SPECIFIC AIMS

Calmodulin is an evolutionarily conserved protein that regulates diverse cellular functions through its interactions with numerous calmodulin-binding proteins (CaMBPs) [1]. Among these, ryanodine receptors (RyRs), which serve as Ca^{2+} release channels in the endoplasmic reticulum (ER) membrane, are well-known CaMBPs [2]. Calmodulin inhibits cardiac RyRs in a Ca^{2+}-dependent manner [2, 3]. Over the past decade, numerous studies have linked calmodulin mutations to specific cardiac arrhythmias and shown that these mutations reduce Ca^{2+} binding affinity, enhance RyR-mediated Ca^{2+} release, and slow Ca^{2+}/calmodulin-dependent inactivation of voltage-gated Ca^{2+} channels (VGCCs) [4, 5]. Intriguingly, patients with calmodulin mutations frequently present with neurological disorders [4, 6, 7]. However, the mechanism by which calmodulin mutations lead to neurological dysfunction remains to be investigated.

In a genetic screen using *C. elegans* to identify suppressors of a sluggish phenotype caused by a gain-of-function (*gf*) KQT-1, an ortholog of mammalian KCNQ1 K^+ channel [8], we isolated a hypomorphic calmodulin mutant, *cmd-1(zw47)*, where a conserved glutamate (E121) before the fourth EF-hand domain is replaced by lysine (K). This mutation resulted in increased synaptic transmission at the neuromuscular junction (NMJ), characterized by a higher frequency of miniature postsynaptic currents (minis) and larger amplitudes of minis and evoked postsynaptic currents (ePSCs) compared to wild type. These effects are opposite to those observed in animals with a neuronal deficiency of *ryr-1*, the sole RYR gene in *C. elegans*, as reported in our previous studies [9-11]. We also observed similar changes in minis and ePSCs in both an *ryr-1* single mutant and the *ryr-1;cmd-1(zw47)* double mutant, suggesting that RYR-1 acts downstream of CMD-1. This study will test the **hypothesis that calmodulin in neurons downregulates neurotransmitter release via RyRs.**

Aim 1. Determine how calmodulin regulates synaptic transmission

We will investigate whether CMD-1 regulates synaptic transmission pre- or postsynaptically by performing cell-targeted rescue experiments in the *cmd-1(zw47)* mutant and CMD-1 depletion experiments in wild-type animals, targeting either motor neurons or muscle cells in each case. We will also assess whether muscle cells in the mutant exhibit abnormal sensitivity to exogenous neurotransmitters. To determine if the *cmd-1* mutation affects calmodulin's Ca^{2+} affinity, we will monitor Ca^{2+}-dependent changes in calmodulin intrinsic fluorescence. Additionally, we will examine whether the mutation causes gross neuronal morphological changes by imaging GFP-labeled motor neurons in live *C. elegans*. Finally, we will assess whether large-amplitude minis and ePSCs observed in the *kqt-1(gf)* strain result from a “sink” effect, where the calmodulin-binding domain of the overexpressed KQT-1(*gf*) protein sequesters endogenous calmodulin, reducing its availability for other interactions.

Aim 2. Investigate how calmodulin regulates RyR function

We will determine whether calmodulin regulates neurotransmitter release through RYR-1 by comparing properties of minis and ePSCs at the NMJ among wild type, and single and double mutants of *cmd-1* and *ryr-1*. To assess whether calmodulin regulates RyR-mediated Ca^{2+} mobilization in motor neurons, we will image and compare Ca^{2+} signals across wild-type and mutant strains. We will also determine if calmodulin regulates RyR function in a Ca^{2+}-dependent manner by analyzing the effects of the *cmd-1(zw47)* mutation on synaptic transmission under different Ca^{2+} concentrations. Additionally, we will investigate whether calmodulin regulates minis and ePSCs via VGCCs by testing if VGCC deficiencies in motor neurons antagonize the synaptic effects of the *cmd-1(zw47)* mutation. Finally, we will determine whether introducing calmodulin mutations associated with human neurological disorders into endogenous *cmd-1* affects minis and ePSCs through RYR-1.

The results of this study will likely provide significant insights into the molecular mechanisms underlying neurological disorders associated with calmodulin mutations.

SIGNIFICANCE

Calmodulin is a small Ca^{2+}-binding protein that is ubiquitously expressed. By binding to Ca^{2+}, calmodulin transduces the Ca^{2+} signal to various calmodulin-binding proteins (CaMBPs) [12-14], affecting many cellular functions. The human genome contains three calmodulin genes, including *CALM1, CALM2,* and *CALM3*, which are located on three different chromosomes but encode proteins with an identical amino acid sequence. The calmodulin protein consists of 149 amino acid residues separated into an amino-terminal lobe and a carboxy-terminal lobe by a long exposed α-helix [15]. Each lobe contains two EF-hands that can coordinate two Ca^{2+} ions (one per EF-hand). The carboxy-terminal lobe of calmodulin binds Ca^{2+} with a much higher affinity than the amino-terminal lobe, allowing calmodulin to sense Ca^{2+} over a wide concentration range [16].

Despite its great physiological importance, calmodulin mutations had not been associated with human disease until 2012, when two different calmodulin missense mutations (N53I and N97S) were linked to catecholaminergic polymorphic ventricular tachycardia (CPVT) [17], a life-threatening heart condition. Since then, a total of at least 18 different calmodulin mutations have been linked to patients with heart problems, the majority being long QT syndrome and/or CPVT, and a smaller number being idiopathic ventricular fibrillation [5]. The identified mutations reduce the affinity of calmodulin for Ca^{2+}, which in turn alters the function of specific CaMBPs important to cardiac function. The most affected CaMBPs in the heart are the ryanodine receptor 2 (RyR2) in the sarcoplasmic reticulum and the α_1 subunit of the voltage-gated Ca^{2+} channel (VGCC) $Ca_V1.2$ in the plasma membrane. Typically, calmodulin mutations result in increased RyR-mediated Ca^{2+} release from the sarcoplasmic reticulum and slower inactivation of $Ca_V1.2$ channels [5, 18]. The inhibitory effect of calmodulin mutations on RyR2 is consistent with a recent cryo-electron microscopy study, which shows that Ca^{2+} binding to calmodulin induces a conformational change in RyR2, leading to the closure of the channel pore [2].

It has become increasingly clear that the pathological effects of calmodulin mutations are not limited to the heart, but also affect other parts of the body, including the nervous system. In an analysis of 111 patients with calmodulin mutations, 20 of them had obvious primary neurological disorders, manifested as autism, seizures, attention deficit hyperactivity disorder, dyslexia, cognitive impairment, abnormal electroencephalograms, etc. [4]. These findings suggest that calmodulin mutations are also an important cause of neurological disorders. However, the molecular mechanisms underlying such disorders remain to be investigated.

C. elegans can be a useful model for studying the role of calmodulin mutations in neuronal function. This organism has a single calmodulin gene, *cmd-1*, which encodes a protein that is almost identical to human calmodulin, differing by only three amino acids (**Figure 1**). In a recent study, *C. elegans* was used to assess the effect of specific calmodulin mutations found in human patients on neuronal function by first editing the *cmd-1* gene to produce a protein identical to human calmodulin and then independently introducing three mutations identified in human patients (N54I, D96V, and N98S) into this “humanized” CMD-1 [18]. All three mutations are associated with cardiac arrhythmias (CPVT and/or Long Q-T syndrome), reduce Ca^{2+} affinity, and cause abnormal function of both $Ca_V1.2$ and RyRs, with the D96V and N98S mutations also being associated with neurological disorders [4]. This study investigated the effects of the mutations on neuromuscular transmission using aldicarb- and levamisole-induced paralysis assays. Aldicarb, a cholinesterase inhibitor, induces paralysis by blocking the breakdown of acetylcholine [19], while levamisole does so by directly activating muscle acetylcholine receptors [20]. The D96V mutation resulted in significantly increased sensitivity to both aldicarb and levamisole compared to the wild-type humanized calmodulin. In contrast, the N54I mutation had the opposite effect, reducing sensitivity to these compounds, while the N98S mutation had no discernible effect in either assay. Unfortunately, it is not possible to draw any meaningful conclusions about the neuronal effects of these calmodulin mutations from these results, although the authors suggest that the mutations may contribute to neurological disorders.

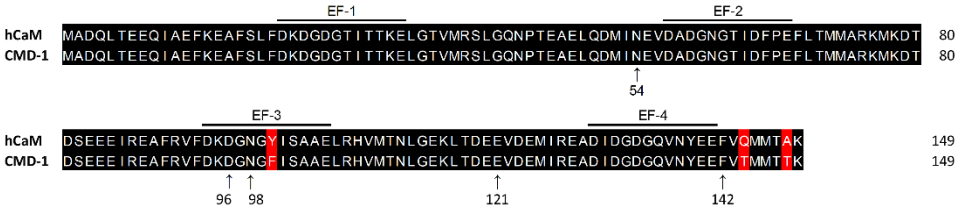

Figure 1. Alignment of amino acid sequences of human calmodulin (hCaM) and *C. elegans* CMD-1. Identical residues are highlighted with a black background, while differing residues are highlighted in red. The numbers below the arrows indicate positions of the mutated residue (E121K) in *cmd-1(zw47)* and the human pathogenic mutations to be studied (N54I, D96V, N98S, and F142L).

We isolated a mutant of *cmd-1*, *zw47*, in a forward genetic screen for suppressors of a sluggish phenotype caused by ectopic expression of KQT-1(R258Q) in cholinergic and GABAergic motor neurons under the control of a chimeric promoter of *Punc-17Δ1* (vesicular acetylcholine transporter) and *Punc-47* (vesicular GABA transporter) (In this proposal, the letter "*P*" before a gene name denotes the gene's promoter). Although *unc-17* is normally expressed in many neurons, we used only the distal part of its promoter sequence (*Punc-17Δ1*) to restrict the promoter activity almost exclusively to ventral cord cholinergic motor neurons [10]. In *cmd-1(zw47)*, a conserved glutamate residue (E121) between the third and fourth EF-hands is replaced by a lysine (**Figure 1**). The *kqt-1(gf)* strain exhibited a much slower locomotion speed compared to wild type, moving at approximately 30% of the wild-type speed. While *kgt-1(gf);cmd-1(zw47)* appeared to be grossly normal in locomotion, expression of wild-type *cmd-1* in the double mutant reinstated the sluggish phenotype of *kqt-1(gf)* (not shown), suggesting that *cmd-1(zw47)* is a loss-of-function mutation. *cmd-1(zw47)* must be a hypomorphic mutant, since global knockdown of *cmd-1* results in lethality [21].

KQT-1(R258Q) was originally designed by Aguan Wei. In our electrophysiological experiments using the *Xenopus* oocyte heterologous expression system, wild-type KQT-1 showed voltage-dependent activation, whereas KQT-1(R258Q) was constitutively active (**Figure 2**). We henceforth refer to KQT-1(R258Q) as KQT-1(*gf*) (*gf* for *g*ain of *f*unction).

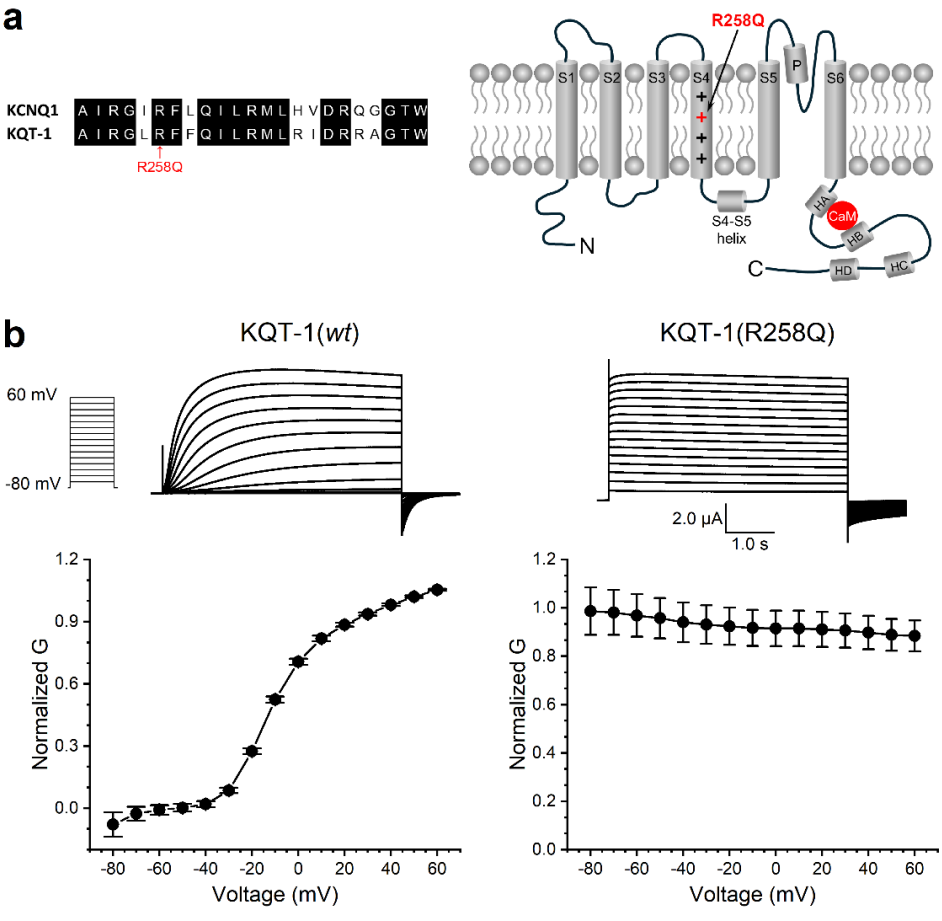

Figure 2. R258Q mutation in KQT-1 renders the channel constitutively active. a. (*Left*) Sequence alignment of the S4 domain of the human KCNQ1 and *C. elegans* KQT-1. The position of R258 in KQT-1 is indicated. (*Right*) Schematic representation of a KCNQ subunit showing the location of the R258Q mutation and the calmodulin binding site. **b.** Representative whole-cell current traces (*top*) and conductance-voltage relationships of wild-type (*wt*) KQT-1 and KQT-1(R258Q), expressed in *Xenopus* oocytes. Conductance values were calculated based on the average currents during the last 1.0 second of the voltage steps.

Electrophysiological analysis of synaptic transmission at the NMJ revealed substantial

increases in the amplitude of evoked postsynaptic currents (ePSCs) and the mean amplitude of miniature postsynaptic currents (minis) in both *kqt-1(gf)* and *cmd-1(zw47)* mutants compared to wild type. However, while the frequency of minis was reduced by approximately 60% in the *kqt-1(gf)* strain, it was increased by 67% in the *cmd-1(zw47)* strain (**Figure 3**). The contrasting effects on mini frequency suggest that KQT-1(*gf*) and CMD-1(E121K) affect minis by different mechanisms.

C. elegans has a single RyR gene, *ryr-1* (also known as *unc-68*). Our previous studies have shown that RYR-1 plays a crucial role in neurotransmitter release and that the loss of RyR-1 function in neurons leads to a reduced frequency of minis, as well as reduced amplitudes of both minis and ePSCs [9-11]. In addition, we found that AIPR-1, an ortholog of the human aryl hydrocarbon-interacting protein (AIP), acts as a stabilizer for the RYR-1 Ca^{2+} release channel, and that a hypomorphic mutation in *aipr-1* results in increased mini frequency and larger amplitudes of both minis and ePSCs [10]. Since the synaptic phenotypes of *cmd-1(zw47)* resemble those of the *aipr-1* mutant, but are opposite to those of *ryr-1* mutants, we investigated whether CMD-1 regulates synaptic transmission via RYR-1 by comparing minis and ePSCs among *cmd-1(zw47)*, *ryr-1(syb216)*, and *cmd-1(zw47);ryr-1(syb216)*. We chose *ryr-1(syb216)* for this experiment because this mutant exhibits neuron-specific loss of *ryr-1* expression [22]. Consistent with our previous findings in different mutant strains [9-11], this mutant exhibited reduced mini frequency and smaller amplitudes of both minis and ePSCs compared to wild type (**Figure 3**). The synaptic phenotypes of the *cmd-1(zw47);ryr-1(syb216)* double mutant were very similar to those of the *ryr-1(syb216)* single mutant, suggesting that CMD-1 regulates synaptic transmission via RYR-1.

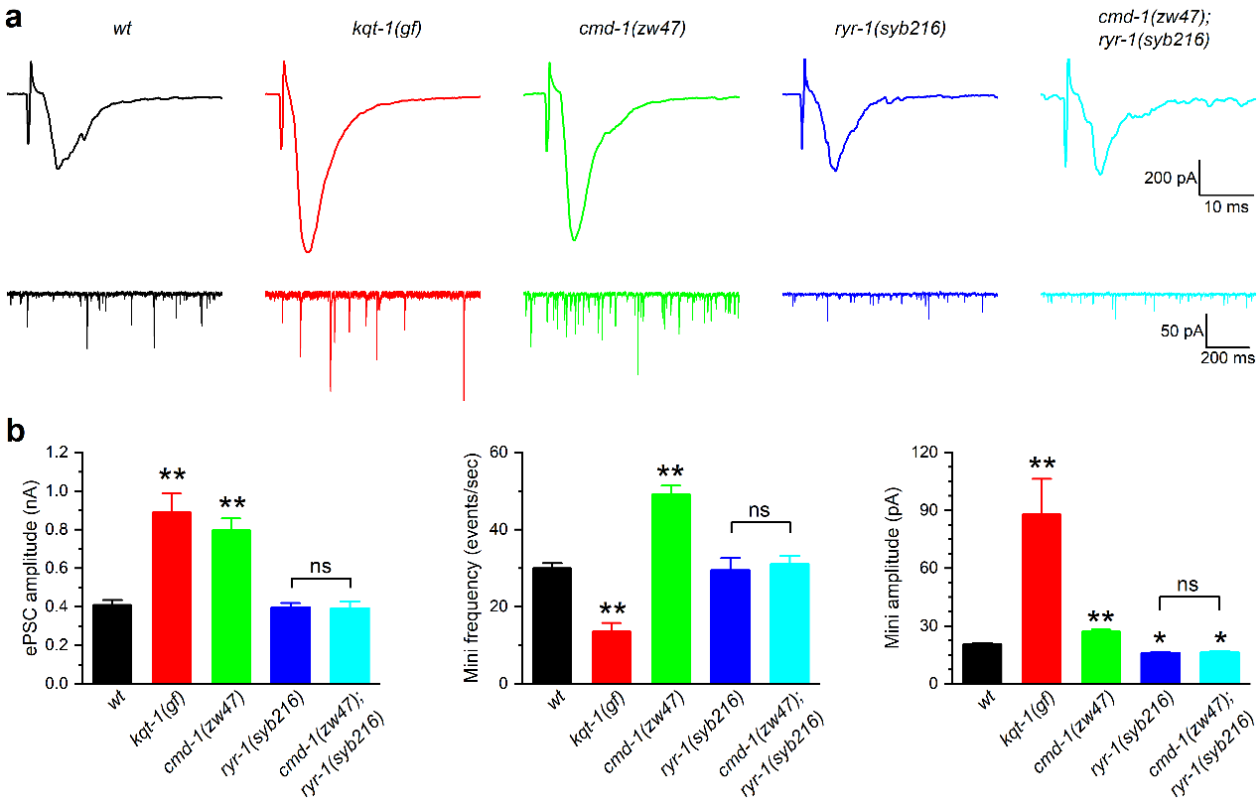

Figure 3. Effects of mutations in *kqt-1*, *cmd-1*, and *ryr-1* on ePSCs and minis at the NMJ. a. Representative traces of ePSCs and minis. **b.** Comparisons of ePSC amplitude, mini frequency, and mini amplitude between the groups. Shown are mean ± SE. The single and double asterisks indicate statistically significant differences compared to wild type (*wt*) at $p<0.05$ and $p<0.01$ levels, respectively, while "ns" means no significant difference between the marked groups (one-way ANOVA with Tukey's post hoc test). The number of experiments performed (*n*) was 5 to 6.

This study will test the hypothesis that calmodulin downregulates neurotransmitter release by inhibiting the function of RYRs. We will use a variety of approaches to examine this possibility. The results will likely reveal a previously unknown role of calmodulin in regulating neurotransmitter release and provide significant insights into the molecular mechanism of neurological disorders associated with calmodulin mutations.

INNOVATION

1. This study aims to uncover a previously unknown physiological function of calmodulin in the regulation of neurotransmitter release, as well as the underlying mechanism.
2. This research will employ a powerful combination of electrophysiological techniques and genetic approaches to provide conclusive insights into calmodulin's role and mechanism in neurotransmitter release regulation.
3. This research will investigate the effects of pathogenic calmodulin mutations identified in human patients on neuronal function, utilizing *C. elegans* as a model organism. These studies are more cost-effective and less time-consuming than equivalent mammalian studies.

APPROACH

Aim 1. Determine how calmodulin regulates synaptic transmission

1. Determine whether calmodulin regulates synaptic transmission by acting presynaptically

The abnormalities of minis and ePSCs at the NMJ in *cmd-1(zw47)* could result from either a pre- or postsynaptic deficiency of the gene. We will perform the following experiments to distinguish between these two possibilities:

a. Express wild-type *cmd-1* specifically in motor neurons and body-wall muscle cells of the mutant using *Punc-17Δ1;unc-47* and *Pmyo-3*, respectively, to determine whether the neuronal or muscle expression can rescue the mutant synaptic phenotypes.

b. Use an auxin-inducible degradation approach [23] to deplete CMD-1 in motor neurons and body-wall muscle cells conditionally. In these experiments, we will first use the CRISPR/Cas9 genome editing technique to tag the carboxyl-terminus of endogenous CMD-1 with degron and then express the TIR1 F-box protein specifically in motor neurons or body-wall muscle cells using the promoters described above. We will transfer L4-stage transgenic worms to NGM plates containing 1 mM auxin and use the adult worms for electrophysiological recording. We have used this approach successfully to achieve conditional degradation of an innexin protein [24].

c. We will determine whether the *cmd-1(zw47)* mutant has altered muscle sensitivities to neurotransmitters compared to wild type by analyzing the amplitude of body-wall muscle whole-cell currents caused by pressure-ejection of exogenous acetylcholine (100 μM) and GABA (100 μM).

We predict that the results of these experiments will indicate that CMD-1 functions presynaptically to regulate neurotransmitter release.

2. Determine whether the CMD-1(E121K) mutation impairs Ca^{2+} binding

We will independently express His-tagged wild-type CMD-1 and CMD-1(E121K) in the BL21(DE3) strain of *E. coli*, followed by protein purification using affinity chromatography. The Ca^{2+} binding affinities of the purified proteins will be evaluated by monitoring Ca^{2+}-dependent changes in intrinsic phenylalanine and tyrosine fluorescence [25]. The resulting data will be fitted to the Adair equation. Binding of Ca^{2+} to the calmodulin C-terminal lobe is expected to increase tyrosine fluorescence, while binding to the N-terminal lobe is expected to decrease phenylalanine fluorescence [25]. This method is widely used to assess the effects of calmodulin mutations on Ca^{2+} binding affinity [5].

3. Determine whether calmodulin mutations alter gross neuronal morphology

We will express GFP in cholinergic and GABAergic motor neurons under the control of *Punc-17Δ1* and *Punc-47*, respectively, and analyze motor neuron gross morphology by fluorescence microscopy. Comparisons will be made between wild type and *cmd-1(zw47)*. The outcomes of these experiments are currently unpredictable.

4. Determine how KQT-1(*gf*) affects synaptic function

It is intriguing that both *kqt-1(lf)* and *cmd-1(zw47)* mutant strains showed substantially larger amplitudes of minis and ePSCs but different effects on mini frequency. Since *cmd-1(zw47)* was identified as a suppressor of the sluggish phenotype of the *kqt-1(gf)* strain, we aim to understand how *kqt-1(gf)* alters synaptic activity by testing two hypotheses:

Hypothesis 1: *KQT-1(gf)* reduces mini frequency by decreasing membrane excitability.

We will analyze the effect of overexpressing wild-type KQT-1 and KQT-1(*gf*) in motor neurons. If the hypothesis is correct, the transgenic strain expressing wild-type KQT-1 will exhibit normal or slightly reduced mini frequency compared to wild type while maintain the higher amplitudes of minis and ePSCs observed in *kqt-1(gf)*.

Hypothesis 2: *KQT-1(gf)* enhances mini and ePSC amplitudes by a "sink" effect on calmodulin.

We will mutate one or two residues critical for calmodulin binding in KQT-1, based on a previous study of KCNQ2, which demonstrated that either I340E or R345E mutation in the HA domain, or combined mutations of K553E/R554E in the HB domain of mouse KCNQ2, abolished physical interactions with calmodulin [26]. We will make similar mutations to the corresponding amino acid residues in KQT-1 (**Figure 4**) to identify a mutation that can disrupt KQT-1 calmodulin binding. If our hypothesis is correct, a transgenic *kqt-1(gf)* strain with this mutation will not exhibit larger amplitudes of minis and ePSCs compared to wild type. However, the inhibitory effect of *kqt-1(gf)* on mini frequency will also likely be abolished since calmodulin binding to KCNQ channels are important to the channel function [27-30].

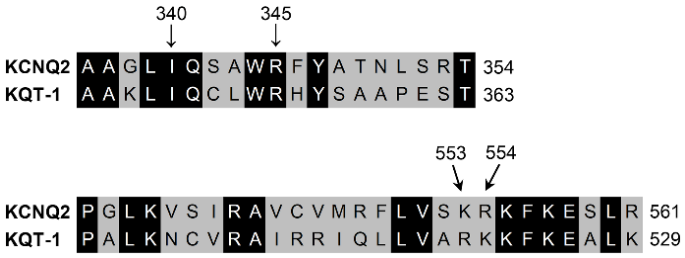

Figure 4. Alignment of calmodulin HA and HB domains between mouse KCNQ2 and C. elegans KQT-1. The amino acid residues critical for calmodulin binding in mouse KCNQ-2 are indicated.

Aim 2. Investigate how calmodulin regulates RyR function

1. Determine whether calmodulin regulates neurotransmitter release through RyRs

We will analyze minis and ePSCs in additional animals of *cmd-1(zw47)*, *ryr-1(syb216)*, and *cmd-1(zw47;ryr-1(syb216)* so that the sample size of each group in **Figure 3** is 8 to 10. The additional experiments will ensure the necessary scientific rigor to support our conclusion that calmodulin regulates neurotransmitter release through RYR-1.

2. Determine whether calmodulin regulates RyR-mediated Ca^{2+} mobilization in motor neurons

We will image spontaneous Ca^{2+} signals in selected motor neurons (VA5, VB6, and VD5) of wild type, *cmd-1(zw47)*, *ryr-1(sby216)*, and *cmd-1(zw47);ryr-1(sby216)* using Furo-2 AM. a ratiometric chemical Ca^{2+} sensor. These neurons are chosen because they include both cholinergic (VA5 and VB6) and GABAergic (VD5) motor neurons, which can be easily identified in our dissected animals for electrophysiology based on their anatomical locations [24, 31]. We will use a chemical Ca^{2+} sensor instead of GCaMP6 because the calmodulin domain in the genetically encoded Ca^{2+} sensor might complicate our analysis. We predict that Ca^{2+} signals will be enhanced in *cmd-1(zw47)* but decreased to a similar degree in *ryr-1(syb216)* and *cmd-1(zw47);ryr-1(syb216)*, which would suggest that calmodulin regulates RYR-1-mediated Ca^{2+} mobilization.

3. Determine whether the effect of CMD-1 on RYR-1 function is Ca^{2+}-dependent

We will assess whether the regulatory effect of CMD-1 on RYR-1 in *C. elegans* neurons is Ca^{2+}-dependent by recording minis in wild type, *cmd-1(zw47)*, and *cmd-1(zw47);ryr-1(syb216)* under varying Ca^{2+} concentrations (50 µM, 100 µM, 250 µM, 500 µM, 1 mM, 5 mM) in the bath solution. The [Ca^{2+}] in the bath solution will be increased cumulatively. If CMD-1's function is Ca^{2+}-dependent, the augmenting effect of the *cmd-1(zw47)* mutation on mini amplitude may show Ca^{2+}-dependent changes, which would not be observed in the double mutant. When interpreting the results, we will note the caveat that the exact effects on internal [Ca^{2+}] remain unknown.

4. Determine whether the presynaptic function of CMD-1 depends on UNC-2 or EGL-19

Since calmodulin mutations in humans enhance the function of $Ca_V1.2$ by slowing its inactivation rate, we will investigate whether calmodulin similarly regulates VGCC function in *C. elegans* neurons. Specifically, we will determine whether the effects of the *cmd-1(zw47)* mutation on minis and ePSCs at the NMJ can be prevented by loss-of-function (*lf*) mutations in *unc-2* or *egl-19*, which encode VGCCs homologous to Ca_V2 and Ca_V1, respectively. In *C. elegans* motor neurons, UNC-2 and EGL-19 mediate the release of distinct pools of synaptic vesicles [11]. We predict that neither *unc-2(lf)* nor *egl-19(lf)* will eliminate the augmenting effects of *cmd-1(zw47)* on minis and ePSCs, unlike *ryr-1(syb216)*, for two reasons 1) *ryr-1(syb216)* completely abolished the augmenting effects of the *cmd-1(zw47)* mutation, and 2) the augmenting effects of the *aipr-1(lf)* mutation on mini and ePSCs cannot be prevented by *unc-2(lf)* [10].

5. Determine how selected pathogenic calmodulin mutations affect synaptic transmission

We will record minis and ePSCs at the NMJ from animals expressing the wild-type "humanized" *cmd-1* (*hCaM*) as well as from animals carrying selected pathogenic human calmodulin mutations introduced into *hCaM*. The mutant strains to be analyzed include *hCaM(N54I)*, *hCaM(N98S)*, *hCaM(D96V)*, and *hCaM(F142L)*. We have already obtained the wild-type *hCaM* strain and the first three mutant *hCaM* strains [18] from Dr. Anders Olsen. We will add *hCaM(F142L)* to our analysis because this mutation has been linked to various neurological disorders, including autism, intellectual disability, epilepsy, and EEG dysrhythmia [4]. This strain will be generated from the wild-type *hCaM* strain using the CRISPR/Cas9 technique. We will also determine whether these mutations affect minis and ePSCs via RYR-1 and whether they alter the gross morphology of motor neurons, using the approaches described earlier for the *cmd-1(zw47)* mutation.

Since all four mutations cause a reduced calmodulin affinity for Ca^{2+} [32-35], we predict that the *hCaM* strains carrying these mutations will exhibit synaptic phenotypes consistent with calmodulin loss-of-function. However, the severity of the synaptic phenotypes may vary.

Scientific Rigor

1) *C. elegans* electrophysiology experiments will be performed on adult day 1 hermaphrodites reared under identical conditions. 2) All *C. elegans* mutants induced by chemical mutagens will undergo at least four rounds of outcrossing with wild type before use. 3) Power analysis will be used to guide sample size. For electrophysiological experiments, we choose sample sizes of 8 to 12 to achieve high statistical power, although 5 to 6 is usually sufficient. 4) Experimenters will perform procedures blinded to genotype. 5) When comparing the effects of a transgene in different genetic backgrounds, we will first express or express and integrate the transgene in wild type and then introduce it into the mutant strains by crossing to maintain consistent transgene dosage.

Statistical Analysis

All quantitative parameters will be compared by either *t*-test (paired or un-paired) or one-way analysis of variance (*ANOVA*) followed by Tukey's post hoc test depending on whether the number of groups for comparison is two (*t*-test) or more than two (one-way *ANOVA*). Statistical analysis and data graphing will be done using OriginPro 2024b (OriginLab, Northampton, MA, USA).

REFERENCES

1. Tidow H, Nissen P. Structural diversity of calmodulin binding to its target sites. FEBS J. 2013;280:5551-65. doi: 10.1111/febs.12296
2. Gong D, Chi X, Wei J, Zhou G, Huang G, Zhang L*, et al.* Modulation of cardiac ryanodine receptor 2 by calmodulin. Nature. 2019;572:347-51. doi: 10.1038/s41586-019-1377-y
3. Balshaw DM, Xu L, Yamaguchi N, Pasek DA, Meissner G. Calmodulin binding and inhibition of cardiac muscle calcium release channel (ryanodine receptor). J Biol Chem. 2001;276:20144-53. doi: 10.1074/jbc.M010771200
4. Crotti L, Spazzolini C, Nyegaard M, Overgaard MT, Kotta M-C, Dagradi F*, et al.* Clinical presentation of calmodulin mutations: the International Calmodulinopathy Registry. Eur Hear J. 2023;44:3357-70. doi: 10.1093/eurheartj/ehad418
5. Chazin WJ, Johnson CN. Calmodulin Mutations Associated with Heart Arrhythmia: A Status Report. Int J Mol Sci. 2020;21. doi: 10.3390/ijms21041418
6. Nimgampalle M, Chakravarthy H, Sharma S, Shree S, Bhat AR, Pradeepkiran JA*, et al.* Neurotransmitter systems in the etiology of major neurological disorders: Emerging insights and therapeutic implications. Ageing Res Rev. 2023;89:101994. doi: 10.1016/j.arr.2023.101994
7. Teleanu RI, Niculescu AG, Roza E, Vladacenco O, Grumezescu AM, Teleanu DM. Neurotransmitters-Key Factors in Neurological and Neurodegenerative Disorders of the Central Nervous System. Int J Mol Sci. 2022;23. doi: 10.3390/ijms23115954
8. Wei A, Jegla T, Salkoff L. Eight potassium channel families revealed by the C. elegans genome project. Neuropharmacology. 1996;35:805-29. doi: 10.1016/0028-3908(96)00126-8
9. Liu Q, Chen B, Yankova M, Morest DK, Maryon E, Hand AR*, et al.* Presynaptic ryanodine receptors are required for normal quantal size at the Caenorhabditis elegans neuromuscular junction. J Neurosci. 2005;25:6745-54. doi: 10.1523/JNEUROSCI.1730-05.2005
10. Chen B, Liu P, Hujber EJ, Li Y, Jorgensen EM, Wang ZW. AIP limits neurotransmitter release by inhibiting calcium bursts from the ryanodine receptor. Nat Commun. 2017;8:1380. doi: 10.1038/s41467-017-01704-z
11. Mueller BD, Merrill SA, Watanabe S, Liu P, Niu L, Singh A*, et al.* CaV1 and CaV2 calcium channels mediate the release of distinct pools of synaptic vesicles. Elife. 2023;12. doi: 10.7554/eLife.81407
12. Yap KL, Kim J, Truong K, Sherman M, Yuan T, Ikura M. Calmodulin Target Database. J Struct Funct Genom. 2000;1:8-14. doi: 10.1023/a:1011320027914
13. Shen X, Valencia CA, Szostak JW, Szostak J, Dong B, Liu R. Scanning the human proteome for calmodulin-binding proteins. Proceedings of the National Academy of Sciences. 2005;102:5969-74. doi: 10.1073/pnas.0407928102
14. O'Connell DJ, Bauer MC, O'Brien J, Johnson WM, Divizio CA, O'Kane SL*, et al.* Integrated Protein Array Screening and High Throughput Validation of 70 Novel Neural Calmodulin-binding Proteins*. Mol Cell Proteom. 2010;9:1118-32. doi: 10.1074/mcp.m900324-mcp200
15. Babu YS, Sack JS, Greenhough TJ, Bugg CE, Means AR, Cook WJ. Three-dimensional structure of calmodulin. Nature. 1985;315:37-40. doi: 10.1038/315037a0
16. Linse S, Helmersson A, Forsén S. Calcium binding to calmodulin and its globular domains. J Biol Chem. 1991;266:8050-4
17. Nyegaard M, Overgaard Michael T, Søndergaard Mads T, Vranas M, Behr Elijah R, Hildebrandt Lasse L*, et al.* Mutations in Calmodulin Cause Ventricular Tachycardia and Sudden Cardiac Death. Am J Hum Genet. 2012;91:703-12. doi: 10.1016/j.ajhg.2012.08.015
18. Jensen HH, Frantzen MT, Wesseltoft JL, Busuioc A-O, Møller KV, Brohus M*, et al.* Human calmodulin mutations cause arrhythmia and affect neuronal function in C. elegans. Hum Mol Genet. 2023;32:2068-83. doi: 10.1093/hmg/ddad042

19. Mahoney TR, Luo S, Nonet ML. Analysis of synaptic transmission in Caenorhabditis elegans using an aldicarb-sensitivity assay. Nat Protoc. 2006;1:1772-7. doi: 10.1038/nprot.2006.281

20. Davis AN, Tanis JE. Measuring Caenorhabditis elegans Sensitivity to the Acetylcholine Receptor Agonist Levamisole. J Vis Exp. 2022. doi: 10.3791/64056

21. Karabinos A, Bussing I, Schulze E, Wang J, Weber K, Schnabel R. Functional analysis of the single calmodulin gene in the nematode Caenorhabditis elegans by RNA interference and 4-D microscopy. Eur J Cell Biol. 2003;82:557-63. doi: 10.1078/0171-9335-00347

22. Marques F, Thapliyal S, Javer A, Shrestha P, Brown AEX, Glauser DA. Tissue-specific isoforms of the single C. elegans Ryanodine receptor gene unc-68 control specific functions. PLoS Genet. 2020;16:e1009102. doi: 10.1371/journal.pgen.1009102

23. Zhang L, Ward JD, Cheng Z, Dernburg AF. The auxin-inducible degradation (AID) system enables versatile conditional protein depletion in C. elegans. Development. 2015;142:4374-84. doi: 10.1242/dev.129635

24. Liu P, Chen B, Mailler R, Wang ZW. Antidromic-rectifying gap junctions amplify chemical transmission at functionally mixed electrical-chemical synapses. Nat Commun. 2017;8:14818. doi: 10.1038/ncomms14818

25. VanScyoc WS, Sorensen BR, Rusinova E, Laws WR, Ross JB, Shea MA. Calcium binding to calmodulin mutants monitored by domain-specific intrinsic phenylalanine and tyrosine fluorescence. Biophys J. 2002;83:2767-80. doi: 10.1016/S0006-3495(02)75286-7

26. Wen H, Levitan IB. Calmodulin is an auxiliary subunit of KCNQ2/3 potassium channels. J Neurosci. 2002;22:7991-8001. doi: 10.1523/JNEUROSCI.22-18-07991.2002

27. Ghosh S, Nunziato DA, Pitt GS. KCNQ1 assembly and function is blocked by long-QT syndrome mutations that disrupt interaction with calmodulin. Circulation research. 2006;98:1048-54. doi: 10.1161/01.RES.0000218863.44140.f2

28. Shamgar L, Ma L, Schmitt N, Haitin Y, Peretz A, Wiener R*, et al.* Calmodulin is essential for cardiac IKS channel gating and assembly: impaired function in long-QT mutations. Circulation research. 2006;98:1055-63. doi: 10.1161/01.RES.0000218979.40770.69

29. Kang PW, Westerlund AM, Shi J, White KM, Dou AK, Cui AH*, et al.* Calmodulin acts as a state-dependent switch to control a cardiac potassium channel opening. Sci Adv. 2020;6. doi: 10.1126/sciadv.abd6798

30. Alaimo A, Villarroel A. Calmodulin: A Multitasking Protein in Kv7.2 Potassium Channel Functions. Biomolecules. 2018;8. doi: 10.3390/biom8030057

31. Liu P, Chen B, Wang ZW. GABAergic motor neurons bias locomotor decision-making in C. elegans. Nat Commun. 2020;11:5076. doi: 10.1038/s41467-020-18893-9

32. Vassilakopoulou V, Calver BL, Thanassoulas A, Beck K, Hu H, Buntwal L*, et al.* Distinctive malfunctions of calmodulin mutations associated with heart RyR2-mediated arrhythmic disease. Biochim Biophys Acta (BBA) - Gen Subj. 2015;1850:2168-76. doi: 10.1016/j.bbagen.2015.07.001

33. Søndergaard MT, Liu Y, Brohus M, Guo W, Nani A, Carvajal C*, et al.* Diminished inhibition and facilitated activation of RyR2-mediated Ca2+ release is a common defect of arrhythmogenic calmodulin mutations. FEBS J. 2019;286:4554-78. doi: 10.1111/febs.14969

34. Søndergaard MT, Liu Y, Larsen KT, Nani A, Tian X, Holt C*, et al.* The Arrhythmogenic Calmodulin p.Phe142Leu Mutation Impairs C-domain Ca2+ Binding but Not Calmodulin-dependent Inhibition of the Cardiac Ryanodine Receptor. J Biol Chem. 2017;292:1385-95. doi: 10.1074/jbc.m116.766253

35. Crotti L, Johnson CN, Graf E, Ferrari GMD, Cuneo BF, Ovadia M*, et al.* Calmodulin Mutations Associated With Recurrent Cardiac Arrest in Infants. Circulation. 2013;127:1009-17. doi: 10.1161/circulationaha.112.001216