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Cornichons control ER export of AMPA receptors to regulate synaptic excitability

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SUMMARY

The strength of synaptic communication at central synapses depends on the number of ionotropic glutamate receptors, particularly the class gated by the agonist AMPA (AMPA receptors). Cornichon proteins, evolutionarily conserved endoplasmic reticulum cargo adaptors, modify the properties of vertebrate AMPARs when coexpressed in heterologous cells. However, the contribution of cornichons to behavior and *in vivo* nervous system function has yet to be determined. Here, we take a genetic approach to these questions by studying CNI-1 – the sole cornichon homologue in *C. elegans*. *cni-1* mutants hyper-reverse, a phenotype associated with increased glutamatergic synaptic transmission. Consistent with this behavior, we find larger glutamate-gated currents in *cni-1* mutants with a corresponding increase in AMPAR number. Furthermore, we observe opposite phenotypes in transgenic worms that overexpress CNI-1 or vertebrate homologues. In reconstitution studies, we provide support for an evolutionarily conserved role for cornichons in regulating the export of vertebrate and invertebrate AMPARs.

INTRODUCTION

Rapid, excitatory synaptic communication that occurs in vertebrate brains, as well as in most other nervous systems, is primarily mediated by the neurotransmitter glutamate. The strength of this communication is dependent on the number of AMPA-class ionotropic glutamate receptors, as well as on their functional properties. Synaptic strength is plastic, and nervous system activity can change the number and properties of AMPARs (Anggono and Huganir, 2012; Kerchner and Nicoll, 2008; Kessels and Malinow, 2009).

While the properties of AMPARs can be modified by RNA editing (Wright and Vissel, 2012) and posttranslational modification of receptors (Lu and Roche, 2012), more recent genetic studies have demonstrated that AMPAR trafficking and function are critically dependent on auxiliary proteins, most prominently the TARP and SOL classes of transmembrane proteins (Jackson and Nicoll, 2011; Wang et al., 2012; Wang et al., 2008;

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Yan and Tomita, 2012; Zheng et al., 2004). Furthermore, proteomic studies have identified a host of new candidate auxiliary proteins (Schwenk et al., 2012; Schwenk et al., 2009; Shanks et al., 2012; von Engelhardt et al., 2010), including the vertebrate cornichon (CNIH) proteins CNIH-2 and CNIH-3 (Schwenk et al., 2009).

Cornichon was first identified in *Drosophila*, where the Cni protein binds to the EGF-like ligand Gurken, thus acting as a cargo receptor for recruitment of Gurken into COPII vesicles. In the absence of Cni, export from the endoplasmic reticulum (ER) and secretion of ligand are disrupted (Bokel et al., 2006; Roth et al., 1995). More recently, affinity purification of native AMPARs from rat brain followed by mass spectroscopy analysis identified CNIH-2 and CNIH-3 as AMPAR-interacting proteins. When coexpressed with AMPAR subunits in heterologous cells, these proteins slowed the kinetics of receptor desensitization and deactivation, and increased surface expression (Coombs et al., 2012; Kato et al., 2010; Schwenk et al., 2009; Shi et al., 2010).

Subsequent studies have revealed additional complexities. While CNIH-2 was found at the cell surface of hippocampal neurons, it did not reach the surface of cerebellar Purkinje neurons in stargazer mice, which lack the γ -2 TARP (Gill et al., 2011). Studies in HeLa cells found that overexpression of CNIH-2 altered the glycosylation pattern of the GluA2 AMPAR subunit, suggesting that CNIH-2 regulates AMPAR maturation in the ER, which may affect AMPAR function at synapses (Harmel et al., 2012). While studies in various heterologous cells have provided important insights into CNIH-2 function, these cells might express different proteins and use different trafficking pathways than those present in neurons. Consequently, studies to date have not fully resolved whether CNIHs primarily have a forward trafficking role in neurons, whether they are also recruited away from the ER-Golgi early secretory pathway to function at synapses, or whether they function as auxiliary proteins at synapses to modify the functional properties of AMPARs. Clearly, a deeper understanding of the contribution of CNIHs to nervous system function and behavior would benefit from *in vivo* studies. Genetic-based studies of cornichon function in neurons are a challenge in vertebrates, which express four cornichon homologues with possible redundant functions. In contrast, only one cornichon homologue is found in the *C. elegans* genome.

The study of synaptic signaling in the simple, well-defined nervous system of *C. elegans* has provided new insights into AMPAR function and behaviors regulated by glutamatergic signaling (de Bono and Maricq, 2005). In *C. elegans*, the GLR-1 AMPAR subunit is required for fast glutamate-gated current in specialized command interneurons that control the switch between forward and backward movement; thus, reversal frequency is disrupted in *glr-1* loss-of-function or gain-of-function mutants (Hart et al., 1995; Maricq et al., 1995; Zheng et al., 1999). Genetic screens for modifiers of this behavior have led to the identification of three classes of auxiliary proteins that associate with GLR-1 and modify the kinetics of glutamate-gated current. These include the evolutionarily and functionally conserved TARP proteins, STG-1 and STG-2 (Walker et al., 2006a; Wang et al., 2008), and the two classes of CUB-domain transmembrane proteins, SOL-1 and SOL-2 (Walker et al., 2006a; Walker et al., 2006b; Wang et al., 2012; Zheng et al., 2006; Zheng et al., 2004).

Sequence analysis of the *C. elegans* genome revealed the presence of a single cornichon homologue (*cni-1*). We found that *cni-1* mutants exhibited a hyper-reversal phenotype in contrast to the hypo-reversal phenotype observed in strains with loss-of-function mutations in either the GLR-1 subunit or in the auxiliary proteins (Brockie et al., 2001b; Mellem et al., 2002; Zheng et al., 2004). Cornichon's well-characterized role in the export of specific proteins from the ER (Bokel et al., 2006; Castro et al., 2007; Herzig et al., 2012; Roth et al., 1995) prompted us to examine the *in vivo* trafficking of GLR-1. Compared to wild type, we

found increased anterograde transport of GLR-1 in *cni-1* mutants, with corresponding increases in synaptic GLR-1 expression and GLR-1-mediated currents. In contrast, trafficking and current were reduced with overexpression of CNI-1, the yeast homolog (Evr14p), or vertebrate CNIH-2. Reconstitution experiments revealed that cornichon proteins have evolutionarily conserved roles in limiting the export of AMPARs and modifying receptor function, thus contributing to the regulation of neuronal excitability.

RESULTS

CNI-1 is the sole cornichon homologue in *C. elegans*

Analysis of the *C. elegans* genome for genes that encode proteins with significant identity to members of the vertebrate cornichon family identified only one candidate homologue (T09E8.3; www.wormbase.org), which had significant identity to vertebrate and invertebrate cornichon proteins as well as to the yeast protein Erv14p (Figures S1A and S1C). Analysis of the primary amino acid sequence predicted three transmembrane domains (TMDs), as well as the topological arrangement of intra- and extracellular regions found in previously described cornichon proteins (Figure S1C). Invertebrate cornichon proteins as well as vertebrate CNIH-1 and CNIH-4 lack the signature 16 amino acid region found in the first extracellular domain of vertebrate CNIH-2 and CNIH-3. The T09E8.3 gene was originally named *cnih-2* (Zhang et al., 2012); but because the protein lacks the signature region, a consensus decision was made to rename the gene *cni-1* (Chris Rongo, personal communication). An available deletion mutation in *cni-1* removes over half of the coding region and disrupts the 3' UTR signal required for polyadenylation (Figure S1B).

Reversal frequency is increased in *cni-1* mutants

C. elegans regulate the frequency of turning and reversals to mediate avoidance and attraction behaviors, and to aid in the exploration of their environs by restricting or expanding the search area (Hills et al., 2004). The neural circuitry that controls these reversals has been identified and includes the command interneurons that express the GLR-1 AMPAR (Brockie et al., 2001a; Chalfie et al., 1985; de Bono and Maricq, 2005; Piggott et al., 2011). Mutations in genes that disrupt glutamate release (*eat-4*) or the function of postsynaptic glutamate receptors (*glr-1*, *sol-1* and *stg-2*) decrease reversal frequency (Figure 1A) (Brockie et al., 2001b; Mellem et al., 2002; Walker et al., 2006a; Wang et al., 2012; Wang et al., 2008; Zheng et al., 2004). In contrast, transgenic “lurcher” worms that express a gain-of-function mutation in GLR-1 have an increased reversal frequency (Zheng et al., 1999). Thus, reversal frequency provides a behavioral readout of the strength of AMPAR-mediated synaptic signaling.

We found that the average forward time in *cni-1* mutants was considerably shorter than in wild type, with a corresponding increase in the frequency of reversals (Figure 1A). This increase suggested that the strength of AMPAR-mediated signaling was increased in *cni-1* mutants. In contrast, we did not observe phenotypes associated with loss of GLR-1-mediated signaling, such as altered avoidance responses to tactile or osmotic stimuli (Figure S2), nor did the *cni-1* mutation suppress the behavioral defects of *glr-1* mutants (Figure 1A and Figure S2A).

To further address whether the increased reversal frequency observed in *cni-1* mutants was dependent on glutamatergic signaling, we examined relevant single and double mutants (Figure 1A). Double mutants with *cni-1* and *eat-4*, which encodes the vesicular glutamate transporter, were indistinguishable from *eat-4* mutants alone, indicating that the hyper-reversal behavior in *cni-1* mutants was dependent on glutamatergic neurotransmission. We next asked whether the hyper-reversal behavior was dependent on the GLR-1 AMPAR.

Compared to wild type, reversal frequency was decreased in *glr-1* mutants (Brockie et al., 2001b), and the *glr-1* mutation suppressed the hyper-reversal phenotype of *cni-1* mutants (Figure 1A). These results indicate that the effects of *cni-1* on reversal behavior primarily depend on AMPAR-mediated synaptic signaling. In support of this hypothesis, we found that the *cni-1* reversal phenotype was also dependent on AMPAR auxiliary proteins. Thus, reversals were suppressed in the double mutants *sol-1; cni-1* and *stg-2; cni-1* (Figure 1A).

The amplitude of glutamate-gated currents is increased in *cni-1* mutants

To evaluate the contributions of CNI-1 to AMPAR-mediated currents, we turned to *in vivo* patch-clamp electrophysiological analysis. Compared to wild type, the amplitude of the glutamate-gated and GLR-1-dependent current in the AVA command interneurons was significantly increased in *cni-1* mutants (Figures 1B and 1C). We found the same relative increase when we used the partial agonist kainate and there was no difference in the efficacy of the two agonists (Figure S3A). In contrast, overexpression of CNI-1 in AVA dramatically reduced the amplitude of the glutamate-gated current. In these transgenic worms, the amount of CNI-1 expressed from the multi-copy transgene is predicted to be much greater than that from the endogenous *cni-1* gene. We next asked whether currents were also modified in transgenic *cni-1* mutants that overexpressed the related vertebrate CNIH-2 or yeast Erv14p cornichon proteins. We found dramatically reduced currents in these transgenic worms, consistent with an evolutionarily conserved role for cornichon proteins in the regulation of AMPAR-mediated synaptic signaling (Figures 1B and 1C).

We also evaluated whether NMDA receptors (NMDARs) were dependent on CNI-1. Peak NMDA-gated currents were increased in *cni-1* mutants and reduced with overexpression of either CNI-1 or CNIH-2 (Figure S3B and S3C). In contrast to what we observed for AMPARs, overexpression of CNI-1 or CNIH-2 caused much smaller changes in NMDA-gated current. These effects are not likely secondary to changes in GLR-1 given that NMDAR-mediated currents in *glr-1* mutants are indistinguishable from those in wild-type worms (Brockie et al., 2001b; Mellem et al., 2002; Zheng et al., 2004). These results, together with our behavioral data, demonstrate that CNI-1's most prominent role in AVA appears to be the regulation of AMPARs. Our findings are consistent with an earlier study in yeast demonstrating that Erv14p regulates multiple classes of proteins (Herzig et al., 2012).

The number of synaptic GLR-1 AMPARs is increased in *cni-1* mutants

The increased current that we observed in *cni-1* mutants might be secondary to an increase in the number of synaptic AMPARs. To evaluate whether the number of receptors at synapses is modified in *cni-1* mutants, we used confocal microscopy to image transgenic strains that expressed a functional GLR-1::GFP fusion protein. Consistent with our earlier behavioral and electrophysiological data, we found that the fluorescence intensity of GLR-1::GFP was greater in transgenic *cni-1* mutants and reduced with overexpression of CNI-1, CNIH-2 or Erv14p (Figures 2A and 2B). We obtained similar results with overexpression of CNI-1 in transgenic wild-type worms (Figure S4A). To address whether the surface expression of GLR-1 was modified by the mutation in *cni-1*, we tagged GLR-1 with the pH-sensitive reporter superecliptic phluorin (SEP). The fluorescence of SEP is quenched by the relatively acidic pH of intracellular vesicles, but exhibits greatly enhanced fluorescence at extracellular pH, thus distinguishing intracellular proteins from those at the surface (Miesenbock et al., 1998; Wang et al., 2012). We also found that the surface expression of GLR-1 was increased in *cni-1* mutants (Figure S4B), which is again consistent with the observed increase in GLR-1-mediated current (Figures 1B and 1C).

We did not find any apparent changes in AVA morphology in *cni-1* mutants, or in transgenic worms that overexpressed CNI-1. In addition, changing the expression of CNI-1

did not affect all components of the GLR-1 signaling complex. Thus, in *cni-1* mutants or transgenic mutants that overexpressed CNI-1, the intensity of GFP::SOL-1 was relatively unaffected compared to the pronounced changes in GLR-1 (Figures 2C and 2D). We also generated transgenic worms that coexpressed GLR-1::GFP and STG-2::TagRFP. Interestingly, we observed a significant increase in STG-2::TagRFP in *cni-1* mutants and a decrease with CNI-1 overexpression (Figures 2E and 2F). However, these changes might be secondary to changes in GLR-1 given that CNI-1 overexpression dramatically reduced GLR-1::GFP fluorescence in the ventral cord (Figure S4C). In contrast, the major effect of CNI-1 overexpression on STG-2::TagRFP was a shift from punctate to more diffuse expression.

Transport of GLR-1 AMPARs is increased in *cni-1* mutants

The increase in synaptic GLR-1 in *cni-1* mutants might be caused by changes in either the anterograde or retrograde transport of GLR-1. To address these possibilities, we obtained streaming confocal images of GLR-1::GFP (Figure 3A). In *cni-1* mutants, the frequency of anterograde transport events in the AVA process was significantly increased compared to wild type (Figure 3B), whereas these events were dramatically decreased in transgenic worms that overexpressed CNI-1 or CNIH-2. Interestingly, the *cni-1* mutation had less effect on retrograde transport (Figure 3C). However, retrograde events were significantly reduced by overexpression of either CNI-1 or CNIH-2, which we suggest was most likely consequent to the dramatic reduction in anterograde transport of AMPARs. Thus, the behavioral and electrophysiological changes in *cni-1* mutants appear secondary to the increased export of AMPARs from the cell body.

CNI-1 is expressed in AMPAR-expressing neurons and localizes to the ER

To determine the cellular distribution of *cni-1*, we used confocal microscopy to examine transgenic strains in which the *cni-1* promoter drove expression of GFP (Figure S5A). We found expression in many tissues, including widespread distribution in the nervous system (Figure 4A and Figure S5B). To address whether CNI-1 is expressed in the same neurons as GLR-1, we coexpressed CNI-1::GFP and mCherry driven by the *glr-1* promoter and found that CNI-1 was expressed in all GLR-1-expressing neurons (Figure S5B). In independent experiments, we confirmed that CNI-1 is expressed in the AVA interneurons using the *flp-18* promoter to identify AVA (Feinberg et al., 2008) (Figure S5C). In neuronal cell bodies, CNI-1::GFP appeared punctate and distinctively clustered at perinuclear sites, suggestive of localization to the ER (Figure 4A).

To better examine the subcellular localization of CNI-1, we coexpressed CNI-1 with either TRAM-1, a marker of rough ER, or PISY-1, a general ER marker that also labels Golgi structures (Leber et al., 1995; Lofke et al., 2008; Rolls et al., 2002). While the distribution of PISY-1 (Figure 4B) was more localized compared to the diffuse reticular distribution of TRAM-1 (Figure 4C), we found that CNI-1 colocalized with both markers, and that CNI-1 and GLR-1 colocalized (Figure 4D). To address whether accumulations of CNI-1 and GLR-1 were near ER exit sites (ERES), we coexpressed either CNI-1::GFP or GLR-1::GFP with the COPII protein, SEC-24 (F12F6.6), tagged with mCherry. In the cell body, both CNI-1::GFP and GLR-1::GFP were found localized adjacent to mCherry::SEC-24 puncta (Figures 4E and 4F) in a pattern similar to that described previously for localization of nicotinic acetylcholine receptors to ERES (Srinivasan et al., 2011). These data show that CNI-1 is expressed in discrete regions of the ER/Golgi where it colocalized with GLR-1.

The colocalization of CNI-1 and GLR-1 also suggests that the two proteins might interact with each other. To test this possibility, we coexpressed HA::GLR-1 and CNI-1::GFP in *Xenopus* oocytes and found an association between the proteins using an

immunoprecipitation strategy (Figure 4G). Together, the biochemical and cell biological data indicate a close association between CNI-1 and GLR-1, a finding that is also consistent with studies that demonstrate an association between CNIH-2 and the vertebrate GluA1 AMPAR subunit (Kato et al., 2010; Schwenk et al., 2009; Shi et al., 2010).

CNI-1 colocalizes with surface GLR-1 in the processes of AVA

In *C. elegans*, ER extends into neural processes (Rolls et al., 2002). We found that CNI-1 was also expressed in the processes of AVA, where it was distributed in a punctate pattern that colocalized with the ER/Golgi marker, PISY-1 (Figure 5A). When we examined the localization of CNI-1::GFP and GLR-1::mCherry in the AVA neural processes, we noted a prominent punctate distribution of both proteins where a subset of GLR-1 puncta colocalized with CNI-1 puncta (Figure 5B). While these experiments suggested that the majority of CNI-1 was intracellular and associated with organelles, they did not distinguish between intracellular and cell-surface localization of CNI-1. Therefore, we also tagged CNI-1 with SEP and GLR-1 with pHTomato, a red-shifted pH-sensitive reporter (Li and Tsien, 2012) and coexpressed these tagged proteins in AVA (Figure 5C). Because of reduced intracellular fluorescence using these pH-sensitive fluorophores, we were able to observe extensive colocalization of CNI-1 and GLR-1 with approximately 40% – 80% of the GLR-1 puncta colocalizing with CNI-1 puncta. Although less dramatic than the colocalization of GLR-1 with STG-2 (Figure 5D) (Wang et al., 2008), the punctate colocalization of GLR-1 with CNI-1 suggests that CNI-1 might also function at synapses. Surface expression of CNI-1 was also supported by *in vivo* antibody labeling experiments (Figure S6). These results indicate that CNI-1 is not restricted to the cell body or intracellular organelles and that CNI-1 is present at synapses.

CNI-1 limits ER export of GLR-1

Our data are consistent with a model in which CNI-1 functions to limit export of GLR-1 to synapses. However, in transgenic worms that overexpressed CNI-1 we also found that the fluorescence intensity of GLR-1::GFP was considerably reduced in cell bodies (Figures 6A and 6B). We reasoned that overexpression of CNI-1 might lead to the shunting of retained GLR-1 to the endoplasmic-reticulum-associated protein degradation pathway (ERAD) and ultimately the proteasome. To test this hypothesis, we treated worms with the proteasome inhibitor MG-132, a drug that is commonly used to evaluate ERAD-mediated degradation of transmembrane proteins (Altier et al., 2011). In transgenic worms that overexpressed CNI-1, incubation with MG-132 markedly increased GLR-1 fluorescence. In contrast, we did not observe an increase in fluorescence in wild-type controls or *cni-1* mutants (Figures 6A and 6B). These results are consistent with the hypothesis that CNI-1 overexpression blocks export of GLR-1, leading to subsequent degradation by the proteasome.

If CNI-1 has a role in ER export then the glycosylation of GLR-1 might be altered in *cni-1* mutants. The glycosylation state of proteins in the ER is distinct from those in the Golgi, and can be distinguished by differential sensitivity to the enzyme Endoglycosidase H (Endo H) (Chun et al., 2008). We found that the relative amount of GLR-1 that was resistant to Endo H was increased in *cni-1* mutants compared to wild-type worms (Figures 6C and 6D). This result is consistent with the increased GLR-1 anterograde trafficking observed in *cni-1* mutants (Figure 3).

Reconstitution experiments support a conserved role for cornichons in limiting AMPAR export from the ER

In *C. elegans*, muscle cells do not express glutamate receptor subunits or known auxiliary proteins for iGluRs, thus providing an ideal system for genetic-based reconstitution experiments. We recorded glutamate-gated currents from muscle cells in transgenic worms

that ectopically expressed GLR-1, SOL-1 and STG-1 and compared them to currents from muscle cells that expressed these three proteins along with either CNI-1 or CNIH-2. Similar to what we observed with overexpression studies in the AVA neurons, the amplitude of glutamate-gated current in muscle was significantly decreased with overexpression of either CNI-1 or CNIH-2 (Figures 7A and 7B). We next asked whether CNI-1 modified glutamate-gated currents mediated by vertebrate AMPARs expressed in *C. elegans* muscle, and found that CNI-1 also reduced the amplitude of currents mediated by GluA1 (Figure S7A), and reduced SEP::GluA1 fluorescence (Figure S7B).

Our evaluation of surface AMPARs in transgenic worms relied on measurements of fluorescence intensity. To more directly compare surface expression and receptor mediated currents, we turned to reconstitution studies in *Xenopus* oocytes. Overexpression of CNI-1 or CNIH-2 reduced GLR-1-mediated currents as well as surface expression of GLR-1 (Figures 7C and 7D). Similarly, overexpression of CNI-1 reduced GluA1-mediated currents and surface expression of GluA1 (Figures S7C and S7D). While coexpression of CNIH-2 with GluA1, GluA2 and the γ -8 TARP auxiliary subunit reduced AMPAR surface expression, it increased the peak glutamate-gated current, suggesting an additional effect of cornichon proteins on AMPAR function (Figures 7E and 7F). In contrast, coexpression of the Neto2 CUB-domain protein had no effect on either AMPAR-mediated current or surface expression (Figures 7E and 7F).

We next extended our reconstitution studies to address whether invertebrate and vertebrate cornichons have conserved roles in limiting the export of vertebrate AMPARs in neurons. Therefore, we coexpressed GFP-tagged GluA1 and γ -8 either with or without a cornichon protein in the AVA neurons. We found that overexpression of CNI-1, or vertebrate CNIH-2 or CNIH-1, dramatically reduced the fluorescence intensity of GFP::GluA1 in transgenic worms (Figures 8A and 8B). Electrophysiological analysis showed a reduction in peak glutamate- or kainate-gated current with overexpression of cornichon proteins (Figures 8C and 8D, black traces).

The reduced currents that we observed in transgenic worms that overexpressed either CNI-1 or CNIH-2 are consistent with cornichon's putative role in the export of R-1 from the ER. However, these reconstitution experiments did not address whether cornichon proteins might have additional effects on receptor function. To address this question, we determined the relative efficacy of cyclothiazide (CTZ), a drug that blocks the desensitization of vertebrate AMPARs, thereby causing potentiation of the peak current in response to the relatively slow speed of pressure application of agonist (Partin et al., 1993). In preliminary studies, we found CTZ strongly potentiated glutamate- and kainate-gated currents in transgenic worms that overexpressed vertebrate GluA1 and γ -8 in the AVA neurons. However, we observed far less potentiation in strains that coexpressed either worm CNI-1 or vertebrate CNIH-2 (Figures 8C and 8D). One interpretation of these data is that coexpression of cornichons slowed AMPAR desensitization or otherwise modified AMPAR properties (Gill et al., 2011; Gill et al., 2012; Schwenk et al., 2009), thus reducing the potentiation by CTZ.

The results from our genetic studies together with our reconstitution experiments in *C. elegans* neurons and muscle cells, and *Xenopus* oocytes demonstrate an evolutionarily conserved role for cornichon proteins in regulating the ER export of AMPARs. Furthermore, cornichon proteins colocalized with AMPARs at synapses and, when overexpressed, modified receptor function either directly or indirectly.

DISCUSSION

CNI-1 regulates ER export of AMPARs in *C. elegans*

The number of functional AMPARs at central excitatory synapses is a critical determinant of synaptic strength, and the strength of synaptic transmission is determined in part by the balance between delivery and removal of synaptic AMPARs. Our study has demonstrated that invertebrate and vertebrate cornichon proteins have conserved roles in the control of AMPAR export from the ER. In *cni-1* mutants, the export of AMPARs is unregulated causing increased transport of receptors, larger synaptic currents, neuronal hyper-excitability and disrupted foraging behavior secondary to an increased reversal frequency. While CNI-1 might affect many proteins (Bokel et al., 2006; Castro et al., 2007; Herzig et al., 2012; Roth et al., 1995), the hyper-reversal phenotype observed in *cni-1* mutants is primarily dependent on synaptic AMPARs, as demonstrated by the strong suppression of the *cni-1* mutant phenotype by mutations in either *glr-1*, *sol-1* or *stg-2*.

In support of our hypothesis that CNI-1 regulates GLR-1 export from the ER, we found that CNI-1 colocalized with ER markers in the AVA neuronal cell bodies. Of particular interest was the colocalization of CNI-1 with GLR-1, and with the ER/Golgi marker PISY-1. We also found that CNI-1 and GLR-1 were in close apposition to SEC-24/COPII puncta, which mark putative ER export sites (Srinivasan et al., 2011). These data suggest that CNI-1 might have a spatially restricted role and act to control the export of GLR-1 at the ER-Golgi interface. In support of this hypothesis, we found a larger percentage of Endo H resistant GLR-1 in *cni-1* mutants suggesting ER export was increased in the mutants compared to wild type. These data are consistent with reconstitution experiments in HeLa cells that demonstrated a CNIH-2-dependent increase in immature-glycosylated GluA2 receptors (Harmel et al., 2012).

Cornichon proteins have a conserved role in limiting the ER export of AMPARs

To address whether vertebrate cornichons had conserved roles in limiting the export of vertebrate AMPARs, we reconstituted GluA1 function in *C. elegans* neurons. We generated transgenic *glr-1; cni-1; stg-2* triple mutants that coexpressed the vertebrate GluA1 AMPAR and the vertebrate γ -8 auxiliary protein. *glr-1* and *stg-2* mutants lack fast glutamate-gated currents in AVA (Brockie and Maricq, 2006; Zheng et al., 2004), thereby facilitating the interpretation of our reconstitution studies. Interestingly, we found punctate expression of GluA1 in neuronal processes and we could record glutamate-gated current, indicating that the vertebrate receptors were transported to the surface and were functional. In these transgenic worms, coexpression of vertebrate CNIH-2 or CNIH-1, or worm CNI-1 dramatically reduced GFP::GluA1 fluorescence in neuronal processes. Furthermore, CNIH-2 and CNI-1 reduced glutamate- and kainate-gated currents, indicating that an evolutionarily conserved role of cornichon proteins is to limit the export of AMPARs.

While our manuscript was in revision, a study was published that evaluated the contribution of CNIH-2 and CNIH-3 to AMPAR-mediated synaptic transmission in CA1 neurons of the vertebrate hippocampus (Herring et al., 2013). In this study, the authors found that genetic perturbation of CNIH-2 and CNIH-3, a subset of the four cornichon proteins expressed in the brain that appear predominant in the hippocampus, was associated with reduced numbers of GluA1 containing AMPARs along with a corresponding decrease in peak glutamate-gated currents. Given the results from our reconstitution studies of vertebrate GluA1 in *C. elegans* neurons, the finding of reduced current in the conditional knockout mice suggests that vertebrate CA1 neurons might express additional quality control machinery. For example, GluA1 receptors not associated with a cornichon protein might be more susceptible to degradation in vertebrate neurons.

CNI-1 differentially regulates the components of the GLR-1 signaling complex

Auxiliary proteins are known to contribute to AMPAR function (Jackson and Nicoll, 2011; Yan and Tomita, 2012). In *C. elegans*, the auxiliary proteins SOL-1, SOL-2 and STG-2 colocalize with GLR-1 at synapses (Wang et al., 2012; Wang et al., 2008; Zheng et al., 2004). These transmembrane proteins did not show the same dependence on cornichon as that observed for GLR-1. Thus, the levels of SOL-1 were not appreciably altered in the processes of AVA in *cni-1* mutants or with overexpression of CNI-1. Although we did find an increase in STG-2 in *cni-1* mutants and a minor decrease when CNI-1 was overexpressed, we suspect that these effects were secondary to changes in GLR-1.

Recent analysis of the yeast cornichon homologue, Erv14p, provides a possible mechanism for cornichon's cargo specificity (Herzig et al., 2012). Erv14p associates with many transmembrane proteins and target specificity appears to be associated with the length of the transmembrane domain(s). However, other mechanisms also contribute to ER export and cargo can be made independent of Erv14p by the addition of different trafficking domains (Herzig et al., 2012).

CNI-1 modifies neuronal excitability by regulating the number of synaptic AMPARs

The study of the *cni-1* mutant along with transgenic rescue and AMPAR reconstitution experiments demonstrate that an ancient evolutionarily conserved role for cornichon proteins is to regulate the ER export of AMPARs. We envision two possible mechanisms. In the first model, CNI-1 binds to AMPAR subunits and blocks export from the ER –perhaps by masking an ER export signal. Binding of a putative Protein X exposes the export signal thereby allowing the complex to exit the ER (Figure 9). In *cni-1* mutants, AMPARs more readily exit the ER, resulting in greater delivery of receptors to the cell surface and synapses. This model is similar to one postulated for the regulation of TGF- β from the ER (Castro et al., 2007). Here, cornichon binds to TGF- β , thus limiting ER export until it associates with the transmembrane protein Star.

In the second model, AMPARs directly bind to COPII proteins for export. Overexpression of CNI-1 limits export of AMPARs by competing for COPII binding sites. Conversely, in the absence of *cni-1* more COPII sites are available for GLR-1 binding resulting in increased ER export. In both models, ER-retained receptors are susceptible to subsequent degradation. Although we favor the first model because we found that AMPARs and their auxiliary proteins do not have the same dependence on CNI-1, discrimination of the two models will require a systematic study of the molecular requirements for ER export of AMPARs.

CNI-1 and CNIH-2 overexpression modifies export and function of vertebrate AMPARs

Importantly, we showed in reconstitution studies in transgenic worms that vertebrate and *C. elegans* cornichon proteins similarly decreased the ER export of vertebrate AMPARs, and decreased glutamate-gated currents. These data provide further evidence for the conservation of cornichon function. Our results also highlight the utility of reconstituting vertebrate receptors in *C. elegans*. This strategy not only reveals conserved functions, but also points out significant differences. For example, the interesting difference between our results and those of Herring et al., suggests that additional machinery contributes to the regulation of AMPAR signaling complexes in vertebrates.

Our data showing that CTZ efficacy was decreased by overexpression of cornichon proteins suggests that cornichons might also function as classical auxiliary proteins to modify either the kinetics of AMPAR desensitization or receptor sensitivity to CTZ. Alternatively, cornichon might modify AMPAR function by interacting with associated auxiliary proteins, or exert indirect effects by modifying the maturation of synaptic receptors, e.g., the

glycosylation state or stoichiometry of the receptor complex. In preliminary studies, we did not find an obvious difference in GLR-1 kinetics in wild type and *cni-1* mutants (data not shown), suggesting that the effects of wild-type CNI-1 on AMPAR function might be subtle compared to those observed with overexpression of cornichon proteins. Unfortunately, we were not able to determine the relative effects of CTZ on GLR-1-mediated currents given that *C. elegans* AMPARs are insensitive to CTZ treatment (data not shown). A third possibility is raised by a recent proteomic study that identified many potential auxiliary proteins (Schwenk et al., 2012). Perhaps cornichon proteins help recruit or assemble these or yet to be identified proteins to the receptor complex.

CNI-1 colocalizes with GLR-1 in neural processes

We also found substantial CNI-1 in neuronal processes. Much of CNI-1 appeared to be intracellular and associated with organelles marked by PISY-1. However, more refined analysis revealed that CNI-1 was also at the surface of neuronal processes and colocalized with surface GLR-1, similar to what we have observed for STG-2, SOL-1 and SOL-2 auxiliary subunits (Wang et al., 2012; Wang et al., 2008; Zheng et al., 2004). Although the changes in peak glutamate-gated current in *cni-1* mutants, and with overexpression of cornichon proteins, can be explained by changes in surface expression, the presence of CNI-1 at synapses is intriguing. Our reconstitution studies demonstrating changes in CTZ sensitivity are consistent with a possible auxiliary role for CNI-1 at synapses. Alternatively, CNI-1 might regulate local trafficking of AMPARs between endosomal compartments and synapses – a possible role that might be analogous to its function as a regulator of GLR-1 export from the ER.

Our results help provide a new mechanistic view of the global regulation of receptor numbers at the postsynaptic membrane. Neurons rely on compensatory homeostatic mechanisms that regulate synaptic strength and optimize neuronal excitability (Davis, 2006; Goold and Nicoll, 2010; Turrigiano, 2008). In *cni-1* mutants, the neural circuit that regulates reversals used for avoidance and foraging behaviors is hyper-excitable secondary to an increase in the number of synaptic AMPARs. We propose that CNI-1 regulates the export of AMPARs in response to external or internal cues, thus contributing to homeostatic processes such as global regulation of neuronal excitability.

Experimental procedures

General methods, genetics and plasmids

All *C. elegans* strains were raised at 20 °C under standard laboratory conditions. Transgenic strains carrying multi-copy transgene arrays were generated using microinjection into the gonad of adult hermaphrodite *lin-15(n765ts)* mutants, wild-type worms, or relevant mutant worms. Transgenic worms were selected by rescue of the *lin-15(n765ts)* mutant phenotype, or by expression of a co-injected fluorescent marker. Single copy transgenic strains were generated following an established protocol (Frokjaer-Jensen et al., 2008). Fluorescently labeled CNI-1 was found to be functional in transgenic experiments that showed the fusion protein had a similar effect on the localization of fluorescently tagged GLR-1 as that of untagged CNI-1. Function of fluorescently labeled STG-2 was confirmed by rescue of *stg-2(ak134)* suppression of the lurcher worm hyper-reversal phenotype. Plasmids, transgenes and mutant strains are described in **Supplemental experimental procedures**.

Confocal microscopy

Confocal images were acquired using a Nikon Ti-eclipse equipped with a WaveFX-X1 spinning disc confocal system (Quorum Technologies), and captured by a Cascade 1024B EMCCD camera (Photometrics). Streaming movies (100 ms exposure) of GLR-1::GFP

transport were acquired in a single focal plane in the AVA processes. Image acquisition and kymographs were generated using MetaMorph 7.7.10 (Molecular Devices). Anterograde and retrograde events were quantified by counting the number of trajectories in each direction with the experimenter blind to the genotype.

Electrophysiological studies

Electrophysiological recordings from AVA interneurons and muscle cells from dissected transgenic worms were performed as described (Jensen et al., 2012; Mellem et al., 2002).

Behavioral analysis

Reversal frequency, nose touch response and osmotic avoidance assays were performed as previously described (Brockie et al., 2001b; Mellem et al., 2002). A reversal was defined as a switch from forward to backward or from backward to forward movement. All behavioral assays were performed blind. Statistical significance was determined by using the standard Student's *t* test.

MG-132 treatment

Proteasome inhibition assays were performed as described (Orsborn et al., 2007). Briefly, adult worms were placed in liquid medium containing 1 μ M MG-132 for 3 hr. Controls were incubated in liquid medium without MG-132. Immediately following incubation, GLR-1::GFP in the AVA cell bodies was imaged by confocal microscopy.

Quantification of fluorescence

Total fluorescence in neuronal processes was measured using a linescan measurement in MetaMorph 7.7.10 (Molecular Devices) and analyzed with a custom written MATLAB script (based on <http://terpconnect.umd.edu/~toh/spectrum/PeakFindingandMeasurement.htm>). A detailed description of the quantification of fluorescence signals can be found in **Supplemental experimental procedures**

Electrophysiology, surface labeling and coimmunoprecipitation studies in *Xenopus laevis* oocytes

Two-electrode voltage-clamp recordings, and surface labeling using HA::GLR-1, HA::GluA1 and anti-HA antibodies were performed as described (Morimoto-Tomita et al., 2009). For coimmunoprecipitations, oocyte membranes were suspended in lysis buffer containing TED (25 mM Tris-Cl pH 7.4, 2 mM EDTA, 1 mM DTT), 1% Triton X-100, Halt protease inhibitors (Pierce) and centrifuged at 100,000 \times g for 30 min (Morimoto-Tomita et al., 2009). The supernatants were then incubated with 5 μ g of anti HA antibody and 30 μ l of protein G sepharose beads. The beads were then washed five times with 1% Triton in TEEN (25 mM Tris-Cl pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl). Bound proteins were eluted by heating the resin in 40 μ l of 1X SDS-PAGE sample buffer and analyzed by SDS-PAGE.

Antibody staining in transgenic worms

Transgenic worms that expressed CNI-1::GFP in the AVA neurons were immunolabeled as previously described (Gottschalk and Schafer, 2006; Zheng et al., 2004). Briefly, anti-GFP polyclonal sera (Molecular Probes) was diluted (1:1000) in injection buffer and injected into the pseudocoelome of transgenic worms. Worms were allowed to recover for two hours before imaging.

Endo H digestion and western blots

Mixed-stage worms were washed off plates with M9 buffer. After an additional wash in M9, excess buffer was removed and worms were resuspended in 2 volumes of protein sample buffer and frozen in liquid nitrogen. Samples were heated at 95 °C for 10 min and the resulting lysates were diluted in reaction buffer for either Endo H or PNGase F (as per protocol, New England BioLabs) and digested with 250 units for 30 min at 37 °C. Products were run on 7% TGX precast gels (Bio-Rad) and transferred to nitrocellulose membranes. Blots were incubated with monoclonal anti-GFP antibodies (Santa Cruz Biotechnology) in Odyssey Blocking Buffer (LI-COR). Bands were detected with IRDye 800CW on the Odyssey Imager (LI-COR). Endo H sensitivity was quantified in ImageJ.

Statistical analysis

The results were analyzed using an unpaired Student's T-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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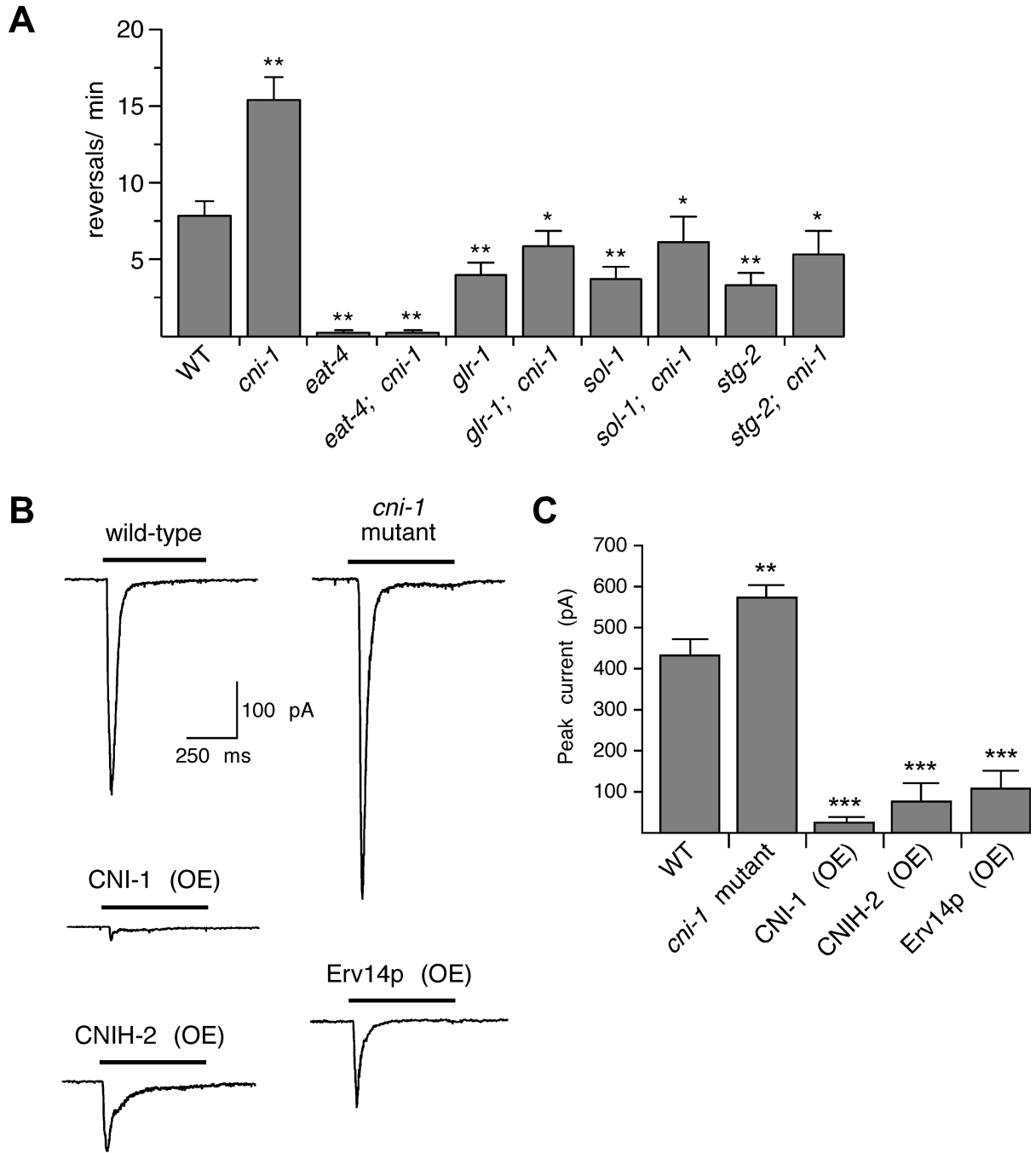


Figure 1.

Reversal frequency and glutamate-gated currents are increased in *cni-1* mutants. (A) Reversal frequency in wild-type worms and various single and double mutants. For wild-type and *cni-1* mutants, $n=8$; for *glr-1*, *sol-1* and *stg-2* single and double mutants, $n=7$; and for *eat-4* single and double mutants, $n=5$. ** Significantly different from both wild type and *cni-1* mutants ($p<0.01$). * Significantly different from *cni-1* mutants ($p<0.05$). (B) Currents measured in AVA neurons in response to pressure application of 3 mM glutamate. Cells were voltage-clamped at -60 mV. (C) Average peak glutamate-gated current in wild-type worms ($n=11$), *cni-1* mutants ($n=11$) and transgenic mutants that overexpressed (OE) either

CNI-1 (n=6), CNIH-2 (n=8) or Erv14p (n=7). Significantly different from wild type (** p<0.01 and *** p<0.001). Error bars indicate SEM.
See also Figures S1, S2 and S3.

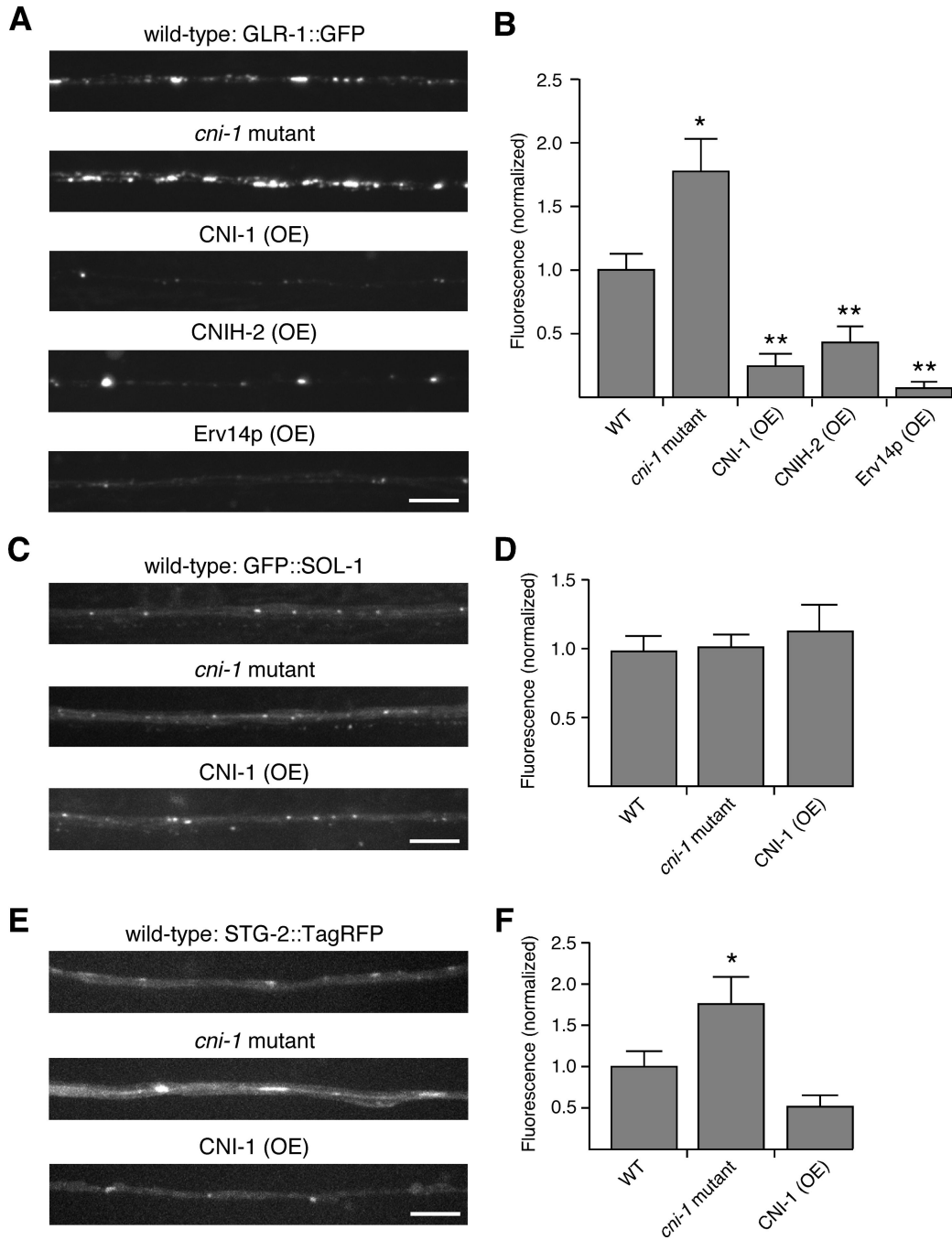


Figure 2.

Synaptic levels of GLR-1::GFP are increased in *cni-1* mutants and decreased by overexpression of cornichon proteins. (A and B) Confocal images of GLR-1::GFP (A) and the total GFP fluorescence (B) in the AVA processes of wild-type worms (n=11), *cni-1* mutants (n=11) and transgenic mutants that overexpressed CNI-1 (n=10), CNIH-2 (n=9) or Erv14p (n=7). (C and E) Confocal images of either GFP::SOL-1 (C) or STG-2::TagRFP (E) in the AVA neurons of transgenic worms. (D and F) GFP (D) or TagRFP (F) fluorescence in transgenic wild-type worms (GFP, n=12; TagRFP, n=15), *cni-1* mutants (GFP, n=11; TagRFP, n=10) and transgenic mutants that overexpressed CNI-1 (GFP, n=5; TagRFP, n=8).

Significantly different from wild type (* $p < 0.05$ and ** $p < 0.01$). Scale bars represent 5 μm .
Error bars represent SEM.
See also Figure S4.

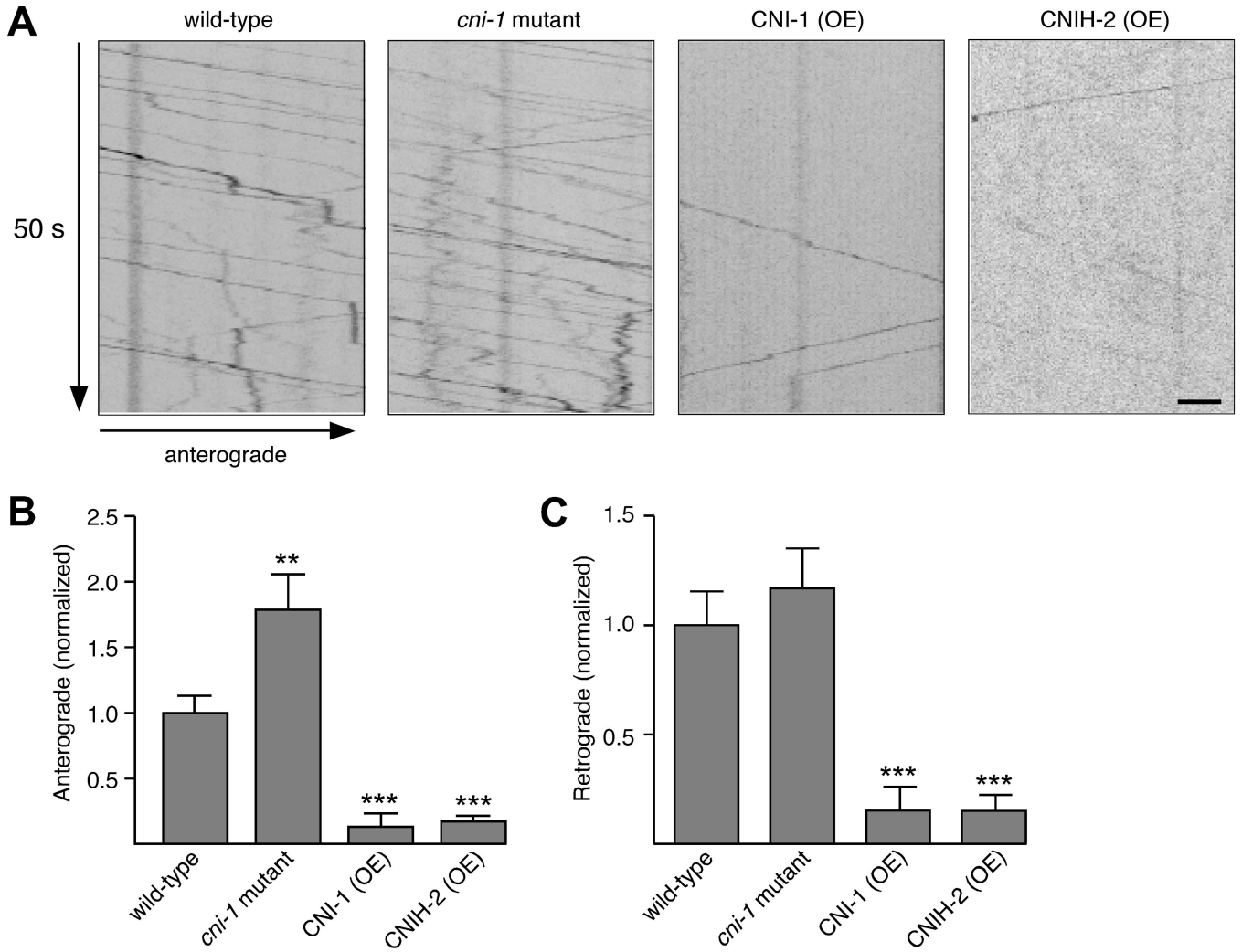


Figure 3. The frequency of GLR-1 anterograde transport is increased in *cni-1* mutants. (A) Kymographs showing the movement of GLR-1::GFP in the AVA interneurons. Scale bar represents 2 μ m. (B and C) Quantification of the number of anterograde (B) and retrograde (C) transport events in wild-type worms (n=13), *cni-1* mutants (n=9), and *cni-1* transgenic mutants that overexpressed either CNI-1 (n=10) or CNIH-2 (n=10). Significantly different from wild type (** p<0.01 and *** p<0.001). Error bars represent SEM.

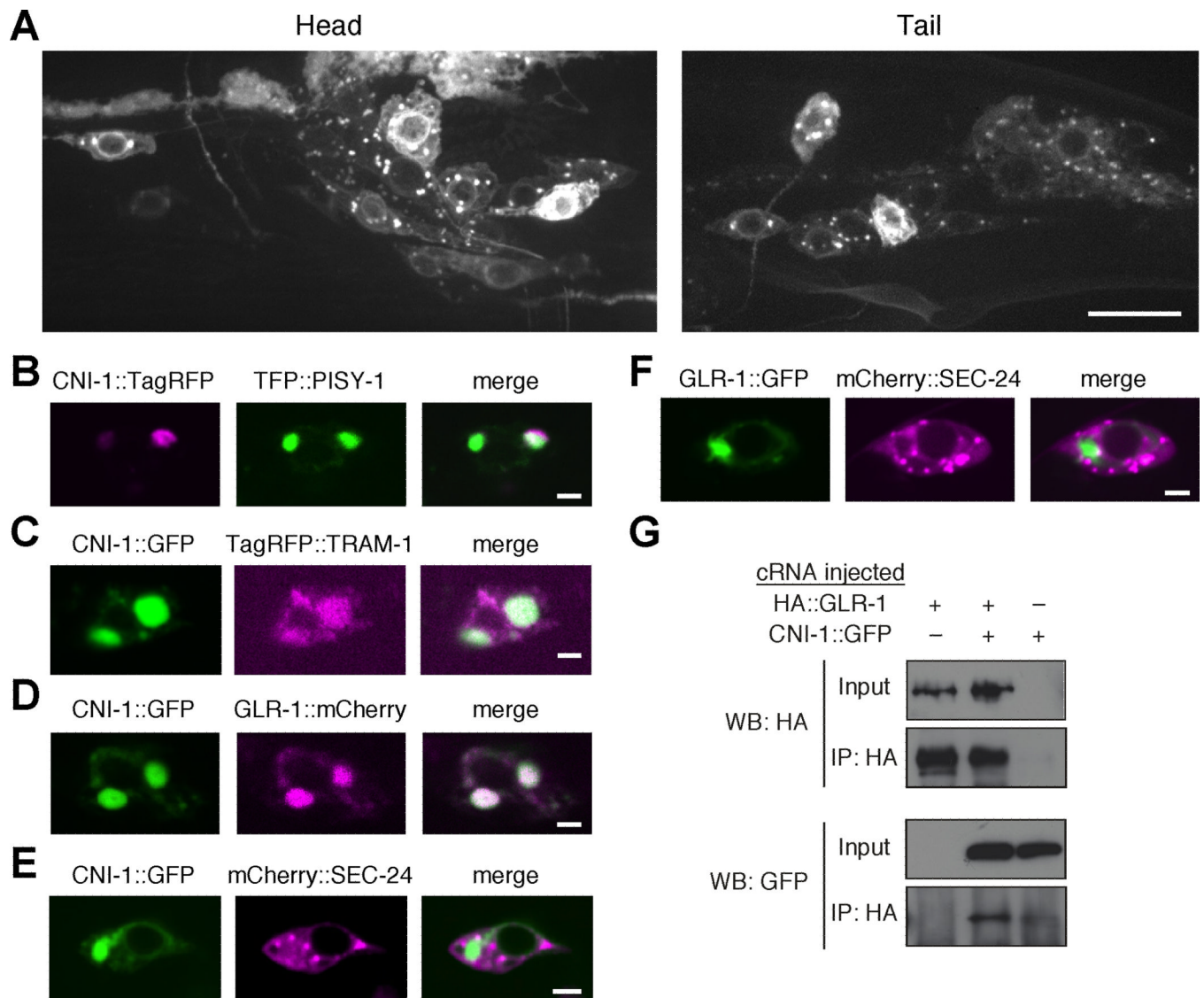


Figure 4.

CNI-1 is widely expressed in the nervous system where it colocalizes with GLR-1 in the ER.

(A) Confocal images of the head and tail region of transgenic worms that expressed the CNI-1::GFP reporter shown in Figure S5A. Scale bar represents 10 μ m. (B–F) Single plane, confocal images of the AVA cell bodies in transgenic worms that expressed various combinations of fluorescently labeled proteins. Scale bars represent 2 μ m. (G)

Immunoprecipitation of HA::GLR-1 and CNI-1::GFP coexpressed in *Xenopus* oocytes.

See also Figure S5.

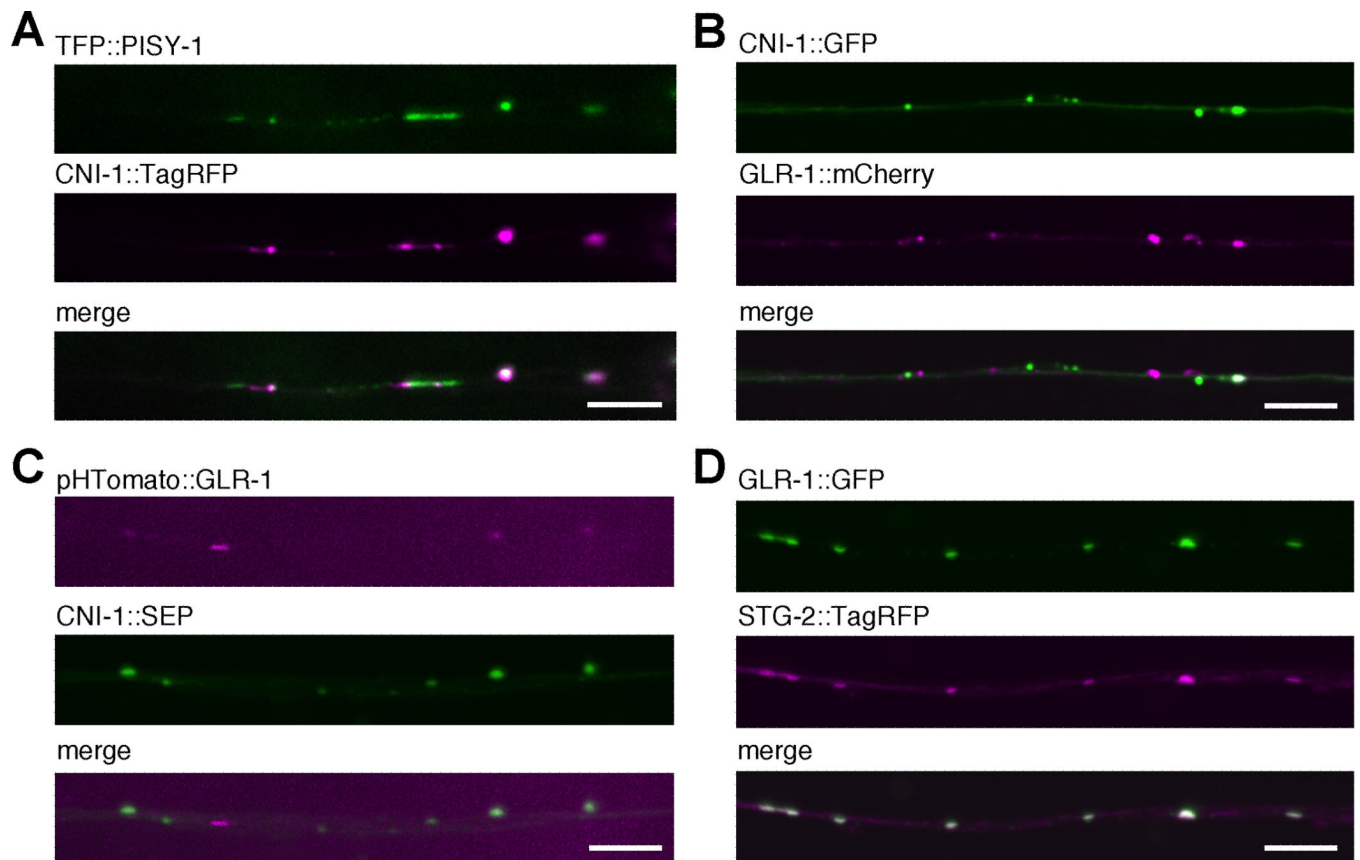


Figure 5. Surface expressed CNI-1 colocalizes with synaptic GLR-1. (A–D) Confocal images of the AVA processes in transgenic worms that expressed various combinations of fluorescently labeled proteins. Scale bars represent 5 μ m. See also Figure S6.

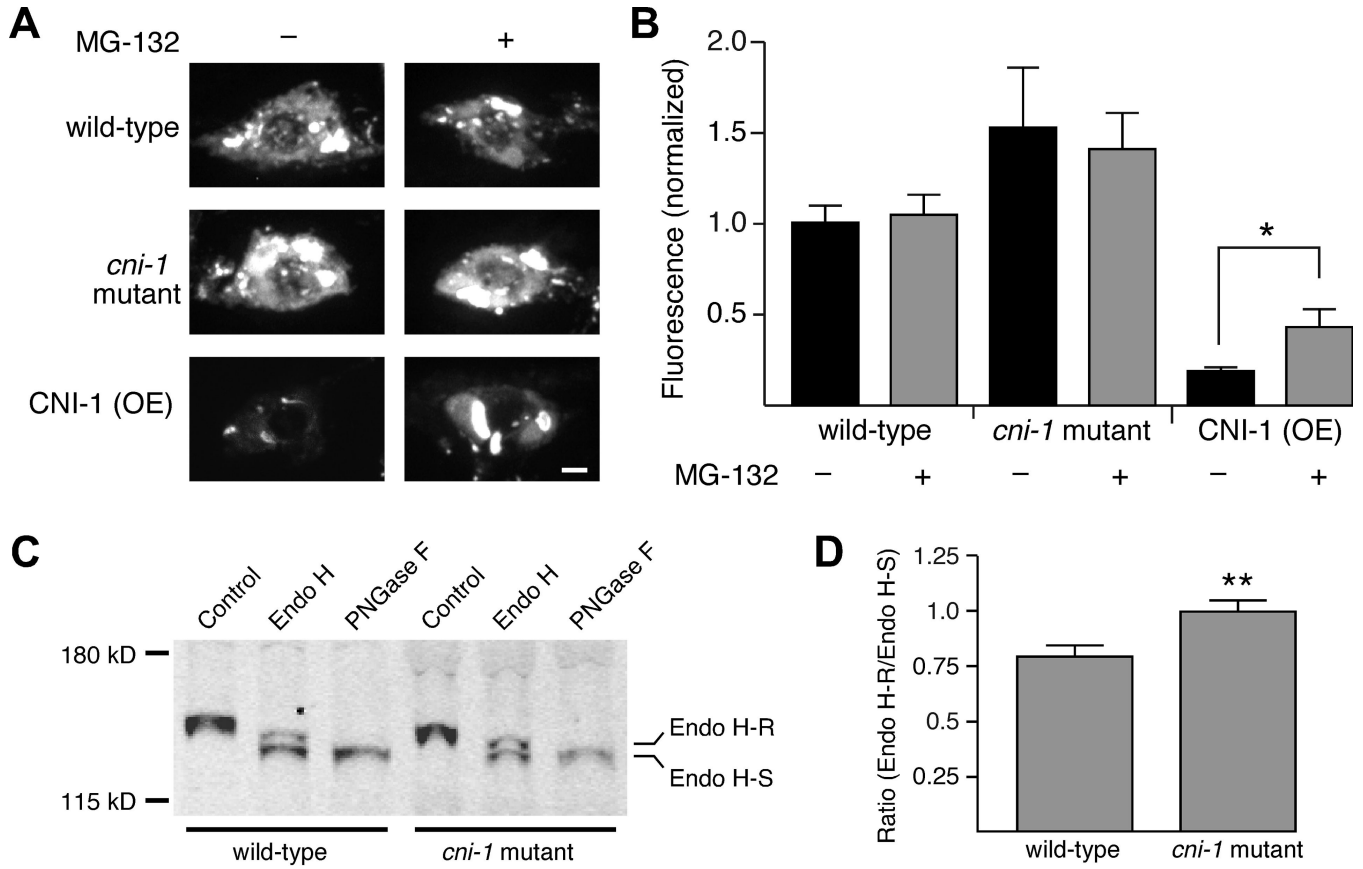


Figure 6. Overexpression of CNI-1 results in GLR-1::GFP accumulation in neuronal cell bodies and its subsequent degradation. (A) Confocal images of GLR-1::GFP in AVA cell bodies in worms either with or without MG-132 treatment. Scale bar represents 2 μ m. (B) Average GFP fluorescence intensity in worms either with (wild type, n=20; *cni-1* mutant, n=12; CNI-1 (OE), n=15) or without (wild type, n=22; *cni-1* mutant, n=13; CNI-1 (OE), n=16) MG-132 treatment. * p<0.05. (C) Western blot showing the relative amounts of Endo H sensitive (Endo H-S) and resistant (Endo H-R) GLR-1::GFP isolated from transgenic wild-type worms and *cni-1* mutants. (D) The ratio of Endo H-R to Endo H-S GLR-1::GFP in wild type (n=11) and *cni-1* mutants (n=10). ** p<0.01. Error bars represent SEM.

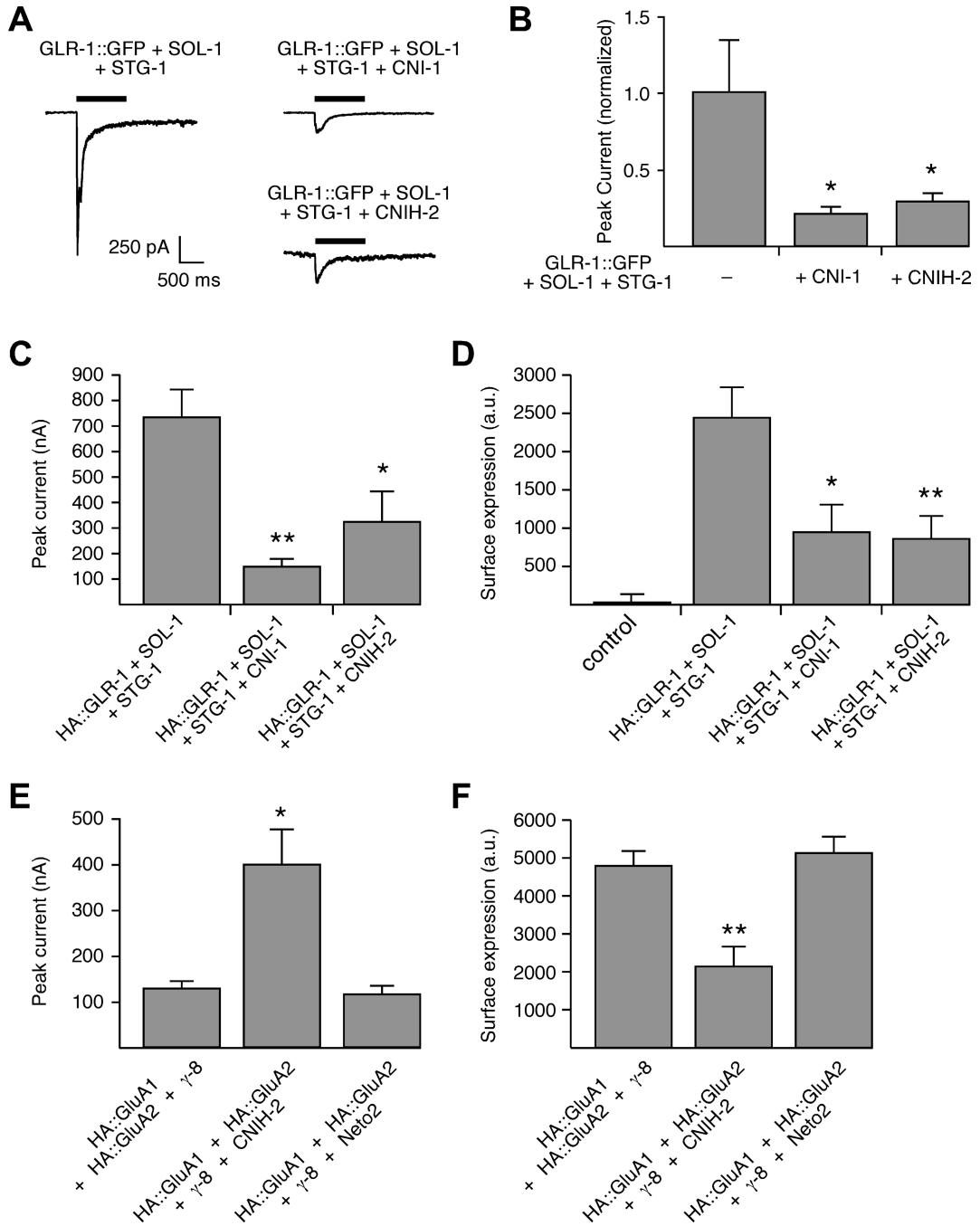


Figure 7.

Overexpressing CNI-1 or CNIH-2 modifies glutamate-gated current and AMPAR surface expression. (A) Glutamate-gated current in transgenic muscle cells in response to pressure application of 3 mM glutamate. Cells were voltage clamped at -60 mV. (B) Average peak glutamate-gated current in muscle cells that expressed GLR-1::GFP, SOL-1 and STG-1 ($n=6$); GLR-1::GFP, SOL-1, STG-1 and CNI-1 ($n=5$); or GLR-1::GFP, SOL-1, STG-1 and CNIH-2 ($n=11$). * Significantly different from GLR-1::GFP + SOL-1 + STG-1 ($p<0.05$). (C and D) Glutamate-gated current in *Xenopus* oocytes (C) and GLR-1 surface expression (D) in non-injected control oocytes or in oocytes that expressed HA::GLR-1, SOL-1 and STG-1 either with or without coexpression of CNI-1 or CNIH-2 ($n=6$ for all conditions).

Significantly different from HA::GLR-1 + SOL-1 + STG-1 (* $p < 0.05$ and ** $p < 0.01$). (E and F) Glutamate-gated current (E) and AMPAR surface expression (F) in *Xenopus* oocytes ($n=6$ for all conditions). Significantly different from HA::GluA1 + HA::GluA2 + -8 (* $p < 0.05$ and ** $p < 0.01$).

Error bars represent SEM.

See also Figure S7.

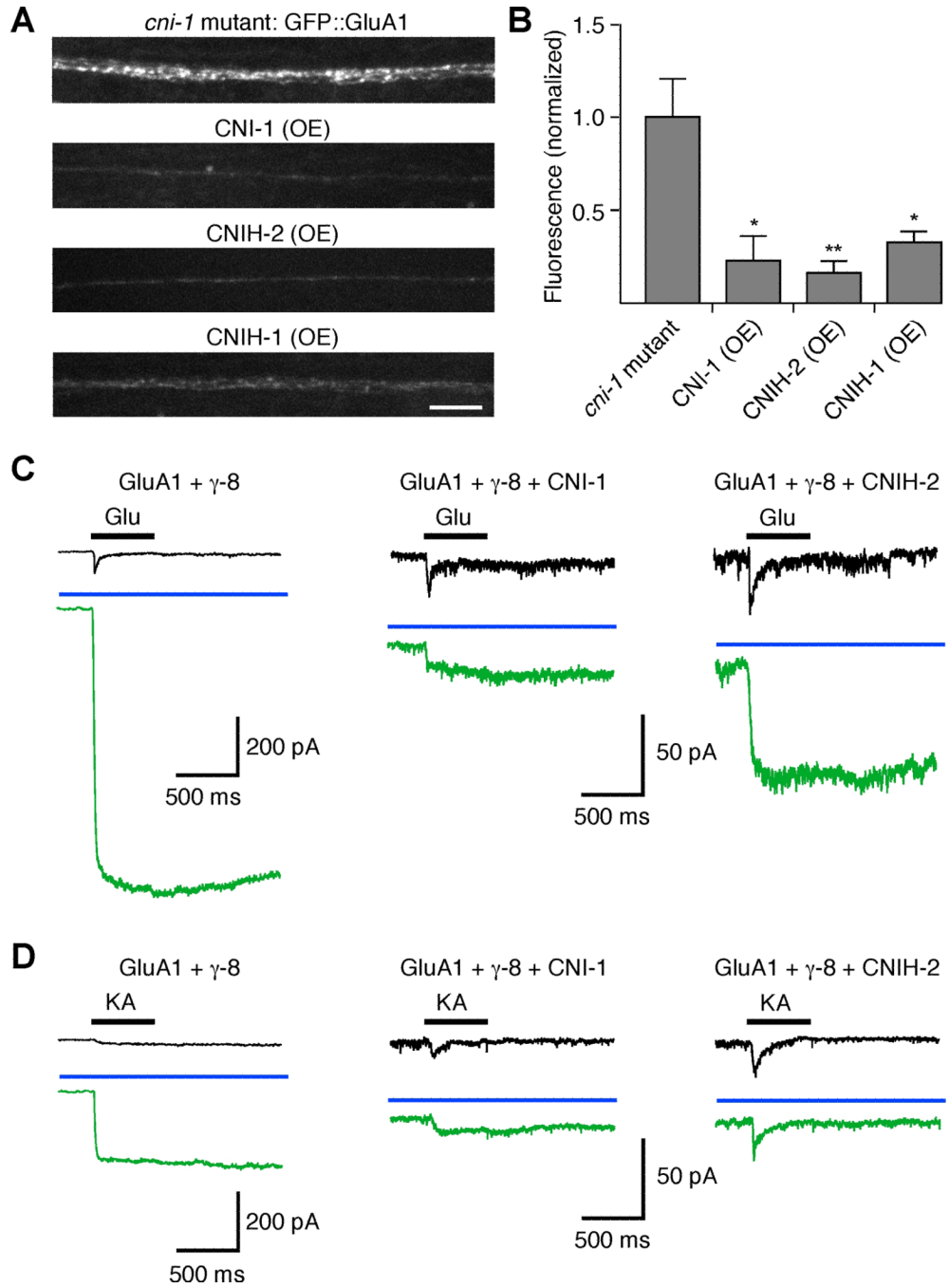


Figure 8. Reconstitution of vertebrate GluA1 receptor function in transgenic *C. elegans*. Cornichon proteins decrease GluA1-mediated current and synaptic GluA1 levels when coexpressed in *C. elegans* AVA neurons. (A and B) Confocal images (A) and quantification (B) of GFP::GluA1 fluorescence in the AVA neurons of transgenic *cni-1* mutants (n=9), or transgenic mutants that also overexpressed CNI-1 (n=4), CNIH-2 (n=5) or CNIH-1 (n=5). Scale bar represents 5 μ m; error bars represent SEM. Significantly different from *cni-1* mutants (* p<0.05, ** p<0.01). (C and D) GluA1-mediated glutamate- (C) and kainate- (D) gated current in the AVA neurons of various transgenic worms both before (black) and after

(green) treatment with 100 μ M cyclothiazide. The blue bar indicates the presence of cyclothiazide.

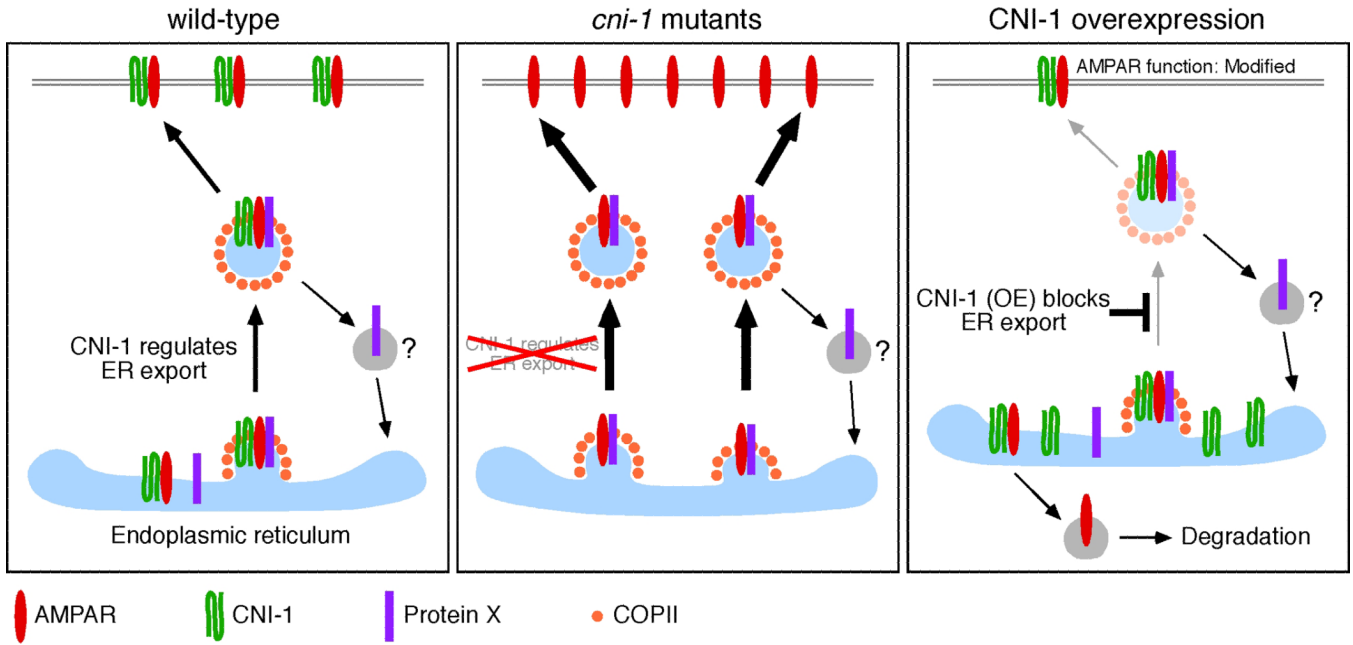


Figure 9. CNI-1 modifies neuron excitability by regulating the export of AMPARs from the ER. In this model, GLR-1 binds to CNI-1, which masks an ER export signal and prevents export. Association of a putative Protein X modifies the complex such that the ER export signal is exposed and the AMPAR can exit the ER. Thus, in *cni-1* mutants AMPARs more readily exit the ER. In contrast, AMPAR exit from the ER is slowed by overexpression of CNI-1. Furthermore, the function of AMPARs that do reach synapses is modified.