RIC-3 phosphorylation enables dual regulation of excitation and inhibition of Caenorhabditis elegans muscle

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ABSTRACT Brain function depends on a delicate balance between excitation and inhibition. Similarly, Caenorhabditis elegans motor system function depends on a precise balance between excitation and inhibition, as C. elegans muscles receive both inhibitory, GABAergic and excitatory, cholineretic inputs from motor neurons. Here we show that phosphorylation of the ER-resident chaperone of nicotinic acetylcholine receptors, RIC-3, leads to increased muscle excitability. RIC-3 phosphorylation at Ser-164 depends on opposing functions of the phosphatase calcineurin (TAX-6), and of the casein kinase II homologue KIN-10. Effects of calcineurin down-regulation and of phosphorylated RIC-3 on muscle excitability are mediated by GABA_A receptor inhibition. Thus RIC-3 phosphorylation enables effects of this chaperone on GABA_A receptors in addition to nAChRs. This dual effect provides coordinated regulation of excitation and inhibition and enables fine-tuning of the excitation–inhibition balance. Moreover, regulation of inhibitory GABA_A signaling by calcineurin, a calcium- and calmodulin-dependent phosphatase, enables homeostatic balancing of excitation and inhibition.

INTRODUCTION A balance between excitation and inhibition is required for normal function of the nervous system. Excitation–inhibition (E-I) imbalance leads to epilepsy, is associated with autism spectrum disorders, and has also been implicated in Alzheimer’s disease (Eichler and Meier, 2008; Lei et al., 2016). Thus understanding mechanisms regulating E-I balance is of great importance. The Caenorhabditis elegans body-wall muscle cells provide an experimentally tractable and well-characterized system in which to study mechanisms regulating E-I balance because they receive innervation from excitatory, cholinergic inputs from motor neurons. Here we show that phosphorylation of the ER-resident chaperone of nicotinic acetylcholine receptors, RIC-3, leads to increased muscle excitability. RIC-3 phosphorylation at Ser-164 depends on opposing functions of the phosphatase calcineurin (TAX-6), and of the casein kinase II homologue KIN-10. Effects of calcineurin down-regulation and of phosphorylated RIC-3 on muscle excitability are mediated by GABA_A receptor inhibition. Thus RIC-3 phosphorylation enables effects of this chaperone on GABA_A receptors in addition to nAChRs. This dual effect provides coordinated regulation of excitation and inhibition and enables fine-tuning of the excitation–inhibition balance. Moreover, regulation of inhibitory GABA_A signaling by calcineurin, a calcium- and calmodulin-dependent phosphatase, enables homeostatic balancing of excitation and inhibition.
muscle nAChRs but not of the GABA\(_{\alpha}\) receptor expressed in these muscles (Halevi et al., 2002); across evolution, RIC-3 regulates functional expression of multiple divergent nAChRs and their close relatives. SHT3 receptors, but shows no effects on other, more distant members of the Cys-loop family of ligand-gated ion channels (Halevi et al., 2002, 2003; Cheng et al., 2005; Lansdell et al., 2005). The key role of RIC-3 in maturation of multiple diverse nAChRs suggested that its regulation enables modulation of cholinergic signaling. Indeed, regulation of RIC-3 quantity via interaction with the CUL-3 adaptor BATH-42 was shown to regulate L-AChR activity in \textit{C. elegans} vulval muscles (Steinigauz et al., 2009). Moreover, a phosphoproteomic screen showed that in \textit{C. elegans}, RIC-3 is phosphorylated at multiple sites in vivo (Zielinska et al., 2009).

Here we examine the function of RIC-3 phosphorylation to show that phosphorylation of RIC-3 Ser-164 enhances \textit{C. elegans} body-wall muscle excitability. We identify TAX-6 (calcineurin A homologue) and KIN-10 (casein kinase II homologue) as likely to function reciprocally in controlling phosphorylation of RIC-3 at Ser-164. Furthermore, we show that effects of TAX-6 and of RIC-3 Ser-164 phosphorylation are mediated by reduced functional expression of the inhibitory GABA\(_{\alpha}\) receptor rather than by enhanced nAChR functional expression. Thus we identify a new target for RIC-3 and a novel mechanism for coordinated regulation of excitatory and inhibitory inputs.

**RESULTS**

Phosphorylation of RIC-3 Ser-164 enables enhanced muscle excitability

To identify targets of signaling pathways regulating body-wall muscle excitability, we examined a database of \textit{C. elegans} phosphorylation sites identified by phosphoproteomics (www.phosida.com; Zielinska et al., 2009) for phosphorylation of muscle-expressed receptor subunits and proteins needed for maturation of these receptors (these proteins and references for their function in \textit{C. elegans} body-wall muscle are listed in Supplemental Table S1). This analysis identifies RIC-3 as a strong candidate for regulation by phosphorylation, as five of its residues are phosphorylated in vivo, whereas for the other proteins examined, no or fewer phosphorylated sites could be identified (except for NLG-1; Supplemental Table S1). RIC-3 is an evolutionarily conserved chaperone of nAChRs shown to affect surface expression and activity of both body-wall muscle nAChRs (Halevi et al., 2002). Thus phosphorylation/dephosphorylation of RIC-3 may affect levamisole responsiveness via regulation of L-AChR functional expression.

To examine the role of RIC-3 phosphorylation in controlling muscle excitability, we generated mutations in RIC-3 phosphorylation sites, focusing on RIC-3 Ser-164, the only phosphorylation site in the minimal rescuing RIC-3 fragment (Figure 1 and Materials and Methods). To eliminate confounding effects due to the phosphorylation state of other RIC-3 residues, we generated Ser-164 mutations within this minimal RIC-3 fragment, a fragment previously shown to fully rescue functional expression of body-wall muscle nAChRs (Biala et al., 2009). Expression of RIC-3 minimal and two Ser-164 mutants—S164A, eliminating phosphorylation, and S164E, mimicking phosphorylation—under a ric-3 promoter in a ric-3(md1181) loss-of-function background rescued the levamisole resistance of ric-3(md1181) animals, demonstrating that all three proteins are functional. Moreover, the S164E mutation led to significant levamisole hypersensitivity within 15 min of exposure (Figure 2A), whereas levamisole responsiveness of animals expressing the S164A mutation was similar to the responsiveness of animals expressing wild-type RIC-3 (Figure 2A).

RIC-3 affects nAChR activity in motor neurons and regulates neurotransmitter release and E-I balance via regulation of this nAChR (Jospin et al., 2009). Thus levamisole hypersensitivity of the S164E mutant could be a result of perturbation of E-I balance due to neuronal expression of this mutant. To examine whether effects of RIC-3 S164E depend on muscle expression of this mutant protein, we compared levamisole responsiveness of animals expressing the RIC-3 S164E mutant protein under a muscle-specific promoter to control animals expressing the wild-type protein under the same promoter. The muscle-expressed wild-type and S164E transgenes were injected into a wild-type background to avoid masking effects due to reduced GABA release by inhibitory motor neurons whose activity depends on the ACR-12 nAChR, which is likely to require RIC-3 function for its expression (Petrasch et al., 2013). Results of this analysis (Figure 2B) showed that muscle-expressed S164E enhances levamisole sensitivity relative to the wild-type protein expressed from the same promoter. Therefore muscle expression of the S164E mutation is sufficient to confer levamisole hypersensitivity to body-wall muscles (Figure 2B). Moreover, effects of the S164E mutation are dominant over wild-type RIC-3. Note that levamisole sensitivities in Figure 2B are lower than in Figure 2A. Indeed, kinetics of the response and overall sensitivity to levamisole in our experimental setup depend on several variables, including temperature and transgene. Thus comparisons between strains or treatments were done within the same experiment (clay) and using appropriate controls; in Figure 2B, the same RIC-3 construct differed by a single amino acid.

Previously we showed that mechanisms regulating RIC-3 quantity affect muscle excitability (Steinigauz et al., 2009). To examine whether the S164E mutation affects RIC-3 quantity, we quantified the fluorescence intensity of green fluorescent protein (GFP)–tagged versions of wild-type and S164E RIC-3 proteins. To better observe muscle distribution of these proteins, we used the muscle-specific

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**FIGURE 1:** Sequence of the \textit{C. elegans} RIC-3. Highlighted in gray is the sequence of the minimal \textit{ric-3}-rescuing fragment. White letters on gray indicate sequence of the alternatively spliced intron missing in the minimal fragment. The transmembrane domains are single underlined, and the coiled-coil domains are double underlined. Residues shown to be phosphorylated by Zielinska et al. (2009) are italicized and indicated by dots. Arrow, Ser-164.
Phosphorylation of Ser-164 enhances muscle excitability and reduces RIC-3 quantity. (A) Levamisole sensitivity of animals expressing the minimal RIC-3 fragment (diamonds) or the same fragment having the S164A mutation (squares), all under a ric-3 promoter in a ric-3(md1181) (loss-of-function) background. Percentage of animals paralyzed at different time points after being placed on plates containing levamisole (0.2 mM). Significance is relative to minimal RIC-3–expressing animals at the same time point; eight plates and four independent experiments. (B) Levamisole sensitivity of animals expressing minimal RIC-3 (dark gray) or minimal RIC-3 S164E (light gray) expressed from a muscle-specific promoter, myo-3p, and in a wild-type background. Percentage of animals paralyzed at different time points, as described in A. Significance is relative to minimal RIC-3–expressing animals at the same time point; six or seven plates and three independent experiments. (C) Quantity and distribution of RIC-3::GFP and RIC-3(S164E)::GFP expressed from a muscle-specific promoter. Top, representative images of GFP-tag fluorescence; scale bar, 5 μm. Bottom left, average intensity; n = 12 or 13, N = 2. GFP intensity is normalized to the average GFP intensity in S164E animals imaged in the same experiment/day. Bottom right, coefficient of variation of fluorescence intensity from the same images. *p < 0.05, **p < 0.01, ***p < 0.001.
Transgenic relative to control, tax-6 loss-of-function mutants not expressing this transgene (Gottschalk et al., 2005). Results of this analysis showed reduced RIC-3 quantity (25.7%) in tax-6 loss-of-function mutants relative to mutants expressing the TAX-6 gain-of-function mutant in muscles. These results are consistent with TAX-6 functioning within body-wall muscle to control RIC-3 quantity (Supplemental Figure S1).

If RIC-3 Ser-164 is dephosphorylated by TAX-6, we expect that mutation of this site either to alanine (eliminating phosphorylation) or glutamate (mimicking phosphorylation but resistant to the effects of TAX-6) will reduce the effects of tax-6 down-regulation by dsRNA. Indeed, both mutations eliminate effects of TAX-6 down-regulation on levamisole responsiveness of body-wall muscles, as seen soon (15 min) after placing the animals on levamisole-containing plates (Figure 3C). At a later time point (30 min), however, effects of TAX-6 are reduced but not eliminated by mutation of Ser-164 (Figure 3D), suggesting that TAX-6 has additional, unknown targets affecting responsiveness to prolonged levamisole exposure. Ser-164, however, is likely to be the only TAX-6 target within RIC-3, as effects of tax-6 knockdown on levamisole responsiveness of animals expressing full-length RIC-3 (wild type) or minimal RIC-3 are similar (Figure 3, C and D). We note that, unlike results shown in Figure 2A, S164E transgenics in dsRNA-feeding experiments fed with control (empty vector)-expressing bacteria are not hypersensitive to levamisole compared with control transgenics on the same food source (Figure 3, C and D). This difference suggests that extrinsic factors—possibly the bacterial strain used as food in dsRNA-feeding experiments—affect muscle excitability to mask effects of this mutation.

Effects of TAX-6 are mediated by GABA<sub>A</sub> receptor inhibition

To better understand the mechanism enabling effects of TAX-6 on levamisole sensitivity, we examined the quantity of synaptic L-AChR using a yellow fluorescent protein (YFP)-tagged knock-in allele of UNC-63, a subunit of this receptor. This knock-in allele was shown to provide a sensitive assay for synaptic expression of L-AChR (Boulin et al., 2012). Results of this analysis showed no significant change in UNC-63::YFP intensity of TAX-6 dsRNA—treated compared with control synapses (Figure 4A). To validate these results and examine the possibility that TAX-6 affects function but not surface expression of L-AChR, we recorded the electrophysiological responses of whole-cell, patch-clamped body-wall muscles in tax-6 loss-of-function mutants. This analysis showed no difference in peak current amplitudes elicited by pressure application of levamisole (100 μM) in tax-6(m) mutants relative to wild-type controls (Figure 4B). Therefore effects of TAX-6 on muscle excitability are not due to enhanced synaptic quantity or levamisole responsiveness of L-AChRs.

Previous analyses showed that reduced inhibitory (GABAergic) inputs to body-wall muscles produced levamisole hypersensitivity (Vashlishan et al., 2008). To examine whether TAX-6 regulates GABA<sub>A</sub> activity, we also recorded the responses of C. elegans body-wall muscles to GABA (100 μM) application in tax-6(m) mutants. Note that under the conditions used in our experiments and as previously...
described, GABA application elicits an outward chloride current, which is unlike the normally inward (inhibitory) currents in intact animals (Richmond and Jorgensen, 1999). Results from this analysis show ~50% reduction in peak current amplitudes in tax-6(lf) compared with wild-type animals (Figure 4C). Thus the effects of TAX-6 on muscle excitability are not due to altered excitatory (nAChR) responsiveness but instead can be explained by altered inhibitory (GABA_A receptor) responsiveness.

**Effects of phosphorylated RIC-3 Ser-164 are mediated by UNC-49 receptor inhibition**

Results in Figure 3 suggest that RIC-3 Ser-164 is a major target of TAX-6, mediating its effects on the acute (early) response to levamisole. Electrophysiological results of GABA application show that effects of TAX-6 seem to be mediated by altered GABA_A receptor activity (Figure 4C). If indeed RIC-3 Ser-164 is a target of TAX-6, its effects should also be mediated by altered GABA_A activity. To examine this possibility, we compared effects of unc-49 (muscle-expressed GABA_A receptor subunit) knockdown on animals expressing minimal RIC-3 (control) or minimal RIC-3 S164E, which mimics phosphorylation. For these experiments, we used the muscle-expressed wild-type and S164E transgenes, as effects of the muscle-expressed S164E transgene are more robust (Figure 2, B compared to A) and, unlike the effects of RIC-3 S164E expressed from the ric-3 promoter, are clearly seen in dsRNA-feeding experiments (Figure 5A compared to Figure 3, C and D). As expected, unc-49 knockdown in transgenics expressing wild-type RIC-3 (control) leads to levamisole hypersensitivity (Vashlishan et al., 2008; Figure 5A). However, in animals expressing the S164E mutant, unc-49 knockdown had no significant effect compared with the same animals fed with control bacteria expressing an empty vector (Figure 5A). Moreover, levamisole responsiveness after unc-49 knockdown in animals expressing wild-type RIC-3 was comparable to responsiveness of S164E animals fed with bacteria expressing empty vector (Figure 5A), consistent with effects of Ser-164 phosphorylation being mediated by reduced functional expression of UNC-49. We expect that loss of UNC-49 function will lead to higher sensitivity to levamisole relative to the RIC-3 S164E mutant; however, dsRNA feeding of unc-49 reduces but does not eliminate UNC-49 function, as seen by its inability to produce the typical loss-of-function, shrinker, phenotype (McIntire et al., 1993).

To further validate inhibitory effects of RIC-3 S164E on UNC-49 (GABA_A receptor), we used heterologous expression in *Xenopus laevis* oocytes. We previously showed that coexpressing RIC-3 mutants with nAChRs in *X. laevis* oocytes enables analysis of their mechanism of action (Cohen Ben-Ami et al., 2005, 2009; Biala et al., 2009). To examine effects of Ser-164 mutants on the body-wall muscle GABA_A receptor, we coexpressed these mutants with the UNC-49B and UNC-49C subunits, which are known to assemble to form this receptor in vivo (Bamber et al., 2003, 2005). Expression of the two UNC-49 subunits leads to robust GABA-dependent currents (Figure 5B). Coexpression of this receptor with the RIC-3 S164A mutant had no significant effect \( p \approx 0.08686 \) on responses to GABA (Figure 5B), a result consistent with our previous results showing that RIC-3 is not needed for UNC-49 function in vivo (Halevi et al., 2002). However, oocytes expressing the receptor with RIC-3 S164E showed significant reduction in GABA responses relative to oocytes expressing the receptor alone or with RIC-3 S164A (Figure 5B), results consistent with direct interaction of phosphorylated RIC-3 with this receptor, in contrast to an indirect effect via cell-specific mechanisms. Coexpression of wild-type RIC-3 had similar effects as coexpression with S164E and unlike effects of S164A (unpublished results), a result consistent with phosphorylation of this protein by casein kinase II, which is known to be strongly expressed in oocytes (Wilhelm et al., 1995). Thus phosphorylation of RIC-3 at Ser-164 enables inhibition of GABA_A functional expression.
KIN-10 functions opposite to TAX-6 to phosphorylate Ser-164

UNC-43, a calmodulin (CaM) kinase II homologue, functions opposite to TAX-6 in the regulation of levamisole sensitivity of vulval muscle (Lee et al., 2004). Thus we examined whether it functions similarly in body-wall muscle. For this, we used unc-43 dsRNA feeding in nonsensitized strains to focus on muscle-mediated functions of unc-43, as we did earlier for tax-6 knockdown. unc-43 knockdown, like tax-6 knockdown, led to a significant increase in the sensitivity of body-wall muscles to levamisole within 30 min of exposure (Figure 6A). Thus UNC-43 is unlikely to function opposite to TAX-6 in regulating body-wall muscle responsiveness to levamisole. Moreover, the RIC-3(S164A) mutation not only did not suppress effects of unc-43 knockdown, instead it synergized with it, leading to a significant increase in sensitivity to levamisole, an increase not seen at this time point in S164A transgenics treated with control (empty vector) dsRNA or after unc-43 knockdown in other ric-3 transgenic strains (wild-type and S164E minimal RIC-3; Figure 6B). Thus UNC-43 is unlikely to target RIC-3 Ser-164.

Casein kinase II (KIN-10) was shown to function opposite to TAX-6 in C. elegans cilia (Hu et al., 2006). Furthermore, bioinformatics (scansite.mit.edu and www.phosida.com; Supplemental Table S2) suggests several casein kinase II targets within RIC-3, including Ser-164. To examine whether KIN-10 is likely to function opposite to TAX-6 in body-wall muscle, we examined its effects on levamisole-dependent paralysis. kin-10 knockdown significantly reduces the effects of levamisole on body-wall muscles (Figure 6C), a result consistent with KIN-10 functioning opposite to TAX-6. To examine whether KIN-10 targets RIC-3 Ser-164, we examined whether mutations in this site reduce the effects of KIN-10 knockdown. Indeed, S164A reverses (15 min) and later (30 min) eliminates (Figure 6D) can be explained by the unmasking of other KIN-10 targets having opposite effects on levamisole responsiveness. Finally,
RIC-3 was previously shown to be a nAChR-specific chaperone (Halevi et al., 2002). Results described here show that phosphorylation of RIC-3 Ser-164 enables inhibitory effects of RIC-3 on GABA_\textsubscript{A} receptors. Thus our work identified a new target for this evolutionarily conserved chaperone. Such promiscuous interactions are consistent with previous work suggesting that RIC-3 is a disordered protein (Cohen Ben-Ami et al., 2009); intrinsically disordered proteins have flexible interaction surfaces, enabling interactions with divergent targets. Moreover, phosphorylation of such proteins strongly affects their specificity and their effects, even switching from positive to negative effects, as reviewed in Tompa et al. (2005).

C. elegans body-wall muscles are an experimentally tractable and well-characterized model for analysis of signaling pathways regulating E-I balance. Here we show that phosphorylation of RIC-3, known to promote maturation of nAChRs mediating excitatory input to muscle, enables inhibition of GABA_\textsubscript{A} receptors mediating excitatory input to muscle. RIC-3 Ser-164 phosphorylation is unlikely to interfere with the positive effects of this protein on L-AChR, mediating excitatory inputs to muscle. Instead, it enables interaction with and inhibition of an additional target. Thus RIC-3 phosphorylation enables coordinated regulation of excitation and inhibition, providing a novel mechanism for fine-tuning E-I balance.

Our results are consistent with phosphorylated RIC-3 Ser-164 inhibiting functional expression of GABA_\textsubscript{A} receptors in C. elegans body-wall muscle. RIC-3 Ser-164 was shown to be phosphorylated in C. elegans by Zielinska et al. (2009). Thus loss-of-function mutations in RIC-3 should lead to higher responses to GABA due to release of body-wall muscle GABA_\textsubscript{A} receptors from inhibition. However, our previous analysis showed no such effect (Halevi et al., 2002). This apparent contradiction can be resolved if, under normal growth conditions, phosphorylation levels of RIC-3 Ser-164 in body-wall muscles are low. This explanation does not contradict findings of the phosphoproteomic analysis, as RIC-3 is expressed widely (Zielinska et al., 2009), and tissue distribution of phosphorylated sites identified in this study is unknown (Zielinska et al., 2009). Moreover, body-wall muscle excitability of RIC-3 Ser-164 transgenics is similar to excitability of transgensics expressing wild-type RIC-3, a result consistent with our suggestion that under normal growth conditions, TAX-6–dependent dephosphorylation of RIC-3 Ser-164 in body-wall muscle is efficient, leading to low-level RIC-3 Ser-164 phosphorylation and no inhibition by RIC-3 of GABA_\textsubscript{A} receptors in body-wall muscle.

**DISCUSSION**

RIC-3 was previously shown to be a nAChR-specific chaperone (Halevi et al., 2002). Results described here show that phosphorylation of RIC-3 Ser-164 enables inhibitory effects of RIC-3 on GABA_\textsubscript{A} receptors. Thus our work identified a new target for this evolutionarily conserved chaperone. Such promiscuous interactions are consistent with previous work suggesting that RIC-3 is a disordered protein (Cohen Ben-Ami et al., 2009); intrinsically disordered proteins have flexible interaction surfaces, enabling interactions with divergent targets. Moreover, phosphorylation of such proteins strongly affects their specificity and their effects, even switching from positive to negative effects, as reviewed in Tompa et al. (2005).

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We note, however, that effects of KIN-10 knockdown on muscle excitability appear to contradict this suggestion. This contradiction is resolved by bioinformatics (Supplemental Table S2) and deletion analysis of RIC-3 (unpublished results), suggesting residues in the C-termine of RIC-3 as major KIN-10 targets mediating much of its effects on muscle excitability—residues that are missing in the minimal RIC-3 fragment used in this study.

Knockdown of both calcineurin (TAX-6) and CaM kinase II (UNC-43) increase muscle excitability. Our work shows that knockdown of calcineurin affects excitability via decreased inhibition. Similarly, it was shown that CaM kinase II loss of function reduced muscle GABA_A receptor synaptic abundance and function (Liu et al., 2007; Vashlishan et al., 2008). Our results showing a synergistic effect of RIC-3 S164A and CaM kinase II knockdown are consistent with interactions between two parallel pathways affecting the same target. This, together with previous work from several labs, suggests a complex network of signaling pathways regulating E-I balance in the C. elegans neuromuscular junction—pathways affecting both neurotransmitter release and postsynaptic receptors (Liu et al., 2007; Vashlishan et al., 2008; Jospin et al., 2009; Stawicki et al., 2013).

Calcineurin activity depends on both calmodulin and calcium, thus serving as a sensor for synaptic activity (Baumgartel and Mansuy, 2012). TAX-6, the C. elegans calcineurin A homologue, was previously shown to physically interact with the muscle nACHR, L-AChR (Gottschalk et al., 2005). Work presented here shows no effect of TAX-6 knockdown or loss of function on L-AChR abundance or function. The increased levamisole sensitivity observed in TAX-6 knockdown animals (Figure 3A) is thus an indirect consequence of reduced GABA_A receptor activity, as tax-6(lf) caused no increase in levamisole-induced currents, yet decreased GABA currents (Figure 4, B and C). In the light of these findings, earlier results showing increased nicotine sensitivity in tax-6(lf) mutants (Gottschalk et al., 2005) are likely due to effects on the GABA_A receptor. We therefore propose that instead of regulating L-AChR function, TAX-6 serves as a sensor for its activity as part of a homeostatic mechanism. Specifically, increased muscle excitation via the highly calcium permeable L-AChR (Boulin et al., 2008) should activate TAX-6, leading to dephosphorylation of its targets such as RIC-3, thus releasing inhibitory GABA_A receptor from suppression by phosphorylated RIC-3 and leading to a homeostatic decrease in muscle excitability.

**MATERIALS AND METHODS**

**Strains and plasmids**

Minimal RIC-3 is a minimal functional fragment containing the conserved RIC-3 domain (two transmembrane domains followed by a coiled-coil domain) but lacking the nonconserved N′-terminus, the C′-terminus domain, which includes a second coiled-coil domain, and the alternatively retained exon (Figure 1). The transgene containing this fragment and the surrounding 5′ and 3′ ric-3 genomic sequences has been described (Biala et al., 2009). RIC-3 S164 present in this fragment was mutated to glutamate to obtain the S164E construct and to alanine to obtain the S164A construct. The mutated fragments were obtained by overlapping PCR using primers glu1-gcgaagagaagaaatttatgtagggag and glu2-catcatcatc-gttcttcctcatcaaaaatattt for S164E and ala1-gcgaagagaagaaatttatgtagggag and ala2- catcatcatctttgcatcaaaaatattt for S164A.

The full-length RIC-3 (wild type), minimal RIC-3, S164E, and S164A constructs were tagged with GFP downstream to the first coiled-coil domain (RIC-3 wild type) and at the end of the RIC-3 fragment (minimal RIC-3, S164A, and S164E) and placed under the myo-3 promoter or the ric-3 promoter. These constructs were injected at 5 ng/μl together with rol-6 DNA (pRF4) as marker at 50 ng/μl. This DNA mix was supplemented with SKII–DNA at 80 ng/μl. Transgenes expressed from the ric-3 promoter were injected into ric-3(md1181) animals, whereas transgenes expressed from the myo-3 promoter were injected into wild-type (N2) animals.

Wild-type (N2 Bristol) and all other strains were grown on nematode growth medium (NGM) plates seeded with OP50 at 20°C (Wood, 1988)

**dsRNA feeding experiments**

Plasmids and bacteria for dsRNA feeding–mediated knockdown were obtained from the Ahringer library (Kamath et al., 2003). Induction of dsRNA expression was done in liquid broth (LB) plus ampicillin using 4 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h. The bacteria (HT115DE3) expressing the desired vector dsRNA were then spun down, resuspended using M9 buffer (0.02 M KH_2PO_4, 0.042 M Na_2HPO_4, 0.085 M NaCl, and 1 mM MgSO_4) plus 5 mM IPTG and seeded on NGM plates. For dsRNA-feeding experiments, L4 animals were transferred to plates seeded with dsRNA-expressing bacteria for one generation at 20°C. Then L4 animals from these plates were transferred to fresh plates with new dsRNA-expressing bacteria 20–22 h before paralysis assays. As a control, the same strain was grown on bacteria-expressing empty vector.

**Levamisole assays**

For paralysis assays, L4 animals were picked 20–22 h before each experiment for growth on fresh plates at 20°C. Levamisole assays were done on 10 young adult animals per NGM plate containing 0.2 mM levamisole. At each time point after placing the animals on the levamisole-containing plate (time zero), each animal was examined for its response to gentle prodding; animals not moving in response to prodding were considered paralyzed.

Experiments were done at room temperature (18–25°C). Because room temperature affects the kinetics of the response to levamisole, we cannot compare response kinetics between experiments, and therefore each experiment includes a control strain.

**Imaging and immunohistochemistry**

Rabbit polyclonal antibodies were generated by injection of the C-terminus of RIC-3 fused to glutathione S-transferase, and the resulting serum was affinity purified on Affigel 15 (Shteingauz et al., 2009). Immunohistochemistry was done after picric acid fixation as previously described (Yassin et al., 2001), using a 1:1000 dilution of the anti-RIC-3 antibody and a 1:300 dilution of Alexa Fluor 488 anti-rabbit antibodies. Before staining, RIC-3 antibodies were incubated overnight with ric-3(md1181) animals to remove nonspecific antibody. RIC-3 was observed and quantified at body-wall muscle and neurons in tax-6 dsRNA–treated worms versus their control or in muscle of tax-6 (p675); lin15/n765ts) animals expressing the transgene inEx92 [myo-3::tax-6 cDNA g.o.f.; lin15+] relative to muscle of tax-6 (p675); lin15/n765ts), which lost the transgene and were identified by having multiple vulva (Gottschalk et al., 2005). Fluorescence intensity in muscle is relative to neurons, as they are weakly affected by dsRNA (Sieburth et al., 2005) or do not express the tax-6(6f) transgene. Similarity between results obtained in the two experiments—one normalizing to wild-type neurons and the other normalizing to tax-6(6f) neurons—are consistent with TAX-6 having no effect on RIC-3 quantity in neurons.

Images and quantification of unc-63::yfp (Boulin et al., 2012) and of ric-3::gfp fluorescence were obtained after mounting in a 5-μl drop of M9 with 25 mM sodium azide placed on an agar pad (2% agar). Worms were transferred to this drop, and after they were paralyzed, a coverslip was placed on top and sealed with 50%/50%
Paraplast/paraffin. To reduce variability due to different imaging conditions, intensity was normalized relative to the average intensity measured in the same day of one of the strains examined.

For imaging, animals were synchronized by eye as L4 or young adults. Images were taken using a Zeiss (Germany) LSM710 confocal microscope using a Plan-Apochromat 63x/1.40 differential interference contrast oil objective (Figures 2–4) or Plan-Neofluar 40x/1.30 objective (Supplemental Figure S1) and a 488-nm laser (Figures 2 and 3 and Supplemental Figure S1) or 514-nm laser (Figure 4). For quantification of GFP fluorescence, we avoided saturation by adjusting the gain on the strain showing higher expression before image acquisition using the systems software, and all images for a specific experiment were taken using the same parameters. These images were analyzed by summing the total fluorescence in a manually defined region of interest, using ZEN 2012 software. To examine altered distribution due to RIC-3 aggregation, we used the coefficient of variation, which is the SD of fluorescence intensity in the region of interest divided by the mean fluorescence intensity in the same region (Shteingart et al., 2009).

Electrophysiology in C. elegans

For electrophysiology experiments, tax-6(p675) was outcrossed twice. Electrophysiology of C. elegans body-wall muscles was done as previously described (Richmond and Jorgensen, 1999; Nagel et al., 2005). Animals were immobilized with Histoacryl glue (B. Braun Surgical, Spain), and a lateral incision was made to access neuromuscular junctions along the ventral nerve cord. The basement membrane overlying the muscles was enzymatically removed by incubation in 0.5 mg/ml collagenase for 10 s (type IV, CS138; Sigma-Aldrich, Germany). Muscle cells were patch clamped in a whole-cell voltage-clamp mode at room temperature (20–22°C) at a holding potential of ~60 mV using an EPC10 amplifier with head stage connected to a standard HEKA (Lambrecht/Pfalz, Germany) pipette holder for glass capillaries (outer diameter, 1.0 mm) using fire-polished borosilicate pipettes (1B100F-4; outer diameter, 1.0 mm; World Precision Instruments, Berlin, Germany) of 4- to 7-MΩ resistance. The bath solution contained NaCl, 150 mM; KCl, 5 mM; CaCl₂, 5 mM; MgCl₂, 1 mM; glucose, 10 mM; sucrose, 5 mM; and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 15 mM, pH 7.3; with NaOH, ~330 mOsm. The pipette solution contained KCl, 120 mM; KOH, 20 mM; MgCl₂, 4 mM; N-Tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid, 5 mM; CaCl₂, 0.25 mM; sucrose, 36 mM; ethylene glycol tetraacetic acid, 5 mM; and Na₂ATP, 4 mM, pH 7.2; with KOH, ~315 mOsm. Using these solutions at holding potential ~60 mV and as previously described (Richmond and Jorgensen, 1999), the chloride gradient in the body wall muscle cells is reversed, and GABA currents therefore appear as inward currents due to chloride efflux through GABA receptor channels. GABA or levamisole (100 μM) was pressure-applied (Picospritzer III; Parker Hannifin, Contamine-sur-Arve, France) onto dissected muscle cells, and corresponding inward currents were measured in whole-cell mode. Data were analyzed by Patchmaster software (HEKA Electronics, Germany).

Heterologous expression and electrophysiology in Xenopus oocytes

UNC-49B and C constructs for oocyte expression were a kind gift from Bruce Bamber (University of Toledo, Toledo, OH) (Bamber et al., 2003). RIC-3 (S164A and S164E) for oocyte expression were generated by mutating the previously described RIC-3 minimal-expressing plasmid using the primers described; these plasmids have a GFP marker downstream and in-frame to RIC-3 (Cohen Ben-Ami et al., 2005). For in vitro transcription, plasmids were linearized with Asp718 I (UNC-49B and C) NheI(ric-3 minimal-WT; and S164E) or NotI(S164A), and cRNA was transcribed using T3 RNA polymerase (Ambion, France) for UNC-49B and C or T7 RNA polymerase (Promega, Fitchburg, WI) for RIC-3 constructs. In vitro synthesis and injection of cRNAs into oocytes was previously described (Cohen Ben-Ami et al., 2005). Briefly, in vitro–transcribed and capped cRNAs were injected at final concentrations of 0.25 ng/oocyte for unc-49B and C and 5 ng for GFP-tagged ric-3 S164A or S164E. At 2–3 d after injections, cells were placed in a 2-ml bath that was perfused with medium and penetrated with two 0.5- to 1.5-MΩ 3 M KCl-filled glass microelectrodes attached to a GeneClamp 500B amplifier (Axon Instruments, Foster City, CA) using a two-electrode voltage clamp with active ground configuration and an HS-2A head stage (Axon Instruments). A Pentium 4 PC system employing the pCLAMP9 (AxoScope) software (Axon Instruments) was used for maintaining voltage clamp. Cells were clamped at ~70 mV. Oocytes with leak currents <250 nA were used.

Electrodes were filled with 3 M KCl. The extracellular recording solution contained ND96 (96 mM NaCl, 2 mM KCl, 5 mM MgCl₂, 5 mM HEPES, pH 7.5). The current and the voltage in the voltage-clamp circuit were recorded simultaneously and saved directly onto the computer. The 1 mM GABA agonist (Sigma-Aldrich) was prepared and used to stimulate oocytes. Results are presented as mean ± SEM, with n the number of oocytes tested and N the number of different frogs in each experiment.

Western analysis

For Western analysis, oocytes were homogenized by repetitive pipetting in 25 μl/oocyte buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Triton X-100) with Complete Mini, EDTA-free, protease inhibitor mixture tablets (Roche Applied Science, Switzerland; 1 tablet/7 ml of buffer). After 30 min on ice, the homogenate was centrifuged at 4°C. The supernatant was aliquoted, and sample buffer (4× Laemmli sample buffer [Bio-Rad, Hercules, CA] + 10% β-mercaptoethanol + 90 mM dithiothreitol) was added at 1:2 buffer-to-sample ratio.

Proteins were separated on 10% SDS–PAGE, transferred to polyvinylidene fluoride membrane and immunoblotted with 1:1000 rabbi anti-GFP antibody (MBL, Woburn, MA) followed by 1:5000 goat-anti rabbit secondary antibody (Jackson laboratories, New Market, UK). Band intensity was quantified by ImageJ software (Schneider et al., 2012).

Statistical analysis

In all experiments, n is number of animals, plates (for levamisole assays), or oocytes examined and N is the number of independent experiments or frogs. Results are given as average ± SEM. Significance was examined using two-sided Student’s t tests.

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