 85–90% of its original volume. After the plates were cooled down to room temperature, a desired amount of ethanol (400 mM), which gives internal ethanol concentration of approximately 50 mM,
2 was applied to the NGM plates. The plates were immediately sealed with parafilm and incubated for an additional 2 hours to equilibrate with the ethanol. Immediately before the assays, three flame-heated copper rings were embedded in the agar surface of the NGM plates. These copper rings allow for the simultaneous comparison of three different genotypes in the same assay plates. We included wild-type and slo-1[Δf] mutant (or ctn-1 mutant) animals in the ethanol assays as positive and negative controls. Ten animals with a given genotype were incubated for 10 min in unseeded plates to remove attached bacteria and then transferred inside a copper ring. After 10 min of exposure to 0 mM and 400 mM ethanol, video frames from three different genotypes were simultaneously acquired from a dissecting microscope fitted with GO-3 camera (Qimaging) with 500 ms intervals for 2 min. For each genotype, the experiments on ethanol plates were repeated three times. We calculated the average speed of the tested animals using the Tracking Objects function in Image-Pro Plus 7.0 (Media Cybernetics, RRID:SCR_016879). The software algorithm automatically generates new tracks when the paths of animals overlap due to collision.

Ethanol-resistant egg-laying behavior assay. All animals used for the egg-laying assays were day 1 adults (30 hours post L4). Unlike the locomotory assays, the egg-laying assay requires bacterial food
7. NGM agar plates were dried without lids for 2 hours in a 37 °C incubator and then seeded in a triradiate shape with E. coli OP50. The next day, three small circular agar plugs (1 cm diameter) were punched out from the outside of the bacterial lawn and ethanol (500 mM) was then added to the holes. The agar plugs were placed back into the holes after the ethanol was halfway absorbed into the agar plate. During this process, we ensured that the ethanol did not come into direct contact with the bacterial food. After being sealed with parafilm, the plates were equilibrated at room temperature for approximately 2 hours. As in the locomotory assay, three copper rings were embedded on the agar surface, but the rings were placed where the bacterial food was spread. These copper rings allow the simultaneous comparison of three different genotypes in the same assay plates. Ten animals were directly transferred to each copper ring, and the number of eggs laid was counted at 60 min. The assay included wild-type and slo-1[Δf] mutant animals in the ethanol assays as negative and positive controls, respectively. The experiments were repeated three times for each genotype.

Microscopy and time-lapse imaging. Images were acquired using a 63x/1.4 numerical aperture on a Zeiss microscope (Axio-Observer Z1) equipped with Spectra X light engine (Lumenroc). Images were captured with an 82% quantum efficiency Zyla 4.2 PLUS sCMOS camera (Andor) using MetaMorph 7.8 (RRID:SCR_002368) at 200 ms intervals with 350 ms exposure time. To quantify the intensity of SLO-1 channel clusters, a 150-pixel line was drawn on the linearly aligned fluorescence clusters in head and body-wall muscles, and its pixel intensity was quantified using a line-scanning method in MetaMorph software package (RRID:SCR_002368). To accommodate the variability within each muscle, data obtained from three neighboring parallel lines were averaged for a single data point. For the quantification in egg-laying muscle, the number of fluorescence clusters was counted from an area of a single vulval muscle cell, which was not overlapped with body wall muscle cells or the ventral nerve cord.

Western blotting and quantification. Mixed-stage worms were lysed and solubilized in SDS lysis buffer (2% SDS, 100 mM NaCl, 10% glycerol, and 50 mM Tris–HCl, pH 6.8) with sonication. Worm extracts were separated by SDS-PAGE and transferred onto PVDF membranes. The separated proteins were probed with anti-GFP (Cell Signaling Technology, #2956, RRID:AB_1196615) or anti-tubulin (Developmental Studies Hybridoma Bank, AA4.3, RRID:AB_579793) antibodies. The pixel intensities of GFP and tubulin bands were quantified using Adobe Photoshop CC 2015.

Experimental design and statistical analysis. The speed data (Figs 1A and 4E) and egg-laying data (Figs 5 and 6B) were analyzed using one-way ANOVA with Tukey’s post hoc analysis, and the number of fluorescence clusters was analyzed using a two-tailed t-test (GraphPad Prism 7, RRID:SCR_002798). Statistical analyses for each experiment are detailed in the figure legends.

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