The Effects of Kavalactones on Cholinergic Signaling and Acetylcholinesterase Activity in *Caenorhabditis elegans*

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Jessie Chappel

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CERTIFICATE OF APPROVAL

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY

Jessie Chappel

ENTITLED

The Effects of Kavalactones on Cholinergic Signaling and Acetylcholinesterase Activity in *Caenorhabditis elegans*

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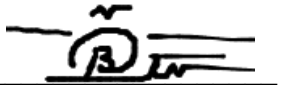
(Signed Signature) 

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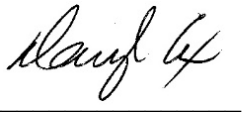
(Signed Signature) 

Dr. Daryl Cox

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Abstract:

Kava is a tranquilizing beverage from the South Pacific Islands that contains lipophilic compounds called kavalactones. Ingestion of these compounds is known to produce sedative and anxiolytic effects; however, the mechanisms underlying these changes are not fully elucidated. In our research studies at Greenville University we have shown that administration of kavalactones enhanced neuromuscular excitation in *C. elegans* nematodes, demonstrated by epileptic-like convulsions and paralysis in a dose-responsive manner. This response suggests an increase in acetylcholine (ACh) transmission at the neuromuscular junction (NMJ). Based on the behavioral responses and our genetic analysis in *C. elegans*, we hypothesized that kavalactones may mediate ACh transmission through the inhibition of acetylcholinesterase (AChE) enzyme. We tested this hypothesis by evaluating the responses of *C. elegans* worms harboring loss-of-function mutations in certain AChE genes. The findings discussed in this thesis seem to support the hypothesis that kavalactones affect the excitatory (cholinergic) system via AChE.

Acknowledgements

I first would like to thank Dr. Bwarenaba Kautu, who has helped me grow immensely both as a person and as a scientist during my time as an undergrad. Working on this project has revealed to me my passion for my research, and I am extremely grateful for his guidance. Next I would like to thank Dr. Daryl Cox who has always been more than ready to listen and answer my innumerable questions and push me out of my comfort zone to pursue new opportunities. I would also like to thank Dr. Eric Nord for his advice and guidance while trying to navigate my way into the computational world. I could not have asked for a better people to teach me over the past 4 years and serve as my thesis committee. Additionally, I would like to thank the rest of the faculty and students in the biology and chemistry departments, all of whom have pushed me to learn and helped make my 4 years at GU very memorable. Lastly, I would like to thank my family for always supporting me and taking interest in my education.

Chapter 1: Introduction

In this thesis, I will investigate the neurological effects induced in the model organism *C. elegans* following treatment with kavalactones, the active chemical constituent present in the South Pacific plant kava. Previous studies have demonstrated that treatment with kavalactones results in overexcitation at the worm's neuromuscular junction, indicative of increased modulation of acetylcholine. In this study, I provide further evidence of this increase, as well as provide a possible mechanism by examining the interaction between kavalactones and acetylcholinesterase. These findings may implicate potential medicinal uses of kavalactones, as many neurological disorders are marked by changed acetylcholine transmission.

1.1 Background of *C. elegans*

The concept of using nematodes as a model organism was first suggested by Sydney Brenner in 1963 (Corsi et al 2015). Concerned that classical problems in molecular biology would be solved within the next decade, he believed the future of the field would be reliant upon extension to other fields, "notably development and the nervous system" (Brenner 1988; Brenner 2002). Originally focused on the nematode *Caenorhabditis briggsae,* he later decided *Caenorhabditis elegans* would be a more suitable model because of the ease with which they grew in the laboratory (Felix 2008). *C. elegans* is now studied in over a thousand laboratories on a global scale (Corsi et al 2015).

C. elegans are free-living and can be found worldwide. Larval worms hatch with a length of approximately 0.25mm and grow to be approximately 1mm long as they reach

adulthood (Corsi et al 2015). Due to their small size, *C. elegans* are typically observed using either a dissecting or compound microscope, which offer up to 100x and 1000x magnification, respectively. Because *C. elegans* is transparent, microscopy techniques can be used to visualize individual cells and subcellular details. Further, fluorescent proteins can be used to tag proteins or other cellular components, aiding in studies of development and function (Chalfie et al. 1994; Boulin et al. 2006; Feinberg et al. 2008).

In addition to multiple to visualization techniques, *C. elegans* have several other characteristics that make them an ideal model organism. Under constant temperature conditions (25^{o}C), a *C. elegans* egg will take approximately 3 days to hatch and grow into a sexually mature adult. *C. elegans* occur mostly commonly as self-fertilizing hermaphrodites, with males occurring at a frequency of less than 0.2%. This high-pace reproductive cycle makes them ideal for eukaryotic genetic studies. Additionally, *C. elegans* have an invariant number of somatic cells, allowing researchers to track individual cells throughout its life-cycle (Sulston and Horvitz 1977; Kimble and Hirsh 1979; Sulston et al. 1983). Using electron micrographs, each of the 302 neurons present in hermaphroditic adult worms have been mapped, leading to the development of one of the most complete "wiring diagrams" of any nervous system (White et al. 2013). *C. elegans* was also the first multicellular organism to have its genome completely sequenced, and through forward and reverse genetics, many molecular, developmental, and cellular processes have been characterized (*C. elegans* Sequencing Consortium 1998).

Conservation of cellular and molecular processes (metabolism, cellular structure, gene function, etc.) in *C. elegans* has made them useful for studying general metazoan

biology. When comparing the *C. elegans* and human genomes, it has been noted that at least 38% of *C. elegans* protein-coding genes have predicted orthologs in the human genome (Shaye and Greenwald 2011), approximately 60-80% of human genes have an ortholog in the *C. elegans* genome (Kaletta and Hengartner), and 40% of genes associated with human diseases have orthologs in the *C. elegans* genome (Culetto and Satelle 2000). These similarities indicate the potential *C. elegans* research has the potential to impact understanding of human health and disease.

1.2 Growth and Maintenance of *C. elegans*

In their natural habitat, *C. elegans* are commonly found in rotting vegetable matter, which is a dense source of bacterial food (Barrière and Félix 2014). To mimic this setting in the laboratory, worms are grown on agar plates seeded with *Escheria coli.* After this food has been consumed, larval stage worms halt development and remain stagnant for up to a month. To preserve worms longer, storage of the stocks at 15ºC may allow worms to persist for up to 6 months. To resume development, a strip of agar from the depleted plate can be transferred to a new plate seeded with fresh bacteria.

C. elegans have several characteristics that allow stocks to be easily maintained. Because a vast majority of worms are self-fertilizing hermaphrodites, a single worm can be used to populate a plate. Freezing protocols can allow these populations to be stored for several years before being used. Because their growth is temperature dependent, researchers can alter reproduction rates by storing worms in temperatures ranging from 12ºC to 25ºC. Reproduction rates have been observed to increase with temperature, decontaminate and synchronize worms, bleaching protocols can be utilized, which kills everything except embryos.

1.3 Life Cycles of *C. elegans*

C. elegans have four distinct larval stages before reaching adulthood. When stored at 20ºC, embryogenesis takes approximately 16 hours (Corsi et al 2015). Embryos grow within the hermaphrodite until reaching the 24-cell stage, at which point the egg is laid to continue developing independently. When the embryo has approximately 588 nuclei, it will hatch and become and a first stage (L1) larva. It will remain in this stage for approximately 16 hours before entering a period of inactivity called lethargus, during which its cuticle will molt, and a new cuticle will form. Lethargus occurs between each transition into a new life-stage. The worm will then spend 12 hours each in the L2, L3, and L4 life-stage. After exiting the L4 stage, the worm is now considered an adult. During the first 2-3 days of adulthood, the hermaphrodite will begin producing progeny until its sperm is depleted; a worm can continue reproducing by mating with a male. Under normal conditions, adult worms will survive a few more weeks after laying eggs before dying. Alternatively, if food sources are limited and the plate is overpopulated, worms may enter an L3 stage called the dauer larva. During this stage, worms will develop a protective cuticle that helps them combat environmental stressors. A worm can remain in the dauer stage for several months. Once the worm is provided with food, it will continue its development and move to the L4 stage (Corsi et al 2015).

1.4: Genetic Characterization of *C. elegans*

One primary reason for the adoption of *C. elegans* as a model organism is because of their genetic amenability. Because *C. elegans* are self-fertilized, mutant alleles can be maintained throughout generations via self-propagating. Additionally, the integrity of these strains can be maintained by freezing populations during periods of disuse, ensuring they won't acquire unwanted suppressors, modifiers, or additional mutations.

Traditionally, mutant strains were identified using forward genetics. This process begins by selecting mutant worms presenting a certain phenotype, and then subsequently identifying the role of the mutated gene by comparing it to the wild-type (Brenner 1974). Once mutant strains have been isolated and demonstrated that they are true-breeding, they can be mapped via classical genetic tools to identify the genotype. While previously this process may have taken up to a year to accomplish, the rate at which this can be done has been expedited significantly due to tools such as whole-genome sequencing. Advances in genome-editing tools, such as CRISPR/Cas9, has also greatly improved the efficiency at which mutant strains can be produced (Corsi et al 2015).

1.5 Movement of *C. elegans*

Deep to the epidermis, *C. elegans* have four quadrants of body-wall muscles that span the length of the body. Patterns of relaxation and contraction of these muscles results in the sinusoidal movement observed in the worms. These muscles are striated and mononucleate with multiple sarcomeres per cell (Moerman and Fire 1997). Innervation of these muscles is accomplished by muscles cells sending extensions to the ventral and dorsal cord, where they receive *en passant* (side by side as neurites pass each other) synapses from motor neurons (White et al. 1986). Along with body-wall muscles, *C. elegans* also have pharyngeal muscles for eating, vulval and uterine muscles and the contractile gonad sheath for egg-laying, enteric muscles for waste excretion, and, in males, tail muscles for mating (Corsi et al 2015).

1.6 The *C. elegans* Nervous System

In adult hermaphrodites, the nervous system is comprised of 302 neurons (Sulston and Horvitz 1977; White et al. 1986). These neuronal cell bodies are primary located in a few ganglia in the head, in the ventral cord, and in the tail. A majority of these neurons have only one or two neurites, except for mechanosensory neurons, which are known to have many branched neurites (Dong et al. 2013). With the exception of sensory dendrites, most neurites in the nervous system cannot be identified as either dendrites or axons, as most processes both send and receives synapses. The nervous system also contains glia-like support cells, which are mainly associated with sensory neurons (Oikonomou and Shaham 2010). Nerve conduction is a primarily passive process and is not dependent on action potentials (Lockery and Goodman 2009).

Altogether, *C. elegans* neurons make more than 7000 chemical synapses, approximately 2000 of which are neuromuscular junctions (White et al. 1986). A distinct characteristic of nematodes is that their muscles send cellular projections to motor neurons to receive synapses, rather than vice versa. In concert with many other organisms, *C. elegans* utilize some of the most common neurotransmitters, including acetylcholine, glutamate, γ-amino butyric acid (GABA), dopamine, and serotonin. However, *C. elegans* are not limited to chemical synaptic and gap junction connections, but also use a number of neuroendocrine signals to modulate neurons (Li and Kim 2008). In contrast with vertebrates, many functions are controlled by a single neuron. The observed multifunctionality is thought to be an evolutionary consequence of the relatively small number of neurons present in the nervous system.

1.7 Acetylcholine Transmission in *C. elegans*

Acetylcholine (ACh) was first discovered in *Ascaris* and other nematodes in 1955 (Mellanby 1955) and was subsequently shown to be an excitatory transmitter at the nematodes neuromuscular junction (NMJ) (del Castillo et al., 1963; del Castillo et al., 1967). The molecule is synthesized by the action of choline acetyltransferase (ChAT) and is then loaded into synaptic vesicles via vesicular acetylcholine transporters (VACht) (Rand 2007). When the vesicle moves into the cytoplasm, the pH gradient acidifies the vesicle lumen, allowing ACh to diffuse within the synaptic cleft and bind to acetylcholine receptors located on post-synaptic cells. In contrast with most other neurotransmitters, the action of acetylcholine is terminated via hydrolysis by acetylcholinesterase (AChE), rather than being removed by a transporter. The resulting choline is then transported back to the presynaptic neuron by a choline transporter, where it can be used to synthesize more ACh (Rand 2007).

ACh mediates a number of behaviors in *C. elegans*, including egg laying, pharyngeal pumping, defecation cycling, male mating, and, most prominently, locomotion (Rand 2007). Locomotion of the worm (crawling on surfaces and swimming through liquids) requires the highest number of cholinergic neurons to execute. To accomplish the smooth, sinusoidal motion that is observed, worms must generate muscle contractions at one side of the body, while relaxing muscles on the opposite side of the body (Jorgenson 2005); During muscle contraction, cholinergic motor neurons send signals to the ventral/dorsal body muscles and GABA to motor neurons. This results in acetylcholine release on one side of the body, which causes muscle contraction, and stimulates the release of GABA onto muscles on the opposite side, which inhibits

contraction (or causes relaxation) (Jorgenson 2005). The antagonistic relationship between these two neurotransmitters results in sinusoidal locomotion.

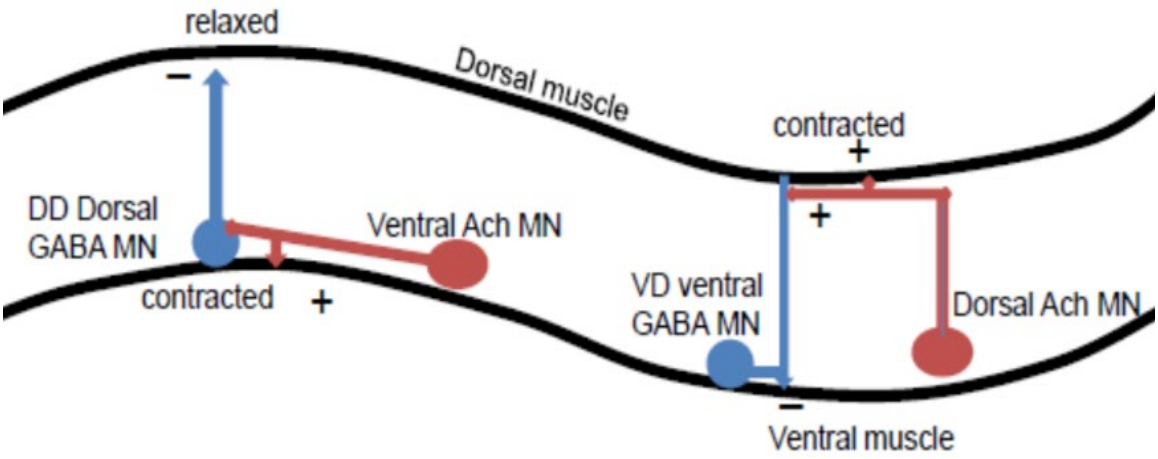

Figure 1.1: The inhibitory-excitatory balance at *C. elegans* NMJ is controlled by the transmission of acetylcholine (red), which causes muscle contraction, and GABA (blue), which causes muscle relaxation.

(Jorgenson 2005)

ACh signaling is also thought to play a role in protein homeostasis in post-synaptic cells. More specifically, worms with cholinergic hyperstimulation have been shown to form premature polyglutamine aggregates in body wall muscle cells (Garcia et al 2007). These aggregates are thought to be the result of overstimulation of post-synaptic cells, which interferes with the cell's folding capacity and results in protein misfolding and aggregation. This finding may be useful in understanding neurodegenerative and neuromuscular disease.

1.8 Expression and Action of Acetylcholinesterase in *C. elegans*

The expression of acetylcholinesterase, the enzyme responsible for the termination of cholinergic nerve transmission, is linked to four genes: *ace-1, ace-2, ace-3,* and *ace-4*, which produce the proteins ACE-1, ACE-2, ACE-3, and ACE-4, respectively. (Johnson et al., 1981; Culotti et al., 1981; Kolson and Russell, 1985; Combes et al., 2000). These enzymes are separated into three separate classes (excluding ACE-4*,* which is not thought to be enzymatically active) based on properties such as substrate affinity, inhibitor specificities, and detergent specificities and differ most notably in their cellular expression pattern (Johnson and Russel, 1983; Kolson and Russel, 1985). Mosaic analysis demonstrated that *ace-1* is primarily expressed in muscle cells, with little to no expression in neurons (Herman and Kari, 1985). *ace-2* is expressed mainly in motoneurons, as well as in the pharyngeal muscles (Combes et al., 2003). *ace-3* comprises very little of the overall AChE activity and is expressed in very few cells (Stern, 1986). However, it does produce enough activity to sustain mutants with *ace*-1 and *ace-2* knock-outs. *ace-4* is transcribed, but the enzyme is not detected *in vivo* (Combes et al 2000).

Worms with a loss-of-function mutation in one of these four genes will be phenotypically similar to the wild-type, but may demonstrate sensitivity to AChE inhibitors, such as aldicarb (Johnson et al., 1981; Culotti et al., 1981; Johnson et al., 1988). Because *ace-1* and *ace-2* are responsible for a majority of AChE's expression, this double mutant is relatively uncoordinated; double mutants with any other combination of the four genes will be behave essentially wild-type (Culotti et al. 1981). The *ace-1; ace-2; ace-3* triple mutant has been shown to be lethal. While embryonic development

appears to occur normally, worms either do not hatch or are paralyzed and developmentally arrested (Johnson et al. 1988).

To evaluate worms with the "Ric" (Resistance to Inhibitors of Cholinesterase) phenotype, the carbamate acetylcholinesterase inhibitor aldicarb has risen as the top reagent for experimentation (Rand, 2007). When inhibition of AChE occurs, the immediate result is a buildup of synaptic ACh. This results in muscle hypercontraction and eventual paralysis due to overexcitation a the NMJ (Rand, 2007).

1.9 Cultural Use of Kava

Kava (*Piper methysticum)* is a small, shrubby plant indigenous to the South Pacific. The plant is consumed (typically in the form of a beverage) in most oceanic cultures in both formal and informal settings and is known to have sedative, anxiolytic, and analgesic properties. (Cox and O'Rourke 1987). While formal uses of kava may differ between cultures, one common theme is the use of kava as a gift while travelling. Travelers may be greeted with kava upon arrival to a village, or travelers may bring kava as an offering. These formal presentations of kava often include some type of ornate rhetoric and ceremony. Informal consumption of kava may occur as a gathering around a kava bowl after work or for get-togethers with friends and family. One may also go to kava bars to consume kava in a more organized setting.

To prepare a kava beverage, the roots or rhizomes are first macerated and then mixed with waters. In some cultures, esteemed individuals, such as the *taupou* (village virgin), may masticate the kava prior to mixing, increasing the significance of the drink. The size of the leaf may also impact the significance of the drink. In Samoan cultures, large rhizomes with the attached root, known as *lupesina*, are given as an honorable gift and is not intended for ingestion. Typically, very small roots or small pieces of rhizome are used to prepare the beverage. Preparation of the drink may also differ depending on if fresh or dried materials are used (Cox and O'Rourke 1987).

1.10 Chemical Composition of Kava

To date, over 40 compounds have been isolated from kava. Active components have been shown to be present in lipid-soluble resin and contains three chemical classes: arylethylene-α-pyrones, chalcones and other flavones, and conjugate diene ketones (Fu et al., 2008). 4-methoxy-5, 6-dihydro-α-pyrones or kavapyrones, also known as kavalactones, are known to be produce the greatest anxiolytic effects (Rowe 2011). Kavalactones comprise approximately 3%-20% dry weight of the kava plant and at least eighteen distinct compounds have been identified from the root. Approximately 95% of the organic extract is accounted for by 6 of these kavalactones, including kavain, dihydrokavain, methysticin, dihydromethysticin, yangonin, and desmethoxyyangonin. When conducting HPLC (high-performance liquid chromatography) on these compounds, individual strains can be identified, yielding over 200 variants of kava (Rowe 2011).

Chapter 2: Materials and Methods

Worm strains and maintenance. The worm strains were cultured and maintained according to standard procedures (Appendix 1) (Brenner 1974).

Kavalactone assays: These methods are described extensively in the *Bioprotocol* manuscript (chapter 3).

Aldicarb paralysis assay: The plates were made according to the protocol described by Locke and his colleagues (Appendix 2) (Locke 2008).

Statistical analysis: This was performed in R. The methods used are described extensively in the manuscript.

Chapter 3: *BioProtocol* Manuscript

The following is a manuscript that was published in the journal *BioProtocol* on September 20th, 2018. The author of this thesis, Jessie Chappel, is the co-first author on this paper. The following is the citation of the manuscript:

Kautu, B. B., Chappel, J., Steele, K., Phillips, J. and Mengarelli, M. S. (2018). A Behavioral Assay to Examine the Effects of Kavalactones on *Caenorhabditis elegans* Neuromuscular Excitability. *Bio-protocol* 8(18): e3008.

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A Behavioral Assay to Examine the Effects of Kavalactones on *Caenorhabditis elegans* Neuromuscular Excitability

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Abstract: Kavalactones are a class of lactone compounds found in kava, a traditional beverage from the South Pacific Islands that is derived from the root of *Piper methysticum*. When consumed, these compounds produce sedative and anxiolytic effects, suggesting their potent actions on the nervous system. Here, we provide a protocol to examine the effects of kavalactones on *C. elegans* neuromuscular excitability. Our methodology could provide insight into the neurophysiological actions of kavalactones.

Keywords: Kava, Kavalactones, Lactone, *C. elegans*, Neuromuscular, Pacific, Beverage

Background

Kava, a tranquilizing beverage from the Pacific Islands, has been consumed by Pacific Islanders for centuries (Rowe et al., 2011; Kautu et al, 2017). Kava contains a group of lipophilic compounds called kavalactones, which are believed to be responsible for the sedative, anxiolytic, and other therapeutic effects of the drink (Rowe et al, 2011; Savage et al, 2015; Kautu et al, 2017). Here, we provide a protocol to examine the effects of kavalactones on neuromuscular activity, using *C. elegans* as a model system (Kautu et al., 2017). In our assay, we showed that administration of aqueous kavalactone solution induced epileptic-like convulsions and paralysis in a dose-responsive manner. These manifestations are suggestive of the modulatory actions of kavalactones on the neuromuscular junction. Thus, our protocol could provide important insight into the neurophysiological actions of kavalactones.

Materials and Reagents:

1. Petri dishes, sterile (Carolina Biological Supply, catalog number: 741248)
2. 2 mL centrifuge tubes (Thomas Scientific, catalog number: 111572LK)
3. P1000 pipette tips, sterile (Carolina Biological Supply, catalog number: 215060)
4. P200 pipette tips, sterile (Carolina Biological Supply, catalog number: 215050)
5. 0.5 L bottle
6. *E. coli* OP50 (Carolina Biological Supply, catalog number: 155073)
7. *C. elegans* N2 (wild-type)
8. Kava pills (Gaia Herbs, catalog number: 90A10060)
9. NaCl (Sigma-Aldrich, CAS: 7647-14-15)
10. Peptone (Sigma-Aldrich, catalog number: 70176-100G)
11. Difco Bacto agar (Carolina Biological Supply, catalog number: 156783B)
12. $CaCl_2$ dihydrate (Fisher Scientific, catalog number: C79 500)
13. $MgSO_4$ (Sigma-Aldrich, CAS: 7487-88-9)
14. Potassium Phosphate Monobasic (KH_2PO_4) (Sigma-Aldrich, CAS: 7778-77-0)
15. Potassium Phosphate Dibasic (K_2HPO_4) (Sigma-Aldrich, CAS: 7758-11-4)
16. Cholesterol (Fisher Science Education, CAS: 57-88-5)
17. Hypochlorite solution (Sigma-Aldrich, CAS: 7681-52-9)
18. NaOH (Sigma-Aldrich, CAS: 1310-73-2)
19. 95% ethanol (Sigma-Aldrich, CAS: 64-47-5)
20. 1 M $CaCl_2$ stock solution (see Recipes)
21. 1 M $MgSO_4$ stock solution (see Recipes)
22. 1 M Potassium Phosphate stock solution (pH 6) (see Recipes)
23. 5 mg/mL Cholesterol stock (see Recipes)

24. Nematode Growth Medium (NGM) Agar plates (see Recipes)

25. Bleach sodium hypochlorite solution (see Recipes)

26. Kava stock solution (5 mg/mL) (see Recipes)

Equipment:

1. P200 and P1000 micropipette
2. Sharp Edged Scissors
3. Autoclave
4. Bench-top Micro Centrifuge (Oxford Lab Products, model: C12V)
5. Meiji EMT Stereomicroscope on PBH Stand (Meiji Techno, models: EMT-1 , PBH Stan d, MA502)
6. Microscope Digital Camera (OMAX Microscope, catalog number: A3550UPA-R75)
7. Platinum wire worm pick (Genesee Scientific, catalog number: 59-AWP)
8. Sterile incubator

Software

1. OMAX ToupView 3.7
2. Microsoft Office 2010 Excel (Microsoft Corporation, Redmond, USA)

Procedure:

A. Synchronization of *C. elegans* via sodium hypochlorite treatment

1. First, allow gravid adult N2 (wild-type) worms to grow at 20 °C and lay embryos on NGM plates seeded with *E. coli* OP50 strain (Brenner, 1974). Once there is a sufficient number of embryos to be used for the assays, the worm stages should be collected, washed, and synchronized. To collect worms, use a P1000 micropipette to dispense 1,000 µl of DI water onto the plate to pool the worms and embryos into one spot. Pipette the water containing the worms into a 2 mL microcentrifuge tube and centrifuge at room temperature for 1 min at 7,558 *x g*. Carefully pipette off the excess liquid without disturbing the worm pellet.

   Note: To avoid strain loss, a few worms should be transferred to a seeded NGM plate prior to collection and synchronization; all worms from this point on will be used in the assay.
2. Prior to synchronizing the worms, dilute 20% stock of bleach sodium hypochlorite solution to 10-15% using distilled water. Synchronize the worms by incubating them in the diluted bleach sodium hypochlorite solution (10-15%) for 3-5 min.
3. Centrifuge the tube at room temperature for 30 sec at 7,558 *x g*. Carefully remove excess bleach sodium hypochlorite solution by pipetting. Resuspend the pellet using 500 µl of DI water. Centrifuge at room temperature for another minute at 7,558 *x g*. Remove excess DI water, leaving the pellet intact.

   Note: Do not incubate the worms for more than 5 min, including time in the centrifuge. Also, we found that carryover bacteria does not interfere with the assay. However, an excessive amount of carryover bacteria must be avoided. An

experimenter can do this by controlling the amount of bacteria that is initially seeded on the plate. In general, we seed 100 x 200 mm NGM plates with 150-200 µl of OP50 bacteria.

4. Collect the embryos and the dead worms from the microcentrifuge tube using a P200 or P1000 micropipette and transfer them to a new NGM plate seeded with OP50 bacteria. It is best to transfer the dead worms and the embryos to the edge of the seeded plate not covered with OP50 bacteria.
5. Allow the embryos to hatch and grow until the L4 to young adult, stage. This will take approximately two days.

B. Treatment of worms with aqueous kavalactone solutions

1. Transfer L4 to young adult worms to 2 mL microcentrifuge tubes. This can be done using the same method of washing and collecting described previously.
2. Prepare 0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL and 1.0 mg/mL kavalactone solutions by diluting the stock solution (5 mg/mL) with distilled water.
3. Suspend worms in 500 µl of the prepared kavalactone concentrations (above).

   Note: Our kavalactone solution was made using a kavalactone supplement purchased from Gaia Herbs, Inc. (Brevard, NC, USA). Stocks of kavalactone solutions can be stored for up to 2 months at 4 °C. It is important to shake and mix the kavalactone solution well before using it.

4. Incubate the worms in the pre-warmed kavalactone solution (in the 2 mL microcentrifuge tubes) for 30 min at room temperature. During incubation gently invert the tubes 5-8 times. Do this 5 times during the 30-min time span.
5. Following the 30-min incubation, centrifuge the tubes at room temperature for 2 min at approximately 7,558 *x g*.
6. Remove excess solution, while leaving the pellet of worms intact.
7. Rinse the worms with 200 µl of distilled water, resuspending the pellet, and centrifuge again at room temperature for an additional 2 min at 7,558 *x g*.
8. Remove the excess liquid, leaving a sufficient amount to transfer the worms. Using a P200 or P1000 micropipette, transfer approximately 40 worms from the kavalactone solution to new, dry 60 mm NGM plates without *E. coli* bacteria.
   Note: Worms can be lost during the resuspension/transfer process, so it's a good idea to start with a higher number of worms than necessary for the assay to account for loss.

C. Scoring worms for convulsions and paralysis

1. Use a dissecting/stereo microscope to count the worms as they are being transferred to the clean 60 mm NGM plate.
2. Allow the worms to acclimate to the new environment for 15 min before scoring. Here, the experimenter can spread out the worms using a worm pick to prevent them from clumping together in one spot.

3. When scoring, observe the worms for convulsions and paralysis. The convulsions manifest as full body repetitive muscle contractions and paralysis. Paralysis is characterized as no visible movement upon observation. Since many convulsing worms progressively become paralyzed over the course of time, we treated convulsions and paralysis as one variable in this particular assay (see Figure 1).

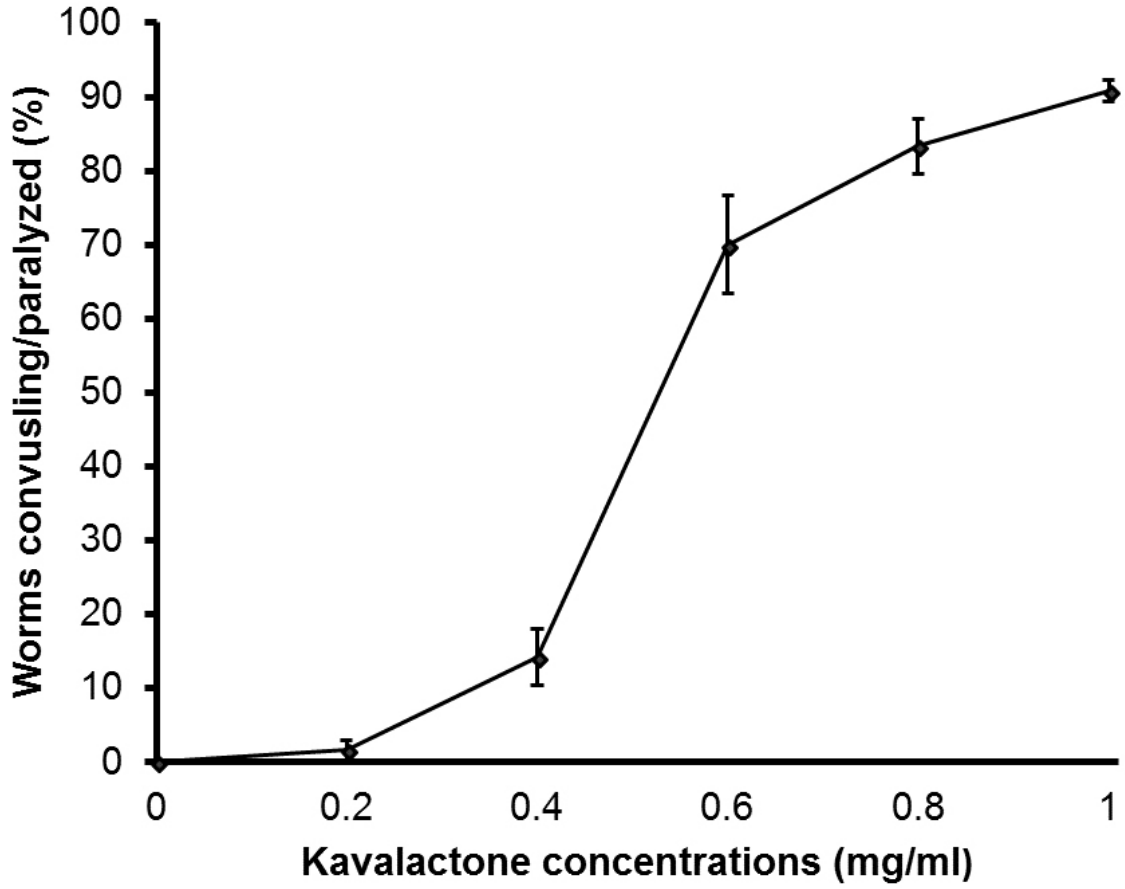

Figure 3.1: A dose-response curve of Kavalactone-induced convulsions and paralysis in *C. elegans* N2 (wild-type) worms. Three independent experiments were performed at each concentration and the response levels of the worms were averaged and converted to percent. For each experiment (at each concentration) 40 worms were scored (n = 120). At 0 mg/mL, *C. elegans* worms were dissolved in DI water without kavalactones (negative control). All error bars indicate standard deviations (SD) for the 3 averaged experiments.

4. Record movies of convulsing and paralyzed worms after kavalactone treatment for a minimum of 30 seconds, using a suitable video camera or device:

Video 1. A wild-type N2 worm exhibiting strong full body convulsions (repetitive anterior-posterior muscle contractions) when treated with 1 mg/mL kavalactone aqueous solution:

Video 2. A wild-type N2 worm showing progression from convulsion to full body paralysis when exposed to 1 mg/mL kavalactone aqueous solution

Video 3. A wild-type N2 worm showing full body paralysis when treated with 1 mg/mL kavalactone aqueous solution

Link to videos: https://bio-protocol.org/e3008

Data analysis

We calculated percent averages and standard deviations for all experiments using Microsoft Excel (2010 version) (Microsoft Corporation, Redmond, USA).

Notes

This protocol can be adapted for other *C. elegans* mutants of interest.

Recipes

1. 1 M $CaCl_2$ stock solution

   Dissolve 7.35 g of $CaCl_2$ in 50 mL of DI H_2O

   Autoclave for 30 min at 121 °C

   Store at room temperature
2. 1 M $MgSO_4$ stock solution

   Dissolve 6.02 g of $MgSO_4$ in 50 mL of DI H_2O

   Autoclave for 30 min at 121 °C

   Store at room temperature
3. 1 M Potassium Phosphate (pH 6) stock solution

   Dissolve 35.6 g of K_2HPO_4 and 108.3 g of KH_2PO_4 in 1 L of DI water

   Autoclave for 30 min at 121 °C

   Store at room temperature
4. 5 mg/mL Cholesterol stock

   Add 250 mg of cholesterol to 50 mL 95% ethanol

Mix on a rotator until fully dissolved

Store the cholesterol solution at room temperature

5. Nematode Growth Medium (NGM) Agar plates-recipes

   Add 1.2 g of NaCl, 1.0 g of peptone, and 6.8 g of Difco Bacto agar to 400 mL of DI H_2O in a 0.5-L bottle

   Autoclave for 25 min at 121 °C

   Allow media to cool to approximately 55-60 °C, then add 400 µl of 1 M $CaCl_2$, 400 µl of 1 M $MgSO_4$, 10 mL of potassium phosphate (pH 6), and 400 µl of 5 mg/mL cholesterol

   Mix gently after adding each component

   Store plates in a sterile 20^{oC} incubator.

6. Sodium hypochlorite solution

   Combine 8.25 mL of DI H_2O, 3.75 mL of 1 M NaOH, and 3.0 mL of sodium hypochlorite

   Store at room temperature

7. Kava stock solution (5 mg/mL)

   Dissolve 1 kava pill in 15 mL of DI H_2O

   Store at 4 °C

   The pill (caplet) can be cut open with scissors

Acknowledgments

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Chapter 4: Manuscript to be Submitted for Publication

The following is a manuscript that will be submitted for publication. The author of this thesis, Jessie Chappel, will be the co-first author on this paper.

A Survey of the Neuromuscular Effect of Kavalactones via Acetylcholinesterase and Postsynaptic Calcium Signalling in *C. elegans*

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Key words: Kavalactones, Acetylcholinesterase, Acetylcholine, Calcium, Caenorhabditis

ABSTRACT

Kava is a plant native to the Pacific Islands whose root extract is widely consumed. The kava extract contains lipophilic compounds called kavalactones. These compounds yield sedative, anxiolytic, and therapeutic effects in humans. How kavalactones bring about such neurobiological effects is not fully understood. Previously, we showed that administration of kavalactones caused muscle hypercontraction in *C. elegans*, suggesting increased or prolonged acetylcholine (ACh) transmission. Based on this result we hypothesized that the neuromuscular-enhancing effect of kavalactones could be mediated by acetylcholinesterase (AChE). This was supported by the result that AChE loss-of-function

(LOF) mutants were hypersensitive to kavalactones. Furthermore, we reasoned that the kavalactone-induced muscle hypercontraction may be a result of sustained calcium influx along the postsynaptic body wall muscles. This was supported by the finding that worms harboring a gain-of-function (GOF) mutation in the L-type calcium channel (EGL-19) were also hypersensitive to kavalactones.

INTRODUCTION

Kava is a tranquilizing beverage derived from the root of *Piper methysticum*, a pepper plant native to the Pacific Islands. This beverage has been used socially and ceremonially for hundreds of years by Pacific Islanders.[1-4] In recent times, the popularity of the kava extract has increased outside of the islands and is now being marketed to several other countries around the world.[4] Chemical analyses of kava indicate that kavalactones are the active ingredients of the beverage. Such compounds are thought to be responsible for the sedative and anxiolytic effects of the shrub.[1-4] The neurological manifestations associated with kava consumption have been attributed to the potency of kavalactones on the nervous system.[1-4] However, how exactly kavalactones exert their effects on the nervous system is still not fully understood. Previously, we provided evidence using *C. elegans* that administration of kavalactones promotes neuromuscular excitation, suggesting an increase in ACh transmission at the NMJ.[5,6] Despite that, how ACh transmission is augmented by the kavalactones is not completely understood.

Additional studies have also shown that some classes of lactones can inhibit AChE, thereby leading to increased or prolonged ACh transmission at the synapse or NMJ.[7] Furthermore, it has been demonstrated that oral administration of kava extract in rats

impacted the levels of AChE in the cortex, hippocampus, and the striatum of the rat brain. For example, in the cortex, administration of kava caused a significant reduction in AChE activity after 2 and 4 weeks of treatment. In the hippocampus, a significant increase of AChE activity was observed after 2 weeks of kava administration. This change was then followed by a downregulation of AChE activity after 4 weeks of treatment.[8] Therefore, based on the aforementioned evidence, we hypothesized that the increase of ACh transmission at the *C. elegans* could be a result of the inhibition of AChE.

To test the hypothesis that kavalactones may impact ACh transmission through AChE, we examined the effects of kavalactones using *C. elegans* mutants lacking *AChE* genes. *C. elegans* is an ideal experimental platform for this study because of its short lifespan, simple and well-defined nervous system, a fully sequenced genome, as well as availability of tools that allows scientists to study the effects of molecules/drugs on *C. elegans* development and physiology. Further, many *C. elegans* mutants are viable and amenable for pharmacological testing.[9-11]

In *C. elegans,* there are four known genes that code for AChE. *ace-1* is expressed mainly in muscle cells and a few motor neurons. *ace-2* is expressed in motor neurons. *ace-3 and ace-4* overall have little to no effect on AChE activity. For our study, we specifically examined the responses of *ace-1* and *ace-2* mutants to kavalactones because these genes contribute to more than 80% of AChE activity in *C. elegans.*[12,13] The results of our experiments indicate that *ace-1* LF mutants are hypersensitive to kavalactones. In addition, we found that *C. elegans* worms harboring a GOF mutation in the L-type calcium channel downstream of ACh receptors were also hypersensitive to kavalactones. These results imply that kavalactones likely promote neuromuscular excitation via AChE and the

downstream postsynaptic calcium signaling pathway.

MATERIALS/METHODS

Worm strains and maintenance:

The following worm strains were used in our study: Bristol N2 (wild type), *ace-1 (ok663), ace-2 (ok2545), ace-1(p1000), ace-2(g72), tom-1 (ok2437)* and *tom-1 (ok285).* Descriptions of these mutants can be found in **Table 4.1**. The worm strains were cultured and maintained according to *C. elegans* standard maintenance protocols or procedures. [14]

Table 4.1: Descriptions of *C. elegans* mutants exposed to Kavalactones

Gene name	Protein identity	Mutant alleles	Effect of mutation on ACh transmission
ace-1	encodes Acetylcholinesterase 1 expressed in *C. elegans* muscle cells	ok663	The LOF allele reduces the function of the acetylcholinesterase enzyme (ACE-1) expressed in muscle cells [12,13]
ace-2	encodes Acetylcholinesterase 2. Exclusively expressed in *C. elegans* neuronal cells	ok2545	The LOF allele reduces the function of the acetylcholinesterase enzyme (ACE-2) expressed in neuronal cells [12,13]
egl-19	encodes an α subunit of L-type calcium channel	ad695	The GOF allele causes sustained Ca^{2+} influx into the postsynaptic muscle cells [18-20]
tom-1	orthologous to mammalian tomosyn protein implicated in synaptic vesicle exocytosis	ok2437, ok285	The LF alleles causes excessive ACh transmission due to failure in inhibition of presynaptic ACh release [17]

Kavalactone-induced convulsion and paralysis assay:

Kavalactone-induced convulsion and paralysis assays were performed using a kavalactone supplement from Gaia Herbs, Inc. [5,6] The supplement was dissolved in DI water and administered to the worms at different concentrations. Worms were synchronized using a hypochlorite bleach solution, leaving only the embryos, which were left to grow until the L4-young adult life-stage. Worms were then washed and centrifuged for 1 minute at 12,000RPM. The excess water was removed, leaving the pellet intact, and 500µL of kavalactone solution with one of the following concentrations was added to the tube: 0.0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, 1.0 mg/mL. The worms were incubated in the kavalactone solution for 30 minutes at room temperature. Worms were then centrifuged for 2 minutes at 13,000 RPM. The kavalactone solution was pipetted out without disturbing the pellet and an additional 200 µL of DI water was added. The tube was centrifuged for an additional 2 minutes and excess water was pipetted off, leaving the worm pellet intact. Approximately 40 of the worms from the pellet were transferred to an unseeded NGM plate via pipette and then given 15 minutes to acclimate. After the worms were acclimated to the new plates, they were scored to see the number experiencing convulsions or paralysis. When scoring, one should look for worms having anterior and/or posterior repetitive muscle contractions, or worms that have no visible movement at all. This assay was repeated 3 times for each concentration of kavalactone solution for the various strains.

Aldicarb-induced paralysis assay

Aldicarb-induced paralysis was conducted according to the protocol provided by Kautu and Phillips et al. 2017. [5,6] This process was repeated every 30 minutes for a total of 180 minutes. Three replicates of this experiment were repeated for each individual strain of worms.

Statistical analysis

All analysis was carried out using R (version 3.6, R Core Team, 2019). The kavalactone-aldicarb induced paralysis assay was analyzed using a general linear mixed-effects model using the package 'lme4'[15] with plate as a random effect to account for possible autocorrelation between repeated measurements of the same plate. Tukey tests was used to test for all possible differences between the three treatment groups using the package 'multcomp'.[16] Logistic regression was used to analyze dose-response data. Proportion paralyzed was modeled as a function of dose, genotype, and the dose × genotype interaction. A Tukey pairwise comparison of slopes was carried out using the package 'emmeans'[17] to test for differences between the logistic regression slopes.[18]

RESULTS

Kavalactone-treated wildtype(N2) worms are hypersensitive to aldicarb-induced paralysis

In our previous study we showed that wildtype (N2) worms treated with kavalactones became more hypersensitive to aldicarb than the untreated N2 worms. Based

on that result we hypothesized that administration of kavalactones may have exacerbated ACh (excitatory) transmission thereby leading to *C. elegans* convulsions and paralysis. To increase our confidence in our previous findings we replicated these experiments using kavalactone concentrations of 0 mg/mL (control), 0.2 mg/mL and 0.4 mg/mL only. We confirmed that wildtype (N2) worms treated with kavalactones at 0.4 mg/mL were indeed more hypersensitive to aldicarb when compared with the controls (p<0.0001) and when compared with N2 worms treated with 0.2 mg/mL (p<0.0001) **(Figure 4.1)**.

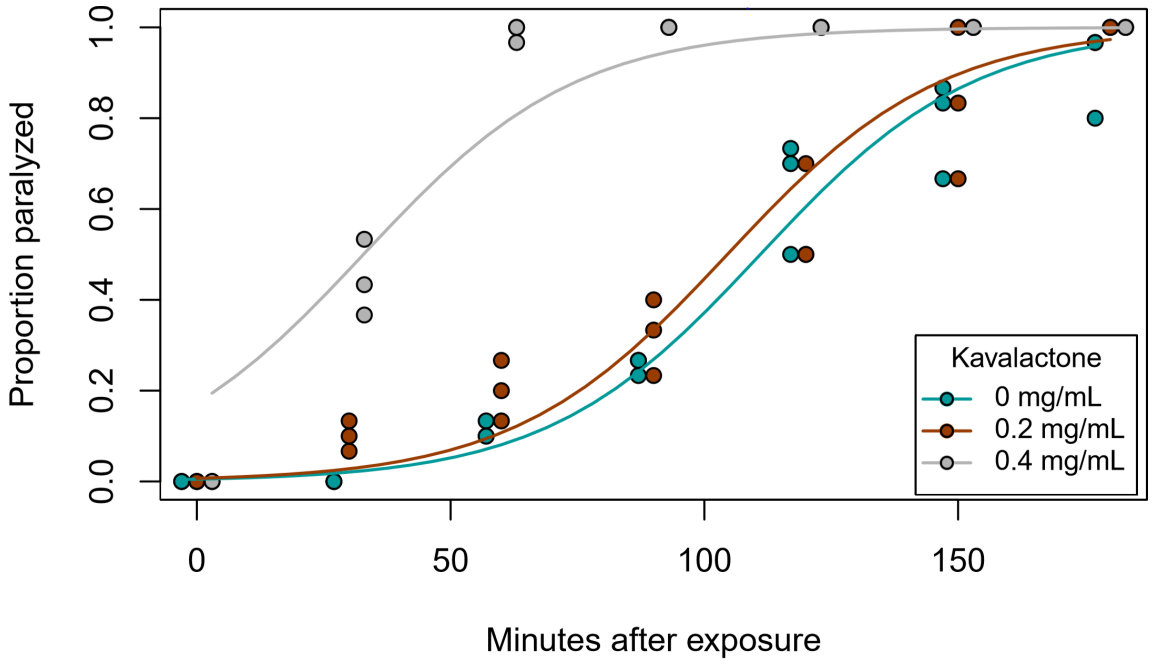

Figure 4.1: An aldicarb-induced paralysis assay to measure steady state ACh transmission at the *C. elegans* NMJ following kavalactone treatment. Wildtype N2 worms were pre-treated with kavalactones at 0 mg/mL (control), 0.2 mg/mL, and 0.4 mg/mL and subsequently exposed to aldicarb (n=30 for each treatment replicated 3 times, n=90). The proportion of paralyzed animals were determined at 30-minute time intervals

over the course of 180 minutes. A slight offset introduced on the *x*-axis to prevent over-plotting of points.

Dose-dependent Responses of AChE and Calcium Signaling Mutants to Kavalactones

In our previous study we also demonstrated that certain ACh signaling mutants with increased ACh transmission displayed enhanced sensitivity to kavalactones in comparison with N2 worms. These results seemed to support the hypothesis that Kavalactones alter the behavioral response of *C. elegans* via ACh signaling. In this present study we wanted to test if the effect of kavalactones on *C. elegans* neuromuscular excitation is specifically altered by a change in the level of AChE and other important downstream components of the ACh signaling pathway. To this end, we exposed AChE LOF mutants *ace-1* and *ace-2* to kavalactones and compared their responses to the positive control *tom-1* mutants[5,6,19] and N2 worms at different kavalactone concentrations. In addition, we also compared the responses of these mutants with *egl-19 (ad695)* mutants. *egl-19* encodes the L-type calcium channel that functions downstream of ACh receptors to promote muscle contraction.[20-22] In *egl-19* GOF mutants there is sustained influx of Ca^{2+} ions into the body-wall muscles of *C. elegans*.[19,20] Because *egl-19* is an important downstream component of ACh signaling, we predicted that *egl-19* GOF mutants will be more hypersensitive to kavalactones than N2 worms. Pairwise comparisons of the slopes obtained from our logistic regression analysis showed that *ace-1* but not *ace-2* mutants were significantly more hypersensitive to kavalactones than N2 worms. Likewise, *egl-19 (ad695)* mutants were also more hypersensitive to kavalactones than N2 worms ($p<0.05$ based on Tukey tests) **(Figure 4.2, Table 4.2)**.

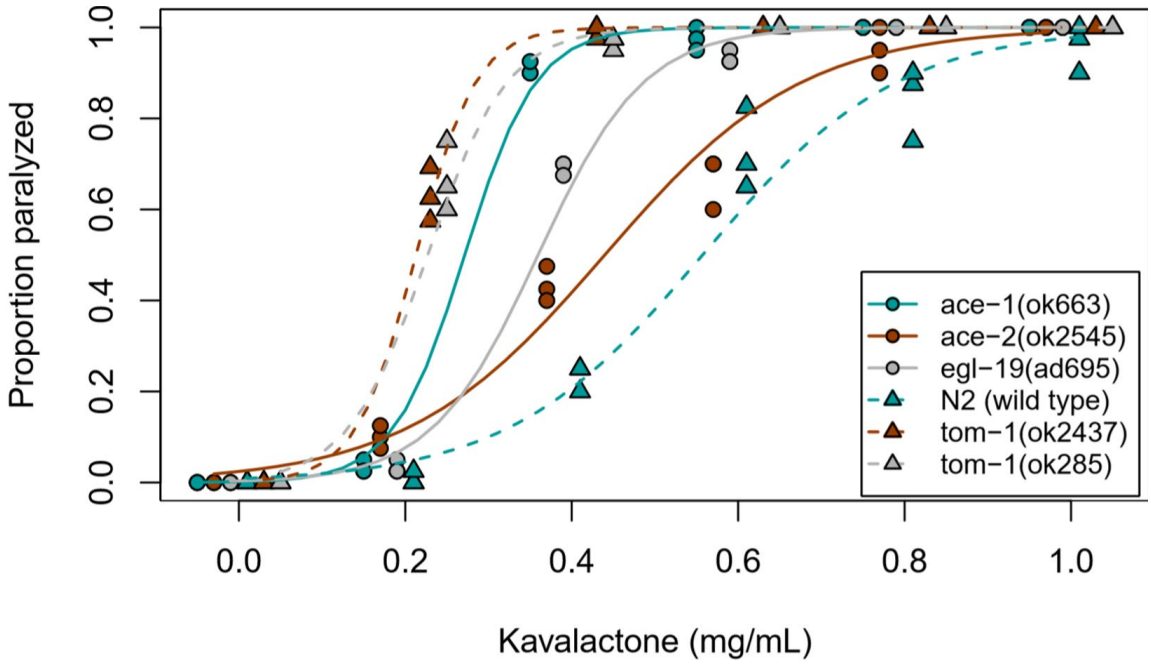

Figure 4.2: Responses of acetylcholinesterase and calcium signaling mutants to Kavalactones. *ace-1* and *ace-2* LOF AChE mutants and *egl-19* (ad695) GOF mutants were treated with different concentrations of kavalactones and their responses were compared with N2 (wildtype) and *tom-1* LOF mutants (positive controls). The proportion of paralyzed/convulsing worms were calculated at different concentrations (n=40 for each experiment replicated 3 times, n=120). Like *tom-1* mutants, *ace-1* and *egl-19* (ad695) mutants were more sensitive to kavalactones than N2 worms. A slight offset was introduced on the *x*-axis to prevent over-plotting of points, all assays were carried out at 0, 0.2, 0.4, 0.6, 0.8, or 1.0 mg/mL kavalactone.

Table 4.2: Statistical summary of kavalactone assay

Genotype	Slope	SE	asymp.LCL
tom-1 (ok2437)	27.947	5.024	18.100
ace-1(p1000)	23.312	2.262	18.878
tom-1 (ok285)	20.980	2.542	15.998
egl-19(ad695)	15.695	1.451	12.852
N2 (wild type)	8.541	0.605	7.356
ace-2(g72)	8.410	0.592	7.249

DISCUSSION

In this present study we wanted to test if altering the level of the enzymatic components of ACh hydrolysis, i.e. AChE, will impact the response of *C. elegans* to kavalactones, thereby suggesting a potential or novel action mechanism for kavalactones in the nervous system. In addition, we wanted to understand if the cholinergic-enhancing effect of kavalactones will also impact the downstream postsynaptic Ca^{2+} signaling, thereby leading to muscle hypercontraction and paralysis in *C. elegans.*

The first question was addressed by exposing different AChE mutants to different concentrations of kavalactones. In this experiment, we observed that *ace-1* mutants were more hypersensitive to kavalactones than wildtype worms while *ace-2* mutants responded similarly to wildtype worms (Figure 1). These results suggest that kavalactones may affect ACh signaling more potently through the muscle acetylcholinesterase ACE-1 than the neuronal ACE-2. Another possible explanation for this result is that ACE-1 is more abundantly expressed than ACE-2, as there is probably more muscle tissue in *C. elegans* than neuronal tissue. Thus, there is more ACE-1 enzymes to interact with kavalactones. Furthermore, it is possible kavalactone absorption or metabolism in the *C. elegans* nervous system occurs at a rate different from its absorption in muscle tissue. Regardless, our results suggest that AChE likely mediates the effect of kavalactones with respect to

neuromuscular excitability.

The second question was explored by examining the effect of kavalactones in *egl-19* GOF mutants. In *C. elegans*, *egl-19* encodes an L-type calcium channel which functions downstream of ACh receptors to promote muscle contraction. Physiologically, opening of the channel, following the binding of ACh to ACh receptors, will allow an influx of Ca^{2+} ions into the postsynaptic muscle cells, leading to muscle contraction. Since wildtype (N2) worms exhibited muscle hypercontraction when exposed to kavalactones, we hypothesized that kavalactones might also promote calcium signaling downstream of the ACh receptors. This hypothesis was supported by the result that *egl-19* mutants with sustained calcium influx were significantly more sensitive to kavalactones than N2 wildtype worms (Figure 2).

Overall, our findings showed that *ace-1* LOF mutations greatly increased *C. elegans* convulsions and paralysis in the presence of kavalactones. In addition, increased calcium signaling via EGL-19 also greatly enhanced the convulsive and paralytic effects of kavalactones in *C. elegans*. These results imply that the response of *C. elegans* to kavalactones is likely mediated by AChE and the downstream postsynaptic calcium signaling of the cholinergic pathway. AChE enzymes are known to be expressed throughout the eukaryotic nervous system, particularly within the cholinergic neuronal system, an important component of the Parasympathetic Nervous System. Our findings in *C. elegans* suggest that the AChE enzymes and the downstream Ca^{2+} signaling pathway could be a potential target of kavalactones. This may have implications on the mechanism underlying the anxiolytic effects of kava. Future studies will need to examine the biochemical interactions between kavalactones and AChE enzymes or other components

of the cholinergic system.

ACKNOWLEDGEMENTS

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Chapter 5: Conclusion

In concert with previous studies, we have demonstrated that treatment with kavalactones induces neuromuscular excitation, indicative of increased or prolonged acetylcholine transmission. Experimentation on *ace-1* and *ace-2* LOF mutants revealed that worms with decreased expression of AChE are hypersensitive to treatment with kavalactones. Further, *ace-1* mutants were shown to be more sensitive than *ace-2*, which may be potentially attributed to *ace-1*'s expression in muscle cells. Similar responses were seen in *C. elegans* treated with aldicarb, suggesting the two treatments may share a similar action mechanism-inhibition of AChE. To provide further evidence for this hypothesis, *in vitro* biochemical assays should be performed using the purified enzyme to further characterize this interaction. *egl-19* GOF mutants, which overexpresses an L-type calcium channel downstream of ACh receptors, were also shown to be hypersensitive to treatment with kavalactones. Because opening of this channel leads to muscle contraction following ACh binding to ACh receptors, we believe the observed hypersensitivity is the result of prolonged opening of the channels due to increased transmission of ACh. Because many neurological disorders are marked by insufficient ACh transmission, it is our hope that the novel insights provided by our results regarding kava's action mechanism may help further evaluate its potential as a therapeutic option for those suffering with various neurological conditions.

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Appendix One: Genetics of C. elegans

From: Brenner S. The genetics of Caenorhabditis elegans. Genetics. 1974;77(1):95– 104.

Media:

1. NG agar: 3 g NaC1, 2.5 g Bactopeptone (Difco) and 17 g Bacto-agar (Difco) are dissolved in 975 mL distilled water. After autoclaving, 1 mL cholesterol in ethanol (5 mg/mL), 1 mL M $CaCl_2$, 1 mL M $MgSO_4$, and 25 mL M potassium phosphate buffer (pH 6.0) are added in order.

2. M9 buffer: 6 g $NaHPO_4$, 3 g KH_2PO_4, 5 g NaCl and 0.25 g MgSO4 per liter.

3. S buffer: 0.1 M NaCl and 0.05 M potassium phosphate (pH 6.0).

4. Standard bacteriological media are used for growth and maintenance of bacterial strains.

Nematode strains:

The nematode used in this work is the Bristol strain of Caenorhabditis elegans. It was originally sent by the late Professor E.C. Dougherty as an axenic culture, but it was transferred to a strain of Escherichia coli B. After some passages on solid media, a culture was found which contained a large number of males. These males could be maintained by mating with hermaphrodites. From this stock, a hermaphrodite was isolated and its progeny used to establish two lines: one, a line of hermaphrodites propagating by self-fertilization; the other, a line with males. These are the founder stocks and carry the code name N2; all mutants have been isolated in these strains.

Maintenance of stocks:

Stocks are maintained on NG plates seeded with OP50, a uracil requiring mutant of E.

coli, and incubated at 15ºC. 9 cm petri dishes are used and cultures require subculturing every 10 days or so. Male cultures are maintained by adding 6 or 7 males to a similar number of hermaphrodites on a seeded NG plate. Several of these stocks, staggered with respect to their subculturing, are held, so that active males are always available for crosses. A uracil-requiring strain of *E. coli* is used to prevent overgrowth of the bacterial lawn. The medium has limited uracil, and the bacteria cannot grow into a thick layer which obscures the worms. These plates are the working stocks for genetical and other experiments. The canonical stocks of the mutants are held frozen in liquid nitrogen. Many experiments on long-term maintenance were carried out without much success. Dr. J Sulston discovered that the worms could be stored in liquid nitrogen, provided that glycerol was present and that the initial freezing took place slowly. The standard method used is as follows: worms are washed off the surface of a petri dish culture using about 1.5 mL of S or M9 buffer. To 1 mL of this suspension is added 1 mL of a 30% solution of glycerol in S buffer, and after mixing, four 0.5 mL aliquots are dispensed into small plastic tubes. These are placed in the holder provided with the Linde liquid nitrogen refrigerators at a level in the vapor phase giving a cooling rate of about lºC/min. After two hours or more, the tubes are mounted in canes and submerged in the liquid nitrogen. The next day, one of the four tubes is removed, thawed. and the contents poured on an NG plate. The plate is examined after a day to make sure that there are viable growing worms. The remaining three cultures are then stored: one in one refrigerator as a master stock, the other two in a different refrigerator as the canonical stocks. If, at any time, the last of these is used, it is immediately replaced so that the master stocks are only used in emergency. With the wild type and most mutants, it is mostly the early larval stages that

survive freezing and thawing; eggs do not survive at all. This method has proved completely reliable. Plate stocks can become contaminated with bacteria and molds. Cultures may be rendered monoxenic in the following way: A culture containing many eggs is suspended in 1.5 mL M9 buffer. 1.5 mL of 4% glutaraldehyde in M9 buffer is added and the suspension allowed to stand at 4ºC for 4 hours. A few drops of a culture of *E. coli* is spread over a half sector of a 9-cm NG S. Brenner plate. The glutaraldehyde-treated suspension is briefly centrifuged, and the sediment taken up in 0.1 to 0.2 mL of M9 buffer and applied to the edge of the uninoculated sector. If necessary, the plate is tilted so as to confine this to one side. The glutaraldehyde kills the worms and most contaminants but does not penetrate the eggs. After one day, these hatch and the larvae cross over to the bacterial lawn. The agar with the debris may then be removed. Induction of mutation with ethyl methanesulphonate (EMS): The animals are washed off the plate in M9 buffer, and to 3 mL of the suspension is added 1 mL of freshly prepared 0.2 M ethyl methanesulphonate in M9 buffer (final concentration 0.05 M). The standard treatment is for 4 hours at room temperature. The suspension is then taken up into a pipette and the warms allowed to concentrate by sedimentation. 0.2-0.5 mL is dripped onto the surface of an NG plate to absorb the excess fluid. The worms move out and can then be picked to initiate clones.

Handling and observation of animals:

Mass transfers of animals on plate cultures are carried out with paper strips. Single animals can be manipulated using a sharpened wooden stick or toothpick, sterilized by autoclaving. Observations of the plates are made using a dissecting microscope illuminated from below.

Appendix 2: Paradigms for Pharmacological Characterization of C. elegans Synaptic Transmission Mutants

From: Locke CJ, Berry K, Kautu B, Lee K, Caldwell K, Caldwell G. Paradigms for pharmacological characterization of C. elegans synaptic transmission mutants. J Vis Exp. 2008;18:837.

Aldicarb Exposure Paradigm

1. On the first day, ensure that at least thirty young adult stage worms of each genotype and of each replicate will be available for aldicarb assays on the second day. It is best if an experimenter selects fifty or more L4 stage worms onto fresh NGM plates (best without nystatin), which contain *E. coli* (preferably OP50) as a food source, and grow them for 12-24 hours at a consistent and permissive temperature (20°C to 22°C is best, although 25°C is okay).
2. On the second day, make a 100mM stock solution of aldicarb with 70% ethanol (EtOH) and 30% ddH_2O. Spread the appropriate amount of aldicarb onto NGM minus nystatin plates with defined volumes to achieve the desired aldicarb concentrations. We consistently use 0.5mM aldicarb by plating 37.5µL of 100mM aldicarb onto 7.5 mL NGM plates. Allow the aldicarb plates to dry for roughly 30-60 minutes at room temperature. It is not necessary to crack the lids. Alternatively, aldicarb can be added to NGM and stored at 4°C for one week.
3. After drying, plate consistent volumes of *E. coli* (preferably OP50) onto the center of each aldicarb plate and dry for another 30-60 minutes at room temperature. We consistently plate 25µL of OP50, which creates a sufficiently sized food lawn to keep the worms concentrated in a small spot without overcrowding.

4. When the food lawn is dry, one may proceed with aldicarb assays. Due to the subjective nature of aldicarb assays, it is highly recommended that experiments be performed "blindly". A colleague of the primary experimenter could re-label the original plates with worms to be assayed. Likewise, the colleague could transfer worms from the original plates to ciphered aldicarb plates immediately before starting a timer. If the experimenter anticipates assaying a particular strain of worms with a characteristic phenotype, such as uncoordination, then there must also be a control with a similar phenotype to reduce bias. Furthermore, it is best if the experimenter assays a wild-type strain, as well as a resistant strain and a hypersensitive strain, in parallel to help standardize experiments. The experimenter should strive to analyze a consistent number of worms for each replicate. We consistently analyze thirty worms of a single genotype for each replicate. We also perform at least three replicates for each experiment. An experienced experimenter should be able to analyze at least six strains at a time.
5. Count the number of paralyzed worms by prodding in a consistent manner each worm with a platinum wire. We consistently prod our worms twice on the head and twice on the tail every 30 minutes for a total of three hours. Cessation of pharyngeal pumping may also be used to define paralysis, but only if the experimenter employs a consistent definition of paralysis over all assays. Also, it is worth noting that some worms, especially those that are resistant to aldicarb, may attempt to crawl off the plate. In this case, the experimenter may spread a consistent amount of palmitic acid, a physical barrier to worm locomotion, around the aldicarb plates. We spread 25µL of 10mg palmitic acid/mL EtOH