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TARP Proteins Have Fundamental Roles in the Gating of Glutamate Receptors and the Tuning of Synaptic Function

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Summary

Neurotransmission in the brain is critically dependent on excitatory synaptic signaling mediated by AMPA-class ionotropic glutamate receptors (AMPARs). AMPARs are known to be associated with Transmembrane AMPA receptor Regulatory Proteins (TARPs). In vertebrates, at least four TARPs appear to have redundant roles as obligate chaperones for AMPARs, thus greatly complicating analysis of TARP participation in synaptic function. We have overcome this limitation by identifying and mutating the essential set of TARPs in *C. elegans* (STG-1 and STG-2). In TARP mutants, AMPAR mediated currents and worm behaviors are selectively disrupted despite apparently normal surface expression and clustering of the receptors. Reconstitution experiments indicate that both STG-1 and STG-2 can functionally substitute for vertebrate TARPs to modify receptor function. Thus, we show that TARPs are obligate auxiliary subunits for AMPARs with a primary, evolutionarily conserved functional role in the modification of current kinetics.

Introduction

Members of the TARP family are tetraspanning transmembrane proteins associated with AMPA-type glutamate receptors (AMPARs). In mice, the surface delivery of AMPARs in cerebellar granule cells is dependent on γ-2, also known as stargazin (Chen et al., 2000), the founding member of the TARP family. Thus, in stargazer mutants no AMPA-mediated current can be evoked in cerebellar granule cells (Chen et al., 2000; Hashimoto et al., 1999). Glutamate-gated currents elsewhere in the mouse brain do not appear to be disrupted by the stargazer mutation, presumably because of the overlapping neuronal expression of multiple redundantly acting TARPs. Initially, four proteins (γ-2, γ-3, γ-4 and γ-8) were classified as TARPs based on sequence similarity and functional characteristics (Tomita et al., 2003). Recently however, γ-7, a protein previously shown to inhibit the expression of calcium channels, was also found to have TARP-like function (Kato et al., 2007), indicating that sequence identity alone is not sufficient to predict whether a tetraspanning protein functions as a TARP.

In vitro experiments have revealed multiple roles for stargazin in vertebrates. First, stargazin serves as an obligate chaperone for AMPARs. In the absence of stargazin, AMPARs are retained in the endoplasmic reticulum and are not expressed on the cell surface (Chen et al., 2000). Second, stargazin is required for AMPAR localization to the synapse. AMPARs that are co-expressed with a stargazin that lacks a consensus PDZ domain binding motif are

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expressed on the cell surface but are not co-localized with the synaptic scaffolding protein PSD-95 (Schnell et al., 2002). More recent experiments have shown that stargazin changes ligand efficacy (Tomita et al., 2006), and rates of receptor deactivation and desensitization (Priel et al., 2005; Tomita et al., 2005; Turetsky et al., 2005; Yamazaki et al., 2004). Much less is known about the *in vivo* contribution of TARPs to receptor function and synaptic signaling. Mice with a deletion mutation in the gene that encodes the γ-8 TARP have a relative decrease in extrasynaptic compared to synaptic AMPARs and a decrease in total AMPAR protein (Rouach et al., 2005).

Previous *in vitro* reconstitution experiments suggested that AMPARs function nearly independently of TARPs; however, more recent studies indicate that AMPARs are directly associated with a TARP (Bats et al., 2007; Nakagawa et al., 2005; Tomita et al., 2004; Walker et al., 2006a). Interestingly, one model suggests that glutamate binding causes the dissociation of the receptor from stargazin (Tomita et al., 2004). The receptor is then thought to be endocytosed after its diffusion to perisynaptic sites (Bats et al., 2007). Thus, two populations of functional AMPARs, one bound to TARPs, are hypothesized to co-exist at the synapse. On the other hand, two studies suggest that all surface AMPARs are bound to TARPs (Bats et al., 2007; Nakagawa et al., 2005). In contrast to the wealth of *in vitro* data, the *in vivo* contributions of TARPs to AMPAR function have been difficult to assess due to their role as either obligate chaperones or their effects on AMPAR number and distribution. Furthermore, *in vivo* analysis of TARP function in mice is greatly complicated by the large number of TARPs and their widespread distribution and apparent redundant function (Kato et al., 2007; Tomita et al., 2003).

Initial efforts to identify TARP homologues in invertebrates were unsuccessful leading to the speculation that TARPs might have roles specialized for the complexities of AMPAR trafficking and plasticity at vertebrate synapses (Vandenberghe et al., 2005). However, we recently identified stargazin-like proteins from *C. elegans*, *Drosophila* and *Apis mellifera* (Walker et al., 2006a). Although distantly related, *C. elegans* STG-1 and vertebrate stargazin have conserved function: in reconstitution experiments, STG-1 and stargazin can act reciprocally to enhance vertebrate and *C. elegans* AMPAR-mediated currents. Invertebrate TARPs appear specifically required for AMPAR function. For example, while no glutamate-gated current can be recorded from *Xenopus* oocytes that express either the *Drosophila* GluRIA or *C. elegans* GLR-1 AMPAR subunits, large currents can be recorded when the receptors are co-expressed with invertebrate TARP proteins (Walker et al., 2006a). These TARP-dependent glutamate-gated currents are not associated with increases in receptor surface expression, suggesting that the primary role of invertebrate TARPs is to promote receptor function.

In *C. elegans*, the AMPAR subunits GLR-1 and GLR-2 (Hart et al., 1995; Maricq et al., 1995; Mellem et al., 2002), together with the AMPAR auxiliary subunit SOL-1 (Zheng et al., 2004), mediate avoidance responses to tactile and osmotic stimuli. Furthermore, mutating either *glr-1*, *glr-2* or *sol-1* disrupts glutamate-gated currents *in vivo* (Mellem et al., 2002; Zheng et al., 2004). Reconstitution of glutamate-gated current in oocytes is dependent on co-expression of three gene products, GLR-1, SOL-1 and STG-1 (Walker et al., 2006a), suggesting that SOL-1 and STG-1, which are co-expressed with GLR-1 in the *C. elegans* nervous system, function as AMPAR auxiliary proteins (Walker et al., 2006a; Walker et al., 2006b).

Here we use a genetic approach to test the requirement for STG-1 in GLR-1 mediated avoidance behaviors and currents. To our surprise, generating a deletion mutation in *stg-1* did not cause appreciable behavioral or electrophysiological phenotypes. However, by conducting a forward genetic screen for mutations that act synthetically with mutations in the *stg-1* gene, we identified a second TARP-like gene (*stg-2*). GLR-1-mediated avoidance behaviors are completely disrupted in *stg-1;stg-2* double mutants and GLR-1 mediated currents are absent,

despite apparently normal surface expression of GLR-1. We also tested the function of vertebrate AMPARs expressed in transgenic worms and found that vertebrate GluR1 is expressed on the cell surface in the absence of TARP-like proteins. However, glutamate-gated currents could not be recorded unless GluR1 was co-expressed with either *C. elegans* STG-1 or vertebrate stargazin. This dependence on a TARP protein could be overcome by introducing a mutation in GluR1 that prevented receptor desensitization. Thus, our genetic and electrophysiological analyses point to an essential, evolutionarily conserved role for TARPs in regulating AMPAR function.

Results

To test STG-1's role in nervous system function, we used standard techniques to first generate a transposon insertion in the *stg-1* gene, and then to detect a rare imprecise excision of the transposon that deleted almost the entire *stg-1* coding sequence (Fig. 1A, B). *stg-1(ak104)* mutants were viable and had no obvious morphological or movement abnormalities. To assess the involvement of STG-1 in AMPAR mediated neurotransmission, we tested the performance of *stg-1* mutants in behaviors mediated by the GLR-1 AMPAR. *C. elegans* recoils in response to mechanostimulation of the anterior tip of the head (nose touch response), and this avoidance behavior is dependent on glutamatergic neurotransmission that activates GLR-1 (Hart et al., 1995; Lee et al., 1999; Maricq et al., 1995). However, we did not find that nose touch avoidance was disrupted in *stg-1* mutants (Fig. 1C). We also examined the hyper-reversal or "lurching" phenotype of transgenic worms that expressed a *glr-1* gain-of-function variant, GLR-1(A687T) (Fig. 1D). The average duration of forward movement before reversing is greatly reduced in lurcher worms compared to wild-type (Zheng et al., 1999) and the suppression by the *stg-1 (ak104)* mutation was only a small fraction of that previously observed for the *sol-1* mutation (Zheng et al., 2004). To directly address whether AMPAR-mediated currents were disrupted in *stg-1* mutants, we used conventional whole-cell patch-clamp techniques (Francis and Maricq, 2006) to record glutamate-gated currents from AVA, an interneuron that is required for backward movement. In contrast to *glr-1* and *sol-1* mutants (Zheng et al., 2004), we found no appreciable differences in glutamate-gated currents between wild-type and *stg-1* mutants (Fig. 1E, F).

stg-1; stg-2 double mutants have synergistic defects

Considering the importance of STG-1 in reconstitution experiments and its co-expression with GLR-1 in the nervous system (Walker et al., 2006a), we considered the possibility that additional TARP proteins may be co-expressed with GLR-1. We identified several additional genes that encode proteins with weak sequence identity to STG-1, including *clc-3*, *clc-4*, and *f53b3.5* (Fig. 2A). However, the *stg-1; clc-3* double mutant had no obvious defects in GLR-1-dependent behaviors, and *clc-4* is not expressed in the nervous system (data not shown). Also, we were unable to record glutamate-gated currents from *Xenopus* oocytes that co-expressed either CLC-3 or F53B3.5 with SOL-1 and GLR-1 (data not shown). Therefore, we used a forward genetic approach to identify additional gene products that may contribute to glutamatergic neurotransmission. We reasoned that screening for genes that acted in concert with *stg-1* might circumvent the potential problem of redundancy. Thus, we screened for synthetic mutations—those that individually did not suppress the GLR-1(A687T) lurcher phenotype but did cause suppression in worms that also contained the *stg-1* mutation.

Our screen identified two mutations that were synthetic with *stg-1* and failed to complement each other. We mapped the mutations to a small interval on LG X and, using standard cosmid rescue techniques, identified mutations in an open reading frame in cosmid F12D9 (Fig. 2B). F12D9.1b is predicted to encode a 279 amino acid (a.a.) protein with no identity to known proteins (wormbase.org). Our analysis of the genome and of the corresponding cDNA indicates

that the coding sequence, which we have named *stg-2*, actually extends an additional 234 bp, encoding a 357 a.a. protein. Like other TARPs and γ-subunits, STG-2 has 4 predicted transmembrane domains but has rather low sequence identity with either *C. elegans* STG-1 or vertebrate stargazin (Fig. 2A, C). The *stg-2(ak134)* allele contains a nonsense mutation following a.a. 108, suggesting that this mutation is a null. The *stg-2(ak138)* mutation contains both a missense mutation and a small deletion (removing a.a. 26–34), resulting in a premature stop codon; however, a plausible initiating methionine exists at a.a. 45 (Fig. 2C). Neither mutation alone significantly suppressed the hyper-reversal phenotype of lurcher worms. In contrast, the duration of forward movement was increased significantly in *stg-1* mutants that also carried either allele of *stg-2* (Fig. 2D). This effect was more pronounced for *stg-1; stg-2 (ak134)* double mutants, consistent with the notion that *ak134* represents a null allele. For this reason, we focused most of our subsequent analyses using this allele. The *stg-1; stg-2* double mutants incompletely suppressed lurching behavior, consistent with previous evidence that GLR-1(A687T) maintains some function in the absence of STGs (Walker et al., 2006a). Lurching behavior was restored in transgenic lurcher; *stg-1(ak104); stg-2(ak134)* double mutants that overexpressed either STG-1 or STG-2 (Fig. 2D).

To address the contribution of *stg-2* to behavior, we outcrossed the mutant worms to remove the GLR-1(A687T) transgene. Compared to *glr-1(ky176)* null mutants, which are slow to avoid osmotic stimuli, *stg-1* or *stg-2* single mutants responded with delays comparable to wild-type worms (Fig. 2E). In contrast, *stg-1(ak104); stg-2(ak134)* double mutants were indistinguishable from *glr-1* mutants, and normal osmotic avoidance behavior was restored in transgenic *stg-1; stg-2* mutants that expressed wild-type copies of either *stg-1* (*stg-1* genomic) or *stg-2* (*stg-2* genomic) (Fig. 2E). *stg-1; stg-2* double mutants also showed comparable defects to *glr-1* mutants in nose touch avoidance (Fig. 2F). However, in this assay, we found that *stg-2* mutants were also significantly different from wild-type worms, whereas *stg-1* mutants were indistinguishable from wild-type (Fig. 2F). Normal behavior was restored in transgenic *stg-2(ak134)* mutants that expressed wild-type copies of the *stg-2* gene.

STG-1 and STG-2 have overlapping but distinct expression patterns

The synthetic phenotypes that we observed in our behavioral analysis suggested that most neurons that express GLR-1 should also express both STG-1 and STG-2; however, the disrupted nose touch response of *stg-2* but not *stg-1* mutants implied that a subset of neurons required for nose touch avoidance may differentially express the STG proteins. To test this hypothesis, we generated transgenic worms that co-expressed GFP under the regulation of the *nmr-1* promoter (*Pnmr-1*::GFP) and the mCherry protein driven by either the *stg-1* (*Pstg-1*:mCherry) or *stg-2* (*Pstg-2*::mCherry) promoter. The *nmr-1* promoter drives GFP expression in a subset of GLR-1-expressing interneurons, including most of the command interneurons that regulate avoidance responses (Brockie et al., 2001a; Brockie et al., 2001b). Using confocal microscopy to examine GFP and mCherry expression in transgenic worms, we found that most of the interneurons that expressed *Pnmr-1::*GFP also appeared to express both *Pstg-1*::mCherry and *Pstg-2*::mCherry, with the notable exception of the AVA command interneuron (Fig. 3A). STG-2, but not STG-1, was strongly expressed in AVA, which is required for backward avoidance responses (Chalfie et al., 1985; de Bono and Maricq, 2005). Both *Pstg-1*::mCherry and *Pstg-2*::mCherry expression were more widespread than *Pnmr-1*::GFP, and were exclusively expressed in neurons. Co-expressing *Pstg-1*::GFP with *Pstg-2*::mCherry revealed considerable overlap in their expression patterns (Fig. 3B). We also generated transgenic worms that co-expressed functional full-length STG-1::mCherry and GLR-1::GFP both under the regulation of the *glr-1* promoter. The fusion proteins co-localized at punctate structures in the ventral cord that are thought to represent postsynaptic sites (Fig. 3C). This suggests that STG-1 and GLR-1 form part of a signaling complex at synapses. We

were unable to confidently assess STG-2 localization because the reporter fusions for the STG-2 protein were not functional.

GLR-1 mediated currents are not detected in *stg-1; stg-2* double mutants

To directly examine GLR-1 function in wild type and mutant worms, we used *in vivo* patch-clamp recording techniques to record glutamate-gated currents from the AVA interneuron. In wild-type worms, pressure application of glutamate evoked an inward current (537 ± 85 pA, n=7) that desensitized in the continued presence of glutamate (Fig. 4A). At least two classes of receptors contribute to the glutamate-gated current; rapidly activating AMPARs that contain the GLR-1 subunit, and slower, rectifying NMDA-type receptors that contain the NMR-1 subunit (Brockie et al., 2001b;Mellem et al., 2002;Zheng et al., 2004); the NMDA receptor mediated component is the predominant current observed in *glr-1* mutants (Fig. 4B). GLR-1-mediated current can be selectively activated using the agonist kainate (342 ± 32 pA, n=4) (Fig. 4A) and no kainate-gated current is detected in *glr-1* mutants (Fig. 4B). In *stg-1* mutants, glutamate-gated currents appeared indistinguishable from those recorded in wild-type worms (Fig. 1E). In contrast, only the NMDA component of the glutamate-gated current was recorded in *stg-2* mutants, and no kainate-gated current was detected (Fig. 4C). Apparently normal currents were recorded in transgenic *stg-2* mutants that expressed a wild-type copy of *stg-2* (*stg-2* genomic) (459 pA ± 117, n=4) (Fig. 4D). *stg-1; stg-2* double mutants appeared indistinguishable from *stg-2* mutants and *glr-1* mutants (Fig. 4E), providing additional evidence that rapid-glutamate-gated currents in AVA were primarily dependent on STG-2. Glutamate-gated current was restored in transgenic *stg-1; stg-2* double mutants that expressed a wild-type copy of *stg-2* (453 pA ± 28, n=3), but rescue was not observed when expressing STG-1 under control of its native promoter (Fig. 4F, G). We did note a very small, rapid inward current that may be a consequence of overexpressing the *stg-1* transgene (Fig. 4F). These data indicate that GLR-1-mediated glutamate-gated currents in AVA are primarily dependent on STG-2, a finding that is consistent with the differential expression of STG-1 and STG-2 in AVA (Fig. 3).

A question raised by these results is whether STG-1 can functionally substitute for STG-2 in AVA. To address this, we recorded glutamate-gated currents from transgenic *stg-1; stg-2* double mutants that expressed STG-1 or STG-2 under control of the *stg-2* and *stg-1* promoters, respectively, i.e., *Pstg-2*::STG-1 or *Pstg-1*::STG-2. These promoter swap experiments showed that rapid, GLR-1-mediated currents were detected when STG-1 was expressed under control of the *stg-2* promoter (Fig. 4H). Our date show that either STG-1 or STG-2 was competent to promote glutamate-gated currents in AVA and that the STG proteins function cell-autonomously. However, the current measured in transgenic animals that express *Pstg-2*::STG-1 was smaller than that for wild type (73 ± 27 pA, n=5; $p < .01$). This difference may reflect variability in transgenic expression or indicate that additional gene products contribute to iGluR function in AVA.

GLR-1 is expressed on the cell surface in the absence of both STG-1 and STG-2

In cerebellar granule cells, the surface expression of AMPARs is greatly reduced in stargazin mutants (Chen et al., 2000). One explanation for the phenotype of *stg-1; stg-2* double mutants is that GLR-1 is not present on the cell surface. To address this possibility, we examined GLR-1 expression in transgenic worms that expressed a functional hemagglutin (HA) epitope-tagged GLR-1::GFP under control of the *glr-1* promoter (HA::GLR-1::GFP). Using anti-HA antibodies under non-permeabilized conditions (see Experimental Procedures), we found similar levels of surface expression in wild-type worms, *stg-1* or *stg-2* single mutants, and *stg-1; stg-2* double mutants (Fig. 5A, B). We also found that vertebrate stargazin did not increase surface expression of GLR-1 in *Xenopus* oocytes, and that neither STG-1 nor STG-2 increased the surface expression of vertebrate GluR1 (Supplementary Fig. 1A, B). These data

are consistent with a previous report that showed GLR-1 is delivered to the cell surface when ectopically expressed in muscle cells of transgenic worms in the absence of STG-1, STG-2 and SOL-1 (Walker et al., 2006a). Together, these results suggest that the GLR-1-mediated behavioral and electrophysiological defects observed in *stg-1; stg-2* double mutants are not due to altered GLR-1 surface expression and that STG-1 and STG-2 are required for GLR-1 function.

To further address this question, we characterized GLR-1 expression and glutamate-gated currents in worms that lacked a *C. elegans* vesicular glutamate transporter, EAT-4 (Lee et al., 1999). We previously demonstrated that the chronic lack of glutamatergic neurotransmission in *eat-4* mutants caused compensatory postsynaptic changes in GLR-1 (Grunwald et al., 2004). Thus, both the abundance of GLR-1::GFP in neuronal processes and the magnitude of GLR-1-mediated currents measured *in vivo* were increased in *eat-4* mutants (315 pA ± 85; n=2) compared to wild-type worms (245 pA ± 48; n=2) (Fig. 5C, D) (Grunwald et al., 2004). The abundance of GLR-1::GFP also appeared to be increased in *eat-4; stg-1; stg-2* triple mutants compared to *stg-1; stg-2* double mutants (Fig. 5C). However, despite this, we could not detect GLR-1-mediated current in the *eat-4; stg-1; stg-2* triple mutants even when GLR-1 was overexpressed (n=3; Fig. 5D). These results indicate that both normal and compensatory trafficking and surface expression of GLR-1 occurs independently of STG-1 and STG-2 and that increased GLR-1 expression does not overcome the functional dependence on *C. elegans* TARP proteins.

STG-1 and STG-2 differentially modify the kinetics of GLR-1 AMPA receptors

Information processing at synapses is greatly influenced by the timecourse of the synaptic current. To address whether STG-1 and STG-2 have different effects on the kinetics of GLR-1-mediated currents and thus GLR-1 function, we recorded glutamate-gated currents in response to rapid application of glutamate. For better control of the genetic background, we expressed GLR-1 and SOL-1 along with either STG-1 or STG-2 in the muscle cells of transgenic *stg-1; stg-2* double mutants. In *C. elegans*, excitatory neuromuscular transmission is mediated by acetylcholine, not glutamate, and no endogenous glutamate-gated currents are present in muscle cells (Walker et al., 2006b). We recorded glutamate-gated currents from cultured muscle cells dissociated from transgenic worms that ectopically expressed components of the GLR-1/SOL-1/STG receptor complex in body wall muscle cells. We previously demonstrated that reconstitution of glutamate-gated current in muscle depends on STG-1 (Walker et al., 2006a). We now find that the timecourse of glutamate-gated current is dramatically influenced by the STG subtype that is co-expressed with GLR-1 and SOL-1 (Fig. 6). Thus, when STG-1 was co-expressed with GLR-1 and SOL-1, glutamate-gated currents incompletely desensitized (Fig. 6A, D, E), whereas full and rapid desensitization was observed when STG-2 was co-expressed (Fig. 6B, D, E). Interestingly, when both STG-1 and STG-2 were co-expressed the peak glutamate-gated current was almost 10 fold greater than that observed with co-expression of either STG-1 or STG-2 alone (Fig. 6C, F), suggesting perhaps that all four proteins may interact to form a signaling complex.

STG-2 promotes glutamate-gated currents mediated by invertebrate and vertebrate iGluRs

To examine whether STG-2 can promote glutamate-gated currents in reconstitution experiments, we measured glutamate-gated currents from *Xenopus* oocytes that expressed combinations of *C. elegans* GLR-1, SOL-1, STG-1 and STG-2. As previously observed (Walker et al., 2006a; Zheng et al., 2006), no glutamate-gated current was detected in oocytes that only co-expressed GLR-1 and SOL-1. In contrast, co-expression with STG-1 or STG-2, or both STG-1 and STG-2 was sufficient to promote glutamate-gated currents (Fig. 7A, B). In contrast to rapid perfusion experiments (Fig. 6), we did not find increased current when both STG-1 and STG-2 are co-expressed. This apparent difference may be secondary to the slow

kinetics of drug application when recording from oocytes. We also found that either STG-1 or STG-2 was competent to promote vertebrate GluR1-mediated currents (Fig. 7C, D), although the *C. elegans* proteins were less efficacious than vertebrate stargazin, with STG-2 having the smallest effect. The apparent difference between STG-1 and STG-2 may reflect differences in protein expression or receptor kinetics. Glutamate-gated currents in oocytes that co-expressed GluR1 and STG-2 desensitized with a time course similar to that observed in oocytes that co-expressed GluR1 and vertebrate stargazin. In contrast, glutamate-gated currents in oocytes that co-expressed GluR1 and STG-1 only partially desensitized (Fig. 7C). Oocytes that co-expressed GluR1, STG-1 and STG-2 desensitized with a time course similar to that observed in oocytes that co-expressed GluR1 and STG-1 (Fig. 7E, F).

Vertebrate GluR1 function is dependent on TARP proteins

Glutamate-gated currents can be recorded from *Xenopus* oocytes injected with cRNA encoding vertebrate GluR1; however, the magnitude of the current is increased with the co-expression of stargazin (Chen et al., 2003). The interpretation of this result is that overexpression of GluR1 can bypass the need for stargazin. Considering that TARPs from different species (and within species) have low sequence identity but conserved function, an alternative hypothesis is that some protein activity in *Xenopus* oocytes promotes GluR1 function. To test this hypothesis, we expressed GluR1 in muscles of transgenic worms and recorded current in response to pressure application of glutamate. We did not record fast glutamate-gated currents from muscles that overexpressed functional GFP::GluR1 alone (Fig. 8A), although GFP::GluR1 was expressed at the cell surface (Fig. 8B). Occasionally, we recorded very small, slow currents (<10 pA). In contrast, we observed large, fast glutamate-gated currents when GFP::GluR1 was co-expressed with stargazin (484 pA ± 187, n=6), and smaller currents when co-expressed with *C. elegans* STG-1 (56 pA ± 17, n=7; Fig. 8A). Surface delivery of GFP::GluR1 appeared equivalent in the presence or absence of either stargazin or STG-1 (Fig. 8B).

Importantly, any possible differences in surface expression cannot explain the large relative differences in glutamate-gated current observed with co-expression of stargazin or STG-1. The larger current observed with stargazin may reflect a greater affinity for vertebrate GluR1. We also found that introducing a point mutation into GluR1 (L507Y) that blocks desensitization (Stern-Bach et al., 1998) restored glutamate-gated current in the absence of either stargazin or STG-1 (403 pA ± 33, n=4; Fig. 8A), providing further evidence for stargazin-independent surface expression of GluR1. Together, these results indicate that the primary evolutionarily conserved role for TARPs is to promote the function of iGluRs.

Discussion

Our study provides the first comprehensive analysis of AMPAR function in mutants devoid of TARP function. Using both reverse and forward genetic approaches, we have identified and deleted two TARP proteins in *C. elegans*, STG-1 and STG-2. In *C. elegans*, we could evaluate the unique contribution of each TARP to receptor function without the confounding variable of possible compensatory TARPs. Furthermore, by characterizing the *in vivo* kinetic properties of AMPARs in the absence of TARPs, we were able to shed light on the possible role of TARPs at synapses. Although STG-1 and STG-2 are distantly related by primary sequence to vertebrate TARPs, both can functionally substitute for vertebrate TARPs in reconstitution experiments. In *stg-1; stg-2* double mutants, GLR-1 receptors are expressed on the cell surface; however, no AMPAR mediated current is detected and the behavioral phenotype mimics that of *glr-1* mutants. Our experiments have identified the minimal set of TARP proteins in *C. elegans* that are required for normal glutamatergic communication and demonstrate that TARPs have essential, evolutionarily conserved roles in regulating the kinetics of AMPARs.

TARPs are required for AMPAR function in *C. elegans*

In *stg-1; stg-2* double mutants, no *in vivo* glutamate-gated current could be elicited by pressure application of glutamate. Reconstitution experiments in transgenic *C. elegans* muscle cells provided similar results. Even when using rapid perfusion techniques we did not observe fast glutamate-gated currents in the absence of TARPs. The lack of current in *stg-1; stg-2* double mutants could not be explained by changes in surface expression of GLR-1 as both the distribution and surface expression of GLR-1 in *stg-1; stg-2* double mutants appeared indistinguishable from that in wild-type worms. The first characterized TARP has significant roles in the surface expression and localization of AMPARs (Chen et al., 2000). Later, three other TARPs were discovered, each with an equivalent ability to promote surface expression (Tomita et al., 2003). However, even the role of vertebrate TARPs now appears more complicated, e.g., a new TARP (γ-7) is quite divergent in sequence and whether it participates in surface delivery or receptor function has not yet been established (Kato et al., 2007). In contrast, there is no apparent role for TARPs in the trafficking or surface expression of AMPARs in *C. elegans*. These differences may reflect evolutionary changes. For example, functions in trafficking and surface expression may have been gained or lost during evolution. Reconstitution experiments in *Xenopus* oocytes and cell lines show that functional AMPARs are expressed on the cell surface in the absence of stargazin, suggesting stargazin independent trafficking. However, the genetic background of these heterologous systems has not been established. At least 5 vertebrate proteins (γ-2, γ-3, γ-4, γ-8 and γ-7) have functional effects on AMPARs, and these proteins are widely expressed. It is possible that heterologous cells express endogenous TARPs that promote receptor surface delivery and function. There is precedent for this notion. The NR1 NMDA receptor subunit was cloned by functional expression in *Xenopus* oocytes (Moriyoshi et al., 1991); however, NMDA receptors are now known to be heteromeric receptors with NR2 subunits and NR1 does not bind glutamate (Dingledine et al., 1999), strongly suggesting that *Xenopus* oocytes must express endogenous NR2-like proteins.

Vertebrate GluR1 function depends on TARPs

We have demonstrated that independent of any TARP effects on surface expression or localization of vertebrate AMPARs, TARPs are required for receptor function. Our reconstitution experiments in *C. elegans* show that vertebrate GluR1 is trafficked to the surface, but that glutamate-gated currents cannot be measured in the absence of a TARP, whether vertebrate or invertebrate, thus demonstrating that TARPs have an evolutionarily conserved role in AMPAR function independent of other roles. A mutation that prevents GluR1 desensitization restores function in the absence of exogenous TARPs, suggesting that TARPs act to regulate the desensitization of the receptor.

TARPs differentially regulate the rate of GLR-1 desensitization

Because all AMPAR-mediated current was eliminated in *stg-1; stg-2* double mutants, we could evaluate the unique contribution of each TARP to receptor function without the confounding variable of possible compensatory TARPs. We found that the rate and extent of glutamate-gated current desensitization was greater with STG-2 than with STG-1. Desensitization is an evolutionarily conserved feature of ligand-gated receptors. We propose that the differential neuronal expression of STG-1 and STG-2 may optimize the bandwidth of synaptic transmission by tuning the kinetics of glutamate-gated currents. The diversity of TARP effects on desensitization can be appreciated by comparing the varying impact of STG-1, stargazin (Fig. 7 & 8), and *Apis* STG1 (Walker et al., 2006b), on currents mediated by vertebrate GluR1. At vertebrate synapses, TARPs may similarly modify synaptic signaling and thus behavior.

The diversity of TARPs

The conserved function of vertebrate and invertebrate TARPs despite the low amino acid identity suggests that 3-dimensional structure is conserved and retains functional effects on quite divergent AMPARs (insect, nematode and vertebrate). The functional properties of AMPARs are influenced by subunit composition, alternative splicing, RNA editing, phosphorylation state, and in dramatic fashion by association with one or more TARPs. Thus, this tremendous combinatorial complexity in receptor function may contribute to the synaptic processing of information. The percent identity between *C. elegans* STG-1 and STG-2 is almost the same as the identity between STG-1 and vertebrate stargazin, highlighting the difficulty in estimating the true number of TARPs in a genome. In vertebrates, the original TARP family is now found to contain an outlier, γ-7, which has significantly lower identity with stargazin. Given that STG-1 and STG-2 can partly substitute for stargazin, it would not be unexpected if additional TARPs, with more limited identity to stargazin, were identified in the mouse genome.

Experimental Procedures

General Methods, Genetics and Germline Transformation

All *C. elegans* strains were raised under standard conditions at 20° C. Transgenic strains were generated by germ-line transformation using *lin-15(n765ts)* mutants and pJM23 as a transformation marker (Huang et al., 1994). The following plasmids were used to generate transgenic lines: pDM797, *stg-1* genomic; pDM1105, *stg-2* genomic, pWR5, *Pglr-1*::HA::GLR-1(A687T)::GFP; pDM1199, *Pstg-1*::mCherry; pDM649, *Pstg-1*::GFP; pDM1122, *Pstg-2*::mCherry; pPB1, *Pnmr-1*::GFP; pDM1233, *Pstg-2*::STG-1; pDM1244, *Pstg-1*::STG-2; pCSW163, *Pglr-1*::STG-1::mCherry; pPB45, *Pglr-1*::GLR-1::GFP; pYZ96, *sol-1* genomic; pWR6, *Pglr-1*::HA::GLR-1::GFP; pYZ318, *Pmyo-3*::HA::GLR-1::GFP; pYZ146, *Pmyo-3*::SOL-1; pDM796, *Pmyo-3*::STG-1; pDM1158, *Pmyo-3*::STG-2; pDM1041, *Pmyo-3*::GFP::GluR1; pDM1099, *Pmyo-3*::GFP::GluR1(L507Y). Additional transgenic lines used were *akIs9*, *Pglr-1*::GLR-1(A687T) (Zheng et al., 1999); *akIs58; Pglr-1*::HA::GLR-1(A/T)::GFP + *Psol-1*::SOL-1; and *nuIs25*, *Pglr-1*::GLR-1::GFP. The full-length *stg-2* cDNA was isolated by PCR amplification from *C. elegans* first-strand cDNA (GenBank accession number EU019551). Analysis of the STG-2 protein was aided by the ExPASy suite of programs (Gasteiger et al., 2003).

We carried out an F2 synthetic suppressor screen to identify *stg-2*. Transgenic *akIs58; stg-1 (ak134)* worms that over expressed *Pglr-1*::HA::GLR-1(A687T)::GFP and *Psol-1*::SOL-1, were mutagenized with 50 mM of EMS at the L4 larval stage. F2 progeny of the mutagenized worms were screened for suppression of the lurcher phenotype as previously described (Zheng et al., 1999). *stg-2* was cloned using standard genetic mapping techniques and transformation rescue of *akIs58; stg-1(ak104); stg-2(ak134)* worms.

Additional Plasmids

The oocyte expression plasmids used in reconstitution experiments were: pDM657, *glr-1*; pDM350, *sol-1*; pDM654, *stg-1*; pDM1116, *stg-2*; p59/2-rat GluR1; pGEMHE-mouse stargazin.

Primary Cultures of Dissociated *C. elegans* Muscle Cells

Muscle cells were dissociated from transgenic worms as described (Walker et al., 2006b). Ectopic expression in muscle cells was achieved using the *myo-3* muscle specific promoter. Expression of *Pmyo-3*::HA::GLR-1::GFP was used as a marker to identify transgenic muscle cells in culture.

Microscopy and Immunolabeling

Immunolabeling in live transgenic worms was achieved as previously described (Zheng et al., 2004). Briefly, anti-HA polyclonal sera (Molecular Probes) was diluted (1:200) in worm injection buffer and injected in the pseudocoelomic space of transgenic worms. Injected worms were allowed to recover for approximately 6 hours before imaging. Wide-field images were acquired using a Zeiss compound microscope with a Roper CoolSnap camera. Confocal images were acquired with Zeiss LSM 510. Quantification of GFP expression and anti-HA staining (Fig. 5B) was achieved using Metamorph software. Briefly, a region was drawn around a section of the ventral cord with GFP expression. Metamorph was used to determine the average fluorescence intensity of both GFP and anti-HA staining in the region. To correct for the background signal, a region of the same area located adjacent to the worm was also measured and then subtracted from the fluorescent signal in the ventral cord.

Electrophysiological Studies

Electrophysiological recordings from the AVA interneuron *in vivo* were made as previously described (Brockie et al., 2001b; Mellem et al., 2002). Rapid perfusion experiments using dissociated muscle cells in culture were performed using previously described protocols (Walker et al., 2006b). The change in open-tip potential was used to measure the rate of solution exchange. Solutions were delivered using a theta tube mounted on the piezoelectric manipulator (Burleigh). Recordings from *Xenopus* oocytes were performed using standard two-electrode voltage clamp techniques (Walker et al., 2006a).

Behavioral Analysis

Nose touch response, osmotic avoidance and the average duration of forward movement (Brockie et al., 2001b; Mellem et al., 2002) were performed using published protocols. The standard Student's *t* test was used to determine statistical significance. Error bars throughout represent the SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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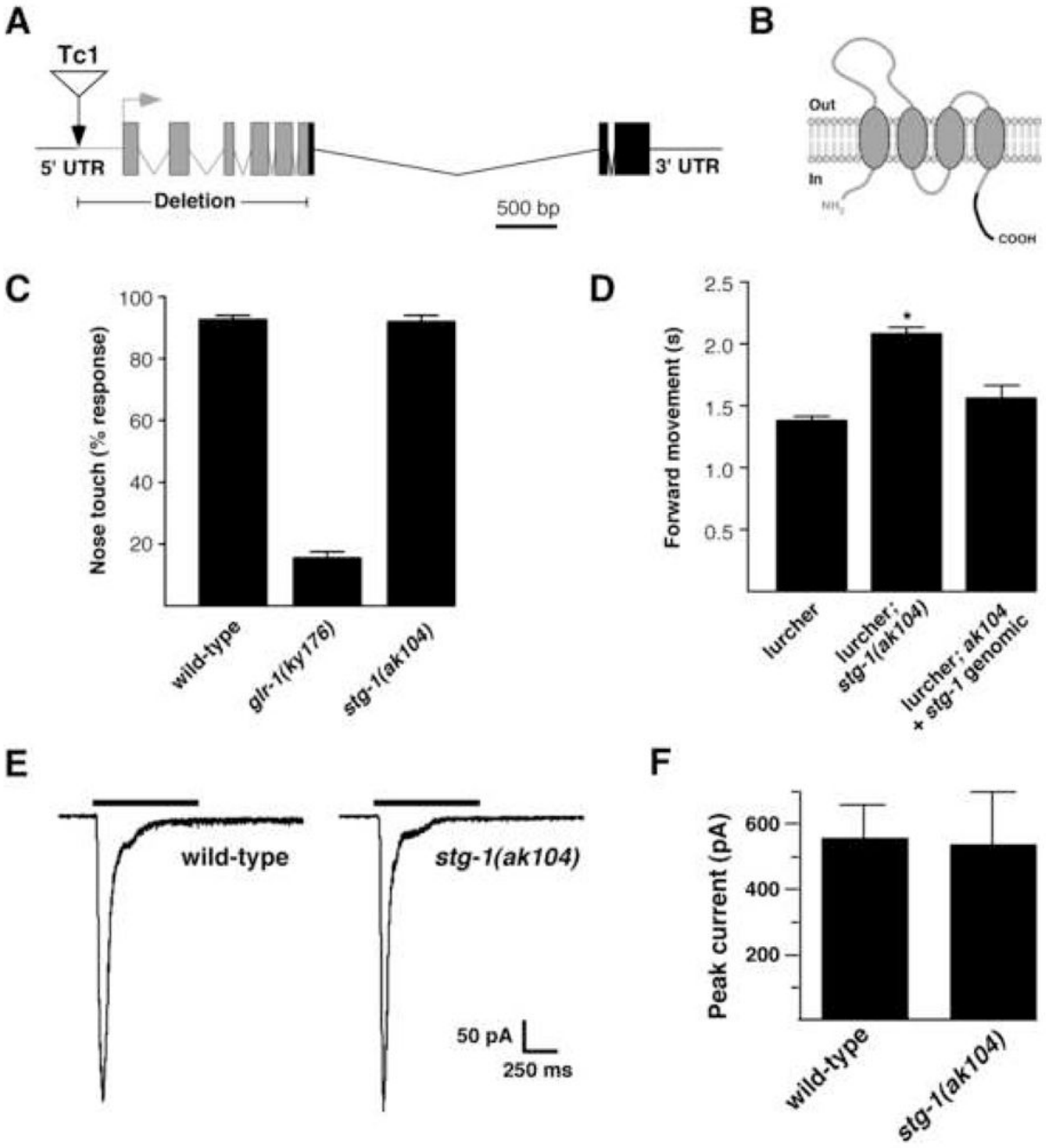

Figure 1.

The STG-1 and STG-2 TARP-like proteins differentially affect GLR-1-mediated behaviors. (**A**) Genomic organization of the *stg-1* locus with exons and introns represented as boxes and lines, respectively. The black arrow indicates the site of the Tc1 insertion and gray shows the region deleted by its imprecise excision. (**B**) Membrane topology of the predicted STG-1 protein showing the 4 transmembrane domains and the intracellular N- and C-terminal domains. Gray represents the region deleted in the *stg-1(ak104)* allele. (**C**) The nose touch response in wild-type, n=16; *glr-1(ky176)* mutants, n=12; and *stg-1(ak104)* mutants, n=15. (**D**) Average duration of forward movement in transgenic lurcher worms, n=10; lurcher; *stg-1 (ak104)* mutants, n=10; and lurcher; *stg-1(ak104)* mutants that expressed an extrachromosomal

wild-type copy of *stg-1*, n=4. *, significantly different from lurcher worms, p<0.001. (**E**) Glutamate-gated currents in response to 3 mM glutamate application recorded from the AVA interneuron in wild-type worms (left) and *stg-1(ak104)* mutants (right). (**F**) Mean peak glutamate-gated current amplitude in AVA of wild-type, n=7, and *stg-1(ak104)* mutants, n=3

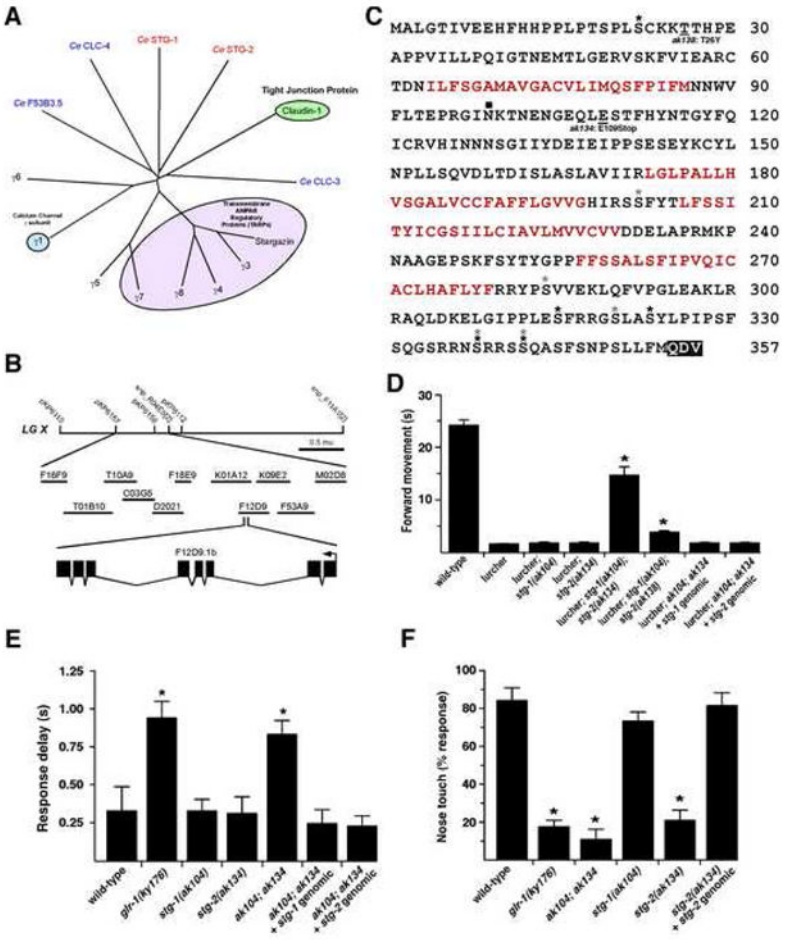

Figure 2.

stg-2 encodes a TARP protein required for GLR-1 mediated behavior. (**A**) Phylogenetic tree of vertebrate TARPs; the calcium channel subunit, γ-1; the tight junction protein, claudin-1; and *C. elegans* STG-1, STG-2, CLC-3, CLC-4 and F53B3.5 (adapted from (Tomita et al., 2003)). (**B**) Region of linkage group (LG) X showing the single nucleotide polymorphisms used to map the *ak134* and *ak138* mutations (top). Cosmids that cover the region are shown. The open reading frame F12D9.1b constitutes the *stg-2* locus, the genomic organization of which is shown with boxes and lines representing exons and introns, respectively (bottom). (**C**) Predicted protein sequence encoded by the *stg-2* gene. Indicated are putative transmembrane domains (red text); N-linked glycosylation site (filled square); PKA phosphorylation sites (gray asterisks); and PKC phosphorylation sites (black asterisks). The sites of the *ak134* and *ak138* mutations are underlined and the black box highlights a putative noncanonical PDZ-domain binding motif. (**D**) Average duration of forward movement for wild-type (n=15); transgenic lurcher worms (n=15); and the following mutants that expressed the lurcher transgene: *stg-1(ak104)* single mutant (n=15), *stg-2(ak134)* single mutant (n=8); *stg-1(ak104); stg-2(ak134)* double mutant (n=7); *stg-1(ak104); stg-2(ak138)* double mutant (n=7). STG-1 (n=7) and STG-2 (n=7) genomic rescue are also shown. *, significantly different from lurcher, $p<0.001$. (**E**) The delay in the response to hyperosmotic stimuli in wild-type, n=9; *glr-1(ky176)*, n=8; *stg-1(ak104)*, n=8; *stg-2(ak134)*, n=8; *stg-1(ak104); stg-2(ak134)*, n=18; *ak104; ak134 + stg-1* genomic, n=7; and *ak104; ak134 + stg-2* genomic, n=10. *, significantly different from wild-type, $p<0.01$. (**F**) The nose touch response in wild-type, n=3;

glr-1(ky176), n=3; *stg-1(ak104); stg-2(ak134)*, n=3; *stg-1(ak104)*, n=4; *stg-2(ak134)*, n=3; and *stg-2(ak134)* + *stg-2* genomic, n=6. *, significantly different from wild-type, $p<0.005$.

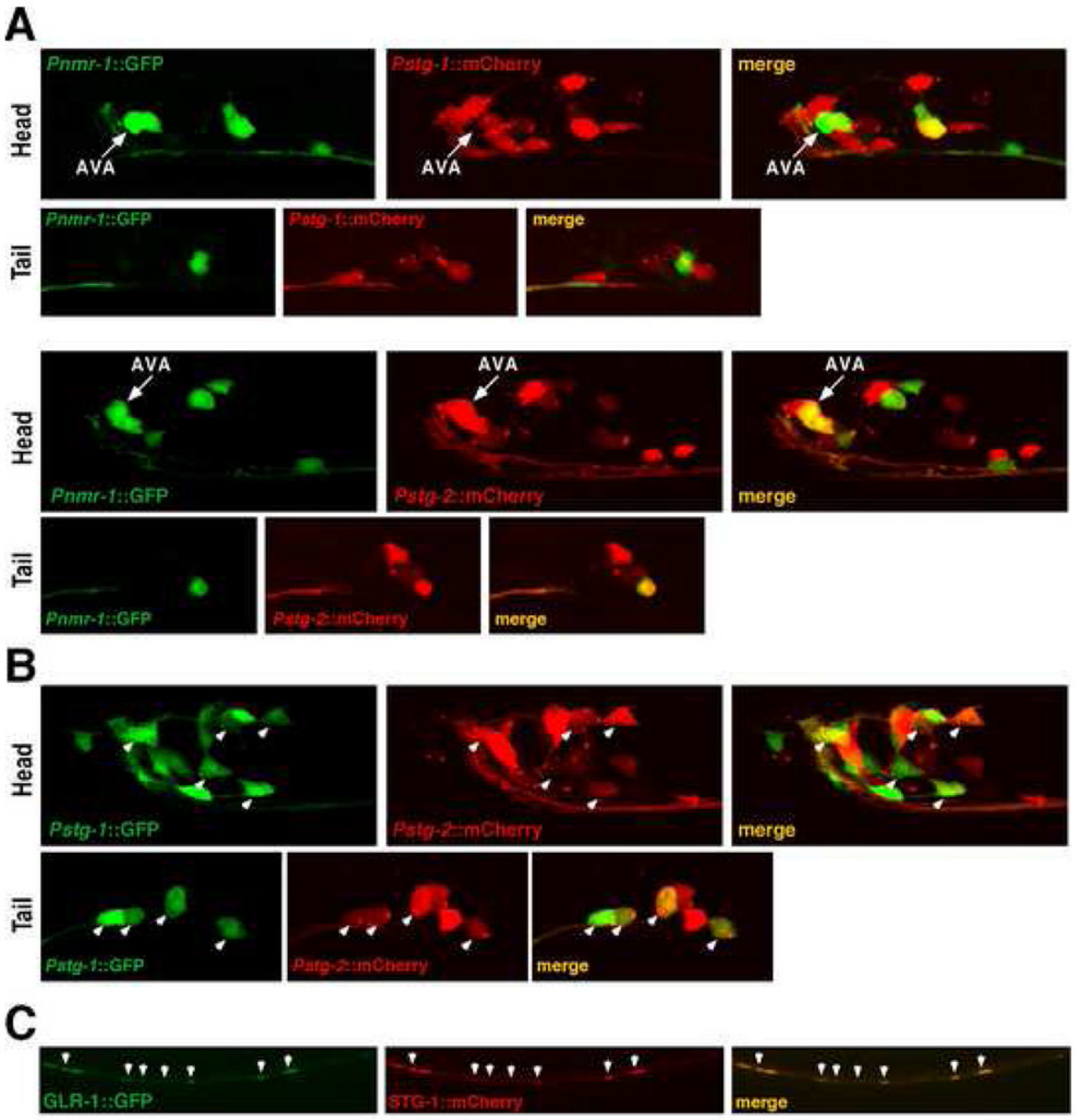

Figure 3.

stg-1 and *stg-2* are differentially expressed in the nervous system. (**A**) Confocal images of transgenic worms that expressed *Pnmr-1*::GFP and *Pstg-1*::mCherry, or *Pnmr-1*::GFP and *Pstg-2*::mCherry. Head and tail regions only. Arrows point to the AVA interneuron. (**B**) Confocal images of a transgenic worm that expressed *Pstg-1*::GFP and *Pstg-2:*:mCherry. Arrowheads indicate a subset of the cells that co-express GFP and mCherry. (**C**) Confocal images of the ventral nerve cord in a transgenic worm that expressed functional GLR-1::GFP and STG-1::mCherry both under the regulation of the *glr-1* promoter. Arrowheads indicate puncta thought to represent postsynaptic sites.

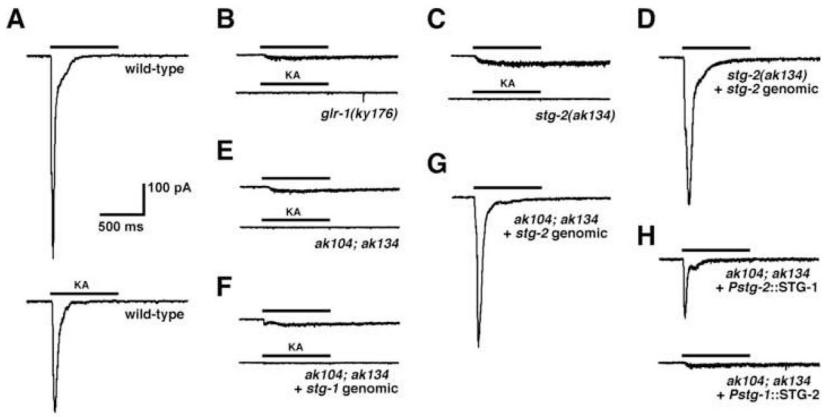

Figure 4.

stg-2 is required for GLR-1-mediated glutamate-gated currents. (**A–H**) Current responses to 1 or 3 mM glutamate or 100 μM kainate (KA) measured in the AVA interneuron in wild-type, mutant, and transgenic mutant worms.

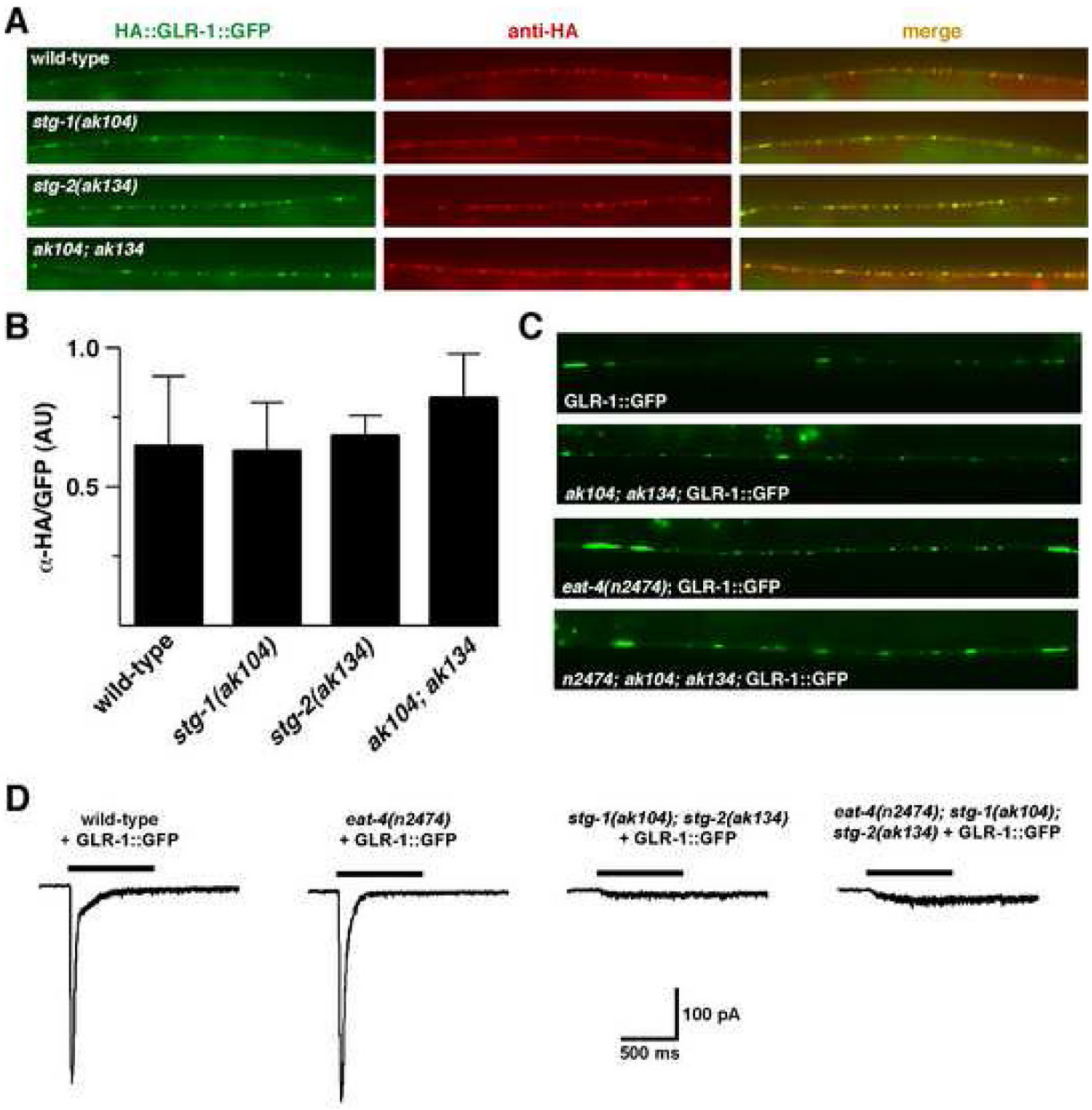

Figure 5.

GLR-1 surface expression is independent of both STG-1 and STG-2. (**A**) Images of transgenic wild-type and mutant worms that expressed HA::GLR-1::GFP under the regulation of the *glr-1* promoter. GFP (left) and anti-HA staining (middle) in the neural processes of the ventral cord are shown. Merged image, right. (**B**) The ratio of anti-HA staining to GFP fluorescence in the ventral cord of transgenic worms shown in (A), arbitrary units (AU). Wild-type, n=3; *stg-1(ak104)*, n=7; *stg-2(ak134)*, n=6; *ak104; ak134*, n=9. (**C, D**) Images of GFP expression in the ventral cord (**C**) and current response to 3 mM glutamate application (**D**) in transgenic wild-type and mutant worms that overexpressed GLR-1::GFP.

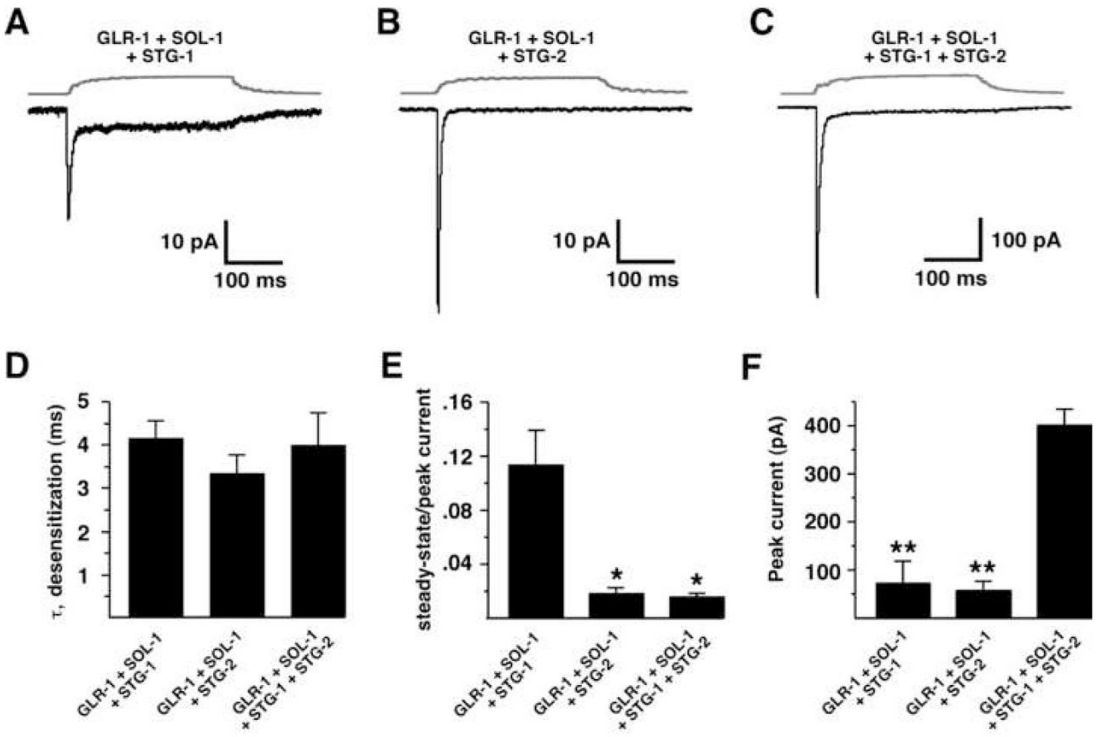

Figure 6.

TARPs differentially regulate *C. elegans* GLR-1 and vertebrate GluR1 receptor desensitization. (**A-C**) Currents measured in response to the rapid application of 3 mM glutamate in cultured muscle cells dissociated from transgenic *stg-1(ak104); stg-2(ak134)* worms that ectopically expressed various combinations of GLR-1, SOL-1, STG-1 and STG-2 in body wall muscle cells. The gray trace represents the open-tip potential. (**D–F**) Rate of receptor desensitization (**D**), steady-state to peak current ratio (**E**), and mean peak current (**F**) for currents measured in dissociated muscle cells described in **A**–**C**. GLR-1 + SOL-1 + STG-1, n=4; GLR-1 + SOL-1 + STG-2, n=6; GLR-1 + SOL-1 + STG-1 + STG- 2, n=4. *, significantly different from GLR-1 + SOL-1 + STG-1, $p<0.01$. **, significantly different from GLR-1 + SOL-1 + STG-1 + STG-2, $p<0.01$.

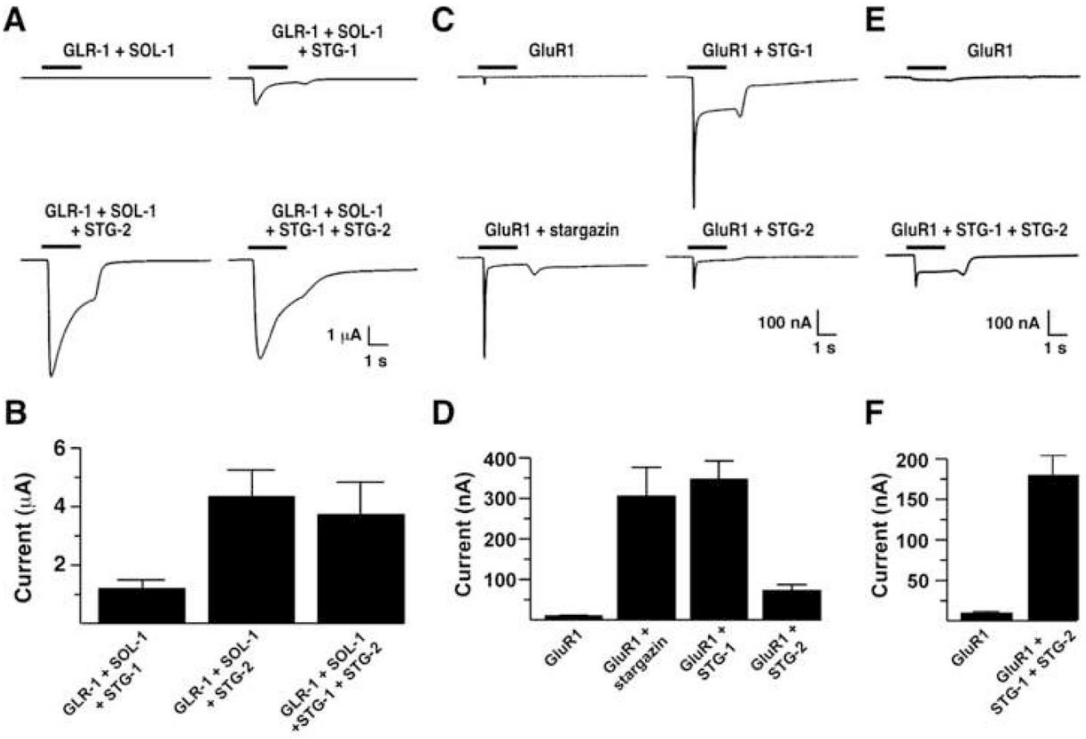

Figure 7.

Both STG-1 and STG-2 can partially substitute for vertebrate stargazin in heterologous cells. (**A, C, E**) Currents measured in response to 1 mM glutamate application in *Xenopus* oocytes that expressed various combinations of GLR-1, SOL-1, STG-1 and STG-2 (**A**) or vertebrate GluR1, stargazin, STG-1 and STG-2 (**C, E**) cRNAs were injected at 8.3 ng (GLR-1, SOL-1, total STG), 0.1 ng (GluR1), 0.56 ng (stargazin). (**B, D, F**) Mean peak current amplitude. (**B**) GLR-1 + SOL-1 + STG-1, n=12; GLR-1 + SOL-1 + STG-2, n=12; GLR-1 + SOL-1 + STG-1 + STG-2, n=7. (**D**) GluR1, n=12; GluR1 + stargazin, n=11; GluR1 + STG-1, n=12; GluR1 + STG-2, n=11. (**F**) GluR1, n=4; GluR1 + STG-1 + STG-2, n=6.

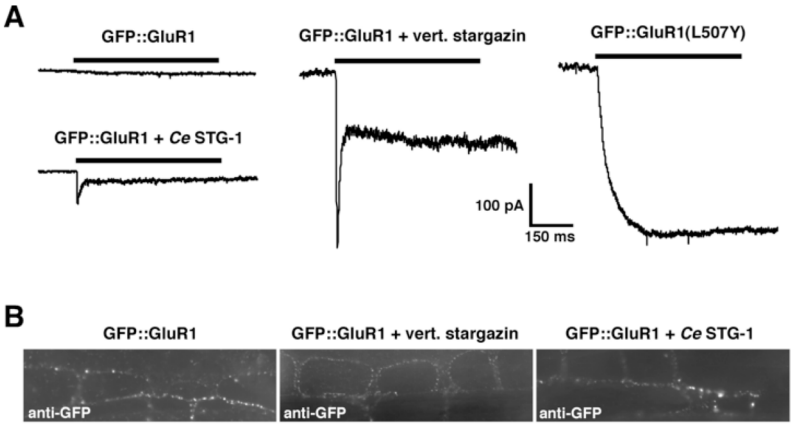

Figure 8.

Vertebrate GluR1 function is dependent on TARP proteins. (**A**) Currents measured in response to pressure application of 3 mM glutamate to body wall muscle cells that expressed vertebrate GFP::GluR1 alone, GFP::GluR1(L507Y) alone, or GFP::GluR1 coexpressed with either *C. elegans* (*Ce*) STG-1 or vertebrate stargazin under the regulation of the muscle specific *myo-3* promoter. (**B**) Images of anti-GFP staining in transgenic wild-type worms that expressed GFP::GluR1 in muscle cells.