Behavioral Deficits Following Withdrawal from Chronic Ethanol Are Influenced by SLO Channel Function in *Caenorhabditis elegans*

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ABSTRACT Symptoms of withdrawal from chronic alcohol use are a driving force for relapse in alcohol dependence. Thus, uncovering molecular targets to lessen their severity is key to breaking the cycle of dependence. Using the nematode *Caenorhabditis elegans*, we tested whether one highly conserved ethanol target, the large-conductance, calcium-activated potassium channel (known as the BK channel or Slo1), modulates ethanol withdrawal. Consistent with a previous report, we found that *C. elegans* displays withdrawal-related behavioral impairments after cessation of chronic ethanol exposure. We found that the degree of impairment is exacerbated in worms lacking the worm BK channel, SLO-1, and is reduced by selective rescue of this channel in the nervous system. Enhanced SLO-1 function, via gain-of-function mutation or overexpression, also dramatically reduced behavioral impairment during withdrawal. Consistent with these results, we found that chronic ethanol exposure decreased SLO-1 expression in a subset of neurons. In addition, we found that the function of a distinct, conserved Slo family channel, SLO-2, showed an inverse relationship to withdrawal behavior, and this influence depended on SLO-1 function. Together, our findings show that modulation of either Slo family ion channel bidirectionally regulates withdrawal behaviors in worm, supporting further exploration of the Slo family as targets for normalizing behaviors during alcohol withdrawal.

KEYWORDS alcohol; ethanol; withdrawal; behavior; *slo-1*; potassium channel

NEURAL adaptation during persistent exposure to ethanol underlies many of the symptoms of withdrawal from chronic alcohol consumption (Koob *et al.* 1998, 2013) . These symptoms include life-threatening conditions such as seizures and rapid heart rate as well as psychological conditions such as anxiety and confusion (Finn and Crabbe 1997). The severity of symptoms, particularly the degree of negative affect, following withdrawal from chronic ethanol use is a driving force for relapse (Winward *et al.* 2014). Uncovering targets that modulate the neural state in withdrawal to more closely match the naïve state is important for developing pharmacological agents that will ameliorate withdrawal symptoms and thus reduce relapse (Becker and Mulholland 2014).

The large-conductance, calcium- and voltage-activated potassium channel, known as the BK channel or Slo1, is a well-conserved target of ethanol across species as diverse as worm, fly, mouse, and man (Mulholland *et al.* 2009; Treistman and Martin 2009; Bettinger and Davies 2014). Across the phylogenetic spectrum, clinically relevant concentrations (10–100 mM) of ethanol alter Slo1 gating in *in vitro* preparations (Chu and Treistman 1997; Jakab *et al.* 1997; Dopico *et al.* 1998; Walters *et al.* 2000; Dopico 2003; Brodie *et al.* 2007). Additionally, impairing Slo1 function influences ethanol-related behaviors, such as acute intoxication and tolerance (Davies *et al.* 2003; Cowmeadow *et al.* 2005, 2006; Martin *et al.* 2008; Kreifeldt *et al.* 2013). In mammalian tissue, prolonged ethanol exposure lowers overall expression of Slo1 and increases abundance of ethanol-insensitive isoforms of the channel (Pietrzykowski *et al.* 2008; Velázquez-Marrero

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et al. 2011; Li *et al.* 2013; N'Gouemo and Morad 2014). These results have made Slo1 a potential target for treating alcohol withdrawal symptoms (Ghezzi *et al.* 2012; N'Gouemo and Morad 2014). Slo1 function appears to contribute to the escalation of drinking in a withdrawal paradigm as revealed in mice lacking nonessential auxiliary subunits of the channel (Kreifeldt *et al.* 2013). However, study of Slo1 in withdrawal directly has been impeded by the behavioral and physiological deficits exhibited by Slo1 knockout mice (*e.g.*, Thorneloe *et al.* 2005; Meredith *et al.* 2006; Pyott *et al.* 2007; Typlt *et al.* 2013; Lai *et al.* 2014).

To surmount the pleiotropic deficits of the Slo1 knockout mouse and directly probe whether Slo1 function contributes to behavioral deficits during alcohol withdrawal, we used the nematode *Caenorhabditis elegans*. Previously, the worm ortholog of the Slo1 channel, called SLO-1, was shown to be critical for acute ethanol intoxication with unbiased forward genetic screens (Davies *et al.* 2003). Ethanol activated the SLO-1 channel in neurons at the same concentration (20–100 mM) as shown for human Slo1 channels (Davies *et al.* 2003; Davis *et al.* 2014). Loss-of-function mutations in *slo-1* rendered worms resistant to intoxication, while gain-of-function mutations in *slo-1* caused worms to appear intoxicated in the absence of alcohol (Davies *et al.* 2003).

Here we show that, in contrast, enhanced SLO-1 function reduced the severity of alcohol withdrawal. Consistent with previous findings in mammalian cells *in vitro* (Pietrzykowski *et al.* 2008; Ponomarev *et al.* 2012; N'Gouemo and Morad 2014), SLO-1 expression declined in some neurons during chronic ethanol exposure *in vivo*. Another member of the large-conductance potassium-channel family, SLO-2 (Yuan *et al.* 2000; Zhang *et al.* 2013), showed a relationship to alcohol withdrawal that was inverse to and dependent upon SLO-1 function. Loss of function in *slo-2* enhanced SLO-1 expression in naïve worms. Our results are consistent with the idea that Slo channels are part of the neural adaptation to chronic ethanol exposure in *C. elegans*. Additionally, increasing SLO-1 channel activity or decreasing SLO-2 channel activity rebalances neural circuits responsible for behaviors impaired during alcohol withdrawal.

Materials and Methods

Animals

C. elegans were grown at 20° and fed OP50 bacteria on Nematode Growth Media (NGM) agar plates as described in Brenner (1974). Worms cultured on plates contaminated with fungi or other bacteria were excluded. The reference wild-type (WT) strain was N2 Bristol. The background for the *slo-1(null)* rescue strains was NM1968, harboring the previously characterized null allele *js379* (Wang *et al.* 2001). The background *slo-1(null);slo-2(null)* double mutant strain was JPS432, obtained by crossing NM1968 with LY100 and confirmed via sequencing. This latter strain harbored the previously characterized *slo-2* null allele *nf100* (Santi *et al.* 2003). Strains NM1630 and LY101 were also used as *slo-1(null)* and *slo-2(null)* reference strains, respectively. JPS1 carried the previously characterized *slo-1* gain-of-function allele *ky399* (Davies *et al.* 2003). The reference strains for *dgk-1(sy428)* and *unc-10(md1117)* were PS2627 and NM1657, respectively.

Transgenesis

Multi-site gateway technology (Invitrogen, Carlsbad, CA) was used to construct plasmids for the *slo-1* rescue and overexpression strains. To drive *slo-1a(cDNA)*::*mCherry-unc-54* UTR expression, 1894 kb of the native *slo-1* promoter (*pslo-1*) was used. *punc-119* was used as a pan-neuronal promoter (Maduro and Pilgrim 1995). All plasmids were injected at a concentration of 20–25 ng/μl for rescue in a *slo-1(js379)* or *slo-1(js379);slo-2(nf100)* background and 5–10 ng/μl for overexpression in a WT background (Mello *et al.* 1991). The co-injection reporter PCFJ90 *pmyo-2:mCherry* (1.25 ng/μl) was used to ensure transformation. Two independent isolates were obtained for most strains to help control for variation in extrachromosomal arrays. The following strains were generated: JPS344 (*pslo-1:slo-1#1* in text) *slo-1(js379) vxEx344* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR pmyo-2*::*mCherry*], JPS345 (*pslo-1:slo-1#2* in text) *slo-1(js379) vxEx345* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR* + *pmyo-2*::*mCherry*], JPS529 *slo-1(js379) vxEx529* [*punc-119*::*slo-1a*::*mCherry*::*unc-54UTR* + *pmyo-2*::*mCherry*], JPS523 *slo-1(js379);slo-2(nf100) vxEx523* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR* + *pmyo-2*::*mCherry*], JPS524 *slo-1(js379);slo-2(nf100) vxEx524* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR* + *pmyo-2*::*mCherry*], JPS521 *vxEx521* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR* + *pmyo-2*::*mCherry*] (injected at 5 ng/μl), JPS522 *vxEx522* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR* + *pmyo-2*::*mCherry*] (injected at 10 ng/μl). Additionally, a *slo-2(+)* extrachromosomal array previously used to rescue a hypoxia response (Wojtovich *et al.* 2011) was crossed onto the *slo-2(nf100)* background to make JPS877 *pha-1(e2123);slo-2(nf100)* rnyEx112 [partial *slo-2*::*mCherry* recombined *in vivo* with linear F56A8 fosmid + *pha-1(+)*]. To image mCherry-tagged SLO-1 protein expression, we first made strains JPS572 *slo-1(null);vsIs48* [*punc-17*::*GFP*] *vxEx345* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR* + *pmyo-2*::*mCherry*], and JPS595 *slo-1(null) vxEx595* [*pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR* + *podr-10*::*GFP*]. JPS854 *slo-1(js379) vxEx854* [*punc-119*::*GFP* + *pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR*], and JPS874 *slo-1(js379);slo-2(nf100) vxEx854* [*punc-119*::*GFP* + *pslo-1*::*slo-1a*::*mCherry*::*unc-54UTR*] were then made with the same extrachromosomal array to allow direct comparison between strains. To determine if the *slo-1* promoter was sensitive to chronic ethanol treatment, we made strain JPS584 *vxEx584* [*pslo-1(rescue)*::*GFP*::*unc-54UTR* + *ptph-1*::*mCherry*].

Ethanol treatment

Methods for assaying ethanol withdrawal were modified from Mitchell *et al.* (2010). Well-populated (>200 worms),

6-cm-diameter plates were bleached to obtain eggs, which were allowed to grow to the mid-to-late-stage L4-larval stage. Age-matched L4 worms derived from the same plate were then divided between an ethanol-infused (+ethanol) and standard control (−ethanol) seeded plate. Standard plates were 6-cm-diameter Petri dishes filled with 12 ml NGM-agar and seeded with OP50 bacteria. Ethanol plates (400 mM) were prepared by adding 280 μl of 200-proof ethanol (Sigma Aldrich) beneath the agar of the standard seeded plates and allowing the ethanol to soak into the agar. The plates were sealed with Parafilm and worms were exposed for 20–24 hr. The ethanol-treated worms were withdrawn on standard seeded plates for 1 hr. Worms kept on the standard seeded plates overnight served as the naïve controls.

Diacetyl-race assay

Methods were modified from Bargmann *et al.* (1993) and Mitchell *et al.* (2010). Race plates were prepared by drawing a start and a goal line on the bottom of standard unseeded, 6-cm-diameter Petri dishes filled with 12 ml NGM-agar. Race plates with low-dose ethanol were infused with 60 mM 200-proof ethanol (Sigma Aldrich) and sealed with Parafilm. This concentration of ethanol was chosen because it was previously shown to minimize withdrawal behaviors (Mitchell *et al.* 2010). The race plates were prepared within 20 min of each race by applying a 10-μl mixture of attractant (1:1000 dilution of diacetyl) and paralytic (100-mM sodium azide) at the goal. Worms were cleaned of bacteria by transferring them to one or more unseeded plates until they left no residual tracks of bacteria, a process that took <10 min. Approximately 25 worms were transferred to the start side of the race plate with a platinum pick. The total number of worms and the number of worms that reached the goal were counted every 15 min for 1 hr to calculate the percent of worms at the goal. Counts were performed with the observer blind to genotype and experimental treatment. The area under the curve (AUC) was calculated for the fraction of worms at the goal *vs.* time for each race. In order to compare the magnitude of impairment during withdrawal between strains, the performance of withdrawn worms was normalized to the performance of the naïve worms run in tandem to generate normAUC values.

Locomotion assay

Worms were cleaned of bacteria as described above and ~15 were moved into a 5/8-inch-diameter copper ring sealed on a standard unseeded plate (see above). Movement was recorded for 2 min at 2 frames/sec with a FLEA digital camera (Point Gray, Richmond, BC, Canada). The distance that the worms crawled during 1 min was measured using a semiautomated procedure in ImagePro Plus (Media Cybernetics, Rockville, MD) to objectively calculate overall speed of individual worms.

Gas chromatography

Internal ethanol measurements were estimated using previous methods (Alaimo *et al.* 2012). Only a fraction of the external ethanol enters worms when treated on NGM-agar plates; but see Mitchell *et al.* (2007) for an alternate view of how ethanol enters worms incubated in liquid Dent's medium. For WT worms, we measured the internal ethanol concentration at 0, 20 min, 3 and 24 hr of ethanol treatment as well as after 1 hr of withdrawal. For other strains, the internal ethanol concentration was measured at 24 and 1 hr after withdrawal. Worms exposed to ethanol as described above were rinsed with ice-cold NGM buffer into a 1.5-ml Eppendorf tube and briefly spun (<10 sec) at low speed to separate the worms from the bacteria. The liquid was removed, replaced with ice-cold NGM buffer and the sample was spun again. All of the liquid was carefully removed to leave only the worm pellet. This pellet was then doubled in volume with ice-cold NGM buffer. The sample went through five rapid freeze-thaw cycles using liquid nitrogen plus 30 sec of vortexing and was finally spun down at high speed for 2 min. Two microliters of the sample was added to a gas chromatography vial. The amount of ethanol was measured using headspace solid-phase microextraction gas chromatography (HS-SPME-GC). Automation of the HS-SPME-GC measurement was obtained using an autosampler (Combi Pal-CTC Analytics, Basel, Switzerland). Ethanol analysis was carried out using a gas chromatograph equipped with a flame ionization detector.

Confocal microscopy

First-day adult worms were mounted on 2% agarose pads, immobilized with 30-mM sodium azide and imaged with a Zeiss laser-scanning microscope (LSM710) using Zen (black edition) acquisition software (Carl Zeiss, Germany). GFP fluorescence and phase contrast images were collected using a 488-nm laser and mCherry fluorescence was collected using a 561-nm laser. Once set, the laser power and electronic gain were held constant for the red and green channels to perform ratiometric analysis. Using a 63× water immersion objective and a 0.9-μm pinhole, neurons were imaged in three dimensions taking slices every 0.8 μm through the *z*-axis. Ratiometric analysis was completed in ImageJ (Schneider *et al.* 2012). Z-stacks through the neurons were summed, and the mean pixel intensity was measured for the red and green channel in the area of interest. Background intensity was measured using the same size region of interest next to the worm. This background measurement was then subtracted from the neuronal measurement.

Quantitative real-time PCR

Whole worm RNA was prepared for nine biological replicates of age-matched, day 1 adult WT and *slo-2(nf100)* null worms that were either naïve or treated with ethanol for 24 hr (see above). Worms were washed 2×, lysed, and mRNA was prepared using the PureLink RNA Mini kit (Thermo Fisher).

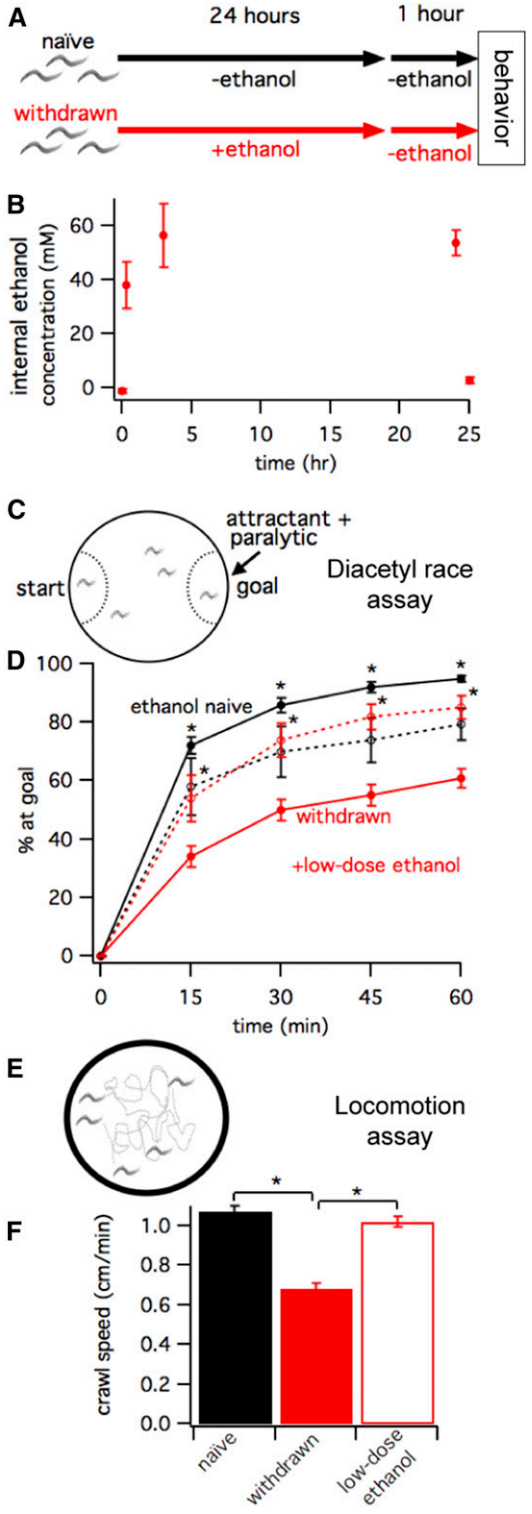

Figure 1 Two behavioral deficits during alcohol withdrawal recovered by low-dose ethanol. Worms withdrawn from chronic ethanol exposure display behavioral deficits. (A) Schematic showing the exposure paradigm used for the two treatment groups, naïve (black) and withdrawn (red), starting with age-matched L4-stage larvae. Worms assayed for behaviors are young adults 25 hr later. (B) Gas chromatography determined internal ethanol concentration after 0, 20 min, 3, and 24 hr of ethanol treatment, and after 1 hr of withdrawal. (C) Schematic of the diacetyl-race assay. Diacetyl was used as a volatile attractant and sodium azide was used as a paralytic trapping worms that reached the goal. (D) The mean fraction of WT worms that reached the attractant ± SEM plotted every 15 min for 1 hr. At all timepoints, withdrawn worms (solid red line) performed less well than naïve worms (solid black line, $^{****}P < 0.001$). Withdrawn worms treated with a low dose of ethanol during the race (dashed red line) performed significantly better than withdrawn worms ($^{*}P < 0.05$). Naïve worms treated with a low dose of ethanol during the race (dashed black line) performed similarly to naïve worms. (E) Schematic of locomotion assay. Worms were allowed to move freely on a blank agar surface within a copper ring. (F) Histogram of mean speed ± SEM. Locomotion was also impaired during withdrawal. Withdrawn worms moved slower than naïve worms (naïve *vs.* withdrawn, 1.10 ± 0.026 *vs.* 0.68 ± 0.028 cm/min; $^{****}P < 0.001$). Again, this withdrawal-induced impairment was improved when worms were placed on low-dose ethanol during the assay (withdrawn *vs.* + low-dose ethanol, 0.68 ± 0.028 *vs.* 1.0 ± 0.025 cm/min; $^{****}P < 0.001$).

Messenger RNA (mRNA) was converted to complementary DNA (cDNA) using the SuperScript VILO master mix (Thermo Fisher). Taqman probes were used to measure transcript expression for *slo-1* (Ce02419368_g1, probe binds to all isoforms) and the control gene *cdc-42* (Ce02435136_g1). To compare transcript expression across the four groups (WT ± ethanol, *slo-2* ± ethanol) the fold change ($2^{-\Delta\Delta Ct}$) was converted to relative transcript expression (Falcon *et al.* 2013; Ozburn *et al.* 2015). Fold change for each individual run was normalized such that the highest was 100. Mean ± SEM for relative transcript expression was calculated for each group.

Statistical analysis

Sigmaplot 12.5 (Systat Software, San Jose, CA) was used for all statistical analyses to determine significance ($P \leq 0.05$, two tailed) between two or more groups. Groups were compared using *t*- or ANOVA tests where appropriate. If needed, *post hoc* multiple comparisons were performed using the Holm-Sidak method. All measures were obtained with the observer blind to genotype and experimental treatment.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Strains are available upon request or through the *Caenorhabditis* Genetics Center.

Results

Behavioral deficits during withdrawal recovered by low-dose ethanol

To test how *C. elegans* behaves during withdrawal from chronic ethanol exposure, we modified a treatment paradigm based on Mitchell *et al.* (2010). In brief, WT, age-matched, L4-stage larvae were treated with ethanol for 24 hr and then withdrawn for 1 hr on seeded control plates (red timeline in Figure 1A, see *Materials and Methods* for details). A control group of naïve worms was set up in parallel (black timeline in Figure 1A). We used gas chromatography to estimate the

worms' internal ethanol concentration at 0, 20 min, 3, and 24 hr of ethanol treatment, as well as after 1 hr of withdrawal. Internal ethanol concentration rose gradually to ~50 mM over 3 hr, consistent with noninstantaneous uptake of the ethanol from the agar substrate (Figure 1B). *C. elegans* only absorbs a fraction of the high external concentration of ethanol (400 mM) when assayed on standard plates (Alaimo *et al.* 2012). The internal ethanol concentration was ~50 mM after 24-hr exposure and returned to baseline values after withdrawal (Figure 1B).

Next, we assayed the behavioral performance of worms in a chemotaxis race to the attractant diacetyl (Figure 1C). Withdrawn worms and ethanol-naïve controls from the same age-matched cohort were raced in tandem on different plates. Similar to findings by Mitchell *et al.* (2010), we found that worms withdrawn from chronic ethanol treatment showed impaired diacetyl-race performance relative to untreated, ethanol-naïve worms (Figure 1D; comparison of AUCs, $P < 0.001$, $N = 24$). The performance of worms withdrawn from chronic ethanol treatment improved on race plates with a low concentration (15% of the chronic dose) of exogenous ethanol (comparison of AUCs, $P < 0.01$, $N = 4$–24), while the same dose did not improve performance for ethanol-naïve worms (Figure 1D; comparison of AUCs, n.s., $N = 5$–24).

In a separate assay without a chemoattractant, we determined that baseline locomotion was also impaired during withdrawal. Crawling on unseeded plates (Figure 1E) was ~40% slower for withdrawn worms than naïve worms (naïve *vs.* withdrawn, 1.10 ± 0.026 *vs.* 0.68 ± 0.028 cm/min, $P < 0.001$; Figure 1F). Again, this withdrawal-induced impairment was improved when worms were treated with low-dose ethanol (withdrawn *vs.* withdrawn + low-dose ethanol, 0.68 ± 0.028 *vs.* 1.0 ± 0.025 cm/min, $P < 0.001$; Figure 1F). Thus, in agreement with Mitchell *et al.* (2010), we find that *C. elegans* displays the fundamental traits of alcohol withdrawal symptoms observed in higher animals including humans, *i.e.*, behaviors are impaired after removal from a prolonged exposure to ethanol, and these impairments can be partly to fully rectified by reexposure to a low dose of ethanol.

Withdrawal impairments worsened by reduced neuronal SLO-1 channel function

The BK channel SLO-1 represents a major target of ethanol in *C. elegans* (Davies *et al.* 2003). To ascertain whether these behavioral impairments during ethanol withdrawal are modulated by changes in SLO-1 activity or expression, we looked at withdrawal behavior in a number of strains with genetically altered *slo-1*. Withdrawn performance was assessed as a function of naïve performance to account for any baseline behavioral effects of the genetic modifications. Two strains carrying the *slo-1* null alleles, *js379* and *js118*, respectively, showed significantly stronger withdrawal-related impairment on the diacetyl-race assay than WT (Figure 2A; *js379 vs.* WT, $P < 0.01$; *js118 vs.* WT, $P < 0.005$). The *slo-1(null)* strains also showed greater withdrawal-induced slowing in locomotion than WT (Figure 2B; *js379 vs.* WT, $P < 0.05$; *js118 vs.* WT, $P < 0.01$). The deleterious effect of losing *slo-1* function on withdrawal behaviors did not appear to affect ethanol uptake or metabolism (Supplemental Material, Figure S2; *slo-1(null) vs.* WT, n.s.).

Next, we explored the severe withdrawal phenotype of the *slo-1(js379)* null mutant. This phenotype appeared to be recessive because a heterozygous *slo-1(+/js379)* strain showed similar withdrawal-related behavioral impairment to WT (Figure 2A; *+/js379 vs.* WT, n.s.). The severity of withdrawal was also minimized by extrachromosomal expression of *slo-1(+)* with different promoters. Rescue with *slo-1(+)* driven by the endogenous promoter (*pslo-1*) or a pan-neuronal promoter (*punc-119*) substantially reduced withdrawal compared to the background *slo-1(null)* strain (Figure 2A; each rescue strain *vs.* *slo-1(null)*, $P < 0.001$). Intriguingly, the diacetyl-race performance of two of these strains appeared unimpaired by ethanol withdrawal (NormAUC ≈ 1). We also found rescue of severe withdrawal with *slo-1(+)* driven by either promoter for locomotion (Figure 2B; *pslo-1*, $P < 0.001$; *punc-119*, $P < 0.05$). These findings suggest that the severe withdrawal behavioral in *slo-1* null can be minimized to WT levels or further by expressing multiple copies of *slo-1(+)* in an extrachromosomal array.

Mutant strains that lack *slo-1* exhibit strong resistance to acute ethanol intoxication (Davies *et al.* 2003). To test if resistance to intoxication relates to severity of alcohol withdrawal, we assayed the *dgk-1(sy428)* diacylglycerol kinase mutant, which is mildly resistant to acute intoxication (Davies *et al.* 2003). The *dgk-1* mutant was unimpaired by ethanol withdrawal (NormAUC ≈ 1), unlike even WT (Figure 2A; *dgk-1 vs.* WT, $P < 0.001$). Thus, resistance to intoxication does not simply correlate with the degree of alcohol withdrawal severity in *C. elegans*.

Withdrawal impairments improved by enhancing SLO-1 channel expression or activity

Thus far our findings showed that reducing SLO-1 channel expression in neurons exacerbated behavioral impairments after withdrawal from chronic ethanol treatment. Next, we tested whether increasing SLO-1 function could improve these withdrawal-related behavior impairments. A strain carrying the previously characterized gain-of-function allele *slo-1(ky399)* showed no withdrawal-related impairment in the diacetyl-race assay (Figure 3; *slo-1(ky399) vs.* WT, $P < 0.001$) and limited withdrawal-related impairment in the locomotion assay (Figure 3B; *slo-1(ky399) vs.* WT, $P < 0.05$). In naïve worms, this gain-of-function strain displayed substantial baseline impairments in crawl speed relative to WT (Figure S1, A and C). However, variance in naïve performance between the *slo-1* strains did not generally predict the degree of behavioral impairment during withdrawal for either assay. Basal performance on the diacetyl race was also not as profoundly impaired for any *slo-1*-related strain as it was for a representative slow strain, the moderately uncoordinated mutant *unc-10(md1117)*.

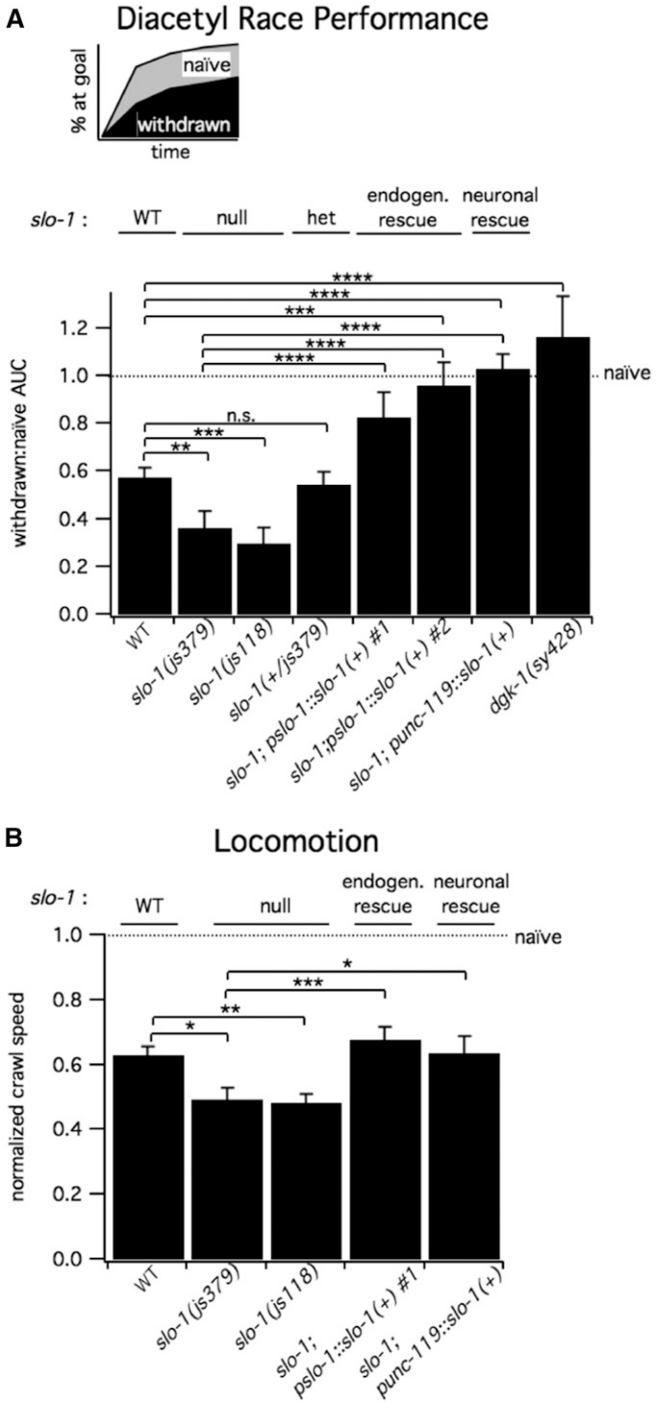

Figure 2 Reduced neuronal SLO-1 channel function exacerbated behavioral impairments during alcohol withdrawal. (A) Schematic above indicates how the time course of performance was quantified by the AUC for the percent of worms at the goal *vs.* time for the diacetyl race. Treatment groups: withdrawn (black area), naïve (gray + black areas). Histogram below shows the mean AUC for withdrawn worms normalized to the mean AUC for naïve worms (dashed horizontal line) ± SEM. The *slo-1* genotype for each strain is indicated above each bar for reference. Two *slo-1* strains with null alleles (*js379* and *js118*) showed more withdrawal-related impairment for the diacetyl-race assay than WT strain N2. A heterozygous *slo-1* (*+/js379*) strain performed similarly to WT. Rescue strains with *slo-1(+)* driven by the endogenous promoter (*pslo-1*; JPS344=#1, JPS345=#2) or a pan-neuronal promoter (*punc-119*) all showed substantially improved withdrawn performance on the diacetyl-race assay compared to the background *slo-1* null strain containing *slo-1(js379)*. Two of these rescue strains (*pslo-1:slo-1(+)* #2, *punc-119:slo-1(+)*) also showed substantially less withdrawal-related impairment than WT. A *dgk-1(sy428)* null strain showed substantially less withdrawal-related impairment than WT or either *slo-1* null strains ($P < 0.001$). (B) Locomotion during withdrawal also worsened with reduced BK channel function. Histogram shows mean speed during withdrawal for different strains normalized to mean speed for naïve worms (dashed horizontal line) ±SEM. Two *slo-1* null strains were more impaired upon withdrawal for locomotion than WT. Rescue strains with *slo-1(+)* driven by the endogenous promoter or a pan-neuronal promoter showed substantially improved performance compared to the background null strain containing *slo-1(js379)*. For A and B, $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.001$.

To test the idea that enhanced SLO-1 function can reduce withdrawal severity without altering baseline performance, multi-copy *slo-1(+)* overexpression strains were made with varying concentrations of injected DNA in a WT background. Overexpression with a low (5 ng/ml) or moderate (10 ng/ml) concentration of *slo-1(+)* showed limited effects on baseline performance in either behavioral assay (Figure S1, A and C). In the diacetyl-race assay, these strains showed little to no withdrawal-related impairment (Figure 3A; both strains *vs.* WT, $P < 0.001$), and showed absolute withdrawn performance that was similar to naïve WT performance (Figure S1, A and B). The *slo-1(+)* overexpression strains also showed less severe withdrawal than WT for locomotion (Figure 3B; both strains *vs.* WT, $P < 0.001$), and showed similar or better absolute performance during withdrawal to WT worms (Figure S1D, $P < 0.05$). These findings indicate that while crawl speed is sensitive to *slo-1(+)* levels, both locomotion and diacetyl-race performance can be improved both relatively and absolutely during withdrawal by *slo-1(+)* overexpression. Just as for the *slo-1* null strains, differences in ethanol uptake or metabolism did not appear to account for the protective effect of enhancing SLO-1 function on withdrawal behavior (Figure S2; *slo-1(+)* overexpression strain *vs.* WT, n.s.). Overall, our findings show that in *C. elegans* eliminating SLO-1 channel function exacerbates withdrawal symptoms, while increasing SLO-1 channel function reduces withdrawal symptoms.

SLO-2, a distinct large-conductance potassium channel, influences withdrawal impairments via a SLO-1 channel-dependent mechanism

Concerted regulation of the activity or tone of distinct ion channels in response to changes in neuronal activity supports homeostatic function of the nervous system (O'Leary *et al.* 2014). Like mammals, worms have >1 large-conductance potassium channel in the Slo family, specifically SLO-1 and SLO-2 (Yuan *et al.* 2000; Santi *et al.* 2003). The SLO-2 channel appears to carry a large portion of outward rectifying current in many worm neurons (P. Liu *et al.* 2014). Physiological evidence suggests that, like SLO-1, *C. elegans* SLO-2 is activated by intracellular Ca^{2+} and depolarization (Zhang *et al.* 2013), suggesting that SLO-2 could play a similar role

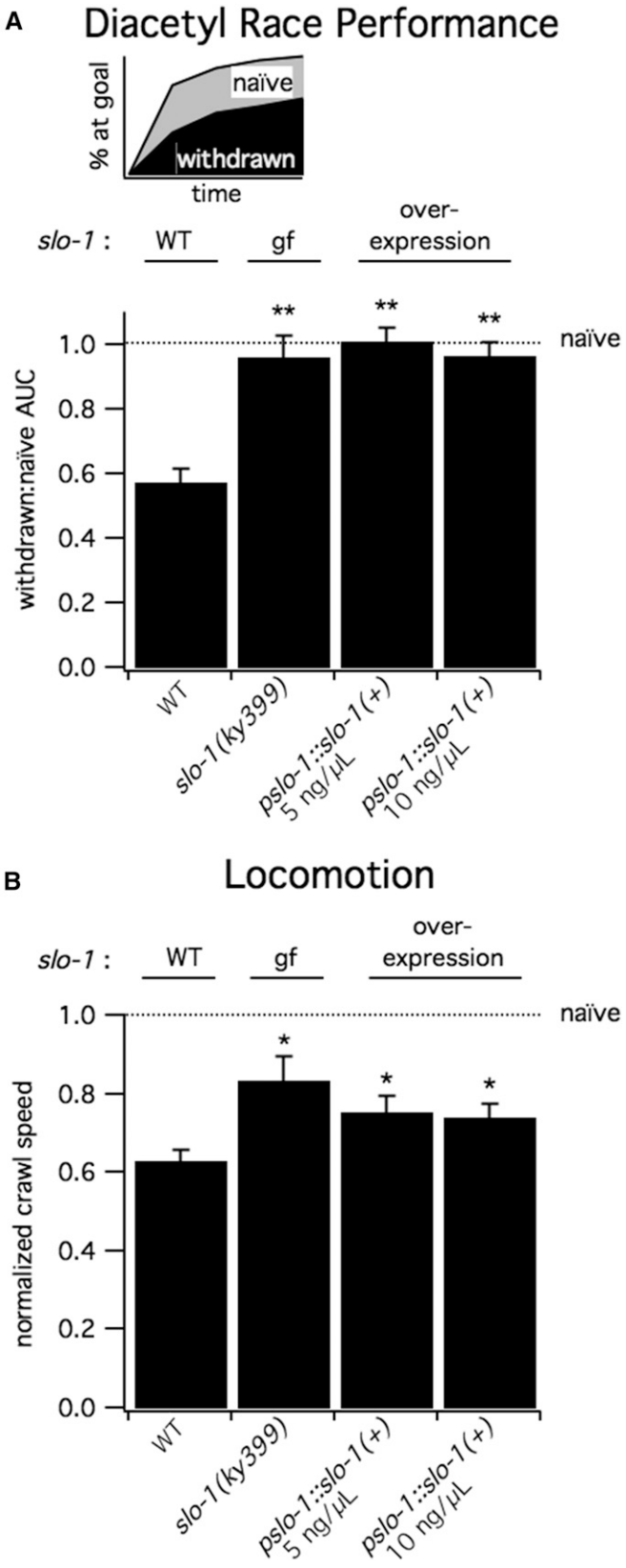

Figure 3 Enhanced SLO-1 channel function ameliorated behavioral impairment during alcohol withdrawal. (A) Schematic above indicates how performance was quantified by the AUC for the percent of worms at the goal *vs.* time for the diacetyl race. Treatment groups: withdrawn (black area), naïve (gray + black areas). Histogram below shows the mean AUC for withdrawn worms normalized to the mean AUC for naïve worms (dashed horizontal line) ± SEM. The *slo-1* genotype for each strain is indicated above each bar for reference. The *slo-1(ky399)* gain-of-function mutant and two strains with *slo-1(+)* overexpressed in a WT background were significantly less impaired upon withdrawal for the diacetyl-race assay than WT strain N2. (B) Enhancing SLO-1 channel function also improved locomotion during withdrawal. Histogram shows mean normalized crawl speed ± SEM. For A and B, $*P < 0.05$, $***P < 0.005$, $****P < 0.001$.

in neuronal function as SLO-1 in worms. Coexpression and coregulation in sensory neurons suggest that these channels could act in concert to regulate behavior (Alqadah *et al.* 2016). Accordingly, we tested whether blocking SLO-2 function influenced withdrawal behavior using the diacetyl-race assay. In reverse of our findings for SLO-1, we found that strains with independent *slo-2* null alleles, *nf100* or *nf101*, showed reduced withdrawal symptoms relative to WT (Figure 4A; *nf100* or *nf101 vs.* WT, $P < 0.001$). The protective effect of eliminating SLO-2 did not appear to be due to differences in ethanol uptake or metabolism (Figure S2). Conversely, reintroduction of *slo-2(+)* under the endogenous promoter (Wojtovich *et al.* 2011) on the *slo-2*(*nf100*) background resulted in severe withdrawal (Figure 4A; *slo-2*; *slo-2(+) vs. slo-2*, $P < 0.001$; *slo-2*;*slo-2(+) vs.* WT, $P < 0.001$). All *slo-2* strains showed similar baseline performance to WT (AUC for N2: 44.7 ± 1.09; *slo-2(nf100)*: 49.3 ± 1.06, *vs.* WT n.s.; *slo-2(nf101)*: 45.4 ± 1.67, *vs.* WT n.s.; *slo-2*; *slo-2(+)*: 40.2 ± 1.81, *vs.* WT n.s.). These findings indicate that, like SLO-1, withdrawal severity is bidirectionally modulated by SLO-2 expression.

We next performed epistasis analysis to probe the genetic relationship between *slo-1* and *slo-2* during withdrawal. Although the *slo-2* null allele *nf100* alone reduced withdrawal symptoms, the *slo-1*;*slo-2* double null mutant showed a level of withdrawal severity similar to the parent *slo-1* null mutant (Figure 4B; *slo-1(js379)*;*slo-2(nf100) vs.* WT, $P < 0.025$; *slo-1(js379)*;*slo-2(nf100) vs. slo-1(js379)*, n.s.). Withdrawal-related impairment was not apparent (NormAUC ≈1) in either double mutant strain with *slo-1(+)* reintroduced under the endogenous promoter (Figure 4B; both rescue strains *vs. slo-1(js379)*;*slo-2(nf100)*, $P < 0.001$). Together these results showed that knocking out the SLO-2 channel protects against withdrawal-related behavioral impairments. Moreover, this protection is dependent upon SLO-1 function.

Chronic ethanol treatment suppresses SLO-1 channel expression in some neurons

In vertebrates, Slo1 channel function is downregulated with chronic alcohol exposure (Pietrzykowski *et al.* 2008; N'Gouemo and Morad 2014). Such a change may underlie behavioral impairments that we observe in *C. elegans* during withdrawal. To investigate differences in SLO-1 protein expression, we used the endogenous promoter for *slo-1* (*pslo-1*) to express mCherry-tagged SLO-1 in a *slo-1(js379)* null background to eliminate the endogenous SLO-1 protein. The

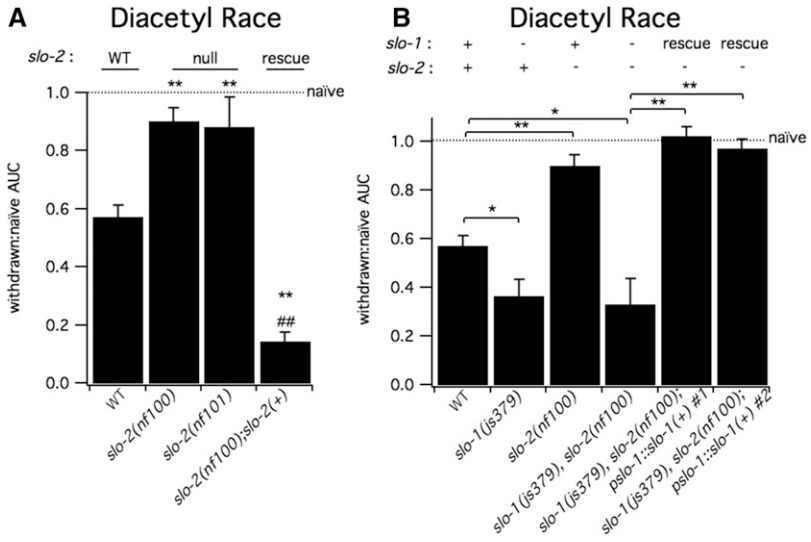

Figure 4 A different large-conductance potassium channel, SLO-2, influences withdrawal impairments via a SLO-1 channel-dependent mechanism. Knockout of *slo-2* improved behavior during alcohol withdrawal. (A) Histogram shows the mean AUC values of different strains for diacetyl-race performance; withdrawn performance normalized to naïve performance (dashed lines) ± SEM. Two *slo-2* strains with null alleles (*nf100* and *nf101*) were significantly less impaired upon withdrawal for the diacetyl race than WT (**$P < 0.001$). A strain with genomic *slo-2(+)* driven by the endogenous promoter (*pslo-2*) on background *slo-2* null strain containing *slo-2(nf100)* showed substantially impaired withdrawn performance on the diacetyl-race <assay compared to the background strain (##$P < 0.001$) and WT (**$P < 0.001$). (B) Epistasis between *slo-1* and *slo-2* for alcohol withdrawal. A strain carrying the null alleles *slo-1(js379)* and *slo-2(nf100)* was more impaired in the diacetyl race during withdrawal than WT, similar to the parent *slo-1(js379)* null strain. Independent rescue strains (#1 and #2) with *slo-1(+)* introduced on the *slo-1(js379);slo-2(nf100)* double null mutant background were less impaired than the parent strain during withdrawal. For B, *$P < 0.025$, **$P < 0.001$.

amount of red fluorescence was expressed as a function of GFP-labeling in representative neurons that participate in locomotion (VC4 and VC5 motorneurons) or odor sensation (AWA sensory neurons) (Bargmann *et al.* 1993; Faumont *et al.* 2011; Vidal-Gadea *et al.* 2011). We found that the red:green ratio decreased by half in motorneurons after ethanol treatment (Figure 5A, $P < 0.0001$), but showed no significant change in sensory neurons (Figure 5B). These findings suggest that SLO-1 expression levels may be decreased, but not abolished, by ethanol exposure in a subset of neurons.

To investigate if the ethanol-induced downregulation of SLO-1 protein could be explained by decreased transcription, we tested whether a *slo-1* transcriptional reporter was sensitive to ethanol. We used the same promoter region from above that was sufficient to rescue or improve behavioral phenotypes to drive expression of GFP. To perform ratiometric analysis, this reporter was coexpressed on the same extrachromosomal array with a second mCherry reporter that labels the same motorneurons as above with a *ptph-1* promoter that was previously shown to be insensitive to a higher dose of ethanol (Kwon *et al.* 2004). We found that expression of the *slo-1* transcriptional reporter was not altered in motorneurons in response to 24 hr of ethanol exposure (Figure 5C). Together, our results suggest that the decrease in mCherry-tagged SLO-1 channel expression after chronic ethanol treatment may arise instead from post-translational processes.

Loss of function in slo-2 alters SLO-1 channel expression

To test if the less severe withdrawal effects displayed by the *slo-2* mutant corresponded to altered SLO-1 expression, we next measured levels of mCherry-tagged SLO-1 in a *slo-2* mutant background. As above, all strains carried a *slo-1(js379)* null mutation to eliminate the endogenous SLO-1 protein. We found that the absence of *slo-2* did not limit the decrease in SLO-1 in motorneurons after ethanol treatment (Figure 6A, $P < 0.001$). However, in ethanol-naïve worms, SLO-1 levels were higher in the *slo-2* mutant (Figure 6A, $P < 0.05$). Red fluorescence alone showed the same difference (normalized mean pixel intensity, *slo-1*: 1.00 ± 0.06 *vs.* *slo-1*;*slo-2*: 1.23 ± 0.10; $P = 0.05$) suggesting that the effect was not caused by genotypic differences in *ptph-1*-driven GFP expression. By contrast, SLO-1 expression after ethanol treatment was similar across backgrounds (Figure 6A, n.s.). Thus, our findings indicate that while loss of *slo-2* may raise SLO-1 expression in naïve worms, it did not alter overall SLO-1 levels in motorneurons after chronic ethanol treatment.

To understand how *slo-2* influences SLO-1 expression, we tested whether *slo-1* transcript levels change as a function of ethanol exposure in the *slo-2* mutant. Consistent with previous findings (Kwon *et al.* 2004) and our results with the transcriptional reporter (above), chronic ethanol treatment did not alter total *slo-1* transcript expression in WT worms (Figure 6B, n.s.). Total *slo-1* transcript expression was not significantly altered in a *slo-2* null mutant, either in naïve worms or after a 24-hr exposure to ethanol (Figure 6B, n.s.). These findings support the idea that modulation of mCherry-tagged SLO-1 expression by chronic ethanol exposure or *slo-2* loss of function may be due to post-translational mechanisms.

Discussion

Here we show that worms withdrawn from chronic ethanol displayed behavioral deficits suggestive of altered nervous system function. Simply increasing SLO-1 channel tone, even selectively in neurons, was sufficient to overcome these behavioral symptoms of withdrawal. Conversely, we found that the extent of withdrawal-induced impairments was far worse

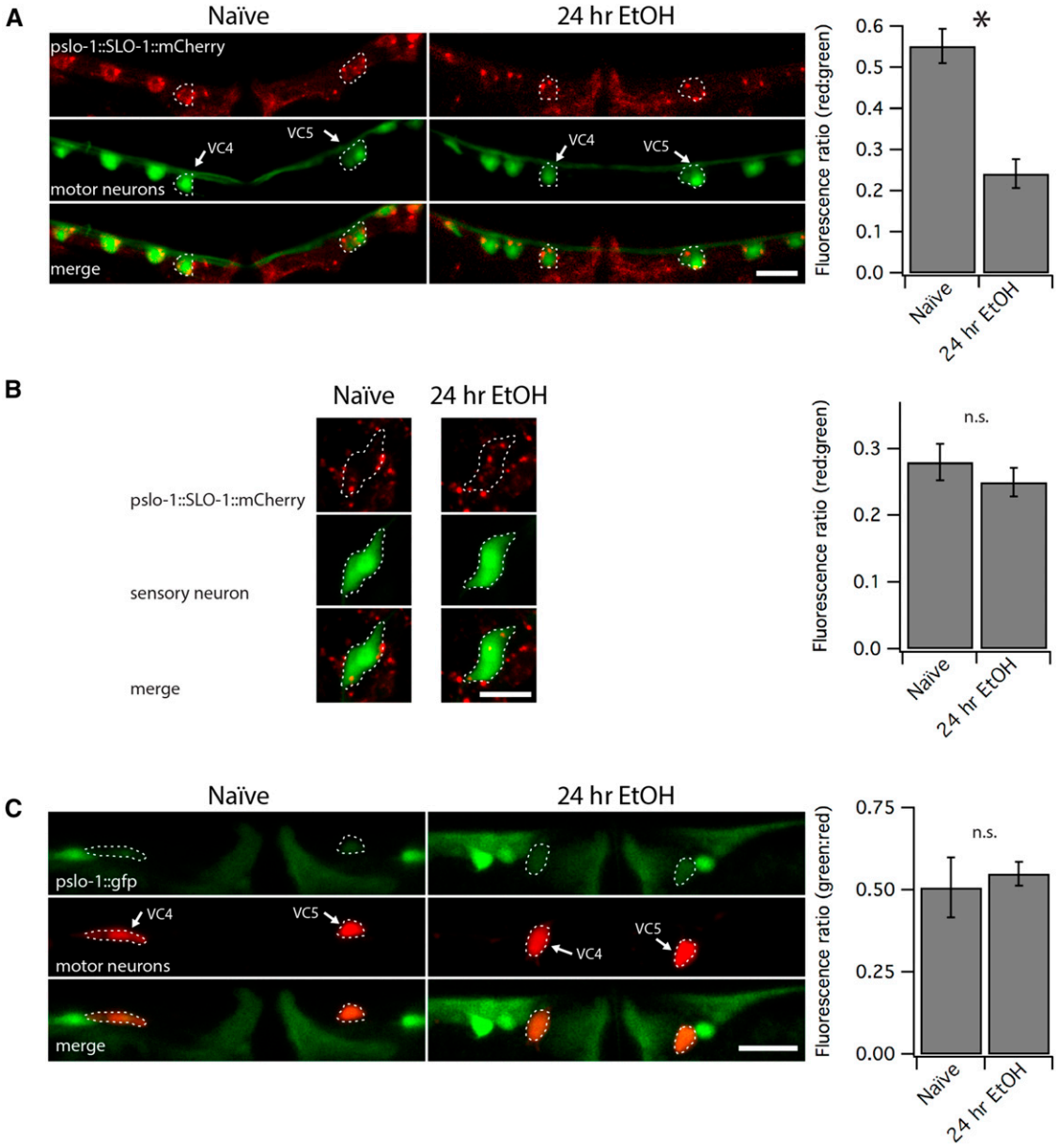

Figure 5 Chronic ethanol treatment suppresses neuronal SLO-1 channel expression. (A and B) Confocal microscopy stacks were summed to produce the photomicrographs showing translational *slo-1* reporter tagged with mCherry in a *slo-1(js379)* null background. The red:green fluorescence decreased by half in GFP-labeled VC4 and VC5 neurons after 24-hr exposure to ethanol (A, ***$P < 0.0001$), but not in GFP-labeled AWA olfactory neurons (B). (C) Confocal photomicrographs showing a GFP transcriptional reporter of *slo-1* in the green channel and mCherry-labeled VC4 and VC5 motorneurons in a WT background. Ratiometric analysis showed no change in whole body green:red ratios in the VC4 and VC5 neurons following chronic ethanol treatment. Bar, 10 μm in A–C.

in the absence of SLO-1 channels. This bidirectional relationship between SLO-1 channel function and withdrawal behavior severity may be explained in part by a decrease in SLO-1 channel function during prolonged exposure to ethanol. The activity of a number of ion channels during neuroadaptive changes to the presence and subsequent removal of ethanol may be linked. We discovered that the extent of withdrawal-related behavioral impairment was modulated oppositely by a second highly conserved member of the large-conductance potassium family, SLO-2, via a *slo-1*-dependent mechanism. These results suggest that the Slo family of ion channels may represent molecular targets to alleviate withdrawal symptoms in higher animals.

Withdrawal as a neuroadaptive response to prolonged ethanol exposure

Many studies support the theory that alcohol abuse disorders including addiction are accompanied, or even caused, by adaptive responses of the nervous system to chronic alcohol consumption (Koob 2013, 2015). Chronic ethanol exposure

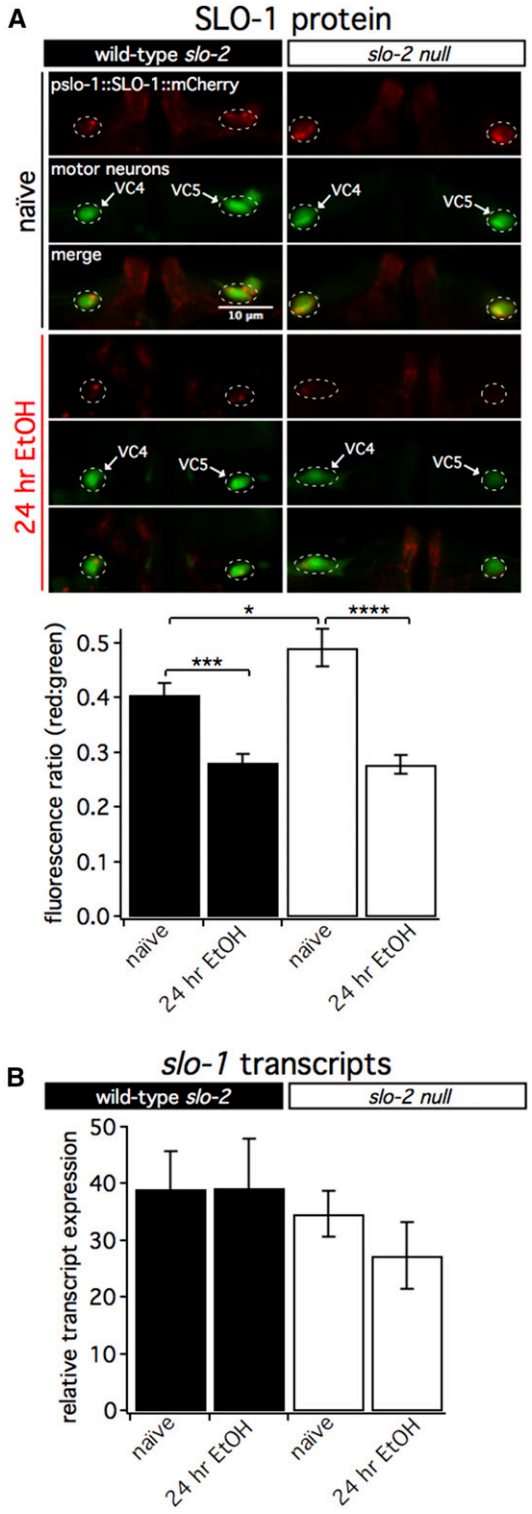

Figure 6 Loss-of-function mutation in slo-2 enhances neuronal SLO-1 channel expression. (A) Confocal microscopy stacks were summed to produce the photomicrographs showing translational *slo-1* reporter tagged with mCherry in a *slo-1(js379)* null (left, solid bar) or a *slo-1(js379);slo-2(nf100)* double null mutant (right, open bar) background. In both strains, the red:green fluorescence decreased in GFP-labeled VC4 and VC5 neurons after 24-hr exposure to ethanol (***$P < 0.005$, ****$P < 0.001$). In naïve worms, the amount of VC4 and VC5 neuron red:green fluorescence was greater in the *slo-1;slo-2* double null mutant than the *slo-1* null background (*$P < 0.05$), while the fluorescence ratio was the same in the strain after a 24-hr ethanol treatment. (B) Relative total *slo-1* transcript expression in whole worm. qPCR measured *slo-1* transcript expression relative to the control gene *cdc-42* in WT (solid bar) and a *slo-2(nf100)* null strain (open bar). Chronic ethanol treatment did not alter *slo-1* transcript expression in either strain. A loss-of-function mutation in *slo-2* did not alter *slo-1* transcript expression in either naïve or chronic ethanol-treated worms.

has been found to change many aspects of nervous system function and whole body physiology in animal models including gene expression in worms (*e.g.*, Kwon *et al.* 2004; Nagy 2004; Lovinger and Roberto 2013; Osterndorff-Kahanek *et al.* 2015). Some of these homeostatic changes may lead to pathological dysfunction when alcohol is removed from the system, contributing to alcohol dependence.

Our results are consistent with the idea that Slo1 expression is regulated as part of neural adaptation to chronic ethanol exposure. Acute ethanol exposure acts directly to modulate the function of the Slo1 channel (Dopico *et al.* 2016). In *C. elegans*, ethanol increases the open probability of the SLO-1 channel both *in vivo* and *in vitro* (Davies *et al.* 2003; Davis *et al.* 2015). Over a longer period, homeostatic downregulation of Slo1 channel function could compensate for prolonged activation of the Slo1 channel in the presence of ethanol but contribute to behavioral dysfunction in the absence of ethanol. Indeed, we found that chronic ethanol exposure decreased SLO-1 channel expression in certain neurons. Moreover, behavioral deficits during withdrawal from ethanol were overcome with either multi-copy overexpression or gain-of-function mutation in the SLO-1 channel. These *slo-1* manipulations could have offset the decrease in SLO-1 channel tone during chronic ethanol exposure and/or led to faster "rebound" from the suppression of SLO-1 expression once ethanol was removed. Interestingly, a strictly endogenous pattern or level of *slo-1* expression was not required for more naïve-like behavioral performance. *C. elegans* expresses a broad array of SLO-1 isoforms (Glauser *et al.* 2011; Johnson *et al.* 2011); however, behavior was normalized even by expressing multiple copies of only a single isoform, *slo-1a*, without the endogenous 5′ regulatory region.

C. elegans likely experiences changes beyond SLO-1 expression in response to chronic ethanol exposure. In mammalian tissue, ethanol has a broad influence on both direct and indirect targets spanning multiple neurotransmitter systems and signaling pathways (Morikawa and Morrisett 2010; Wu *et al.* 2014). Previous work in *C. elegans* found that two neuromodulatory signaling genes were required for ethanol withdrawal phenotypes (Mitchell *et al.* 2010): *npr-1*, a worm ortholog to the vertebrate neuropeptide-Y receptor, and *egl-3*, a propeptide convertase required for cleavage of hundreds of neuropeptides (Mitchell *et al.* 2010). The CRF-like receptor as well as the serotonergic and dopaminergic transmitter systems were found to modulate ethanol withdrawal behaviors after only 4 hr of ethanol exposure (Lee *et al.* 2009; Jee *et al.* 2013). SLO-1 could act as a master regulator and/or a

major downstream target of neuroadaptive mechanisms. As a master regulator, a loss or reduction in SLO-1 channel function could *promote* dysregulation of nervous system function, whereas multi-copy expression and gain-of-function *slo-1* mutants could counteract this dysregulation. As a downstream target, a lack of SLO-1 function in *slo-1* null worms may simply overcompensate other imbalances in the nervous system during withdrawal. Loss of *slo-1* alone cannot explain the impairment in behaviors, however, because naïve *slo-1* null mutants perform better than withdrawn WT worms.

Mechanisms for SLO-1 regulation by chronic ethanol

How might Slo1 function be lowered during chronic ethanol exposure? We found that for *C. elegans*, one way chronic ethanol appears to downregulate Slo1 channel tone is to reduce expression in select neurons. Ratiometric analysis showed a reduction in mCherry-tagged SLO-1 channels in the soma of certain motorneurons but not sensory neurons. The SLO-1 channel is expressed throughout the nervous system and muscle (Wang *et al.* 2001). Adaptive neuronal changes in SLO-1 channel expression may only occur in some neurons.

Given the evidence for varied modulation of Slo1 channel function by ethanol in other systems (Ron and Jurd 2005; Pietryzykowski *et al.* 2008; Velázquez-Marrero *et al.* 2011; Ponomarev *et al.* 2012; Dopico *et al.* 2014; N'Gouemo and Morad 2014; Shipston and Tian 2016), we suspect that SLO-1 channel function is also downregulated with chronic ethanol exposure via multiple mechanisms in worms. The reduced expression of mCherry-tagged SLO-1 without a corresponding decrease in *slo-1* transcriptional reporter in the same neurons strongly suggests regulatory mechanisms at the protein level. In mammals, kinases and other signaling pathways influenced by ethanol alter Slo1 function post-translationally (Ron and Jurd 2005; Dopico *et al.* 2014; Shipston and Tian 2016). Ethanol exposure could also enhance Slo1 degradation and/or impair distribution to active sites [reviewed in Kyle and Braun (2014)]. For example, seizure activity causes Slo1 ubiquitination and subsequent degradation in the ER (J. Liu *et al.* 2014). Similar mechanisms may decrease Slo1 function or expression to normalize circuit activity in the face of chronic ethanol.

In mammalian tissue, both total and specific Slo1 isoform transcript levels are modulated by chronic ethanol exposure, balancing the effect of ongoing ethanol activation of the channels (Pietryzykowski *et al.* 2008). However, our lack of evidence for total *slo-1* transcriptional response to chronic ethanol exposure in *C. elegans* is consistent with a previous report showing no overall ethanol-induced downregulation of *slo-1* transcription in whole worms or evidence of a consensus sequence for an ethanol-responsive element in the *slo-1* promoter (Kwon *et al.* 2004). It remains to be tested whether ethanol exposure alters the expression profile of the 10 *slo-1* isoforms in *C. elegans* (Johnson *et al.* 2011; LeBoeuf and Garcia 2012). Given the importance of splice variation in Slo1 expression, function, and sensitivity to ethanol in mammals (Dopico *et al.* 2014; Shipston and Tian 2016), a future investigation of ethanol-induced transcriptional regulation of *slo-1* is warranted. Based on our finding that SLO-1 expression is differentially regulated in specific neurons, a complete understanding of ethanol-induced splice regulation may require (1) differentiation between transcripts from the adult nervous system *vs.* those from other tissues and the developing worms harbored in eggs within the adult, and (2) isolated measurements of expression changes within specific neurons.

The influence of slo-2 function on neuroadaptation to chronic ethanol

Intriguingly, we found that a second highly conserved member of the large-conductance potassium family, the SLO-2 channel, also bidirectionally modulates neural adaptation upon alcohol withdrawal. The effect of *slo-2* on withdrawal behavior requires intact SLO-1 channel function. Mammalian Slo2 channels are expressed in neurons where they influence action potential propagation and shape synaptic integration (Bhattacharjee and Kaczmarek 2005). Because SLO-1 and SLO-2 channels are coexpressed in neurons and muscle in worms, and share means of channel activation, they may influence behavior in concert. For example, SLO-1 and SLO-2 channels show redundant regulation of the terminal fate of asymmetric sensory neurons in worms (Alqadah *et al.* 2016). However, SLO-1 and SLO-2 function are not entirely overlapping as shown by a role for SLO-2 but not SLO-1 channels in the regulation of hypoxia (Zhang *et al.* 2013). Here we show another interaction between these channels with anti-correlated regulation of alcohol withdrawal.

It is not yet clear whether we have found an example of SLO-1/SLO-2 channel direct coregulation or just a shared influence on neuromuscular circuitry. Our data suggest that *slo-2* loss of function increases baseline SLO-1 expression but does not restrict the decline in SLO-1 expression during chronic ethanol treatment. We cannot rule out a *slo-2*-mediated influence over *slo-1* isoform expression during ethanol exposure, though neither genotype nor ethanol influenced total *slo-1* transcript levels. One possibility, then, is that *slo-2* loss of function alters ethanol-related compensatory changes. This could be driven by the higher expression of SLO-1 in naïve *slo-2* null worms or via SLO-2-specific mechanisms. In turn, the compensatory changes in response to ethanol may be less maladaptive once ethanol is removed than in WT worms, allowing for the improved behavioral function during withdrawal exhibited by *slo-2* null worms. A second possibility is that *slo-2* loss of function improves rebound from neuroadaptation to ethanol during withdrawal. For example, differences in post-translational processing of SLO-1 in the *slo-2* null background could speed the recovery of SLO-1 tone during withdrawal without altering the initial suppression of SLO-1 expression during chronic ethanol treatment. Further work will be necessary to elucidate the specific mechanisms through which SLO-1 and SLO-2 shape neuromuscular function during withdrawal from chronic ethanol exposure.

Slo1 plays a central role in responses to ethanol across behaviors

Previously, through two large, independent, unbiased forward genetic screens, the *slo-1* gene encoding the SLO-1 channel was found to represent the most important single gene required for acute intoxication in *C. elegans* (Davies *et al.* 2003). Our new findings show that the SLO-1 channel also plays an important, but opposite role in neuronal plasticity during alcohol withdrawal in worms. Analogous opposite short- and long-term functional roles of the Slo1 channel in alcohol-related behaviors may be expected in higher animals.

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