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Title: CaMK (CMK-1) and O-GlcNAc transferase (OGT-1) modulate mechanosensory responding and habituation in an interstimulus interval-dependent manner in *Caenorhabditis elegans*

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Short Title: CaMK and OGT modulate mechanoresponding and learning

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Abstract (250 words)

Many aspects of neural physiology, including processes that underlie learning (*e.g.* neurotransmitter release and long-lasting changes in synaptic strength), are regulated by brief and local changes in [μm] levels of free intracellular Ca^{2+}. On this scale, changes in [Ca^{2+}] are known to activate many Ca^{2+}-sensors, including the Ca^{2+}/calmodulin-dependent kinases (CaMKs). Although CaMK4 is known to function in long-term memory and its paralog, CaMK1, in nervous system development, there is no evidence indicating that they function in learning acquisition. Here we reveal that the *Caenorhabditis elegans* ortholog of CaMK1/4, CMK-1, regulates responses to mechanical stimuli and learning, specifically habituation, a conserved form of non-associative learning. The habituation phenotypes of *cmk-1* mutants are sensitive to interstimulus interval (ISI), such that cmk-1 mediates habituation rate at short ISIs and habituation level at long ISIs. This is the first *in vivo* evidence that CaMK1/4 functions to modulate learning acquisition in awake, behaving animals and provides direct evidence to support the hypothesis that different mechanisms mediate habituation learning at different ISIs. From catalytic site analysis of the human and *C. elegans* CaMKs, we predicted potential CaMK phosphorylation targets and, through mutation studies, identified one of these, O-linked N-acetylglucosamine (O-GlcNAc) transferase, OGT-1, as also being necessary for wild-type responses to mechanical stimuli and learning. Detailed behavioral analysis of single and double mutants suggests that CMK-1 and OGT-1 function in parallel pathways that may converge on a common substrate to modulate the tap response. Our results provide the first evidence of a role for CaMK and O-GlcNAc post-translational modification in responding to mechanical stimuli and learning, which are fundamental biological processes present in all animals.

Introduction

Learning is a fundamental biological process by which organisms update and modify their behavioral output to sensory stimuli based on past experience. This important phenomenon allows organisms to adapt their behavior to best suit the current conditions in the inconstant environment. Brief and local changes in micromolar concentration levels of free intracellular Ca^{2+} play a critical role in modifying many aspects of neural physiology such as learning. Changes in [Ca^{2+}] on this scale are known to activate many Ca^{2+}-sensors, including the important and ubiquitously expressed protein calmodulin (CaM). Once bound with four Ca^{2+} ions, CaM (Ca^{2+}/CaM) is known to regulate many different signaling proteins, including the CaM-kinase (CaMK) family of protein-serine/threonine kinases, which are highly expressed in the nervous system. Within the larger CaMK group (consists of ~ 23 kinase families) the CaMK1 family (consisting of CaMKK, CaMK1 and CaMK4; reviewed in 1) has been shown to play important roles in the context of nervous system development and plasticity.

CaMKK is purported to function upstream of both CaMK1 and CaMK4 by phosphorylating Thr residues in the activation site of these kinases to increase their Ca^{2+}/CaM-dependent phosphotransferase activities (2, 3). The substrate recognition motifs of numerous protein kinases have significant overlap and correct target specificity is thought to also be regulated by localization of the kinase and its substrates within the cell (4); this has been established for CaMK1 and CaMK4 (5).

CaMK4's expression is usually restricted to the nucleus (6-8), where it has been shown to regulate gene transcription during the induction of long-term synaptic plasticity and long-term memory in rodents (9-11). In contrast, CaMK1's expression has been observed to be cytoplasmic in most cases (12). Many studies have demonstrated an important role for CaMK1 in the developing mammalian nervous system, specifically regulating axonal growth cone motility and axonal outgrowth (13), dendritic arborization (14-16), and formation of dendritic spines and synapses (17).

More recent studies have attempted to ascertain whether CaMK1 plays a role in plasticity of the nervous system; Schmitt *et al.* (18) investigated the role of CaMKK, CaMK1 and CaMK4 in long-term potentiation (LTP) and demonstrated that CaMKK and CaMK1, but not CaMK4, play a role in activating Ras-extracellular signal-regulated protein kinase (Ras-ERK) signaling during early-phase LTP in hippocampal neuron cultures. Using the same experimental preparation, Guire *et al.* (19) went on to demonstrate that CaMK1 also functions during LTP to recruit calcium permeable AMPA receptors. These studies indicate that similar to CaMK4, CaMK1 might also function in plasticity and perhaps learning and memory, but unlike CaMK4 it most likely functions near the synapse and in shorter forms of plasticity. To date, no one has directly tested whether CaMK1 plays a role in plasticity or learning and memory in any organism *in vivo*.

The nematode *Caenorhabditis elegans* responds to a non-localized mechanosensory stimulus, a tap to the side of the Petri plate it inhabits, by performing a reversal (changing from forward to backward locomotion). In wild-type worms, repeated administration of the tap stimulus results in habituation, a form of non-associative learning, which can be observed as a decrease in both the size of the reversal (response magnitude) and the likelihood of responding (response probability) (20, 21).

Ca^{2+} has been shown to be an important modulator of tap habituation in *C. elegans* (22, 23). Repeated mechanical stimulation resulted in an attenuation of the Ca^{2+} transient that followed each mechanical stimulus (23). Chelation of the tap-induced Ca^{2+} transient resulted in more rapid habituation, as did mutations in the genes encoding the calcium signaling molecules calreticulin (*crt-1*) and the inositol triphosphate receptor (*itr-1:* 22). Despite the obvious importance of Ca^{2+} signaling in this phenomenon, components of the CaMK cascade have yet to be tested for their role in habituation. The catalytic activity of proteins in this signaling cascade are dependent upon increases in intracellular Ca^{2+} concentration (which occurs in response to tap), are highly expressed in the nervous system, and have been shown to be important for cellular models of plasticity and long-term memory (reviewed in 24). Thus we hypothesized that this cascade may also function in learning in *C. elegans*, specifically in

tap habituation.

The *C. elegans* genome encodes a single homolog each of CaMKK, *ckk-1,* and CaMK1/4, *cmk-1* (25). Both genes are expressed in the nervous system and strains with mutations in these genes appear superficially wild-type (26), however several exciting studies have recently characterized molecular mechanisms underlying behavioral deficits related to experience-dependent thermotaxis and heat avoidance (27; 28; 29). We found that *C. elegans* strains carrying mutations in *cmk-1,* but not *ckk-1*, exhibited larger responses to mechanical stimuli and a higher final habituated level of responding than wild-type animals when stimuli were presented every sixty seconds. Intriguingly, the initial response and final habituated level phenotypes of *cmk-1* mutants were genetically dissociable and sensitive to the interstimulus interval (ISI) at which the mechanosensory stimuli were presented. This is the first *in vivo* evidence that CaMK1/4 functions to modulate learning acquisition in awake, behaving animals and provides direct evidence to support the hypothesis that different mechanisms mediate habituation learning at different ISIs (Rankin and Broster, 1992). A screen for downstream targets of CMK-1 predicted from bioinformatics analysis of the human and *C. elegans* CaMK catalytic domains led to the identification of the *C. elegans* O-linked N-acetylglucosamine (O-GlcNAc) transferase homolog, OGT-1, as also functioning in responding to mechanical stimuli and tap habituation. Thus, we demonstrate novel roles for posttranslational O-GlcNAc modification of proteins in responding to mechanical stimuli and *in vivo* learning.

Results

CMK-1 Modulates the Rate of Habituation at Short Interstimulus Intervals (10s) and the Final Habituated Level of Responding at Long Interstimulus Intervals (60s)

To determine whether CaMKs function in learning we examined habituation to repeated tap stimuli by the *C. elegans* putative null mutants of mammalian CaMK1/CaMK4, *cmk-1(oy21*), and CaMKK, *ckk-1(ok1033),* homologs. Wild-type worms habituate to repeated tap stimuli by decreasing

the distance they reverse (20, 21). *ckk-1(ok1033)* mutant worms responded initially and habituated similarly to wild-type worms at both 10 and 60 s ISIs (pNS for all, Fig. 1A-B). In contrast, *cmk-1* mutant worms displayed larger initial responses to tap ($p<0.001$, Fig. 1A) and habituation deficits dependent on the rate at which stimuli were delivered. If stimuli were administered at a 60 s ISI, a larger tap response was apparent across training ($p<0.001$; Fig. 1A), however if stimuli were presented every 10 s, the *cmk-1* mutant habituated faster (greater rate of response decrement) than wild-type, but to an asymptotic level indistinguishable from wild-type (pNS; Fig. 1B). Therefore, the elevated final reversal distance habituation level in *cmk-1* mutants (Fig. 1A) is not caused by a general increase in responding, but instead is specific to the stimuli being delivered at a 60s ISI. Wild-type habituation to tap stimuli presented at a 60s ISI was restored in *cmk-1(oy21)* mutant worms expressing wild-type CMK-1 cDNA under control of its endogenous promoter (N2 *vs.* rescue pNS; Fig. 1C); confirming *oy21* as the causative allele.

Age is an important factor that modulates habituation (30), but the habituation phenotype of *cmk-1* mutants was unlikely to be due to differences in growth or aging, as the mutant worms began egg-laying at a similar time as wild-type worms (data not shown). Additionally, the difference in habituation between *cmk-1* mutant and wild-type worms at a 60s ISI was present regardless of which day of adulthood the worms were tested on ($p<0.05$; Fig. S1 and S2). Note that in younger adults, the initial responses are of normal size, but the habituated responses are larger (Fig. S1), dissociating the initial and final response metrics. This further suggests that *cmk-1* modulates habituation in an ISI-dependent manner, as the *cmk-1* mutant displayed an altered final habituated level of responding at a 60s ISI and altered rate of habituation at a 10s ISI, even at an age where the increased initial response phenotype of *cmk-1* mutants is not present (Fig. S1).

Loss of CMK-1 phosphorylation site (T179) recapitulates null allele

Although previous studies suggested that CaMKK stimulates the kinase activity of CaMK1/4, our results indicate that CMK-1, but not CKK-1, functions in habituation. Consistent with this assertion, the expression pattern of *cmk-1* is broader than *ckk-1* (26) and we found that *cmk-1*, but not *ckk-1*, was expressed in the touch receptor neurons and interneurons of the tap withdrawal circuit (Fig. S3). This suggests that either *(i)* activation of CaMK by calmodulin alone may be sufficient to activate the kinase in the context of some biological signaling, and/or *(ii)* CaMK is activated via phosphorylation by another unidentified kinase. To test these hypotheses we took advantage of a *cmk-1* point mutant *(gk691866)* whose conserved CaMKK phosphorylation site, Threonine-179 (T179; 2, 3), was mutated to an isoleucine (I) and could therefore no longer be phosphorylated. We performed a complementation test between the *cmk-1(gk691866)* point mutant, T179I, and the *cmk-1(oy21)* null mutant and reasoned that if these alleles complemented in the context of habituation it would indicate that phosphorylation of T179 was not required for wild-type habituation. Interestingly, we observed that compared to wild-type worms and *cmk-1(oy21/+)* heterozygotes, *cmk-1(oy21/gk691866)* heterozygote mutants showed significantly increased responding to the initial tap ($p=0.05$ and $p<0.001$, respectively) and a higher final level of habitation to tap stimuli ($p<0.01$ and $p<0.001$, respectively); indicating that these alleles failed to complement (Fig. 2). Thus, these data support a role for phosphorylation of T179 as being necessary for wild-type responding to mechanical stimuli and habituation. Furthermore, because we demonstrated above that CKK-1 does not function in habituation, CMK-1 must be activated via T179 phosphorylation by another, as yet unidentified, kinase.

Genetic dissociation of initial response and habituation phenotypes

In mammals, the orthologs of CMK-1, CaMK1 and CaMK4, localize to different subcellular compartments (the cytoplasm and the nucleus, respectively). Consistent with previous reports (Schild et al., 2014), we found that CMK-1::GFP fusion proteins localize to the cytoplasm in cell bodies and neurites and are largely excluded from the nucleus (Fig. 3A). In a screen for noxious heat avoidance

defects, Schild et al. (2014) isolated a *cmk-1* gain-of-function allele, *pg58*, which encodes a truncated protein lacking most of its regulatory domain and a nuclear export sequence (NES), but with an intact kinase catalytic domain. As had been observed with a similar mutation in mammalian CaMKI (Stedman et al., 2004), Schild et al. (2014) found that truncated CMK-1(1-304) abnormally accumulated in the nucleus. In their model, CMK-1 shuttles between cytoplasm and nucleus to modulate noxious heat avoidance. To test whether a similar process mediated habituation to tap, we evaluated learning in the *cmk-1*(*pg58*) mutant. Although they had a large initial response to tap ($p<0.05$; Fig. 3B) the final habituated response size of *cmk-1*(*pg58*) was indistinguishable from wild-type (pNS; Fig. 3B), suggesting appropriate subcellular localization is essential for setting naïve responsivity to tap, but not necessarily modulating it. By dissociating the initial response and habituation phenotypes of the *cmk-1* mutant, results from this allele confirm they are independent metrics.

Identification of Candidate CMK-1 Target Proteins Through Analysis of Evolutionarily Conserved Predicted CaMK Phosphosites

Protein kinases recognize the specific Ser/Thr/Tyr amino acid residues that they phosphorylate on their target substrates based upon the sequence of residues that flank the phosphoacceptor site (comprises the kinase consensus sequence). This is due to kinase-substrate binding following a lock and key model, whereby the peptide sequence flanking the phosphosite on the target protein fits into the catalytic domain of the kinase, because of the presence of specificity-determining residues located there which often directly interact with the side chains of amino acid sequences surrounding phosphosites in substrates (32). These principles have been previously used to predict the kinase substrate specificities of 492 human protein kinases in silico (33).

The computational methods have now been further improved with refinements of the original algorithms and training data from over 10,000 kinase-protein phosphosite pairs and 8,000 kinase-

peptide phosphosite pairs. We used these updated methods to generate a kinase substrate specificity prediction matrix (KSSPM) for *C. elegans* CMK-1 based on the primary amino acid sequence of its catalytic domain, and then used this KSSPM to query all of the 20,470 known *C. elegans* protein sequences to identify those proteins that featured the top 600 highest scoring predicted phosphosites (Table S1). Next, we identified the closest human cognate proteins that featured similar phosphosites, and then scored the human phosphosites with KSSPMs for all four human CaMK1 isoforms and CaMK4 (which share 65% and 44% sequence identity, as measured by Blastp, with the *C. elegans* CMK-1 protein, respectively; Fig. S4). Of particular interest were those *C. elegans* protein and phosphosites that were highly conserved in *Homo sapiens* and predicted to be targeted by human CaMK1 isoforms and CaMK4. Such high evolutionary conservation would support important functional roles for these kinase-substrate pairs. More information about the predicted phosphorylation of these human phosphosites by human protein kinases and their evolutionary conservation in over 20 other species is available in the PhosphoNET website at www.phosphonet.ca.

The above-generated list of 600 phosphosites in 373 *C. elegans* proteins predicted to be CMK-1 targets was used to prioritize candidates. Candidates that had been previously shown to interact with CaMKs for which testable knockout mutant alleles were available were of highest interest, as well those ranked within the top 20 by p-site score and assayed them for habituation at a 60 s ISI. Out of the 22 mutants tested to date, 17 were observed to show an initial response and/or habituation phenotype (Fig. S5, S6 and (Table 1). A Venn diagram was generated that grouped genes that when mutated showed similar behavioral phenotypes (Fig. 4).

O-GlcNAc transferase, OGT-1, Functions in Habituation and is Expressed in the Nervous System, including the Touch Receptor Neurons

ogt-1 mutants showed an initial mechanosensory response and habituation phenotype that was strikingly similar to *cmk-1* mutants; *ogt-1(ok430)* mutants were significantly more responsive to the

initial tap ($p<0.001$), and showed significantly higher final level of habituation to stimuli delivered at a 60s ISI ($p<0.001$; Fig. 5A, Table 1). Because of this we further investigated the role of this protein in habituation by assaying whether OGT-1 was also similar to CMK-1 in its ISI dependency (*i.e.* whether it mediated distinct aspects of habituation at a 10s ISI versus a 60s ISI). When we habituated *ogt-1(ok430)* mutants at a 10s ISI instead of a 60s ISI, we observed that they were still more responsive to the initial tap ($p<0.001$), but they habituated more rapidly and to the same final level as wild-type worms in the measure of reversal distance (pNS; Fig. 5B). Thus, *ogt-1* mutants robustly phenocopy *cmk-1* mutants in all measures tested. Importantly, Hanover *et al.* (34) has demonstrated that the *ogt-1(ok430)* allele is a true null and results in the complete loss of function of this transferase; in *ogt-1(ok430)* mutants O-glycNAc is absent. This was shown via direct measurement of O-GlcNAcitol released after alkaline β-elimination and by O-GlcNAc antibody staining (34).

We next confirmed that the mutation in *ogt-1, ok430*, was indeed the mutation which caused the mechanoresponding and habituation phenotypes observed above by testing a second null allele of *ogt-1, tm1046*. Tm1046 is a 466 bp deletion, resulting in a frameshift and an early stop after 392 amino acids. *ogt-1(tm1046)* mutants displayed an initial response ($p<0.001$) and habituation phenotype ($p<0.001$) similar to *ogt-1(ok430)* null mutants (Fig. 5; data not shown), thus demonstrating that OGT-1 function is critical for wild-type responses to tap and habituation.

To evaluate the expression pattern of *ogt-1* we created a transcriptional reporter that consisted of ~2 kb of the *ogt-1* promoter fused to GFP (P*ogt-1*::GFP) and injected this into wild-type worms. Imaging of this reporter revealed that OGT-1 is expressed quite broadly across the nervous system, including the touch cells, in addition to muscles and seam cells (Fig. 5C).

***cmk-1* and *ogt*-1 Exhibit a Complex Genetic Interaction**

To test whether *cmk-1* and *ogt-1* interact genetically we assayed the habituation of *cmk-1(oy21)*; *ogt-1(ok430)* double mutants. When these double mutants were habituated at a 60s ISI they acted

additively to give significantly larger initial responses ($p<0.001$ for both) and larger reversal distance habituation phenotypes than that of either single mutant ($p<0.01$ for both; Fig. 6A). The most parsimonious explanation of this data is that each mutation contributes to the habituation phenotype through disruption of independent genetic pathways. This result was surprising given that the individual mutant phenotypes were so strikingly similar and that the mammalian homologues of these proteins have been shown to interact (35, 36). Because the response distance variable is actually composed of two different measures, reversal speed and reversal duration, we decided to further analyze their relationship by breaking reversal distance down into these two components and examining their habituation (Fig. 6B and 6C).

For the initial response to tap, the *cmk-1* single mutant exhibited significantly faster reversals (Fig. 6B) of increased duration (Fig. 6C) compared to wild-type. In contrast, the *ogt-1* mutant reversed at a speed similar to wild-type (Fig. 6), but with a duration longer than even the *cmk-1* mutant (Fig. 6C). Thus, the single mutant data indicate that the similar reversal distance phenotype of unhabituated *cmk-1* and *ogt-1* mutants was caused by distinct processes, with CMK-1 primarily influencing speed of reversals and modestly influencing reversal duration, and OGT-1 influencing duration, but not speed. However, *cmk-1; ogt-1* double mutants performed significantly slower reversals than *cmk-1* single mutants ($p<0.001$), almost indistinguishable from wild-type ($p=0.04$), indicating that *ogt-1* may act as a suppressor of *cmk-1* for reversal speed in naïve animals (Fig. 6B). Thus, in the context of responding to an initial mechanical stimulus, there appears to be some interaction between the *cmk-1* and *ogt-1* pathways.

In the case of habituated animals, both *cmk-1* and *ogt-1* single mutants exhibited significantly faster reversals compared to wild-type and a consistent, but not significant trend towards increased duration, phenotypes which were additive, giving the rapid and long-lasting final responses of the *cmk-1; ogt-1* double mutant.

Discussion

We report here that the *C. elegans* CMK-1, but not CKK-1, mediates habituation to mechanosensory stimuli in an ISI-dependent manner. Through catalytic site analysis of CaMKs we predicted and screened potential CaMK phosphorylation targets to identify the null OGT mutant, *ogt-1*, as also functioning in this form of learning. We showed that similar to *cmk-1*, *ogt-1* mutants altered habituation in an ISI-dependent manner. Finally, we assayed the tap habituation of *cmk-1(oy21)*; *ogt-1(430)* double mutants and found that these two genes have a complex genetic interaction in the context of naïve responding, yet exhibit an additive habituation phenotype.

While many previous studies have suggested that CaMK1/4 and CaMKK can function in the same pathway, our results indicate that CMK-1, but not CKK-1, functions in responses and habituation to mechanical stimuli are not the first findings to indicate that CaMK1/4 can function independently of CaMKK. In *C. elegans*, Kimura *et al.* (26) found that CMK-1 is expressed in more neurons than is CKK-1 and that although CKK-1 enhanced the CMK-1- dependent phosphorylation of a transcription factor (CREB), CMK-1 was able to phosphorylate CREB in the absence of CKK-1. Similarly, Satterlee *et al.* (37) found that CMK-1, but not CKK-1, functioned to regulate AFD sensory neuron specific gene expression in *C. elegans.* These findings also appear to be consistent with studies in vertebrate organisms where CaMK1 is known to have a wider expression profile than CaMKK (reviewed in 38). These data indicate that either in some cases *(i)* activation of CaMK1/4 by calmodulin alone may be sufficient to activate the kinase in the context of some biological signaling or *(ii)* CaMK1/4 is activated via phosphorylation by another unidentified kinase. Although these possibilities are not necessarily mutually exclusive, our results that showed that the *cmk-1(gk691866)* point mutant, T179I, and the *cmk-1(oy21)* null mutant fail to complement in the context of naïve mechanosenory responding and habituation indicate that, at least in this organism and these phenotypes, CMK-1 is activated via phosphorylation by another as yet unidentified kinase. Future work is aimed at identifying the cellular

and subcellular site of action for CMK-1 and OGT-1. A mutant with mislocalized CMK-1 responded initially with large reversals, but habituated normally, suggesting a shuttling of CMK-1 into the nucleus does not mediate tap habituation, as it does heat avoidance (28).

To find downstream targets of CMK-1 in *C. elegans* we used a novel bioinformatics approach to generate a list of protein candidates predicted to be phosphorylated by CMK-1 to screen for tap response and habituation phenotypes, similar to those observed in *cmk-1* mutants. Our bioinformatics approach to generate a list enriched in proteins that were likely to function in either responding to mechanical stimuli and/or mechanosensory habituation used an effective kinase substrate prediction algorithm developed by Safaei and colleagues (33). Behaviorally screening strains with mutations in our top candidates, we found that this list was indeed enriched for mutations which cause tap response and/or habituation deficits, as 17/22 strains deviated from wild-type in some aspect of these behaviors. Thus this approach appears to be an effective method to identify downstream candidates for phosphorylation targets of kinases in the context of mechanosensory responding and learning.

By screening knockout mutants from among the top candidates of the CaMK phosphosite substrate prediction, we found that the *C. elegans* O-GlcNAc transferase (OGT) mutants, *ogt-1*, displayed strikingly similar phenotypes compared to *cmk-1* mutants. O-GlcNAc glycosylation is a unique and dynamic cytosolic and nuclear carbohydrate post-translational modification in which β-N-acetylglucosamine is covalently attached to serine or threonine residues of proteins. In contrast to other forms of glycosylation, O-GlcNAc glycosylation occurs intracellularly, is rapid (occur as quickly as 1-5 min after cellular stimulation; 35, 39) and is not further modified into complex glycans. Hence, it is thought to be more akin to phosphorylation than to other forms of glycosylation. Interestingly, O-GlcNAc glycosylation sometimes occurs on or near serine or threonine residues that are also known to undergo phosphorylation (reviewed in 40). Both OGT and O-GlcNAcase (enzyme that functions in opposition to OGT to remove β-N-acetylglucosamine residues; OGA) are highly expressed in the brain (41, 42) and enriched at synapses within neurons (43,44).

In *C. elegans*, although OGT-1 is expressed in the embryonic nervous system (45) no role has yet been described for this protein in responding to mechanosensory stimuli or learning. Instead, studies have shown that it functions in macronutrient storage (34), dauer formation (46), lifespan (47), the glucose stress response (48) and proteotoxicity in *C. elegans* neurodegenerative disease models (49). In other species a role for O-GlcNAc glycosylation has yet to be demonstrated *in vivo* for learning acquisition, but it has been shown to function in long-term memory (50) and in cellular models of plasticity such as: long-term potentiation (LTP), long-term depression (51) and paired-pulse facilitation (52). Thus, our evidence of an *in vivo* requirement for OGT-1 in learning in *C. elegans* will very likely be conserved in learning in other species, including mammals.

Epistasis experiments measuring reversal distance revealed the *cmk-1(oy21)*; *ogt-1(430)* double mutant phenotype to be additive for both naïve and habituated states. Additivety is most often thought to be suggestive of a sign of the absence of a functional relationship between the mutated genes under study but in some cases it may be due to both gene products converging on the same downstream target(s), and/or non-linear dynamics of regulatory networks (53).

Although, clearly, the most parsimonious explanation for the additive genetic interaction between null mutations in *cmk-1* and *ogt-1* is that each mutation contributes to the habituation phenotype through disruption of at least two contributing independent linear biochemical pathways; data from previous studies have revealed that there is much complex cross talk between O-GlcNAc and phosphorylation signaling, and thus it is tempting to speculate that the relationship between CMK-1 and OGT-1 may be more intimate. Given that *(i) cmk-1* and *ogt-1* mechanosensory responding and two different habituation phenotypes were so strikingly similar, *(ii)* mammalian CaMK4 and OGT interact (CaMK4 phosphorylates OGT (35), and OGT can add O-GlcNAcs to CaMK4 (36)), *(iii)* mammalian CaMK4 and OGT are known to share several common substrates (CREB, serum response factor, the CREB-binding protein, and OGT itself; 36, 54, 55-57), and *(iv)* virtually every other O-GlcNAcylated

protein identified (> 250) has also been shown to be a phosphoprotein (i.e. it is phosphorylated by a kinase; 58, 59) we hypothesized that the additive phenotypes of *cmk-1; ogt-1* double mutants may be caused by CMK-1 and OGT-1 sharing a downstream substrate and/or functioning in a non-linear genetic pathway.

To test this hypothesis, we further analyzed this genetic interaction by separating reversal distance into its two components, reversal speed and reversal duration, for both naïve and habituated mechanosensory responses. The clearest conclusion can be drawn from the double mutant data in the context of responding to tap stimuli. When naïve reversal speed is measured, *cmk-1; ogt-1* double mutants perform significantly slower reversals than *cmk-1* single mutants, very similar to wild-type, indicating that *ogt-1* acts as a suppressor of *cmk-1*. Because the *cmk-1(oy21)* allele used in these experiments is very likely a null allele, these data support the existence of *ogt-1* functions downstream of *cmk-1* to negatively regulate the pathway in wild-type animals. When naïve reversal duration is measured, the *cmk-1; ogt-1* double mutant phenotype is synergistic. Synergy in double mutants affected in two non-homologous genes can be a consequence of genes disrupting steps of pathways that converge at some node of a regulatory network, such as sharing a common substrate (reviewed in 60), suggesting that CMK-1 and OGT-1 share a common downstream target(s).

Additionally, a synergistic phenotype is also observed for the baseline locomotory speed in *cmk-1; ogt-1* double mutants measured before mechanosensory stimuli were administered (Fig. S7); providing additional phenotypic support to our hypothesis that these genetic pathways intersect. In the context of habituation, the additive habituation phenotypes regardless of the measure in *cmk-1; ogt-1* double mutants indicate that in this context *cmk-1* and *ogt-1* function in two parallel pathways, which may converge on a common downstream substrate.

Using all available published data on CaMK1/4 and OGT as well as from the experiments performed in this study, we have a devised a model for this non-linear genetic network in *C. elegans*

(Fig. 7). Biochemical studies, outside of the scope of this work, will be required to validate this hypothesis.

To our knowledge, *cmk-1* and *ogt-1* represent the first reported mechanosensory hypersensitive mutants in *C. elegans* despite several pioneering genetic screens characterizing harsh and soft touch mechanosensory defective mutants by Chalfie and colleagues (61,62). Further, we have shown that *cmk-1(pg58)* mutants also display mechanosensory hyperesponsivity, suggesting that wild-type shuttling of cmk-1 between the cytoplasm and the nucleus is necessary for setting response level to mechanical stimuli – similar to it's effects on thermotaxis and heat avoidance (27, 28, 29, 37). Further characterization of *cmk-1* interacting proteins may identify additional mechanosensory hypersensitive mutants, an as yet largely uncharacterized family of genes regulating mechanosensation.

Finally, our findings identify the first proteins that specifically alter habituation differently at different ISIs: CMK-1 and OGT-1. Early detailed parametric studies of *C. elegans* mechanosensory habituation found that the rate and asymptotic level of habituation differed depending on what ISI stimuli were presented; mechanosensory stimuli which were presented at a 10s ISI resulted in faster and deeper habituation than stimuli presented at a 60s ISI (20). These experiments led to the hypothesis that habituation at different ISIs may recruit/require different molecular mechanisms. Our findings that *cmk-1* and *ogt-1* modulate the final level of habituation at a 60s ISI and the rate of habituation but not the final level at a 10s ISI are the first published evidence to support this hypothesis.

It is important to note that the 60s ISI tap habituation phenotype of *cmk-1* mutants could be interpreted as a general hyperesponsivity to stimuli throughout the training session, rather than an alteration in habituation – which is the ability of animals to learn to decrement their responding to repeated stimulation (63). However, several lines of evidence from this work suggest that CMK-1 alters habituation. First, when worms were tested at 72 hours post egg lay (Fig. S1; as opposed to 96 hours) *cmk-1* mutants displayed wild-type initial responses to mechanical stimuli, but a higher final level of responding at a 60s ISI, dissociating these metrics. Second, *cmk-1* mutants display a normal habituated

final level when trained at a 10s ISI – a general hyperesponsive phenotype should be present regardless of the stimulus interval used (Fig. 1 A-B). Third, the *cmk-1(pg58)* mutant displayed larger initial responses, but wild-type habituated responses, genetically dissociating these response metrics (Fig. 3B). Taken together, these results suggest that initial and final habituated levels of mechanosensory responding to tap stimuli are independent metrics and that CMK-1 mediates habituation in an ISI-dependent manner.

In conclusion, our results provide the first *in vivo* evidence of a role for CaMK and O-GlcNAc post-translational modification in responding to mechanosensory stimuli and learning. Additionally, our work provides the first direct evidence in support of the hypothesis that multiple mechanisms mediate habituation at different ISIs and underscores the importance of detailed behavioral genetic analysis to dissect multiple mechanistically independent components of a seemingly simple learned behavior. Finally, our findings will help draw much-needed attention to a common form of post-translational modification that is required for mechanosensory responding and learning, two fundamental biological processes present in all animals.

Methods

Strains and maintenance. Worms were cultured on Nematode Growth Medium (NGM) seeded with *Escherichia coli* (OP50) as described previously(64). The following strains were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Minneapolis, MN): N2 Bristol, PY1589 *cmk-1(oy21)*, VC691 *ckk-1(ok1033)*, RB1468 *dkf-2(ok1704)*, VC567 *arf-1.2(ok796)*, VC127 *pkc-2(ok328)*, KG532 *kin-2(ce179)*, RB918 *acr-16(ok789)*, RB818 *hum-1(ok634)*, RB781 *pkc-1(ok563)*, RB1447 *chd-3(ok1651)*, RB830 *epac-1(ok655)*, HA865 *grk-2(rt97)*, NW1700 *plx-2(ev773)*; *him-5(e1490)*, PR678 *tax-4(p678)*, KG744 *pde-4(ce268)*, RB758 *hda-4(ok518)*, RB1625 *par-1(ok2001)*, DA596 *snt-1(ad596)*, XA406 *ncs-1(qa406)*, CB109 *unc-16(e109)*, RB653 *ogt-1(ok430)*, BC10002 *dpy-5(e907)* and VC40557 (which harbors *cmk-1(gk691866)* among many other mutations (65)). The

following strains were obtained from the National Bioresource Project for the nematode (School of Medicine, Tokyo Womens Medical Hospital, Shinjuku-ku, Japan): FX01046 *ogt-1(tm1046)*, FX01282 *T23G5.2(tm1282)*, FX03075 *pdhk-2(tm3075)*, FX00870 *nhr-6(tm870)*, FX04733 *syx-6(tm4733)*, FX05136 *R11A8.7(tm5136)*, and FX02653 *rab-30(tm2653).*

Transgenic strains. The transgenic *C. elegans* strain VH905 hdIs30[*Pglr-1*::DsRed2] was a gift from H. Hutter (Simon Fraser University, Burnaby, BC). The plasmid containing *Pmec-7*::mRFP was a gift from J. Rand (University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma). The transgenic *C. elegans* strains YT1128 *lin-15(n765);* tzEx[*Pckk-1*::GFP; *lin-15*(+)] and YT2016 tzIs2[*Pcmk-1*::GFP; *rol-6(su1006)*] and plasmids containing *cmk-1* cDNA were gifts from Y. Kimura (Mitsubishi Kagaku Institute of Life Sciences, Japan). Please see supplemental methods for the primer sequences for PCR fusion constructs generated for this study.

The following strains were created for this work: VG183 yvEx64[*Pcmk-1*::GFP; *Pmec-7*::mRFP], VG12 hdIs30[*Pglr-1*::DsRed2]; tzIs2[*Pcmk-1*::GFP; rol-6(su1006)], VG19 tzEx[*Pckk-1*::GFP; *lin-15*(+)]; hdIs30[*Pglr-1*::DsRed2], VG92 *cmk -1(oy21)*; yvEx49[*Pcmk-1*::CMK-1; *Pmyo-2*::GFP], VG100 *cmk -1(oy21)*; yvEx57[*Pcmk-1*::CMK-1; *Pmyo-2*::GFP], VG260 yvEx73[P*ogt-1*::GFP; P*mec-7*::RFP; *rol-6(su1006)*], VG214 yvEx70[P*ogt-1*::GFP; *rol-6(su1006)*] and VG261 yvEx74[P*ogt-1*::GFP; P*mec-7*::RFP; *rol-6(su1006)*], VG271 *cmk-1(oy21); dpy-5(e907),* VG279 *cmk-1(gk691866); dpy-5(e907),* VG245 *cmk-1(oy21); ogt-1(ok430).*

Imaging procedures. Adult worms were anesthetized in 100 mM NaN_3 dissolved in M9 buffer containing sephadex beads (G-150-50, Sigma-Aldrich, St. Louis, MO) on glass microscope slides, and then covered with a 1.5 thick coverslip. An Olympus Fluoview 1000 Confocal microscope was used for imaging. GFP was excited using a 488 nm wavelength laser setting with light emitted collected through a 491-515 nm bandpass filter. dsRed and mRFP were excited using a 543 nm wavelength laser setting with light emitted collected through a 600-630 nm bandpass filter. Optical sections of 0.5 μm thickness were collected using a 60x oil immersion lens (Olympus). Final figures were generated using Image J

version 1.41o (National Institutes of Health, Bethesda, MD) and Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Behavioral testing of mutant strains. Worms were synchronized for behavioral testing by picking 5 gravid adults onto a Petri plate containing nematode growth media (NGM) seeded with 50 µl of a liquid culture of OP50 *E. coli* 12-24 hours earlier and letting them lay eggs for 3-4 hours before they were removed. These eggs were allowed to develop for 96 hours (unless otherwise stated) in a 20°C incubator. Plates of worms were placed into the tapping apparatus (66) and covered with an optically transparent lid constructed from a Petri plate lid, non-fogging cover-glass and wax. After a 100s acclimatization period, 30 taps were administered at either a 60s or a 10s ISI.

For CMK-1 rescue strains twelve hours prior to testing, 40-60 worms carrying the selection marker were transferred using a platinum pick to a fresh NGM plate. Plates were seeded with 50 µl of a liquid culture of OP50 *E. coli* 16-20 hours beforehand.

Complementation test. Wild-type or *cmk-1(oy21)* males were mated with *dpy-5(e907)* hermaphrodites homozygous for one of the three *cmk-1* alleles (wild-type, *oy21*, or *gk691866*). Tap habituation behavior of non-*dpy* F1 progeny was evaluated.

Image acquisition of behavior, Behavioral scoring and statistical analysis. Stimulus delivery and image acquisition to record the behavior of the worms was done using the Multi-Worm Tracker (version 1.2.0.2) (66) as described previously (30). Offline data analysis was performed using Choreography analysis software (version 1.3.0_r1035 software package) (66) as described previously (30).

Reversal distances and durations in response to tap were compared across strains by statistical analysis of variance and Tukey honestly significant difference (HSD) tests. Genotype was modeled as a fixed effect. Petri plate (on which the worms were tested; minimum of 3 Petri plates of ~ 50 worms per

experimental condition) was modeled as a random effect nested within the fixed effect. For all statistical tests an alpha value of 0.05 was used to determine significance. All analysis was performed using the statistical software programming language, R, and the following R packages: tidyverse, stringr, nlme, broom and multcomp.

Analysis code can be accessed here: https://github.com/ttimbers/CaMK_and_OGT-1_modulate_mechanoresponding_and_learning/tree/master/src

Kinase and phosphosite prediction and evolutionary analyses. The kinase substrate specificity prediction matrices (KSSPM) for *C. elegans* CMK-1, human CaMK1 isoforms and human CaMK4 were generated using an updated version of the algorithm originally described in Safaei *et al*. (33). The *C. elegans* CMK-1 KSSPM was used to score all of the hypothetical peptides predicted surrounding each of the serine and threonine residues in the 20,470 known *C. elegans* protein sequences. The top 597 scoring phosphopeptides were examined for their conservation in humans using the algorithm described in Safaei *et al*. (33). The identified human phosphosites were then scored with the KSSPMs for human CaMK1 isoforms and human CaMK4.

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Table 1. Summary of mechanosensory and learning phenotypes for predicted CMK-1 phosphorylation targets.

Human Protein	*C. elegans* gene	allele	Reversal Distance			
			Initial tap p-value	score	habituation asymptote p-value	score
PRKD1	*dkf-2*	ok1704	NS		< 0.01	- -
ARF6	*arf-1.2*	ok796	< 0.001	- - -	< 0.001	- - -
PRKCG	*pkc-2*	ok328	< 0.01	+ +	NS	
PDK4	*pdhk-2*	tm3075	0.05	-	NS	
PRKAR1B	*kin-2*	ce179	< 0.001	- - -	NS	
CHRFAM7A	*acr-16*	ok789	< 0.001	+ + +	NS	
MYO1F	*hum-1*	ok634	NS		NS	
PRKCH	*pkc-1*	ok563	NS		NS	
CHD3	*chd-3*	ok1651	< 0.001	- - -	< 0.001	- - -
RAPGEF3	*epac-1*	ok655	NS		NS	
STX6	*syx-6*	tm4733	< 0.001	- - -	NS	
ADRBK2	*grk-2*	rt97	< 0.01	+ +	NS	
PLXNA3	*plx-2*	ev773	< 0.01	- -	NS	
RAB30	*rab-30*	tm2653	NS		NS	
CNGA2	*tax-4*	p678	< 0.001	- - -	NS	
PDE7A	*pde-4*	ce268	< 0.001	- - -	NS	
HDAC4	*hda-4*	ok518	NS		< 0.05	+
HUNK	*par-1*	ok2001	< 0.001	+ + +	NS	
SYT6	*snt-1*	ad596	< 0.001	+ + +	< 0.001	+ + +
CHP2	*ncs-1*	qa406	NS		NS	
SPAG9	*unc-16*	e109	< 0.001	- - -	< 0.001	- - -
OGT	*ogt-1*	ok430	< 0.001	+ + +	<0.001	+ + +

Figure Legends

Figure 1. CMK-1, but not CKK-1, modulates response to mechanosensory stimuli and habituation. Response to mechanical stimuli and tap habituation of wild-type, *cmk-1(oy21)*, and *ckk-1(ok1033)* mutants. (A) Reversal distance habituation to tap stimuli presented at a 60s ISI. (B) Reversal distance habituation to tap stimuli presented at a 10s ISI. (C) Response to mechanical stimuli and tap habituation of wild-type, *cmk-1(oy21)*, and genetic rescue with CMK-1 cDNA in *cmk-1(oy21)* mutants. Error bars represent 95% confidence intervals (Clopper–Pearson method for binomial data).

Figure 2. CMK-1 Thr-179 plays a role in wild-type habituation. Response to mechanical stimuli and tap habituation of wild-type, *cmk-1(oy21)*, *cmk-1(oy21/+)*, and *cmk-1(oy21/gk691866)* worms when stimuli are presented at a 60s ISI and reversal distance is measured. Error bars represent 95% confidence intervals.

Figure 3. Genetic dissociation of initial response and habituation phenotypes. (A) CMK-1::GFP fusion proteins localize to the cytoplasm in cell bodies and neurites and are largely excluded from the nucleus. (B) Reversal response distance and habituation of wild-type, and *cmk-1(pg58)* mutants to repeated tap stimuli presented at a 60s ISI.

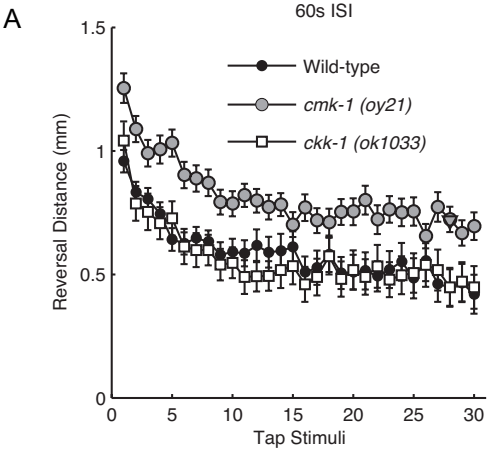
Figure 4. Mechanosensory and habituation phenotypes of predicted CMK-1 downstream phosphorylation targets. Venn diagram grouping genes whose mutant alleles showed similar behavioral phenotypes when given 30 taps at a 60s ISI. Grouping was based on the statistical analysis presented in Table 1.

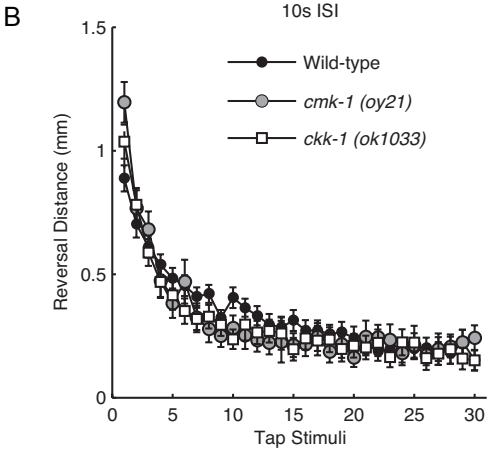
Figure 5. OGT-1 modulates response to mechanical stimuli and habituation. Response to mechanical stimuli and tap habituation of wild-type, and *ogt-1(ok430)* mutants. (A) Reversal distance habituation to tap stimuli presented at a 60s ISI. (B) Reversal distance habituation to tap stimuli

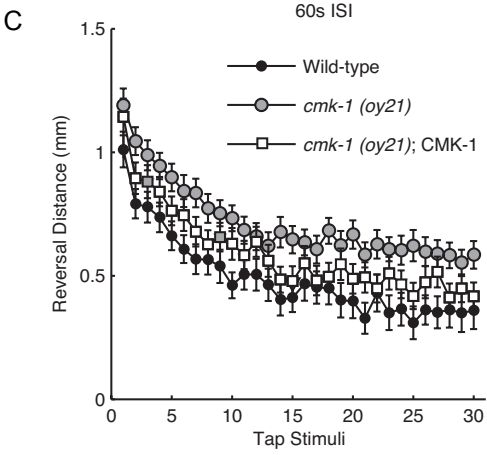
presented at a 10s ISI. (C) Mechanosensory neurons, ALMs and AVM, (visualized with *Pmec-7::mRFP)* express OGT-1 (visualized with *Pogt-1::GFP).* Anterior is at left.

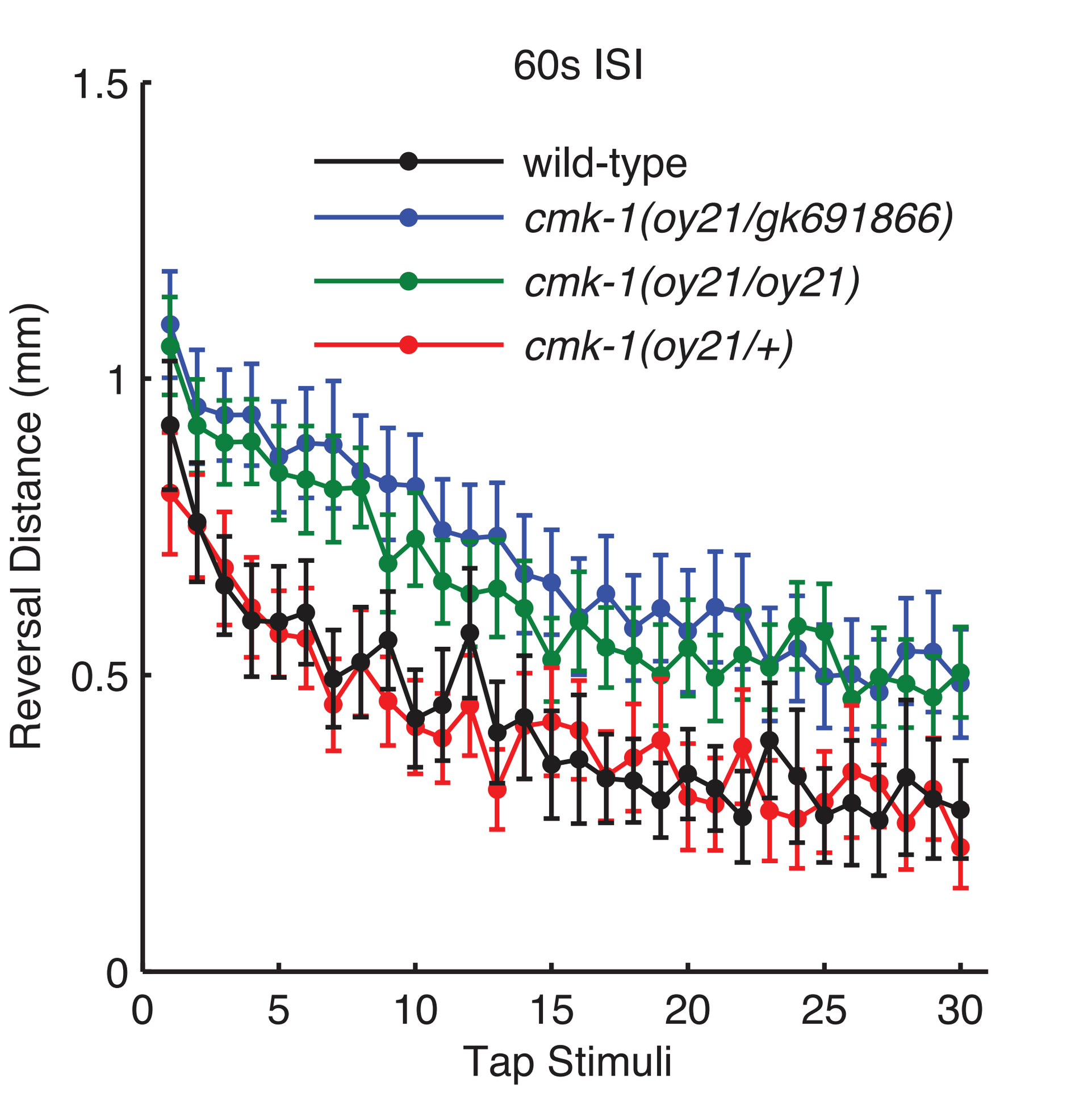
Figure 6. *cmk-1* and *ogt-1* function in a genetic network. Response to mechanical stimuli and tap habituation of wild-type, *cmk-1, ogt-1* and *cmk-1;ogt-1* double mutants when stimuli are presented at a 60s ISI. (A) Reversal distance habituation to tap stimuli. (B) Reversal speed habituation to tap stimuli. (C) Reversal duration habituation. Error bars represent 95% confidence intervals.

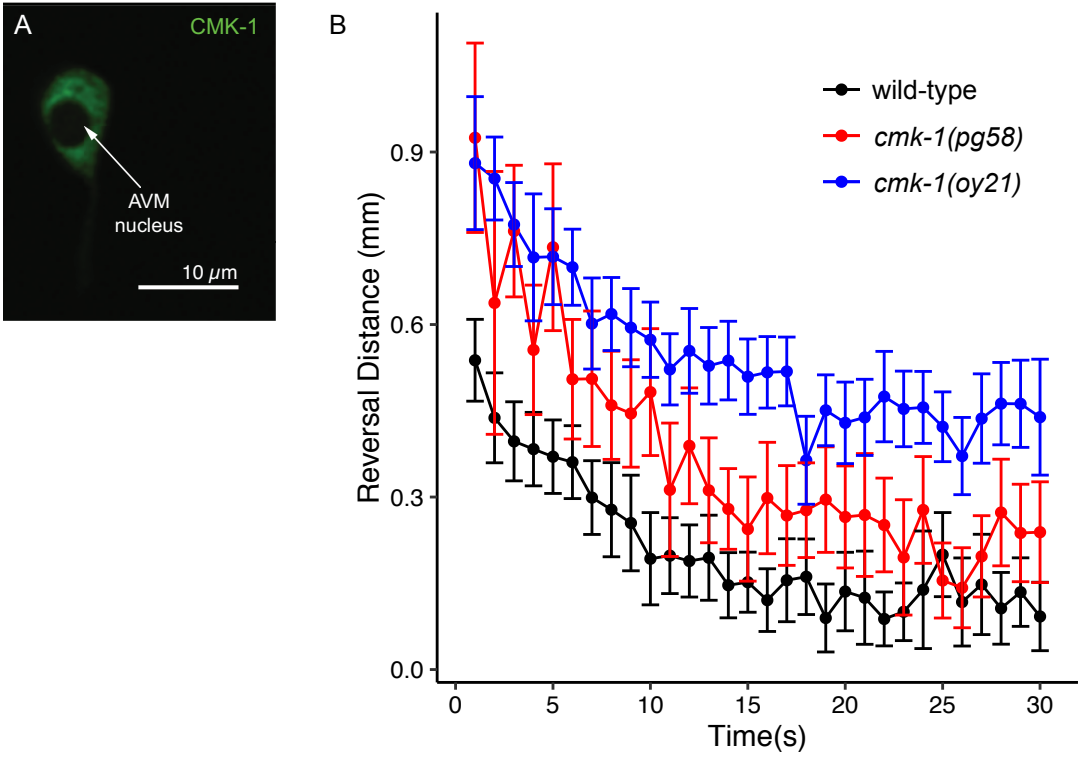
Figure 7. CMK-1 and OGT-1 functional interaction model. Proposed functional model that explains the genetic interaction between *cmk-1* and *ogt-1* mutants. Model aspects supported by data from this study are represented in blue.

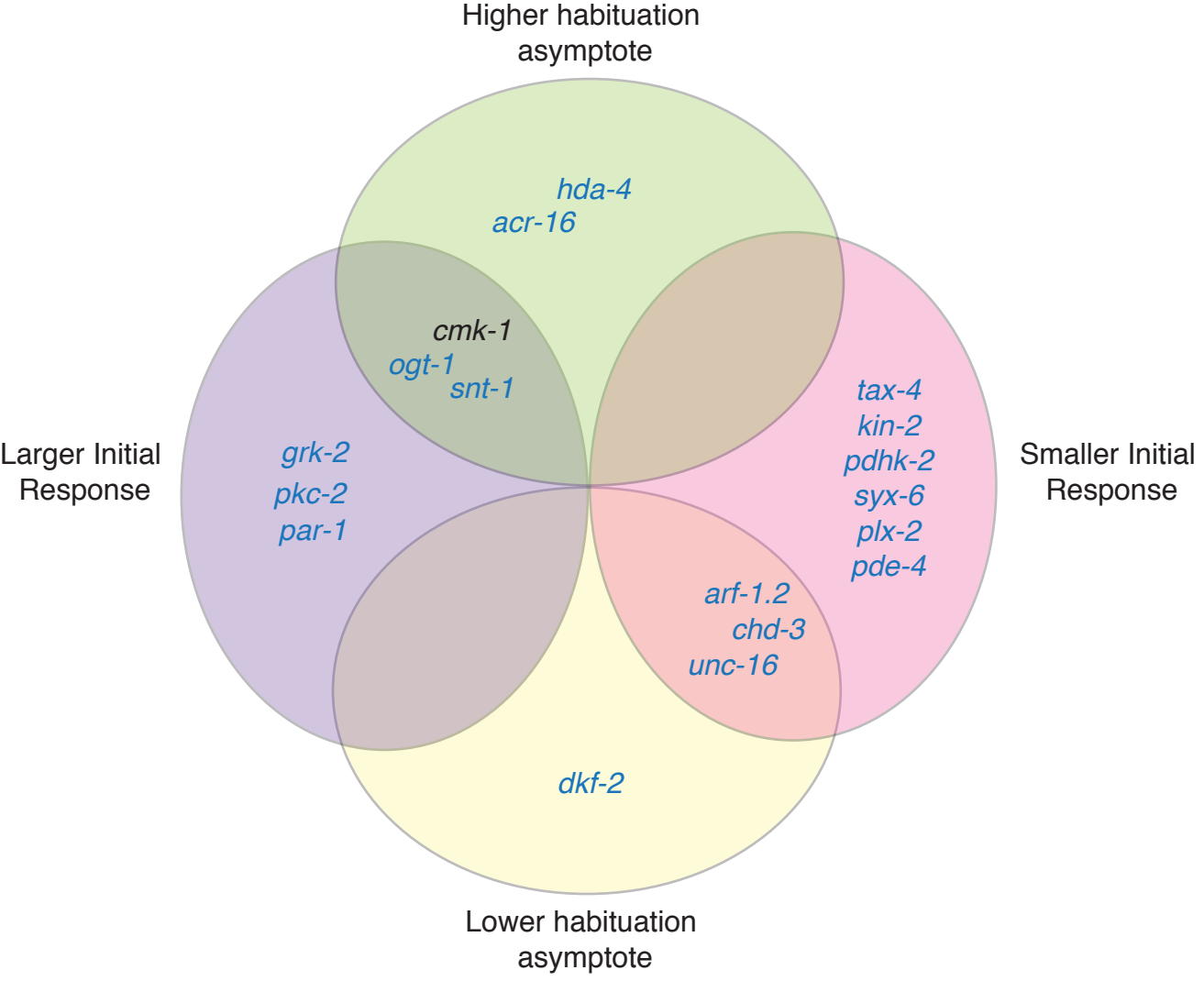

A
60s ISI
Wild-type
cmk-1 (oy21)
ckk-1 (ok1033)
Reversal Distance (mm)
Tap Stimuli
0
0.5
1
1.5
0
5
10
15
20
25
30

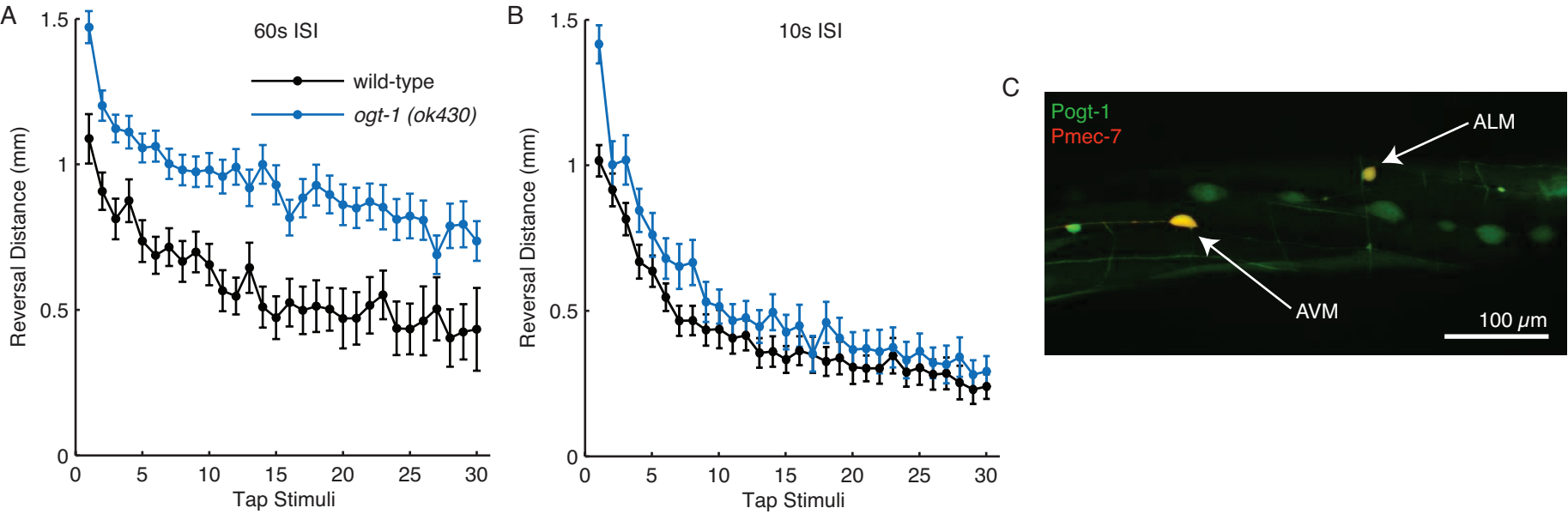

B
10s ISI
Wild-type
cmk-1 (oy21)
ckk-1 (ok1033)
Reversal Distance (mm)
Tap Stimuli
0
0.5
1
1.5
0
5
10
15
20
25
30

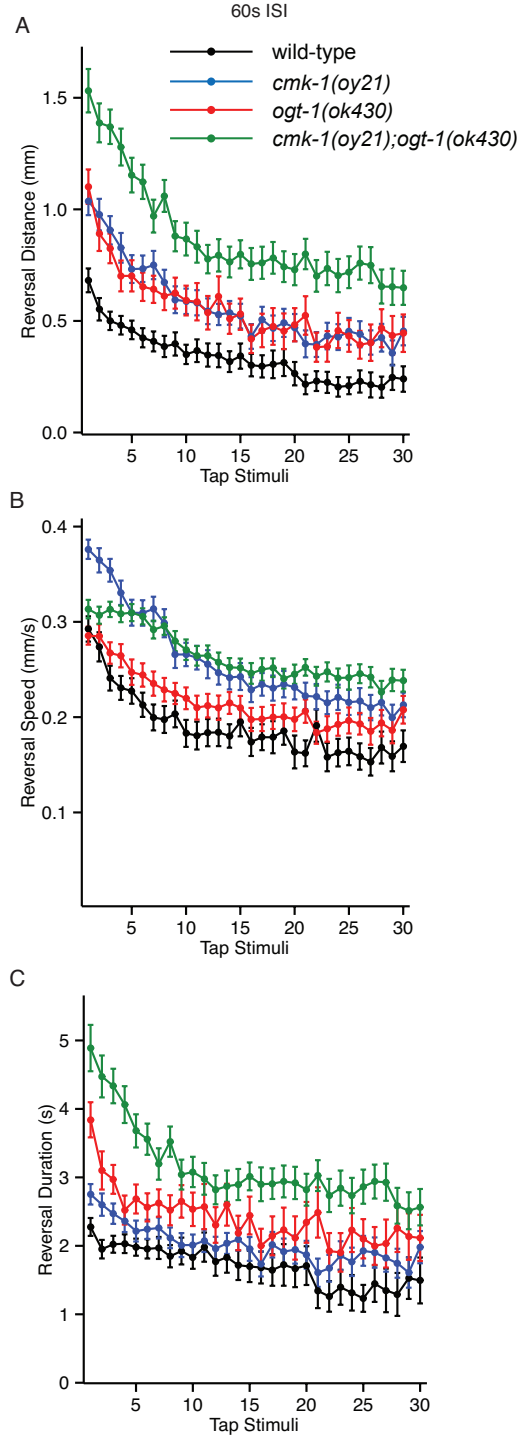

C
60s ISI
Wild-type
cmk-1 (oy21)
cmk-1 (oy21); CMK-1
Reversal Distance (mm)
Tap Stimuli
0
0.5
1
1.5
0
5
10
15
20
25
30

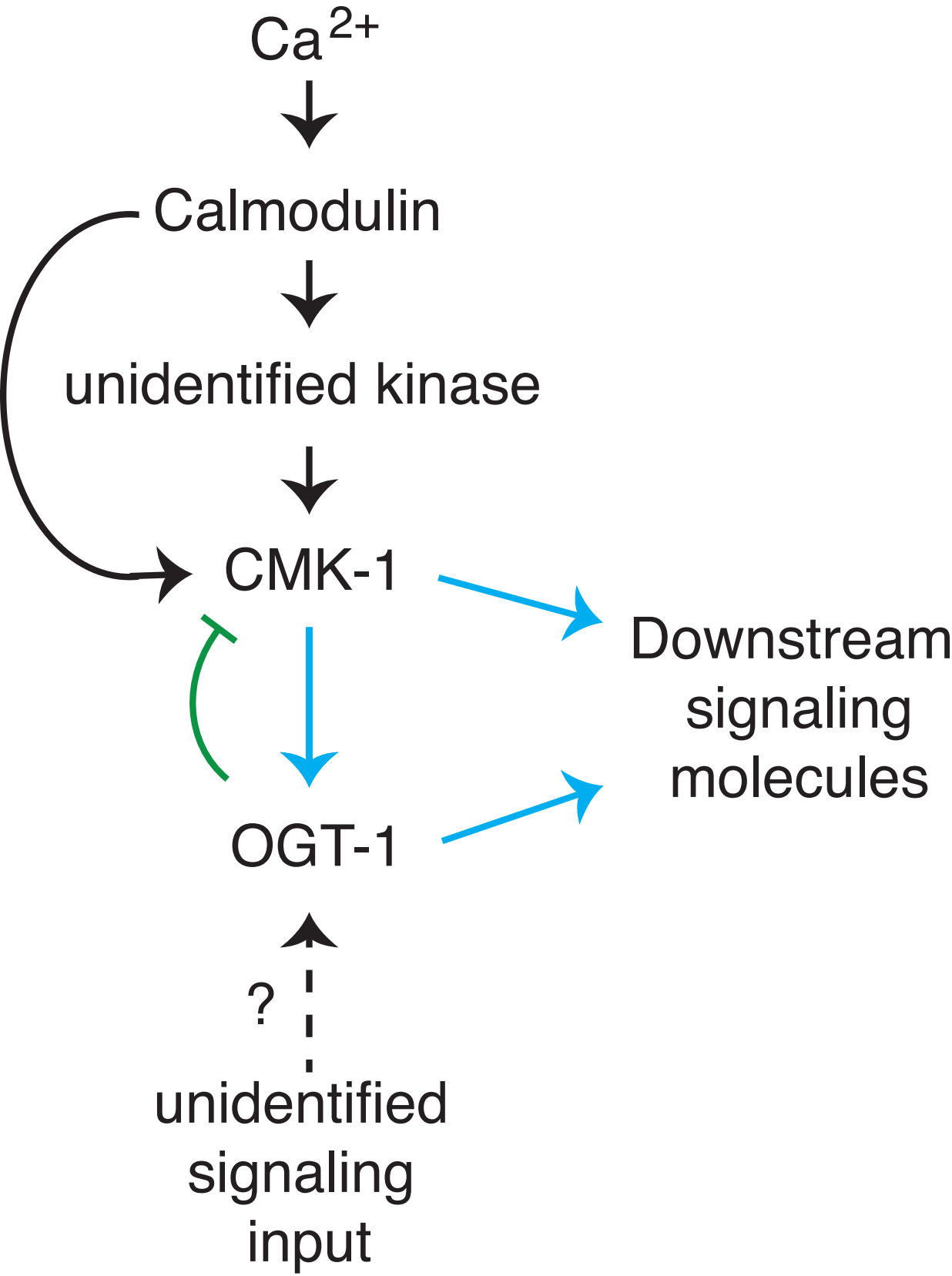

60s ISI
wild-type
cmk-1(oy21/gk691866)
cmk-1(oy21/oy21)
cmk-1(oy21/+)
Reversal Distance (mm)
1.5
1
0.5
0
0
5
10
15
20
25
30
Tap Stimuli


A
CMK-1
AVM
nucleus
10 μm
B
wild-type
cmk-1(pg58)
cmk-1(oy21)
Reversal Distance (mm)
0.0
0.3
0.6
0.9
0
5
10
15
20
25
30
Time(s)


Higher habituation asymptote
hda-4
acr-16
cmk-1
ogt-1
snt-1
Larger Initial Response
grk-2
pkc-2
par-1
Smaller Initial Response
tax-4
kin-2
pdhk-2
syx-6
plx-2
pde-4
arf-1.2
chd-3
unc-16
dkf-2
Lower habituation asymptote


A
60s ISI
wild-type
ogt-1 (ok430)
Reversal Distance (mm)
Tap Stimuli
B
10s ISI
Reversal Distance (mm)
Tap Stimuli
C
Pogt-1
Pmec-7
ALM
AVM
100 μm


60s ISI
A
wild-type
cmk-1(oy21)
ogt-1(ok430)
cmk-1(oy21);ogt-1(ok430)
Reversal Distance (mm)
Tap Stimuli
B
Reversal Speed (mm/s)
Tap Stimuli
C
Reversal Duration (s)
Tap Stimuli


Ca2+
Calmodulin
unidentified kinase
CMK-1
OGT-1
Downstream signaling molecules
?
unidentified signaling input