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UBC-9 Acts in GABA Neurons to Control Neuromuscular Signaling in C. elegans

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ABSTRACT: Regulation of excitatory to inhibitory signaling balance is essential to nervous system health and is maintained by numerous enzyme systems that modulate the activity, localization, and abundance of synaptic proteins. SUMOylation is a key post-translational regulator of protein function in diverse cells, including neurons. There, its role in regulating synaptic transmission through pre- and postsynaptic effects has been shown primarily at glutamatergic central nervous system synapses, where the sole SUMO-conjugating enzyme Ubc9 is a critical player. However, whether Ubc9 functions globally at other synapses, including inhibitory synapses, has not been explored. Here, we investigated the role of UBC-9 and the SUMOylation pathway in controlling the balance of excitatory cholinergic and inhibitory GABAergic signaling required for muscle contraction in Caenorhabditis elegans. We found inhibition or overexpression of UBC-9 in neurons modestly increased muscle excitation. Similar and even stronger phenotypes were seen with UBC-9 overexpression specifically in GABAergic neurons, but not in cholinergic neurons. These effects correlated with accumulation of synaptic vesicle-associated proteins at GABAergic presynapses, where UBC-9 and the C. elegans SUMO ortholog SMO-1 localized, and with defects in GABA-dependent behaviors. Experiments involving expression of catalytically inactive UBC-9 (UBC-9(C93S)), as well as co-expression of UBC-9 and SMO-1, suggested wild type UBC-9 overexpressed alone may act via substrate sequestration in the absence of sufficient free SUMO, underscoring the importance of tightly regulated SUMO enzyme function. Similar effects on muscle excitation, GABAergic signaling, and synaptic vesicle localization occurred with overexpression of the SUMO activating enzyme subunit AOS-1. Together, these data support a model in which UBC-9 and the SUMOylation pathway act at presynaptic sites in inhibitory motor neurons to control synaptic signaling balance in C. elegans. Future studies will be important to define UBC-9 targets at this synapse, as well as mechanisms by which UBC-9 and the SUMO pathway are regulated.

KEYWORDS: SUMO, E:I balance, inhibitory, synapse, presynaptic, Caenorhabditis elegans

Significance statement
- Identifies a presynaptic role for the SUMO enzyme system in inhibitory neurons during neuromuscular signaling.
- Shows SUMO E2 UBC-9 localizes to GABAergic motor neuron presynapses and affects vesicle localization and muscle excitation.
- Aids understanding of conserved UBC-9 functions in E:I balance, which is lost in neurological diseases.

Introduction
Excitatory to inhibitory (E:I) signaling balance is critical for nervous system function, and many neurological diseases, including epilepsy, autism, and numerous neurodegenerative conditions, involve E:I imbalances.1,2 Hundreds of proteins reside at pre- and postsynaptic sites to control the amount and timing of signaling at specific synapses, contributing to regulation of this balance.3 A variety of post-translational modifications, including phosphorylation, ubiquitination, and palmitoylation tightly control the functions of synaptic proteins.4-8 Many of these modifications are activity-dependent and serve to modulate the localization, surface expression, activity, and/or complex formation of synaptic proteins to impact synaptic vesicle release and neurotransmitter signal reception.4-9-15 The extent of these modifications, as well as the specific enzymes and target proteins involved, remain incompletely understood.

Protein SUMOylation is a post-translational modification, conserved from yeast to humans, that has emerged as a key regulator of neuronal and synaptic biology.8,16-18 During SUMOylation, proteins are modified by the covalent attachment of small ubiquitin-like modifier (SUMO) polypeptides. These approximately 100-amino acid globular SUMO molecules (SUMO-1, -2/3, and -4 in humans) are similar in size and shape to ubiquitin and are likewise added to lysine residues in target proteins through the activity of a multi-step enzymatic cascade. For SUMO, this involves activation of mature SUMO molecules by the SUMO-activating enzyme Aos1-Uba2 /SAE1-SAE2 followed by SUMO transfer to the E2 SUMO-conjugating enzyme Ubc9.16,19 Ubc9 alone, or in combination with any of a number of E3 ligases, mediates the attachment of SUMO to the target protein.19 Removal of SUMO peptides from substrates, as well as activation of immature SUMO peptides, occurs via the activity of SUMO isopeptidases, also known as ubiquitin-like-specific proteases (Ulp) or sentrin-specific proteases (SENPs).19,20

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Originally identified for its role in regulating transcriptional activity and other nuclear functions, SUMOylation is now known to modulate the activity and localization of diverse cytosolic proteins, including many involved in neuronal and synapse physiology. Several known SUMOylated neuronal proteins, including huntingtin, amyloid precursor protein, ataxins, tau, alpha-synuclein, and BACE1, are linked to neurodegenerative diseases. SUMO-1 has also been detected in synaptosomes from mouse models of Alzheimer’s Disease, and global SUMOylation levels are reported to be differentially affected by varying levels of oxidative stress, a component of age- and infection-related neurodegeneration. Thus, the SUMOylation pathway has been proposed as a therapeutic target in these diseases. Biochemical studies of synaptic fractions from rat hippocampi indicate the presence of multiple SUMOylated synaptic proteins, although the identities of many of these remain unknown. Synthetic proteins shown to be regulated by SUMOylation, primarily at mammalian glutamatergic synapses, include postsynaptic scaffold proteins CASK and GISP, along with a number of presynaptic regulators of vesicle release. Data from other studies indicate certain K+ and Na+ channels also are regulated either directly or indirectly via SUMOylation to promote general neuronal excitability, as is the AMPA receptor regulator Arc. Together these data demonstrate the importance of protein SUMOylation in controlling synaptic function at central synapses.

Because it is the sole E2 SUMO conjugating enzyme in any organism, Ubc9 represents a critical component of the SUMO pathway. Ubc9 activity itself is regulated by SUMOylation, as well as by phosphorylation and acetylation. Ubc9 typically localizes to sites where SUMO is found, and organisms lacking Ubc9 are not viable, suggesting the importance of SUMOylation in development. Multiple studies also have implicated Ubc9 in synaptic function and plasticity. Work in Drosophila uncovered a genetic requirement for Ubc9 in long term memory formation where it appears to act with histone deacetylase 4 (HDAC4). Studies in rat hippocampal neurons demonstrated the ability of Ubc9 to interact with GluR6 kainate receptor subunits, which are SUMOylated following kainate application to promote postsynaptic receptor internalization and decreased kainate-evoked currents. Activity-dependent pre- and post-synaptic localization of Ubc9, as well as other SUMOylation and deSUMOylation machinery, at glutamatergic central synapses was confirmed by several other studies. These reports underscore the importance of Ubc9 and SUMOylation to synaptic function. However, questions as to whether Ubc9’s role is conserved across phylogeny and at diverse synapse types, including at inhibitory or peripheral synapses, remain. Just one study to date has described a role for the SUMO system at GABAergic synapses, where it regulates the postsynaptic GABA<sub>A</sub> receptor scaffold protein gephyrin, and a single paper identified SUMOylation of a muscarinic acetylcholine receptor in heterologous cell culture. Moreover, only a few papers have explored any neuronal SUMO or Ubc9 functions in non-mammalian organisms.

The neuromuscular junction (NMJ) of Caenorhabditis elegans is an ideal system in which to investigate the broader regulation of synaptic signaling and E:I balance by the SUMO pathway. The body wall muscles of these microscopic round-worms contain both excitatory inputs from cholinergic motor neurons that promote muscle contraction and inhibitory inputs from GABAergic motor neurons that promote muscle relaxation by counteracting the excitatory signals. Tight regulation of the balance of excitatory and inhibitory transmission is required for proper muscle contraction and movement of the worms. Behavioral assays, including sensitivity to paralysis induced by the acetylcholinesterase inhibitor, aldicarb, can be used to measure muscle excitation as a readout for neuromuscular signaling balance. In the presence of aldicarb, populations of worms paralyze over time. Animals carrying mutations that increase cholinergic signaling or decrease GABAergic signaling show increased paralysis relative to wild type worms, whereas those with decreased cholinergic or increased GABAergic signaling exhibit decreased paralysis. Numerous genes now known to regulate E:I signaling balance across phylogeny were identified based on the aldicarb phenotypes of mutant worms. C. elegans contain homologs of the SUMO E1 (AOS-1 and UBA-2 subunits) and E2 (UBC-9) enzymes, as well as homologs of several E3 ligases and SENPs (called ULPs in C. elegans). A single C. elegans SUMO ortholog, SMO-1, exists and has structural and functional similarity to SUMO-1 and −2 in humans. The smo-1 gene is essential and expressed throughout development, and animals lacking smo-1 expression that survive due to maternal contributions are sterile with abnormal somatic gonads, vulvas, and germlines. Other studies have identified SUMOylated C. elegans proteins, and current estimates indicate as much as 15% to 20% of the worm proteome is SUMOylated at any given time, similar to estimates in human cells.

In this study, we set out to assess the potential requirements for the SUMO pathway in controlling E:I balance at the C. elegans NMJ. Given the localization of Ubc9 to synapses in mammalian neurons and the general importance of presynaptic protein SUMOylation for neurotransmitter release at glutamatergic hippocampal synapses, we hypothesized that SUMO pathway enzymes are required in motor neurons to maintain appropriate neuromuscular E:I signaling balance and muscle excitation. Here, we show that the SUMO-conjugating enzyme UBC-9 can act in GABAergic neurons to regulate neuromuscular function. Overexpressed UBC-9 localized to presynaptic sites in GABAergic motor neurons, caused accumulation of GABA-containing synaptic vesicles, and increased muscle contraction, changes that correlated with defects in a pharmacologic test of GABAergic signaling. Similar results were seen with overexpression of the SUMO E1 subunit AOS-1,
supporting a role for the SUMO pathway beyond just UBC-9. Enhanced muscle contraction also was seen with inhibition of UBC-9 or AOS-1 expression, as well as with overexpression of a SUMO conjugation-deficient UBC-9, consistent with dominant negative substrate sequestration effects. Overexpression of SMO-1 also modestly increased muscle excitation in a manner non-additive with UBC-9 overexpression, suggesting UBC-9 and SMO-1 act in a common pathway to impact neuromuscular function and further underscoring the need for precise control of SUMO enzyme activity at this synapse. Based on these data, we propose that endogenous UBC-9 acts in GABAergic neurons, likely via a SUMO-dependent pathway, to promote appropriate levels GABA neurotransmitter release and prevent excess muscle contraction. Our results are consistent with those of prior studies that demonstrate the critical need for tight regulation of synaptic protein SUMOylation and SUMO enzyme function for proper synaptic signaling.

Materials and Methods

C. elegans strains

All strains were grown on nematode growth medium (NGM) agar plates spotted with OP50 Escherichia coli and maintained at 20°C according to standard protocols. Young adult hermaphrodite worms were used for all experiments. Table 1 lists the strains used in this study.

Plasmid and strain generation

The Psnb-1::ubc-9 (pJRK22) and Psnb-1::aos-1 (pJRK23) plasmids were made by inserting ubc-9 or aos-1 cDNA, respectively, into the PD49.26 plasmid containing the Psnb-1 promoter (pFJ19). To do this, the cDNAs were amplified from the Vidal Orfeome clones (F29B9.6 and C08B6.9) using forward and reverse primers engineered with NheI and KpnI restriction sites. The amplified PCR products and plasmid pFJ19 were digested with NheI and KpnI, then the ubc-9 or aos-1 cDNA was inserted into pFJ19 using T4 DNA ligase. To make Punc-25::aos-1 (pJRK39), pJRK23 was digested with SphI and BamHI to remove the Punc-25 promoter. The Punc-25 promoter was isolated from the pJRK8 (Punc-25::yfp::emb-27) plasmid using the same enzymes, then ligated into pJRK23. The Punc-25::ubc-9 (pJRK43) and Punc-25::ubc-9 (pJRK44) plasmids were made by inserting the Punc-25 and Punc-17 promoters into the PD49.26 plasmid containing ubc-9 cDNA. To do this, plasmids pJRK39 and pJRK42 (Punc-17::aos-1) were digested with SphI and BamHI to remove the unc-25 and unc-17 promoters, and these were ligated between the SphI and BamHI sites in pJRK22 following removal of Psnb-1. To generate the Punc-25::ubc-9(C93S) plasmid (pJRK69), QuikChange site-directed mutagenesis (Agilent) was performed using primers designed to introduce a single basepair mutation into the ubc-9 sequence (forward primer: 5’-cccacaggtcagtGACctatctctcctgga-3’; reverse primer: 5’-cccaagagagatTAcggactgtgagctatctcttcgga-3’) and performed according to the manufacturer’s instructions. The Punc-25::gfp::smo-1(GG) (pJRK81) plasmid was made by inserting gfp::smo-1(GG) amplified from pJRK72 [previously generated from plasmid #462] into plasmid pJRK39 using gfp forward and smo-1 reverse primers containing NheI and KpnI restriction sites, respectively. The PCR products and pJRK39 were digested with NheI and KpnI. This removed aos-1 from pJRK39 and allowed for the subsequent insertion of gfp::smo-1 into pJRK39 using T4 DNA ligase. The final pJRK81 plasmid was confirmed by sequencing. The Punc-25::gfp::ubc-9 (pJRK85) plasmid was constructed in 2 steps. First, ubc-9 cDNA containing an internal NotI restriction site at the N-terminus was generated via PCR amplification of ubc-9 from pJRK43 using forward and reverse primers containing NheI and SacI restriction sites, respectively. Plasmid pJRK39 and the amplified [ubc-9(NotI)] products were then digested with NheI and SacI to remove the aos-1 cDNA from pJRK39 and to create restriction sites on the ubc-9(NotI) insert, and the two fragments were ligated to create Punc-25::ubc-9(NotI) (pJRK80). Second, pJRK80 and pJRK50 (Pfhr-1::gfp::fshr-1) were digested with NotI to remove gfp. Following heat inactivation, the digested pJRK80 was treated with calf intestinal alkaline phosphatase. pJRK85 was created by ligating gfp into the NotI site in pJRK80.

Transgenic strains with panneuronal, GABAergic, or cholinergic neuron–specific UBC-9, UBC-9(C93S), AOS-1, or SMO-1(GG) overexpression were isolated following standard microinjection of the plasmids into the gonads of gravid N2 adult worms as described previously and at concentrations that generated viable lines for each transgene. Psnb-1::ubc-9 (pJRK22) and Psnb-1::aos-1 (pJRK23) were each injected at a concentration of 1 ng/µL, along with 10 ng/µL of Pmyo-2::NLS::gfp (co-injection marker) to generate strains JRK71 and JRK50, respectively. Punc-25::aos-1 (pJRK39) was injected at 25 ng/µL, along with 10 ng/µL of Pmyo-2::NLS::gfp to generate strain JRK70. Punc-25::ubc-9 (pJRK43) was injected at a concentration of 10 ng/µL, along with 10 ng/µL of Pmyo-2::NLS::gfp (co-injection marker) to generate strain JRK72 or with 50 ng/µL of Ptna3::dsRed to generate JRK137. Punc-17::ubc-9 (pJRK44) was injected at 20 ng/µL, along with 10 ng/µL of Pmyo-2::NLS::gfp to generate strain JRK102. The Punc-25::ubc-9(C93S) (pJRK69) plasmid, Punc-25::gfp::smo-1(GG) (pJRK81), and Punc-25::gfp::ubc-9 (pJRK85) were each injected at a concentration of 10 ng/µL, along with 10 ng/µL of Pmyo-2::NLS::gfp (co-injection marker) to generate strains JRK110, JRK136, and JRK134, respectively. The mock overexpression (OE) control strain (JRK64) was generated by injection of 10 ng/µL of Pmyo-2::NLS::gfp alone.

RNA interference

RNA interference (RNAi) was performed as follows using the feeding method in RNAi-enhanced strains. Panneuronal
RNAi was performed using the \textit{uIs60};\textit{nre-1(hd20)} \textit{lin-15b(hd126)} strain, which expresses SID-1 RNA channels under a neuron-specific promoter.\textsuperscript{59,60} RNAi clones were obtained from a genome-wide library consisting of HT115(DE) \textit{E. coli} carrying gene fragments for double-stranded RNA production in the T7-tailed L4440 vector.\textsuperscript{61} Bacteria were maintained in the presence of 50\,$\mu$g/ml ampicillin and 15\,$\mu$g/ml tetracycline for plasmid selection.\textsuperscript{62} RNAi feeding plates were prepared as follows. First, 3\,ml overnight cultures of bacteria carrying an RNAi plasmid targeting either \textit{ubc-9}, \textit{aos-1}, or \textit{gfp} (negative control) were grown in Luria broth (LB) containing 50\,$\mu$g/ml ampicillin. Cultures were spotted (150\,$\mu$l each) onto 35\,mm NGM agar plates containing 50\,$\mu$g/ml ampicillin and 5\,mM IPTG. Plates were dried open for 2 hours, then closed.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|}
\hline
\textbf{STRAIN NUMBER} & \textbf{GENOTYPE} \\
\hline
N2 (Bristol) & Wild type \\
NM467 & \textit{snb-1(md247)} \\
RB1500 & \textit{ulp-1(ok768)} \\
FJ413 & \textit{ulp-3(tm1287)} \\
FJ415 & \textit{ulp-1(tm1174)} \\
JRK10 & \textit{ulp-5(tm3071)}\textsuperscript{*2} \\
JRK18 & \textit{ktEx4 (unc-25::mcherry::snb-1; Pmyo-2::NLS::mcherry)}\textsuperscript{48} \\
JRK24 & \textit{ulp-1(ok768)}\textsuperscript{*3};\textit{ulp-3(tm1287)} \\
JRK25 & \textit{ulp-1(ok768)}\textsuperscript{*3};\textit{ulp-5(tm3071)} \\
JRK34 & \textit{uls60 (unc-119::yfp; Punc-119::sid-1; nre-1(hd120) lin-15(hd126)} \\
JRK48 & \textit{ulu1*1} \\
JRK50 & \textit{ktEx12 (Psrb-1::aos-1; Pmyo-2::NLS::gfp)} \\
JRK64 & \textit{ktEx20 (Pmyo-2::NLS::gfp)} \\
JRK68 & \textit{ulu152*2} \\
JRK70 & \textit{ktEx21 (unc-25::ao-1; Pmyo-2::NLS::gfp)} \\
JRK71 & \textit{ktEx11 (Psrb-1::ubc-9; Pmyo-2::NLS::gfp)} \\
JRK72 & \textit{ktEx22 (unc-25::ubc-9; Pmyo-2::NLS::gfp)} \\
JRK77 & \textit{ktEx11; ulu152} \\
JRK82 & \textit{ktEx12; ulu1*2} \\
JRK85 & \textit{ktEx21; ulu1*2} \\
JRK86 & \textit{ktEx22; ulu1*2} \\
JRK89 & \textit{ktEx22; ulu152} \\
JRK102 & \textit{ktEx29 (unc-17::ubc-9; Pmyo-2::NLS::gfp)} \\
JRK110 & \textit{ktEx31 (unc-25::ubc-9(C93S); Pmyo-2::NLS::gfp)} \\
JRK134 & \textit{ktEx34 (unc-25::gfp::ubc-9; Pmyo-2::NLS::gfp)} \\
JRK135 & \textit{ktEx35 (unc-25::gfp::smo-1(GG); Pmyo-2::NLS::gfp) line #1} \\
JRK136 & \textit{ktEx36 (unc-25::gfp::smo-1(GG); Pmyo-2::NLS::gfp) line #2} \\
JRK137 & \textit{ktEx37 (unc-25::ubc-9::ttx-3::dsRed)} \\
JRK138 & \textit{ktEx35; ktEx37} \\
JRK139 & \textit{ktEx4; ktEx34} \\
JRK141 & \textit{ktEx4; ktEx36} \\
\hline
\end{tabular}
\caption{\textit{C. elegans} strains used (*indicates additional times outcrossed to wild type).}
\end{table}
and left overnight at room temperature. For panneuronal RNAi, a <1-generation protocol was used to avoid embryonic lethality caused by potential knockdown in non-neuronal tissues. Worms were synchronized by bleaching gravid adults to hatch and recover L1s, which were then plated onto RNAi-containing plates and grown at 20°C for 3 days. For all experiments, RNAi-treated worms were grown to the young adult stage for use in aldicarb assays.

**Aldicarb assays**

NGM agar plates (35 mm) containing 1 mM of aldicarb (Sigma-Aldrich) were prepared, then spotted with 150 μL of OP50 *E. coli*. After 1 day, approximately 20 young adult worms were placed on each plate in 2-minute intervals, then measured for total paralysis every 25 minutes. Total paralysis was defined as no physical movement of the worms when prodded 3 times with a platinum wire on the nose. Three plates were tested for each strain of worms per experiment with the experimenter double-blinded to genotype. The average percentage of worms paralyzed for each strain at each time point ± s.e.m. was calculated using Microsoft Excel. Data from a total of 3 to 15 plates were pooled from experiments taken at the same time intervals over several days. Statistical analyses were performed using JMP 14 software to compare the average percentages of worms of each strain paralyzed at the timepoint with the experimenter double-blinded to genotype. One-way ANOVAs then were used to assess statistical significance of the differences in the means between groups (α < 0.05), followed by a Tukey's post hoc test (α < 0.05 for all). Connecting letters reports, as well as p value results of ordered difference reports are provided.

**Pentylenetetrazole assays**

NGM agar plates (35 mm) containing 10 mg/ml pentylenetetrazole (PTZ, Sigma-Aldrich) were prepared on the day of the experiment, allowed to dry open for 1 hour at room temperature, then spotted with 25 μL of OP50 *E. coli*. After 2 additional hours of drying, approximately 20 (and no less than 15) young adult worms were placed onto each plate in 4-minute intervals, then measured for anterior convulsions (“head bobs”) after 50 minutes. These anterior convulsions were accompanied by full body paralysis in some animals. Two plates were tested for each strain of worms per experiment with the experimenter double-blinded to genotype. One plate of worms of each genotype was also tested on plates containing 0 mg/ml PTZ to ensure the absence of convulsions. The average percentage of worms exhibiting anterior convulsions for each strain at each time point ± SD was calculated using Microsoft Excel. Data from a total of 8 to 10 plates were pooled from experiments taken at the same time intervals over several days. Statistical analyses were performed using JMP 14 software, with data tested for normality and equality of variances followed by a one-way ANOVA (α < 0.05) and Tukey's post hoc test (α < 0.05 for all). Connecting letters reports and p value results of ordered difference reports are provided.

**Fluorescence microscopy**

For all imaging experiments, young adult worms were immobilized in 30 mg/ml 2, 3-butanedione monoxamine (Sigma-Aldrich) for 5 to 7 minutes, then mounted on 2% agarose pads. Their dorsal nerve cords halfway between the vulva and the tail were imaged with a Leica DMLB microscope (Leica Microsystems) with a 100x Plan Apochromat (1.4 NA) objective equipped with green and red fluorescence filters. Images were captured using an Exi Aqua cooled CCD camera (Qimaging) with Metamorph (v7.7) software (Molecular Devices) as described previously.

Quantitative imaging of GFP::SNB-1 fluorescent puncta in the dorsal nerve cord of worms overexpressing UBC-9 in all neurons (kjrEx11) or only in GABA neurons (kjrEx22) was performed by generating maximum intensity projections of z-series stacks (0.2 μm steps, 1μm total depth). Exposure settings, gain, and binning were set to fill a 12-bit dynamic range without saturation. These settings were identical for all images taken of a given fluorescent marker [i.e., GFP::SNB-1 in GABAergic (ju141) or cholinergic (nuIs152) neurons] for each imaging set. Linescans of dorsal nerve cord puncta were generated using Metamorph (v7.1) software, and the linescan data were analyzed with Igor Pro (WaveMetrics) using custom written software as previously described. Mercury arc lamp output was normalized by measuring the intensities of 0.5 μm FluoSphere beads (Invitrogen Life Technologies) for each imaging day. Puncta intensities were calculated by dividing the average maximal peak intensity by the average bead intensity for the corresponding day. Interpunctal axonal intensities were similarly calculated using the average baseline fluorescence value within the dorsal nerve cord. Puncta widths were determined by measuring the width of each punctum at half the maximum peak fluorescence. Puncta densities were determined by quantifying the average number of puncta per 10 μm of the dorsal nerve cord. For all data, an average of the values for each worm in the data set ± s.e.m. is reported. Statistical significance of any differences between wild type and transgenic strain values was determined in Igor using a one-way ANOVA with Tukey's post hoc test (P ≤ .05). Graphs of puncta intensities, interpunctal axonal fluorescence, and width show data normalized to wild type values. Images were cropped and set to matching input levels using Adobe Photoshop.

For colocalization studies, z-stacks were obtained of worms co-expressing mCherry::SNB-1 and GFP::UBC-9 (kjrEx4;kjrEx34) or mCherry::SNB-1 and GFP::SMO-1 (kjrEx4;kjrEx36) over a 2 μm total depth (0.2 μm steps), and
quantification of colocalization was performed using Metamorph 7.1. An overlaid z-stack image file was created by merging images taken in each of the red and green channels. Then, the individual plane in best focus from the merged stacks of both red and green channel images for each worm was selected for analysis. Regions containing in-focus puncta were selected from these single plane images, and the software’s linescan function was used to measure intensities across these portions of the image in both the red and green channels. Red peaks generated from the puncta were counted and recorded, and individual green peaks were counted and recorded separately. Then, the regions where red and green peaks overlapped, indicating colocalization of the two fluorophores, were counted. Only regions at least 5 a.u. greater than the background intensity value were considered as peaks, and red and green peaks that overlap at least 75% were counted for the colocalization analysis. The mean percentage of mCherry::SNB-1 peaks overlapping GFP::UBC-9 or GFP::SMO-1 and the percentage of GFP::UBC-9 or GFP::SMO-1 overlapping with mCherry::SNB-1 were determined from these counts. To control for potential bleed-through effects, worms overexpressing just mCherry::SNB-1 (kjrEx4), just GFP::UBC-9 (kjrEx34), or just GFP::SMO-1 (kjrEx36) were also imaged under both red and green filters using the same parameters as for the double-transgenic strains (see Supplemental Material). Images were cropped and set to matching input levels for each channel using Adobe Photoshop.

Results
We began by assessing whether SUMO pathway enzymes were required for neuromuscular signaling. To do this, we tested animals with reduced expression of the genes encoding individual SUMO-activating subunits (AOS-1 and UBA-2), the SUMO-conjugating enzyme (UBC-9), the E3 SUMO ligase GEI-17, or the SUMO proteases (ULP-1, -2, -3, -4 and -5) for paralysis responses induced by the acetylcholinesterase inhibitor, aldicarb. In these experiments reduction of gene expression was induced by either feeding RNAi (aos-1, uba-2, ubc-9, gei-17, ulp-2, and -4) or genetic mutation (ulp-1, -3, and -5). Individual inhibition of each of the 5 the proteases led to wild type aldicarb phenotypes. Wild type phenotypes were also observed for ulp-1;ulp-3 and ulp-1;ulp-5 genetic double mutant animals, potentially due to redundancy of protease function (data not shown). Whereas no consistent results were observed with knockdown of uba-2 and gei-17, modest aldicarb hypersensitivity was observed with knockdown of ubc-9 or aos-1 relative to control-treated worms (Figure 1), indicative of increased muscle contraction. We also observed weak but variable aldicarb hypersensitivity following cell type-specific knockdown of aos-1 or ubc-9 only in GABAergic but not in cholinergic neurons (data not shown), suggesting that ubc-9 activity may be required specifically in GABAergic neurons to prevent excess muscle excitation. Due to the modest effects and the variability inherent in doing cell type-specific RNAi, we centered our subsequent investigations on the possible effects of SUMO enzyme overexpression on neuromuscular function, with a particular focus on the SUMO-conjugating enzyme UBC-9.

Since inhibition of SUMO enzyme expression led to increased muscle contraction, we expected overexpression would cause aldicarb resistance, consistent with decreased muscle contraction. However, overexpression of UBC-9 in all neurons or only in GABAergic neurons also led to increased muscle excitation, indicated by aldicarb hypersensitivity, relative to wild type worms or to negative control worms expressing green fluorescent protein (GFP) in pharyngeal cells as a co-injection marker (Figure 2a and b, Figure S1A). In contrast, a wild type aldicarb paralysis phenotype was observed for

Figure 1. Knockdown of ubc-9 or aos-1 expression in neurons causes increased muscle excitation. Aldicarb assays were performed on animals neuronally sensitized to RNAi. Worms were fed RNAi-containing bacteria targeting gfp as a negative control, (A) ubc-9, or (B) aos-1 from the L1 stage. Approximately 20 young adult RNAi-treated worms per plate were exposed to 1 mM aldicarb and paralysis was measured in response to gentle prodding on the nose with a platinum wire. Representative assays testing 3 plates per strain are shown. Error bars denote s.e.m.
UBC-9 overexpression exclusively in cholinergic (ACh) neurons (Figure 2b, Figure S1B). Together, these data suggest that too much or too little UBC-9 expression in GABA neurons can shift the balance of neuromuscular signaling toward excitation. Similar results were observed for GABA neuron-specific overexpression of AOS-1, suggesting the effects may be due to too much or too little UBC-9 expression in GABA neurons.

Given the effects of neuronal UBC-9 expression on muscle function and the reported roles for SUMOylation on presynaptic and synaptic vesicle release at other synapse types, we used quantitative fluorescence imaging of GFP-tagged SNB-1/synaptobrevin, a synaptic vesicle associated v-SNARE protein, to test whether neuronal UBC-9 overexpression alters synaptic vesicle localization in GABAergic or cholinergic motor neurons. Previous studies have shown that GFP::SNB-1 expressed under cell type-specific neuronal promoters marks presynaptic sites and correlates with the number of vesicles at synapses. For this reason, quantitative imaging of GFP::SNB-1 has been used extensively to measure synapse density, as well as serving as a readout of synaptic vesicle accumulation that typically correlates with changes in synaptic vesicle cycling. Worms with increased GFP::SNB-1 puncta intensity tend to have more vesicles clustered at synapses due to decreased release, whereas decreased GFP::SNB-1 puncta intensity (often coupled with increased interpunctal axonal fluorescence) correlates with increased vesicle exocytosis and more signaling. Compared to wild type worms, animals overexpressing UBC-9 in all neurons or only in GABA neurons had significantly brighter (~40-50%) GFP::SNB-1 puncta intensity tend to have more vesicles clustered at synapses due to decreased release, whereas decreased GFP::SNB-1 puncta intensity (often coupled with increased interpunctal axonal fluorescence) correlates with increased vesicle exocytosis and more signaling.

Figure 2. GABAergic neuron-specific overexpression of UBC-9 or AOS-1 causes increased muscle excitation. Aldicarb assays were performed on wild type (WT) animals, animals overexpressing a control plasmid (Mock OE), and animals overexpressing (A) UBC-9 in all neurons (UBC-9 Neuron OE), (B) UBC-9 in GABAergic neurons only (UBC-9 GABA OE), UBC-9 in cholinergic neurons only (UBC-9 ACh OE), or (C) AOS-1 in GABAergic neurons only (AOS-1 GABA OE). (Upper panels) Representative timecourses showing the average percentage of young adult worms paralyzed per plate (15-20 worms per plate, n=3 plates) on 1 mM aldicarb. (Lower panels) Bar graphs showing cumulative data for worms paralyzed at the 75 minute timepoint (indicated by * in upper panels). Error bars for all graphs denote s.e.m. of the corresponding dataset. n = total # plates per strain. All bars sharing common letters (a-b) were determined to be statistically similar using a one-way ANOVA, followed by a Tukey's post hoc analysis. Neuronal UBC-9 overexpression. Figure 2 shows how neuronal UBC-9 overexpression affects muscle paralysis in young adult worms. Worms overexpressing UBC-9 in all neurons (UBC-9 Neuron OE) were significantly more paralyzed than both WT and Mock OE worms. Similarly, overexpression of UBC-9 in GABAergic neurons (UBC-9 GABA OE) caused increased paralysis compared to WT and Mock OE worms. Overexpression of AOS-1 in GABAergic neurons (AOS-1 GABA OE) also led to increased paralysis, suggesting a role for SUMOylation in regulating synaptic function.
Since the aldicarb and imaging results were consistent with decreased GABA signaling, we tested for direct effects on GABA-dependent behaviors by assessing the UBC-9 and AOS-1 GABA neuron overexpressing worms for their sensitivity to the GABAA receptor antagonist, PTZ. PTZ is epileptogenic in mammals and induces seizures in *C. elegans* with reduced GABAergic transmission. Such seizures do not occur in wild type animals or in those with defects only in cholinergic signaling. The combination of PTZ-induced seizures and aldicarb hypersensitivity is routinely used to identify mutants with GABAergic signaling defects. While we observed some background anterior “head bobbing” behavior in approximately 25% of PTZ-exposed wild type and Mock OE animals under our assay conditions, 95% of *snb-1(md247)* mutants, in which general synaptic transmission is reduced, showed convulsions on PTZ, as expected (Figure 4). Although not as robust as the response seen with *snb-1* mutants, nearly 50% of animals with UBC-9 GABAergic neuron overexpression and 67% of worms with AOS-1 GABAergic neuron overexpression exhibited PTZ-induced convulsions (Figure 4). These effects are in line with the moderate hypersensitivity we observed with the UBC-9 and AOS-1 overexpressing animals in the aldicarb assay (Figure 2) and provide further support that animals with SUMO enzyme overexpression have reduced GABAergic signaling, likely due to decreased GABA release.

Synaptic proteins, as well as many transcription factors and other nuclear proteins, are known to be SUMOylated. To test whether UBC-9 might be able to act directly at synapses, we imaged animals expressing either GFP::UBC-9 or the processed form of *C. elegans* SUMO GFP::SMO-1 along with our synaptic vesicle marker mCherry::SNB-1 and performed colocalization analyses. We found that 85.3% of green GFP::UBC-9 and 87.9% of green GFP::SMO-1 peaks colocalized with red.
mCherry::SNB-1 peaks (Figure 5). Similarly, 84% and 83.4% of red mCherry::SNB-1 peaks colocalized with green GFP::UBC-9 and green GFP::SMO-1 peaks, respectively. This strong colocalization was not due to bleed-through of fluorescent probes between channels (Figure S2). Thus, while there was not perfect colocalization, well over three quarters of UBC-9 and SMO-1 puncta in the dorsal nerve cord were found with SNB-1-labeled synapses; likewise, the vast majority of SNB-1 sites contained UBC-9 and SMO-1.

Since UBC-9 and SMO-1 both localized to synapses where they could interact to regulate synaptic protein SUMOylation, we next asked whether the catalytic activity of UBC-9 was necessary for the NMJ effects observed in worms overexpressing UBC-9. To do this, we mutated the active site of UBC-9 at cysteine 93, which forms a thioester bond with the activated SUMO,75,77 to serine (C93S) and compared the aldicarb sensitivity of worms overexpressing wild type UBC-9 and UBC-9(C93S) in GABAergic neurons to each other and to that of wild type animals. The C93S mutation has been shown to render UBC-9 catalytically inactive by preventing thioester bond formation with activated SUMO.77 Worms overexpressing UBC-9(C93S) in GABAergic neurons showed hypersensitivity to aldicarb-induced paralysis compared to mock overexpressing worms and wild type animals, this hypersensitive phenotype was similar to that seen with wild type UBC-9 overexpression (Figure 6a; Figure S3A).

Because overexpression of UBC-9 lacking its SUMO-conjugating ability resulted in the same neuromuscular phenotype as did overexpression of wild type UBC-9, the active site of UBC-9 may not be necessary for the increased muscle contraction observed in UBC-9 overexpressing worms. On the other hand, because SUMO levels are limiting in cells,78 overexpressing UBC-9 alone may lead to SUMO-limiting effects where proteins are bound to excess UBC-9 in the presence of too little free SUMO, causing insufficient SUMOylation and a UBC-9 dominant negative phenotype. To mitigate any SUMO-limiting effects, we co-overexpressed wild type UBC-9 with SMO-1(GG) and again performed aldicarb assays. Co-overexpression of wild type UBC-9 with SMO-1 in GABAergic neurons yielded a hypersensitive phenotype relative to wild type worms (Figure 6b, Figure S3B). The phenotype is slightly, but not significantly, higher than the levels observed in worms overexpressing only wild type UBC-9 or only SMO-1. The more modest effects observed with UBC-9 overexpression in this experiment compared to previous experiments may be due to small differences in expression levels, as different lines of worms had to be used to accommodate the co-expression.

Overall, however, this non-additive phenotype suggests UBC-9 and SMO-1 may be acting in the same pathway in GABAergic neurons to impact muscle contraction and that ultimately either too much or too little SUMO enzyme activity may cause E:I imbalance leading to excess muscle excitation.

**Discussion**

SUMOylation is an essential part of synaptic transmission for all animals, as it controls the activity of many proteins necessary for synapse formation and for pre- and post-synaptic function.8,17 Most studies to date, however, have focused on the role of the SUMO pathway at excitatory glutamatergic synapses. As a result, potential functions for SUMO enzymes in controlling signaling at different synapse types and the effects of SUMOylation on E:I balance remain largely unexplored, despite links between several known SUMOylated neuronal proteins and diseases involving E:I imbalances. Here, we show the SUMO-conjugating enzyme UBC-9 can act in inhibitory motor neurons, where it localizes with SUMO to presynaptic sites and controls synaptic vesicle localization, to modulate E:I balance and muscle excitation at the C. elegans NMJ.

**Tight regulation of UBC-9 activity is required in GABAergic neurons to maintain E:I balance**

Overall, our data show that misregulation of UBC-9 and SUMO expression in GABAergic neurons disrupts
neuromuscular activity. Based on our results, we favor a model in which UBC-9 acts, likely via its SUMO-conjugation activity, to regulate GABAergic vesicle release and prevent excess muscle excitation. Several lines of evidence support our proposed model. First, our over- and under-expression experiments suggest tight control of neuronal UBC-9 expression is critical to maintain proper muscle excitation. In our initial screen, neuronal knockdown of the SUMO enzyme genes \textit{ubc-9} or \textit{aos-1} led to increased muscle contraction compared to wild type worms (Figure 1), indicating \textit{ubc-9} and \textit{aos-1} are necessary to inhibit muscle excitation. Similar increases in muscle contraction were seen in animals with panneuronal or GABAergic neuron-specific overexpression of UBC-9, but not with UBC-9 overexpression in cholinergic neurons (Figure 2), supporting a specific effect of these enzymes in GABAergic neurons. Second, overexpression of UBC-9 in all neurons or just in GABAergic neurons caused synaptic vesicle accumulation at GABAergic motor neuron presynapses, as well as sensitivity to the GABA\textsubscript{A} receptor antagonist, PTZ (Figures 3 and 4). These results are consistent with decreased GABA release and with the increased muscle contraction observed in these animals.48-50 Third, both UBC-9 and SMO-1 co-localized with synaptic vesicle markers in GABAergic neurons (Figure 5), implying UBC-9 may act locally at presynaptic sites where it could SUMOylate known or novel synaptic targets to control synaptic vesicle release. Fourth, the increased muscle contraction seen with overexpression of wild type UBC-9 or the UBC-9(C93S) mutant indicates the active site of UBC-9 overexpression (Figure 6a). Given these are overexpression experiments and that levels of free SUMO in cells are reportedly low, both UBC-9 variants may cause loss of function phenotypes due to binding and sequestration of UBC-9 targets unable to be SUMOylated because of catalytic inactivity [e.g., UBC-9(C93S)] or insufficient free SUMO (e.g. wild type UBC-9).78,79 The fact that overexpression of AOS-1 in GABAergic neurons also led to increased muscle contraction, synaptic vesicle accumulation and PTZ-induced convulsions (Figures 2–4).
further supports this model in which SUMO:enzyme stoichiometry is disrupted in the overexpression context rather than a specific SUMO-independent effect of UBC-9. Fifth and finally, the non-additive increases in muscle excitation caused by UBC-9 and SMO-1 co-overexpression suggest these proteins act in the same pathway to impact neuromuscular function and that too much, as well as too little, SUMOylation can increase signaling for muscle contraction (Figure 6b). Together, these findings support a role for UBC-9 in GABAergic neurons to control muscle excitation, where it is necessary and sufficient in all neurons, and specifically in GABAergic neurons, to control muscle excitation, where it is also sufficient to cause PTZ-induced seizures and to alter synaptic vesicle localization in a manner consistent with decreased GABA release (Figures 1, 2, 3, 4, 6). While it is possible the knockdown phenotype we observed is due to inhibition of ubc-9 or aos-1 in non-neuronal cells, the strain used for the RNAi experiments expresses high levels of SID-1 dsRNA channels in neurons, which biases RNAi effects toward these cells.59 When examining specific neuronal subtypes relevant for NMJ control, we found that overexpression of UBC-9 in GABAergic neurons increased muscle excitation, whereas there was no effect of UBC-9 overexpression in cholinergic neurons. The reason for this GABAergic neuron expression effect remains unknown; however, our data do not rule out a role in excitatory cells. One possible explanation for the cell type-specific effects is that differing levels of UBC-9 expression are achieved with each of the cell type-specific promoters used for the

**Figure 6.** UBC-9 overexpression effects on NMJ signaling are independent of UBC-9 catalytic activity and are non-additive with co-expression of SMO-1. Aldicarb assays were performed on wild type (WT) animals, on animals overexpressing a control plasmid (Mock OE), and animals overexpressing (A) UBC-9 or UBC-9(C93S) or (B) UBC-9, SMO-1(GG), or UBC-9+SMO-1(GG) in GABA neurons. (Upper panels) Representative timecourses showing the average percentage of young adult worms paralyzed per plate (15-20 worms per plate, n = 3 plates) on 1 mM aldicarb. (Lower panels) Bar graphs quantifying cumulative data for worms paralyzed at a single timepoint (indicated by * in upper panels). Error bars for all graphs denote s.e.m. of the corresponding dataset. n = total # plates per strain. All bars sharing common letters (a-b) were determined to be statistically similar using a one-way ANOVA, followed by a Tukey’s post hoc analysis. (A) ANOVA: F(3,32) = 17.2912, P < .0001; Tukey’s post hoc WT versus UBC-9(C93S), P < .0001; Mock OE versus UBC-9(C93S), P < .0001; WT versus UBC-9, P = .0002; Mock OE versus UBC-9, P = .0002; UBC-9 versus UBC-9(C93S), P = .9184; WT versus Mock OE, P = .4934. (B) ANOVA: F(3,40) = 8.4909, P = .0002; Tukey’s post hoc WT versus UBC-9+SMO-1, P < .0001; UBC-9 versus UBC-9+SMO-1, P = .0589; SMO-1 versus UBC-9+SMO-1, P = .2976.
overexpression studies. Similar variability in expression also could explain the lack of \textit{ube-9} knockdown effects in animals engineered to allow RNAi only in cholinergic versus only in GABAergic neurons in our preliminary studies (data not shown). Alternatively, it is possible there is more compensation for effects on neuromuscular transmission in the cholinergic neurons. Unlike GABAergic neurons, which only synapse with postsynaptic muscle, cholinergic motor neurons directly signal to both GABAergic motor neurons and to muscle cells to maintain E:I balance.\textsuperscript{82} Similar GABAergic neuron-specific effects to those we observed were seen with inhibition of the anaphase promoting complex and EEL-1 ubiquitin ligases, the F-box protein MEC-15 FBXW9, as well as the cell adhesion molecule CASY-1, in prior studies at this synapse.\textsuperscript{48,80,81,83} These collective results demonstrate GABAergic neuron-specific regulation is possible, and even common, and that the aldicarb paralysis assay may be particularly sensitive to defects in inhibitory neurons.\textsuperscript{50}

We found that GABAergic neuron-specific overexpression not only of wild type UBC-9 but also the C93S mutant increased muscle excitation (Figure 6a), suggesting UBC-9’s effects may be independent of its catalytic activity; however, because of the overexpression context, we believe this does not necessarily reflect a SUMOylation-independent role for endogenous UBC-9 at this synapse. Rather, the increased muscle contraction seen with overexpression of either wild type UBC-9 or UBC-9(C93S) may actually be due to a loss of function of the enzyme in both cases due to sequestration of UBC-9 targets unable to be SUMOylated. In support of this idea, a yeast two-hybrid assay performed by Eloranta and Hurst (2002) showed the human Ubc9(C93S) mutant is severely compromised in its interaction with SUMO-1 but can still interact with its SUMOylation target, the AP-2 transcription factor, as efficiently as wild type UBC9, leading the authors to suggest a dominant negative mechanism.\textsuperscript{79} Similarly, because free SUMO levels are reported to be limiting in cells,\textsuperscript{26} it is possible that, when overexpressed alone, wild type UBC-9 binds to a greater number of its protein targets but lacks sufficient free SUMO to SUMOylate those proteins efficiently, resulting in a similar dominant negative effect. Further supporting this possibility, we also found that UBC-9 and SMO-1 co-expression in GABAergic neurons caused non-additive increases in muscle contraction (Figure 6b), indicating these proteins likely exert their effects on NMJ function through a common pathway, which would be expected to involve SUMOylation. Our additional observation that neuronal knockdown or GABAergic neuron overexpression of the E1 SUMO-activating enzyme subunit AOS-1 also resulted in increased muscle contraction, synaptic vesicle accumulation, and PTZ sensitivity (Figures 1–4) further indicates that the expression and activity of multiple SUMO enzymes must be precisely regulated for proper NMJ function. Because the Aos1-Uba2 heterodimeric E1 enzyme must bind Ubc9 to enforce SUMO transfer to the E2,\textsuperscript{32} the UBC-9 and AOS-1 overexpression phenotypes in our system also could be due to an imbalance in UBC-9:AOS-1-UBA-2 stoichiometry again leading to dominant negative-type substrate sequestration effects. Together, while SUMOylation-independent effects of UBC-9 have been previously demonstrated\textsuperscript{84–87} and cannot fully be ruled out, these data suggest the phenotypes seen with wild type UBC-9 overexpression in our study are most likely due to SUMO-limiting effects and/or incorrect stoichiometry of SUMO pathway enzymes rather than indicating an endogenous non-catalytic UBC-9 function at this synapse.

When considering our data in light of a model involving UBC-9’s SUMOylation activity, it appears that either too much or too little SUMOylation can lead to increased muscle excitation. Too little SUMOylation could be occurring in the case of \textit{ube-9} knockdown and in the case of UBC-9 or UBC-9(C93S) expression alone through the dominant negative and substrate sequestration mechanisms discussed above. Conversely, if UBC-9 acts in a common pathway with SMO-1 when the 2 are overexpressed together, it is likely due to excess SUMOylation, since conjugation-independent effects of SMO-1 and UBC-9 would not necessarily be expected to depend on one another. Previous work in \textit{C. elegans} showed that although muscle-specific overexpression of the non-conjugatable SMO-1(GA) or human SUMO-2(GA) caused some defects in \textit{C. elegans} body morphology, these were less severe than the defects seen with wild type SMO-1 or SUMO-1 overexpression.\textsuperscript{45} This suggests the majority of effects of SMO-1 overexpression result from increased target SUMOylation, which has been detected with SUMO overexpression in other systems,\textsuperscript{79} rather than non-specific or indirect effects. Other studies described additional cases in which either the excess or absence of synaptic protein SUMOylation causes similar defects in synapse formation or function. For example, SUMOylation of the active zone protein, Rim1α, is required for action potential-evoked synaptic vesicle exocytosis and calcium entry, whereas non-SUMOylated Rim1α aids in synaptic vesicle docking and priming. Thus, either too much or too little Rim1α SUMOylation can decrease synaptic vesicle release.\textsuperscript{10} Likewise, SUMOylated syntaxin1A slows endocytosis, generally under intense stimulation, while non-SUMOylated syntaxin1A is a critical part of the SNARE complex required for membrane fusion during exocytosis.\textsuperscript{9} As our experiments focused on steady state neuromuscular activity in adult organisms rather than muscle excitation achieved under specific stimulation conditions, it is possible subtle differences in the nature of the defects caused by excess or reduced SUMOylation, if both occurred in our system, all manifested as increased muscle excitation. Future studies examining the SUMOylation levels of specific proteins in GABAergic neurons under knockdown and overexpression conditions and using electrophysiology or calcium imaging to more directly measure synaptic transmission will be needed to fully delineate the
mechanisms by which UBC-1 and SMO-1 affect muscle excitation.

**UBC-9 can control GABAergic vesicle localization potentially through local presynaptic effects**

Although we did not explore specific SUMOylation targets in this study, our imaging data provide some clues as to the potential mechanism by which UBC-9 misexpression impacts GABAergic signaling to control E:I balance. Using quantitative imaging of GFP::SNB-1, we found worms with panneuronal or GABAergic neuron-specific UBC-9 overexpression accumulated synaptic vesicles relative to wild type worms in GABAergic but not in cholinergic motor neurons (Figure 3a). Increased GFP::SNB-1 puncta intensity typically reflects decreased exocytosis of vesicles into the synaptic cleft and correlates with decreased signaling.\(^{48-50}\) Conversely, increased interpunctal fluorescence often coincides with endocytic defects, as this fluorescence is indicative of GFP::SNB-1-containing vesicles that have fused with the membrane, allowing the GFP::SNB-1 to diffuse away from the synapse.\(^{49}\) The fact that we observed increases in both punctal and interpunctal GFP::SNB-1 fluorescence could indicate that the UBC-9 overexpressing animals actually have increased release and/or increased levels of GABA-containing vesicles. Alternatively, it is possible UBC-9 overexpression affects aspects of both synaptic vesicle exo- and endocytosis. While we cannot exclude either of these possibilities, or more complicated multicellular effects on both cholinergic and GABAergic signaling, a net decrease in GABA release is most consistent with the increased muscle contraction and the GABA-dependent seizure behaviors we observed in the aldicarb and PTZ assays, respectively, following UBC-9 overexpression (Figures 2 and 4). Accordingly, as loss of function of either the calcium channel subunit UNC-2 or the anaphase-promoting complex ubiquitin ligase, both of which are required for synaptic vesicle release from motor neurons, exhibit similar increases in both GFP::SNB-1 punctal and interpunctal fluorescence,\(^{48,88}\) it is possible the increases we observed in UBC-9 overexpressing animals resulted from synaptic vesicle buildup that led to spillover beyond synaptic sites. Such spillover may or may not be due to overall increases in the total number of vesicles and/or to defects in vesicle trafficking or maintenance at synapses.\(^{48}\)

Importantly, these presynaptic effects may be accompanied by postsynaptic changes in GABA receptor levels or sensitivity, as mutants lacking UNC-49 GABA\(_A\) receptors are both aldicarb hypersensitive and exhibit PTZ-induced convulsions.\(^{50,72}\) Increases in GABA\(_A\) receptors have been observed in response to decreased GABA release in *C. elegans* lacking the function of the anaphase promoting complex, and the opposing homeostatic responses to increases in GABA release have been reported in other systems.\(^{48,89}\) While it is possible increased GABA release could be leading to decreased GABA receptor expression or sensitivity (or vice versa, if there are cell non-autonomous effects of our neuronally expressed SUMO enzymes) to cause the behavioral responses we observe, such changes would be expected to maintain relatively wild type levels of GABAergic signaling. Thus, while further exploration of the homeostatic effects of neuronal UBC-9 overexpression on muscle excitation, as well as additional roles for SUMO enzymes within the muscle cells themselves, will be of interest to explore, our current data most directly support a presynaptic role for the overexpression effects.

Not only do our data suggest UBC-9 expression levels critically regulate synaptic vesicle localization and potentially release, but by imaging worms co-expressing GFP::UBC-9 or GFP::SMO-1 with mCherry::SNB-1 in GABAergic neurons, we observed strong localization of UBC-9 and SMO-1 at presynaptic sites (Figure 4). Given that we also observe GFP::UBC-9 and GFP::SMO-1 in the cell bodies (data not shown), as well as at synapses and more diffusely along the dorsal and ventral nerve cords, our results do not preclude the function of overexpressed UBC-9 in the nucleus or elsewhere within the motor neurons in this context. However, the fact that UBC-9 and SMO-1 can localize to synapses is consistent with a model in which UBC-9 and SMO-1 regulate overall neuromuscular activity and synaptic vesicles in GABAergic neurons by acting directly at presynaptic sites through effects on synaptic proteins. This model is supported by prior studies demonstrating stimulation-dependent localization of mammalian Aos1, Ubc9, as well as other SUMOylation and deSUMOylation machinery, to hippocampal presynaptic sites, where increased SUMOylation of synaptic proteins and effects on synaptic vesicle release are also observed.\(^{14,15,25}\) In hippocampal spines, activity-dependent Ubc9 localization occurs in response to an increase in its Protein Kinase C (PKC)-phosphorylated substrates and is dependent upon its catalytic cysteine.\(^{31}\) It will be of interest to determine whether activity-dependent localization of *C. elegans* SUMOylation machinery to GABAergic presynapses also occurs and what domains are required for this localization. Mammalian Ubc9 is also known to be regulated by acetylation and SUMOylation, modifications that can alter its substrate specificity and thus potentially its localization.\(^{30,90}\) Whether *C. elegans* UBC-9 is similarly regulated remains to be explored.

As noted above, work done at glutamatergic synapses in other systems uncovered several SUMOylated presynaptic proteins, including Rim1\(_\alpha\), synaptotagmin-1, synapsin1A, and syntaxin1A, which could mediate the effects of UBC-9 at GABAergic presynapses to regulate synaptic vesicle release, supporting the relevance of UBC-9 activity to synaptic plasticity\(^{6,10,27,28}\) and to learning and memory.\(^{37}\) Defects in synaptic protein SUMOylation are also linked to neurological and neurodegenerative diseases in which E:I imbalances occur.\(^{16,91}\) For example, the A548T mutation in synapsin1A, which is strongly associated with autism and epilepsy,\(^{71}\) causes reduced...
synapsin1A SUMOylation leading to a reduction in the releasable synaptic vesicle pool following neuronal stimulation in mouse hippocampal neuron cultures. It will be of interest to determine whether the defects in vesicle exocytosis due to loss of synapsin1A SUMOylation contribute to the disease phenotype in patients with the A548T mutation and whether SUMOylation of synapsin1A or other synaptic vesicle-associated proteins is critical for transmission at GABAergic synapses.

Conclusions and Future Directions
Overall, our data are consistent with a role for the SUMO pathway, and specifically, the SUMO-conjugating enzyme UBC-9, in promoting E/I balance at the C. elegans NMJ by acting locally at presynaptic sites in GABA neurons to control GABAergic signaling to prevent excess muscle excitation. Although additional studies are required to determine the precise mechanism underlying UBC-9 and other SUMO enzyme effects at this synapse, to our knowledge, this work is the first to demonstrate presynaptic function and localization of both UBC-9 and SMO-1 at inhibitory GABAergic synapses in any organism. It also further supports the increasingly prevalent notion that tight regulation of the SUMO enzyme system is critical for E/I balance within the nervous system. Given that AOS-1 overexpression causes similar effects to that seen with UBC-9, additional investigation into the effects of other SUMOylating and de-SUMOylating enzymes will be of interest to ascertain the full extent of SUMO pathway influence at the NMJ. Future studies identifying targets of SUMO-dependent and/or independent functions of UBC-9 at this synapse, associated E3 SUMO ligases and/or deubiquitinating enzymes, and potential regulation of UBC-9 and other SUMO pathway components by activity, oxidative stress, and hormone signaling, all of which can impact neuronal SUMOylation, will be critical for understanding how UBC-9 regulates presynaptic function in GABAergic neurons. Such studies will inform further investigation into the roles Ubc9 and the SUMO system play in mammalian GABAergic neurons and, in so doing, will contribute to our understanding of E/I balance misregulation in neurological and neurodegenerative diseases in which the SUMO pathway is disrupted.

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