Electrophysiological studies of *eag* mutants with defective regulation by CaM and CaMKII

Master’s Thesis

Presented to

The Faculty of the Graduate School of Arts and Sciences
Brandeis University
Department of Biochemistry
Leslie Griffith, Advisor

In Partial Fulfillment of the Requirements for

Master’s Degree

By
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May 2013
Acknowledgements

Thank you Peter Bronk for guiding me through the projects, expanding my understanding about research, and encouraging me to try my best.

Thank you Leslie Griffith for advising me.

Thanks you Jessica Hutcheson for creating the T787A mutants.

This thesis would not be possible without your help.
Abstract

Electrophysiological studies of *eag* mutants with defective regulation by CaM and CaMKII

A thesis presented to the Biochemistry Department

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The *Drosophila melanogaster* ether-à-go-go (EAG) protein is a voltage gated potassium channel that has a C-terminal tail that binds proteins, such as Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and calmodulin (CaM). The interaction between EAG and CaMKII is involved in learning and regulating neuronal excitation (Wang et al., 1994). To study the interaction of EAG and CaMKII, point mutations inserted by homologous recombination into the native *eag* gene, T787A and F732S/F735S, were investigated in electrophysiological experiments. T787A eliminates the CaMKII phosphorylation target on EAG. F732S/F735S, blocks CaM binding at the C-terminus of
EAG. Excitatory junction potentials (EJPs) or action potentials were recorded in larval body wall muscles during 1) current injection, 2) fictive crawling motor pattern and 3) nerve stimulation. F732S/F735S was expected to have a decrease in neuron excitability based on a similar mutation in hEAG (Schonherr et al., 2000), while T787A was expected to have an increase in neuron excitability based on oocyte expression (Wang et al., 2002).

When action potentials were evoked by injecting current, the threshold of one line of F732S/F735S was significantly lower than its control, but the other line had lower, but not significantly lower, threshold. The half decay rate was higher for CaM8 than its three genetic controls, but the genetic control for T787A and F732S/F735S responded differently. The results in the current injection experiment were so far inconclusive. The blocked CaM binding site in EAG might increase neuron excitability and have stronger EAG current. In contrast to the controls, the motor patterns in F732S/F735S CaM mutants were not evoked after separating the ventral nerve cord from larval brain and subesophageal ganglion and adding pilocarpine. The increased excitability across the circuit might lead to increased suppression to compensate particular parts of the circuit. When the nerve was stimulated, there was no significant difference in the shape or number of EJP between the T787A mutant and its control. Electrophysiological studies were used to analyze F732S/F735S and T787A mutants, but more experiments are needed to characterize the interaction between EAG and CaMKII.
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Introduction

The *Drosophila* Ether-à-go-go (EAG) protein is a voltage-gated potassium channel subunit (Warmke et al., 1991) with an extended C-terminal tail containing several protein binding domains, such as Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and calmodulin (CaM) (Sun et al., 2004; Schonherr et al., 2000) (Figure 1). The *eag* mutant got its name due to a leg shaking phenotype similar to the “go-go” dance when ether is used for anesthesia. Flies with an inversion, *In(1)sc29*, having a breakpoint at the EAG locus display an electrophysiological defect characteristic of EAG mutants with spontaneous postsynaptic responses at the neuromuscular junction without stimulation (Drysdale, 1991). EAG recruits proteins to the membrane at synapses, which can contribute to learning and memory (Griffith et al., 1994).

**Figure 1: EAG C-terminus structure.** The EAG membrane protein has a C-terminus tail with domains to bind proteins, such as CaMKII and CaM (Schonherr et al., 2000). In the presence of Ca\(^{2+}\)/CaM, Eag and CaMKII bind together to activate CaMKII and CaMKII also activates Eag.
CaMKII is a conserved protein amongst vertebrates and invertebrates with a broad substrate specificity that can precisely regulate particular pathways. It also transmits calcium signals to many ion channels and controls neuronal excitability (Hudman and Schulman, 2002). Flies expressing a transgene inhibitor of CaMKII cannot maintain the effects of conditioning in both associative and non-associative learning in courtship behavior, which suggests that CaMKII is involved in learning (Griffith et al., 1993). The spontaneous activity at the neuromuscular junction of eag mutants was phenotypically similar to the ala CaMKII inhibitory peptide transgene, which shows that CaMKII might be a regulator of EAG (Griffith et al., 1994). When bound to EAG, CaMKII is close to the plasma membrane protein, which allows local signaling complexes of the neuron to independently regulate membrane and synaptic properties based on specific inputs to that region. This might be important in integrating synaptic activity that contributes to neuronal plasticity (Sun et al., 2004).

CaMKII might be involved in long term memory of previous cellular activity, because it can maintain an active or inactive state through auto-phosphorylation of nearby subunits (Hanson et al., 1994). After exciting a neuron, calcium binds to CaM, a calcium sensor. In the presence of Ca\(^{2+}/\text{CaM}\), the auto-inhibitory domain of CaMKII is displaced, and Eag and CaMKII bind together to activate CaMKII, which allows for both substrate phosphorylation and auto-phosphorylation (Sun et al., 2004). Long term activation of CaMKII independent of Ca\(^{2+}/\text{CaM}\) results from auto-phosphorylation of nearby subunits at T287, which allows the binding to continue even after Ca\(^{2+}\) level returns to normal (Fink and Meyer, 2002). Dephosphorylation depends on calcium oscillation and
phosphatases. Since the state is maintained even after calcium level returns to normal, this is proposed to be a molecular memory of previous cellular activity of Ca\(^{2+}\) influx.

While EAG activates CaMKII, CaMKII also regulates EAG activity and increases EAG channel conductance by phosphorylating T787 (Wang et al., 2002). The behavior and physiological phenotypes between *ala* flies and *eag* mutants were similar, which suggests that the function of EAG is regulated by CaMKII (Griffith et al., 1993; Griffith et al., 1994). When wild type T787 was phosphorylated by CaMKII, T787 increased EAG current amplitude in *Xenopus* oocytes (Wang et al., 2002). CaM binding to CaMKII affects phosphorylation of T787 and CaMKII and EAG binding, because this exposes the CaMKII catalytic domain that EAG binds (Sun et al., 2004).

Auto-phosphorylation of CaMKII regulates multiple neuronal functions including the intrinsic properties of neurons (Park et al., 2002). Repolarization following action potentials is mostly controlled by potassium currents. In the presence of potassium current inhibitors, there is no significant repolarization during current injection. Phosphorylation by CaMKII was shown to affect muscle action potentials. T287D had both calcium-dependent and -independent activity, while T287A had only calcium-dependent activity. T287D was less excitable than T287A, which led to lower potassium conductance, lower threshold and higher half decay rate of the action potential. The constitutively active form of CaMKII might help mediate homeostatic plasticity that prevents destabilization of neural networks.

CaMKII inhibition and Eag loss of function mutants have similar phenotypes in electrophysiology experiments (Griffith et al., 1994). While the wild type larvae exhibited one evoked junctional potential response per stimulus, CaMKII inhibition

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exhibited spontaneous junctional potentials, even long after the end of stimulation. This suggests CaMKII and Eag are required for repolarization of the nerve terminal after stimulation. Supernumerary discharges after 5 Hz stimulation were observed in eag and heat shocked ala flies, which expressed CaMKII inhibitory peptide transgene, but not CS, which indicates both CaMKII and EAG were required for repolarization (Griffith et al., 1994).

In order to study the interaction of EAG and CaMKII, the T787A and F732S/F735S eag mutants were created (Bronk and Hutcheson, unpublished). T787A eliminates a CaMKII phosphorylation target on EAG. T787A is expected to show hyperexcitability, because neuron repolarization after action potential is expected to be compromised. The other mutant, F732S/F735S, blocks CaM binding in the C-terminus of Drosophila EAG. F732 and F735 are located in the CaM-binding domain, which extends from residues 773 to 794 (Sun et al. 2004). When the Ca$^{2+}$/CaM complex binds to each hEAG potassium channel tetramer, current inhibition can be induced (Schonherr et al., 2000). Therefore, F732S/F735S is expected to be less excitable than wild type, because current inhibition might decrease when CaM binding is blocked.

These mutants had to be made using homologous recombination, a technique that allows the endogenous gene to be altered because the wild-type eag cDNA transgene failed to rescue the eag electrophysiology phenotype. These cDNA transgenes had reduced expression level compared to wild-type and were mis-localized (Bronk, unpublished observations). As an alternative, the eag mutants were made by knock-in mutations with homologous recombination into the endogenous eag locus. This ends-out homologous recombination used both FLP/FRT and Cre/LoxP systems to introduce point
mutations with minimal genome disruption (Figure 2) (Staber et al. 2011). This technique allowed the mutant protein to express under the control of the native promoter, retain the introns, and allow wild-type splicing, RNA-editing, and cellular trafficking. This mutant generation strategy avoided the possible problems that contributed to previous aberrant transgene expression, but did not allow tissue specificity in expression of the mutant protein. To reveal more about the EAG and CaMKII interaction in the T787A and F732S/F735S mutants, excitatory junction potentials (EJPs) or muscle action potentials were recorded in larval body wall muscles when 1) current was injected, 2) fictive crawling motor pattern was exhibited, and 3) nerve was stimulated.
Materials and Methods

Mutant Generation

The mutants were generated prior to my participation in the Griffith laboratory by Peter Bronk and Jessica Hutcheson (Figure 2). The mutations, T787A and F732S/F735S, were introduced into sequences (arm 1 or 2) spanning the CaMKII or Calmodulin binding domains respectively. These sequences were obtained by PCR of wild type genomic DNA and then cloned into a P[w25.2] vector. Arms 1 and 2 are homologous to the regions of the eag gene to promote recombination with endogenous eag gene. The P-element construct was randomly incorporated into the genome. Once candidate lines with correct integration of the homologous regions were confirmed, Cre recombinase excised the white+ marker and the fly lines were outcrossed. The controls for the mutants went through the same ends out homologous technique as the mutant except a mutation was not introduced in arm 2. The control for T787A was WT8.3, while the control for CaM 17 and CaM8 were WT12.6, WT8.7, and WT6.3. The different numbers after WT and CaM indicate independent fly lines derived from the same respective construct.
Figure 2: Mutant Generation. The mutation (star) was introduced in arm 2. Cre recombinase excised the white+ marker and outcrossing led to the mutant (Staber et al. 2011).

Biochemical Assay

Protein was extracted from 90 µL of fly heads, around one to two hundred fly heads, each for the T787A mutant, eag null line, and their controls, WT8.3 and f02960, respectively with homogenization and solubilization buffers. The eag protein was bound to 100 µL calmodulin beads from Amersham Biosciences and incubated at 4°C. The pellet was washed 3 times with a mixture of homogenization/2X solubilization buffer with 1 M CaCl₂. SDS-PAGE on an 8% acrylamide gel of the protein extracts and flow-through of the protein not binding to the beads was performed. The protein was visualized by primary antibody to EAG’s CaM binding domain and secondary anti-rabbit horseradish peroxidase.
**Electrophysiology**

For the current injection, motor pattern and nerve stimulation experiments, the larvae were dissected on the dorsal side to reveal the body wall muscles (Figure 3). EJP or action potentials of third instar larvae were recorded intracellularly by impaling the muscles with glass microelectrodes filled with 3 M KCl solution (tip resistance: 15-25 MΩ). Muscle 6 of abdominal segments 2-6 at room temperature were recorded.

**Current Injection**

The nerves were severed from the brain and the brain was removed from the larvae. Action potentials of muscles were recorded in hemolymph-like solution Modified A saline (Feng et al., 2004) with 2.4 mM CaCl₂ in current clamp. The resting potential of the muscle was held at -50 mV by adding or subtracting current. Action potentials were evoked by injection of 8 nA current ramp for 450 ms through the recording electrode. The threshold was determined at the inflection point.

Action potentials were also induced by injecting 3 steps of current 2.33, 4.67, and 7.00 nA for 450 ms each. The t1/2 of decay was calculated by measuring the time interval between the peak of the action potential and the point where the amplitude is half the full amplitude. Calculations were performed in Matlab scripts written by Bronk.

**Fictive Motor Patterns**

The ventral nerve cord with nerves still attached was severed from the larval brain and subesophageal ganglion (Figure 3). The preparation was perfused with 5 µM pilocarpine and 1 mM Ca²⁺ in HL3.1 1 minute after the brain was dissected. EJPs were
recorded with electrodes that were designed to be flexible to follow small muscle contractions without losing the recording (Cattaert et al., 2001).

**Nerve stimulation**

The nerves were severed from the brain and the brain was removed from the larvae. EJPs were recorded in HL3.1 with 0.8 mM CaCl$_2$. A suction pipette with a tip opening of about 10 µm was used to stimulate the segmental nerve (stimulation duration = 0.1 ms, strength= 10 V, rate = 40 Hz). Recordings with -60 mV resting potential were analyzed.

![Figure 3: Body Wall Muscles Recording](image)

**Figure 3: Body Wall Muscles Recording.** In motor pattern recording, the brain (not shown) was removed from the ventral nerve cord. The electrode recorded from body wall muscle 6. The current injection and nerve stimulation experiments had the same dissection except the ventral nerve cord was removed from the nerves (Figure created by Bronk).
Results

A Western blot was performed to determine if the full length EAG protein was present in the mutants and their controls (Figure 4). T787A and its control WT8.3 had two bands in the 150 to 160 kDa region. Since these bands run similarly to wild type EAG, CS (Figure 4B), it suggests that the EAG protein is present and looks like the full length protein. The two bands corresponding to EAG might be two splice variants or contain different post-translational modifications. The Δfull did not have a band, because the fly has a complete deletion of the eag gene. The genetic control for Δfull had one band, so it might only have one of the splice variant or be differently modified. This line is not a true wild type since it has the FRT element, which might also explain the occurrence of one band. In the flow-through, the absence of bands in the 150-160 kDa region suggests that there was no EAG and the purification was successful.
A) **Legend:**

- f = f02960, control for Δfull
- TA5.4 = T787A mutant
- WT8.3 = control for T787A mutant
- Δfull = full genetic deletion of eag
- fFT = flow through of control for Δfull
- TAFT = flow through of T787A mutant
- WTFT = flow through of WT8.3
- CS = Canton-S, wild type

**Figure 4: Western Blot.** A) T787A and WT8.3 had two bands in the 150 to 160 kDa region corresponding to EAG. The Δfull does not have a band and the control for Δfull had one band. In the flow-through, the bands in the 150-160 kDa region were absent. B) The Canton-S, wild type, displays 2 bands in the region corresponding to EAG. The CS band was provided by Bronk.

**Current Injection**

To investigate calcium dependent action potentials in F732S/F735S CaM mutant, action potentials were evoked by injecting current in a 8nA ramp (Figure 5A) or with 3 steps of 2.33, 4.67, and 7.00 nA (Figure 6A). In both variations of injecting current, one to three action potentials were generated in the 8nA ramp and 7 nA step 3 with no correlation to the genotype. The action potential was truncated at the end of the current injection at 450 ms. Using one way ANOVA and a posthoc Tukey-Kramer test, the threshold of CaM8 (4.97 +/- 0.24 mV) was significantly lower than its control WT12 (6.03 +/- 0.25 mV) and wild type w- (6.49 +/- 0.19 mV), but CaM 17 (5.55 +/- 0.23 mV) was
not. Since CaM8 and CaM17 both have the F732S/F735S mutation that blocked CaM binding in EAG, they are expected to produce the same response.

Figure 5: Current Injection in 8nA Ramp. A) Sample evoked action potential in WT12. B) Using the Tukey-Kramer test, the threshold of CaM8 was significantly lower than WT12 and W, but CaM 17 was not. The error bars represent standard deviation.
When a 7 nA step current was injected, F732S/F735S CaM8 was significantly different from its genetic controls, WT8.7, WT12.6, and WT6.3 (p=0.0003) (Methods). 7 nA step current injection was used to investigate the half decay rate because a consistent current was injected, which contrasted with the 8 nA ramp that injected a continuous increase in current, so the half decay time was not as reliable. In the 7 nA step current injection, the action potential did not form in step 1 (2.33 nA), started to appear as a bump in the step 2 (4.67 nA), and propagated in step 3 (7 nA) when enough current was injected to reach threshold (Figure 6A). The half decay rate of WT12 was different from CS, but less so from W, so different lines were used as genetic controls to check if there was something wrong with specifically WT12.6.1. Since all the alleles in the CaM control were similar in the lines, there must be a more general difference in genetic background. The half decay rates were very different for WT12.6 (21.0+/−7.9 ms), the genetic control for F732S/F735S, and WT8.3 (10.55+/−1.1 ms) (not shown), the genetic control for T787A, even though they went through the same process without the mutation and the loxP site should not be a contributing factor. WT8.3 should be more similar to the other genetic controls than CS, which had a different genetic background.
Figure 6: Current injection in 3 Steps up to 7 nA. A) Sample evoked action potential in step 2 (4.67 nA) and step 3 (7.00 nA). In step 2, the action potential started to form. One to three action potentials were formed in step 3. B) The half decay rate was significantly different between CaM8 F732S/F735S and its control, but not from W and CS. The error bars represented standard deviation.
To reveal potential explanations for the current injection experiment, results from whole cell patch clamp of motor neurons performed by Bronk are considered (Figure 7). The delayed calcium-dependent potassium currents were reduced. As reported (Gho and Mallart 1986), calcium-dependent potassium currents have a lower threshold than other potassium currents, so using voltage steps below -15 mV allows isolation of calcium-dependent currents. The responses are from slow calcium dependent currents instead of slowpoke current from calcium dependent potassium channel. The excitability of neurons between CaM mutant and its control was compared. The CaM mutants had higher spike frequency for each current step and potassium current density was reduced in the mutants per voltage step. These data indicates that the CaM mutant had increased excitability compared to the control. In muscles, the CaM17 mutant exhibits reduced slow calcium-dependent potassium currents.
Figure 7: Whole Cell Patch of Motor Neurons. A) Spike Frequency versus Current injection from whole cell patch of motor neurons in 1.8 mM Ca^{2+} (Bronk). The mutants were CaM 8 and the control is a combination of WT8.7.1 and WT12.6.1. The CaM mutants had higher spike frequency for each current step. B) Slow Ca-dependent currents from muscle. The mutants were CaM 17 and the controls were WT12.6.1. Potassium current density was reduced in the mutants at voltage steps above -35 mV (Bronk).
Motor pattern

To study behaviorally relevant physiology, the larval motor pattern was recorded. While the cellular physiology experiments reveal details of mutant phenotypes, it is useful to tie these phenotypes to a relevant behavior. At first, attempts to record normal motor patterns failed because the contractions were too strong and the electrodes popped out of the muscles even after trying many different types of electrodes. Experiments in larval crawling have demonstrated that rhythmic motor activities are controlled by central pattern generators (CPG) located in the ventral nerve cord ganglia (Johnston and Levine, 1996). Motor patterns disappeared after the central ganglions CGs were separated from the ventral cord (VC), but was partially restored when muscarinic agonist oxotremorine was applied after separation, which indicates activity was generated in the ventral nerve cord (Cattaert and Birman 2001) (Figure 8). As a solution to the strong muscle contractions, evoked junctional potentials were recorded after separating the CG from the VC and applying pilocarpine, a muscarinic receptor agonist, 1 minute afterward.
Figure 8: Motor pattern with oxotremorine. Motor pattern disappeared after central ganglions (CG) were separated from ventral cord (VC). However, if oxotremorine was applied after separation, motor pattern reappeared (Cattaert and Birman 2001).

The wild type flies exhibited motor patterns after the CG and VC were separated and pilocarpine was added, but the CaM mutants F732S/F735S did not (Figure 9). A combination of ten CaM8 and CaM17 mutants that were recorded over multiple days did not have rhythmic bursts. The recordings of CaM mutants and CS or WT12.6.1 were alternated. These conditions suggest that the phenotype was not by chance. The average spike per burst was similar in CS (22.1+/-9.7) and WT12.6.1 (22.1+/-8.9). The average spike frequency was higher in CS (11.0+/-1.7 Hz) than WT12.6.1 (7.3+/-1.9 Hz).
Figure 9: Motor pattern. A) The wild type flies exhibited motor pattern after the CG and VC were separated and pilocarpine was added, but the CaM mutants F732S/F735S did not. B) The average spike per burst was similar in CS and WT12.6.1. The average spike frequency was higher in CS than WT12.6.1.
Nerve Stimulation

In initial findings, TA5.4 mutants showed increased after-hyperpolarization compared to WT8.3 following nerve stimulation (4 muscles in TA5.4 and 3 in WT8.3). The nerve was stimulated at 40 Hz instead of 5 Hz, because 5 Hz was not enough to create at least one response per stimulation. A year later, the experiment was performed again on a bigger population size and the phenotype disappeared. There was no significant difference in the shape or number of EJPs when the nerve was stimulated among the T787A mutant TA5.4, WT8.3, MHC, and OK371. To determine if the EJP phenotype produced during nerve stimulation was caused by muscle or motor neuron, the responses were compared in larvae expressing the *ala* CaMKII inhibitory peptide (Griffith et al., 1993) in either muscles or motor neurons. The GAL4 fly line, MHC, drives expression of the *ala* transgene in muscle, and the OK371 Gal4 line drives expression of the *ala* transgene in motor neurons. If the CaMKII T787A mutant had a response more similar to MHC-GAL4 driven expression of *ala*, then the phenotype was caused by the mutant protein expression muscles. In contrast, if the mutant was more similar to OK371-GAL4 driven expression of *ala*, then the phenotype was caused by the expression in motor neurons. The reasoning was that if Eag was modified by CaMKII, multiple spikes will occur even after the stimulation stops.
Figure 10: Nerve stimulation. There was no significant difference in the shape or number of EJP when the nerve was stimulated among the T787A mutant TA5.4, WT8.3, MHC, and OK371. MHC and OK371 were used to determine if the response was caused by the muscle or neurons respectively.
Discussion

Current Injection

The results are so far inconclusive until genomic differences among the lines are examined. When action potentials were evoked by injecting current in an 8nA ramp, the threshold of F732S/F735S CaM8 is significantly lower than its control WT12 and wild type W, but CaM17 is not even though CaM8 and CaM17 have the same mutation (Figure 5). In the 7 nA step current injection experiment, the half decay rate is significantly higher for CaM8 than its three genetic controls (Figure 6). However, WT8.3, the genetic control for T787A, is more similar to CS than the genetic controls of F732S/F735S even though the controls went through the same ends-out homologous recombination process without the mutation. More experiments are required to investigate the genomic differences between the controls.

One possible interpretation is that the significantly lower threshold of CaM8 mutant compared to WT12 suggests that the mutation increases neuron excitability, which is opposite of the hEAG-based prediction. Current inhibition can be induced when Ca$^{2+}$/CaM complex binds to each of the hEAG potassium channel tetramer in *Xenopus* oocyte (Schonherr et al., 2000). This suggests that when CaM binding is blocked in EAG, the potassium currents will be less inhibited and this will lead to decreased excitability. The lower half decay rate of CaM8 compared to its genetic control indicates the muscle repolarizes faster and might suggest there are stronger EAG currents when CaM binding is blocked in the C-terminal of EAG. The increase in neuron excitability in CaM8 was
also supported by the motor neuron whole patch experiment, in which the CaM mutants had higher spike frequency for each current step and potassium current density was reduced in the mutants per voltage step (Bronk unpublished) (Figure 7).

The result might be opposite of the hEAG *Xenopus* oocytes experiment because hEAG in oocytes might behave differently from the channel expressed in its native environment, because real cells may have specific proteins that regulate the process. Another possible explanation is that EAGs in vertebrates and invertebrates are different. This is true for things such as the kinetics of channel activation. Compared to *Drosophila*, rEAG channel activation depends strongly on holding membrane potential; hyperpolarization slows down the kinetics of activation; conversely depolarization accelerates the kinetics of activation (Ludwig et al., 1994).

The results are so far inconclusive. The genetic differences between the lines need to be examined and more samples needs to be tested. Patch clamp of human embryonic kidney (HEK) cells expressing EAG with the CaM binding mutation can be performed to confirm effect of CaM on EAG currents.

**Motor Pattern**

The motor patterns in F732S/F735S CaM mutants were not created even after adding pilocarpine to the sectioned preparation, which contrasts with the controls. The motor rhythm generator is located in the ventral nerve cord, but requires an interaction with the brain to cause motor activity (Cattaert and Birman 2001). The pharmacologically induced rhythm was more irregular and disrupted than the control activity before separating the central ganglions from the ventral cord. Endogenous rhythm stopped when
CG and VC were separated, which suggests neural networks in the CG are needed for locomotor activity (Cattaert and Birman 2001). In CS and WT12, the CaM mutant control, rhythmic bursts reappeared after separating the brain and adding pilocarpine, which validates previous studies that the CPG is located in the VC (Figure 9).

The increased excitability across the circuit might lead to increased suppression to compensate in particular parts of the circuit, perhaps in the ventral nerve cord in the CaM mutants. The blocking of CaM binding in EAG might change the neuronal circuits to compensate for the defect. To reveal more about motor patterns in CaM binding mutants, possible experiments in the future include patch clamp of motor neurons to record motor patterns and K⁺ currents. Other mutants can also be analyzed with the same method to compare motor patterns.

**Nerve Stimulation**

The results are different from expected possibly because the mutants are expressed differently from the Griffith experiment. There is no significant difference in the shape or number of EJPs when the nerve was stimulated among the T787A mutant TA5.4, WT8.3, MHC, and OK371. To determine if the increase in after hyperpolarization in the mutant from initial findings is caused by the muscle or neurons, the MHC Gal 4 line, which drives expression of the *ala* transgene in muscle and the OK371 Gal4 line, which drives expression of the *ala* transgene in motor neurons, were compared with the CaMKII mutant.

Removing the phosphorylation of EAG in T787A mutant is expected to show an increase in activity in the muscle and nerve compared to the control. In the T787A mutant, EAG has no CaMKII phosphorylation site so EAG will not be activated. In T787A flies,
the mutated protein is expressed throughout development. In contrast, the *ala* flies with CaMKII inhibitory peptide transgene is expressed conditionally. Protein expression is temporally controlled through heat shock promoter, so inhibitory peptide can be expressed during a specific development stage.

If compensation for the T787A mutation masked the phenotype, the technique to generate the mutants can be modified, so protein expression can be induced when needed. Instead of using constitutive Cre, Cre recombinase fused to the ligand-binding domain of the human estrogen receptor (*UASP-cre-EBD*) regulated by estrogen (Heidmann and Lehner 2001) can be used to excise the white+ gene, which cause the mutant phenotype. The knock-out background can be rescued with wild-type or mutant EA\textsubscript{G} in specific cells with the conditional Cre transgenic. The flies will be fed food containing estrogen, which allows temporal control of protein expression. Using estrogen dependent Cre recombinase, *eag* expression can be induced right before recording, which is more similar to *ala* flies that are controlled by heat shock proteins. This might elicit the phenotype that was lost in the technique used.

Electrophysiological studies were used to analyze F732S/F735S and T787A mutants. From the current injection experiment, the blocked CaM binding site in EAG might increase neuron excitability and speed up repolarization, which is supported by results from whole cell patch clamp performed by Bronk. The absence of motor patterns in F732S/F735S CaM mutants with sectioned brains might be due to the increased excitability across the circuit that leads to increased suppression to compensate particular parts of the circuit. When the nerve was stimulated, compensation for the mutation might
mask the phenotype. More experiments are needed to characterize the interaction between EAG and CaMKII.
Bibliography


Appendix

Introduction

*D. melanogaster* uses a heart to circulate fluids. EAG potassium channels’ role in cardiac muscles could possibly reveal part of the mechanism in cardiac diseases. Pupae heart recording and optically counting heart rate demonstrated that *eag* mutant had an irregular heart and lower heart rate compared to the wild type (Figure 11) (Johnson et al., 1998). To study behaviorally relevant physiology, the action potentials in cardiac muscle of *eag* mutants and their controls were recorded.

![Canton S and eag heart recording](image)

**Figure 11: CS and eag heart recording.** The *eag* mutant had an irregular heart compared to the wild type. The heart rate was 2.68 Hz for CS and 1.65 Hz for *eag* (Johnson et al., 1998).

Materials and Methods

Third instar larvae were dissected on the ventral side since the heart is on the dorsal side (Figure 12). Cardiac action potentials were recorded intracellularly with glass microelectrodes filled with 3 M KCl solution (tip resistance: 15-25 MΩ). The posterior region of cardiac muscles was recorded in HL3.1 solution with 0.1 mM CaCl.
**Figure 12: Cardiac muscle dissection.** The electrode recorded the posterior portion of the heart (image modified by Bronk from Lavalee et al. 2006).

**Results**

The shape of the action potential was the same for the *eag* mutants and wild type. The heart rate was not significantly different among the *eag* mutants, *eag*Δfull (2.92+/−0.50 Hz) and T to A CaMKII mutant (2.47+/−0.18 Hz), and their respective controls, e03618 (2.655+/−0.0093 Hz) and CaMKII control (2.76+/−0.60 Hz). The half decay rate for *eag*Δfull (33.0+/−6.1 ms) was not significantly different from its control e03618 (28.9+/−9.3 ms). The half decay rates were similar for T787A CaMKII mutan (38+/−13 ms) and its control (39.12+/−13 ms).
A) Sample cardiac action potential. B) The heart rate and half decay rate were not significantly different between the eag mutants and wildtype.

**Discussion**

The eag mutants did not exhibit a strong heart phenotype (Figure 13). This contrasts with the pupae imaging experiment in which the eag mutants had a slower heart rate than the control (Johnson et al., 1998). Heart rate and action potential in third instar larvae that could crawl and pupae that were already immobilized might be different due to metamorphosis. Future intracellular recording could be performed in the cardiac muscles of larvae during the initial pupal stage.