A Behavioral Survey of the Effects of Kavalactones on *Caenorhabditis elegans* Neuromuscular Transmission

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ABSTRACT: Kava is a plant root extract that is widely consumed by Pacific Islanders. Kava contains a class of lactone compounds called kavalactones. The sedative and anxiolytic effects of kava are likely attributed to the efficacies of kavalactones on the nervous system. Although some studies have implicated the potencies of certain kavalactone species on γ-aminobutyric acid transmission, evidence supporting the action of kavalactones on the eukaryotic neuromuscular junction (NMJ) and acetylcholine (ACh) transmission is scant. Here, we used behavioral assays to demonstrate the effects of kavalactones at the *Caenorhabditis elegans* NMJ. Our results suggest that kavalactones disrupt the inhibitory-excitatory balance at the NMJ. Such perturbation of NMJ activity is likely due to excess or prolonged ACh transmission. In addition, we found that kavain, a major constituent of kava, induced worm paralysis but not convulsions. Hence, the modulatory action of kavain could be distinct from the other kavalactone species.

KEYWORDS: Acetylcholine, *Caenorhabditis*, kava, kavalactones

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Introduction

For thousands of years, Pacific Islanders have used kava as a ceremonial and medicinal drink.[1–7] The beverage is traditionally prepared from the ground root of the Oceanic plant *Piper methysticum*.[1–7] This peculiar plant is widely cultivated in the Pacific and respected by many native Pacific Islanders. When metabolized in the human body, kava yields a variety of biological effects including, but not limited to, sedation and anxiolysis.[2,3] Scientific studies revealed that kavalactones (a class of lactone compounds) are the active ingredients of kava.[1–7] Notably, the neurobiological effects associated with kava consumption are presumably attributed to the efficacies of kavalactones on some aspects of the nervous system.[5] Despite that, how kavalactones modulate the nervous system is not completely understood. Although some studies have demonstrated the potencies of certain kavalactone species on γ-aminobutyric acid (GABA) transmission,[3,6] evidence supporting the effect(s) of kavalactones at the neuromuscular junction (NMJ) of a living eukaryote, and acetylcholine (ACh) transmission is scarce. To date, at least 18 different kavalactones have been identified,[7] yet, their modes of action are not fully understood.

In this study, we employed behavioral assays to investigate the effects of kavalactones on an intact *Caenorhabditis elegans* NMJ. *C. elegans* possesses many remarkable characteristics that make it an ideal experimental system for our study. First, the entire anatomical connectivity of the worm's nervous system has been mapped by serial section electron microscopy.[8,9] Second, many powerful tools and resources are available for interrogating the cellular and molecular physiology of the *C elegans* nervous system. Third, due to the availability of a complete and annotated genomic sequence, many genes and pathways affecting neurotransmission have been characterized in the worm. Remarkably, most neurotransmission mutants are viable and highly amenable for behavioral and pharmacological experimentations.[10]

In our investigation, we found that treatment of *C elegans* with kavalactone solution resulted in a disruption of normal neurotransmission activity at the NMJ. Our results suggest that such disruption of neuromuscular activity is likely due to increased or prolonged ACh transmission. Such aberrant increase in ACh transmission leads to muscle hypercontraction, which manifests in worm convulsions and paralysis. Using function-altering mutations affecting ACh signaling, we produced additional evidence to support the hypothesis that kavalactones act to promote ACh transmission at the *C elegans* NMJ. For instance, in our experiments, we found that a loss-of-function (LF) mutation in the *C elegans* tomosyn (*tom-1*) gene, a negative regulator of synaptic vesicle priming, resulted in enhanced hypersensitivity to kavalactone-induced convulsions and paralysis. In addition, we observed that gain-of-function (GF) and LF mutations in the neuronal nicotinic receptor ACR-2, significantly altered the sensitivity of *C elegans* to kavalactones, respectively, thus further implying the modulatory effects of kavalactones on cholinergic transmission and neuromuscular excitability.

While searching for specific modifiers of neuromuscular activity in the kavalactone mixture, we noticed that treatment

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of *C elegans* with kavain (one of the most abundant kavalactones)[1] induced paralysis but not repetitive muscle contractions or full-body convulsions. Hence, the repetitive and intense muscle contractions induced by kavalactones in *C elegans* may not be attributed to kavain. Such revelation raises the possibility that the action mechanism of kavain could be distinct from other kavalactone species.

Materials and Methods

Worm strains and maintenance

The following worm strains were used in our study: Bristol N2 (wild type), *acr-2* (*ok1887*), *acr-2* (*n2420*), *tom-1* (*ok 2437*), *unc-17* (*e113*). All worm strains were cultured and maintained according to standard procedures.[11]

Kavalactone-induced convulsion and paralysis assay

Kavalactone-induced convulsion and paralysis assays were performed using a kavalactone supplement that was purchased from Gaia Herbs, Inc. (Brevard, NC, USA). The kavalactone supplement was dissolved in distilled water and administered to worms at various concentrations. We chose this kavalactone supplement because it has been used and characterized in previous studies.[12,13] Staged late larvae (L4) to young adult hermaphrodite worms were used in all convulsion and paralysis assays. To perform the assays, worms were washed in the plates with distilled water and then transferred to 2-mL microcentrifuge tubes. The tubes were then centrifuged at approximately 12 000 to 13 000 rpm for 2 minutes. Excess water was removed from the tubes (leaving the worm pellet undisturbed), and then kavalactone solutions with concentrations of 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL were added to the different microcentrifuge tubes containing worms. The worms were then incubated at room temperature in the kavalactone solutions for 30 minutes and then centrifuged again. The excess kavalactone solution was removed, leaving the pellet of worms intact. The worms were then rinsed with water and centrifuged. The excess liquid was extracted and the worms were transferred to new nematode growth media (NGM) plates to be assayed. Worms were allowed to acclimate on the new NGM plates for 15 minutes before they were scored for convulsions/paralysis. Dissecting microscopes were used for visual inspection and scoring of worms. Worms that experienced kavalactone-induced epileptic-like convulsions displayed one or more of the following responses: anterior repetitive muscle contractions, posterior repetitive muscle contractions, and full-body repetitive muscle contractions. Kavalactone-induced paralysis is defined as when a worm has completely ceased movement following kavalactone treatment (without prodding a worm with a pick). Three independent experiments were performed for this assay. Representative movies of worm convulsions and paralysis were recorded in real time using a digital video camera. These movies are provided in the supplementary movie files (see supplementary movie files 1-3).

Kavalactone-induced and aldicarb-induced paralysis assay

An aldicarb-induced paralysis assay was used in this study to examine the effect of kavalactones at the *C elegans* NMJ. The purpose of this assay was to determine whether administration of kavalactones exacerbates ACh transmission at the NMJ. In this assay, worms were pretreated with kavalactone solutions of 0, 0.1, 0.3, and 0.5 mg/mL. Then, 30 worms were transferred from the NGM plates to aldicarb plates containing 0.5 mM concentration of aldicarb (Sigma-Aldrich, Milwaukee, Wisconsin, USA). The aldicarb plates were prepared according to the same protocol described by Locke and colleagues.[14,15] The worms were then observed and scored for paralysis on aldicarb plates at 30-minute intervals for a period of 180 minutes. To score paralysis, worms were prodded consistently (twice on the head and twice on the tail) every 30 minutes with a worm pick made of platinum wire. Worms that failed to respond to the prodding were considered paralyzed. Three independent experiments were performed for this assay.

Kavain-induced paralysis assay

To maximize solubility, kavain (Sigma-Aldrich) was dissolved in 0.6 mg/mL of egg L-α-phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA). A concentration of 1.0 mg/mL kavain solution was administered to the worms. Worms were observed under a dissecting microscope for a response to kavain. For this assay, a total of 40 worms were analyzed per experiment. Three independent experiments were performed (n = 120).

Statistical analysis

The kavalactone-induced and aldicarb-induced paralysis data were analyzed as a mixed-effects model in R version 3.2[16] using the function "lme" in package "nlme."[17] The time to paralysis for each worm was modeled as a function of treatment, with the plate as a random effect, to account for possible autocorrelation. Tukey tests for all possible differences between the 4 groups were calculated using the function "ghlt" from the package "multcomp."[18] Nonparalyzed worms were not included in this analysis, as the response was time to paralysis. A student *t* test was used to compare the effect of kavain on treated worms versus control worms.

Results

Kavalactone-induced convulsions and paralysis in C elegans

To study the effects of kavalactones on *C elegans*, we exposed wild-type L4 to young adult wild-type (N2) worms to various concentrations of kavalactone solutions. The concentrations of

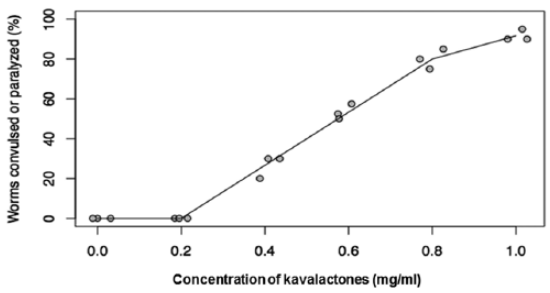

Figure 1. Aqueous kavalactone solution–induced convulsions and paralysis in wild-type (N2) worms. N2 worms were exposed to kavalactone solution concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL (*x*-axis). The worms were scored as convulsed or paralyzed (*y*-axis). The response of worms is commensurate with increasing concentrations of kavalactone solution. The response level of worms is presented as a percentage of worms convulsed/paralyzed per total sample size (n = 40-42). Three independent experiments were conducted (n is approximately 120). A small amount of offset was added to the *x*-axis values to prevent overplotting of points.

the kavalactone solutions ranged from 0 to 1 mg/mL. At 0 and 0.2 mg/mL, we found that 0% of kavalactone-treated worms responded to the kavalactones. However, worms treated with 0.4, 0.6, 0.8, and 1.0 mg/mL displayed epileptic-like convulsions and paralysis in a dose-responsive manner (Figure 1). The convulsing worms exhibited anterior and full-body repetitive muscle contractions (supplementary movie file 1). Interestingly, we also noticed that many convulsing worms progressively become paralyzed over the course of time (supplementary movie file 2). In addition, we observed that some worms became immediately paralyzed following exposure to kavalactones. Instant worm paralysis was seen more frequently at higher kavalactone concentrations (supplementary movie file 3). Thus, in general, the worm's response to kavalactones shows progression from convulsion (repetitive muscle contractions) to complete immobility. For this reason, we decided to treat convulsion and paralysis as one variable (shown on the *y*-axes of the corresponding graphs).

Prior studies have postulated that worm convulsions and paralysis are an indication of an imbalance between inhibitory and excitatory neurotransmission at the *C elegans* NMJ. For instance, it has been shown that increased ACh transmission, caused by the effect of an activating mutation in the nicotinic receptor ACR-2, led to worm convulsions and paralysis.[19,20] In addition, mutant worms with enhanced excitatory (ACh) transmission, albeit diminished inhibitory neurotransmission, tend to exhibit increased sensitivity to an acetylcholinesterase (AChE) inhibitor, aldicarb, when compared with wild-type worms. Interestingly, some of these mutants also displayed epileptic-like convulsions when exposed to the epileptogenic agent pentylenetetrazole.[14,15] These findings prompted us to hypothesize that administration of kavalactones exacerbates ACh transmission at the *C elegans* NMJ. We proceeded to test this hypothesis by examining the response of kavalactone-treated worms on aldicarb.

Kavalactone-treated worms are hypersensitive to aldicarb-induced paralysis

Aldicarb is a reagent of choice for measuring the levels of steady-state ACh release and neuromuscular signaling in a living worm.[21,22] Aldicarb inhibits AChE, thereby promoting the levels of ACh at the *C elegans* NMJ and paralysis.[21] For this reason, scientists have used aldicarb to isolate and characterize numerous mutations and genes involved in ACh transmission in *C elegans*.[21] A mutant worm lacking ACh at the NMJ tends to display resistance to aldicarb-induced paralysis, in comparison with a wild-type worm.[22] By contrast, a mutant worm with elevated levels of ACh tends to exhibit hypersensitivity to aldicarb-induced paralysis, in comparison with a wild-type worm.[14,21–23] To test whether kavalactones perturb the inhibitory-excitatory balance by exaggerating ACh transmission, we exposed kavalactone-treated worms to aldicarb and measured the time course of acute paralysis.

We observed that kavalactone-treated worms were significantly more hypersensitive to aldicarb-induced paralysis than the control group (Figure 2A). In this experiment, worms were treated with lower concentrations of kavalactones (0.1-0.5 mg/mL) to avoid potential toxicity that may arise from the combined effects of kavalactones and aldicarb. Our results indicated that kavalactones significantly increased the paralytic effects of aldicarb at 0.3 and 0.5 mg/mL (Figure 2A). As a positive control, we used *tom-1* (*2437*) LF mutants because loss of TOM-1 causes excessive ACh signaling at the NMJ.[14,15,24,25] As expected, *tom-1* LF mutants were also hypersensitive to aldicarb-induced paralysis (Figure 2A). The response level of *tom-1* LF mutants to kavalactones was not statistically different from the response levels of wild-type worms treated with 0.3 and 0.5 mg/mL of kavalactone solutions, respectively (Figure 2A). In the absence of aldicarb, we also observed that *tom-1* LF mutants were more hypersensitive to kavalactone-induced convulsions and paralysis than wild-type worms (Figure 2B). These results support the hypothesis that kavalactones augment ACh transmission at the *C elegans* NMJ.

ACh-signaling mutants exhibit altered sensitivities to kavalactones

To further support the hypothesis that kavalactones act to promote ACh transmission at the *C elegans* NMJ, we also tested various *C elegans* mutants with aberrant ACh transmissions. As summarized in Table 1, the ACh-signaling mutants tested in this assay were *unc-17* (*e113*) hypomorphs, *tom-1* (*ok2347*) LF, *acr-2* (*n2420*) GF, and *acr-2* (*ok1887*) LF. The genetic lesions harbored in these mutant animals produced different effects on ACh transmission at the *C elegans* NMJ (described in Table 1).[19,20,23–27] For example, *unc-17* hypomorphs have reduced ACh transmission due to impairment in presynaptic loading of ACh into synaptic vesicles.[26] Previous studies have also shown that *unc-17* (*e113*) mutants are resistant to aldicarb-induced

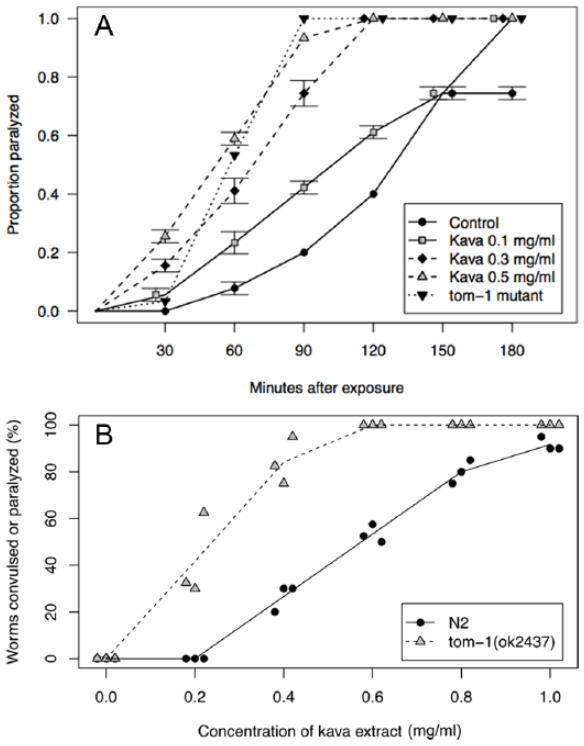

Figure 2. (A) Pretreatment of N2 worms with kavalactone (kava) solution exacerbated aldicarb-induced paralysis. N2 worms were exposed to kavalactone solution concentrations of 0, 0.1, 0.3, and 0.5 mg/mL. The worms were subsequently placed on aldicarb nematode growth media plates containing 0.5 mM aldicarb. The response level of worms on aldicarb was expressed as a proportion of paralyzed worms per total sample size (n = 30) for each 30-minute time point over the course of 180 minutes. Three independent experiments were performed for each kavalactone treatment (n = 90). Error bars show 95% confidence interval for the mean. Time to paralysis for the control (0 mg/mL) and 0.1 mg/mL did not differ ($P = .99$), but all other treatments differed from the control ($P < .001$). A loss-of-function mutant with excess acetylcholine-signaling *tom-1* (*ok2437*) was used as a positive control for the aldicarb-induced paralysis assay. A small amount of offset was added to the *x*-axis values to prevent overplotting of points. (B) Response of *tom-1* (*ok2437*) loss-of-function (LF) mutants to kavalactones (kava). *tom-1* (*ok2437*) LF mutants were exposed to various kavalactone concentrations ranging from 0 to 1 mg/mL. *tom-1* LF mutants are more hypersensitive to kavalactones compared with N2 wild-type worms ($P < .001$).

paralysis.[23] Such response is consistent with diminished ACh transmission. However, loss of TOM-1 in *C elegans* resulted in excessive ACh transmission due to a failure in negative regulation of ACh synaptic vesicle priming or exocytosis.[27] Our results (and prior studies) also indicate that *tom-1* LF mutants are hypersensitive to aldicarb-induced paralysis, thereby implying excess ACh transmission (Figure 3).[24,27] The ACh receptor mutants investigated in this study carry activating and LF mutations in *acr-2*, respectively (Table 1). The *acr-2* gene of *C elegans* encodes 1 of the 5 subunits of a neuronal ACh receptor, which functions to regulate the balance of muscle inhibition and excitation in *C elegans*.[19] We selectively included *acr-2* mutants in this study because earlier studies showed that a GF mutation in *acr-2* causes spontaneous convulsions and paralysis in *C elegans*.[19,20] In addition, *acr-2* GF mutants were found to be hypersensitive to aldicarb-induced paralysis, suggesting that there is elevated ACh transmission in these mutant animals.[19] The behavioral responses and phenotypes demonstrated by *acr-2* GF mutants resemble what we saw with kavalactone-treated worms. These observations led us to speculate that the convulsive and paralytic effects of kavalactones on *C elegans* could be partially mediated by ACR-2 or the entire pentameric nicotinic channel.

The responses of the aforementioned ACh-signaling mutants were quantified following kavalactone treatment. When we investigated the effect of kavalactones on *tom-1* LF mutants, we discovered that these mutant animals were significantly more hypersensitive to kavalactones than the control group (Figure 3). This result implies that kavalactones exacerbate the effect of ACh at the NMJ. To further support this argument, we also tested *unc-17* (*e113*) hypomorphic mutants, which are defective in ACh secretion or signaling.[23,26] If kavalactones act to promote ACh signaling at the NMJ, then one would expect *unc-17* (*e113*) mutants to exhibit resistance to the effect of kavalactones. To our surprise, we found that the dose-response curve for *unc-17* (*e113*) hypomorphs did not statistically differ from the control group, although at 0.4 mg/mL, the average response of *unc-17* mutants displays a nonsignificant trend toward resistance. Because *unc-17* (*e113*) is a hypomorphic mutant allele, it is impossible to conclude based on this result alone if kavalactones modulate presynaptic ACh signaling. We were not able to analyze null mutant alleles of *unc-17* because they are lethal.[26] Regarding *acr-2* mutants, we discovered that *acr-2* LF mutants were resistant to the effect of kavalactones, whereas *acr-2* GF mutants were significantly more hypersensitive to kavalactone-induced convulsions and paralysis than the control group (Figure 3). Overall, these data support the hypothesis that kavalactones positively modulate ACh transmission at the *C elegans* NMJ.

Kavain induces paralysis but not repetitive muscle contractions in C elegans

One of the most abundant constituents of the kavalactone mixture is kavain.[1,3] Kavain has been shown to affect a number of proteins, including components of the inflammatory signaling response.[28] With respect to neurotransmission, Chua et al[3] showed recently that kavain also potentiates $GABA_A$ receptor subtypes in vitro. Thus, kavain likely targets a plethora of signaling proteins, enzymes, and channels.

In this study, we were also interested in testing the effects of kavain on *C elegans*. To this end, we exposed wild-type worms to 1 mg/mL kavain solution and measured their responses. We used 1 mg/mL in this experiment because the responses of worms to kavain at this specific concentration were reproducibly observed. Following exposure to kavain, we were surprised to see that worms displayed paralysis but not epileptic-like convulsions (repetitive muscle contractions). As shown in

Table 1. Descriptions of acetylcholine-signaling mutants treated with kavalactones.

GENE NAME	PROTEIN IDENTITY	MUTANT ALLELES	EFFECT OF MUTATION ON ACH TRANSMISSION
unc-17	Vesicular ACh transporter	*e113*	Hypomorph allele impairs presynaptic ACh release[23,26]
tom-1	Orthologous to mammalian tomosyn protein implicated in synaptic vesicle exocytosis	*ok2437*	Loss of tomosyn (TOM-1) in *C elegans* causes excessive ACh transmission due to failure in inhibition of presynaptic ACh release[14,15,24,25,27]
acr-2	Encodes a subunit of a non-α nicotinic receptor expressed in the *C elegans* motor neurons	*n2420*	GF allele enhanced hyperexcitability of the cholinergic motor neurons, resulting in an increase in ACh transmission[19]
acr-2	Encodes a subunit of a non-α nicotinic receptor expressed in the *C elegans* motor neurons	*ok1887*	LF allele reduced hyperexcitability of the cholinergic motor neurons, resulting in diminished ACh tranmission[19]

Abbreviations: ACh, acetylcholine; GF, gain-of-function; LF, loss-of-function.

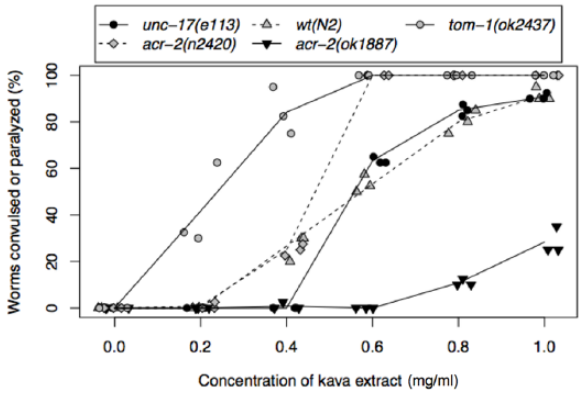

Figure 3. Acetylcholine-signaling mutants displayed altered sensitivities to kavalactones (kava). Different ACh-signaling mutants were exposed to various concentrations of kavalactones ranging from 0 to 1 mg/mL. N2 worms were treated with 0 mg/mL of kavalactones and used as a control. The response level of worms is presented as a percentage of worms convulsing/paralyzed per total sample size (n = 40-42). Three independent experiments were conducted, thus the combined sample size for all 3 experiments is approximately 120 worms. The dose response of *unc-17* (*e113*) mutants did not differ from the control ($P > .4$). *acr-2* (*ok1887*) loss-of-function (LF) mutants were more resistant to kavalactones than the control group ($P < .001$). *acr-2* (*n2409*) LF and *tom-1* (*ok2437*) mutants were more hypersensitive to kavalactone than the control group ($P < .001$). A small amount of offset was added to the *x*-axis values to prevent overplotting of points.

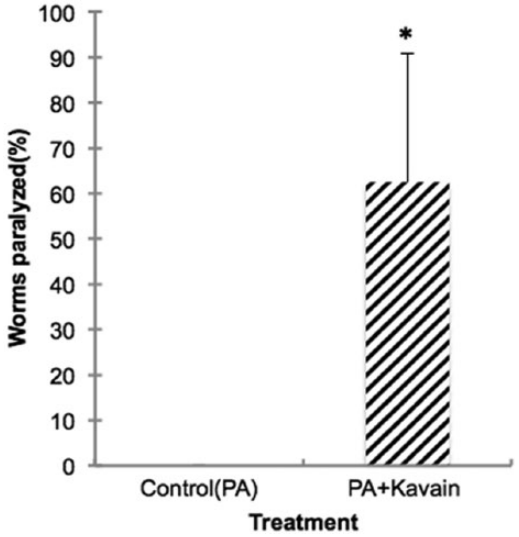

Figure 4. Kavain-induced paralysis in *Caenorhabditis elegans*. Kavain (1 mg/mL) was dissolved in phosphatidylcholine (PA). Worms were treated with kavain and PA (control). The response level of worms is presented as a percentage of worms paralyzed per total sample size (n = 40). Three independent experiments were performed for each treatment (n = 120). Control worms (treated with PA) were not paralyzed. With kavain, 62.5% of the worms were paralyzed ($P < .05$).

Figure 4, 0% of the control worms showed paralysis when treated with the control solvent (phosphatidylcholine). When treated with kavain, approximately 62% of the worms were paralyzed but not convulsing. This result implies that other kavalactone species likely contribute to the convulsive effect of the kavalactones in *C elegans*. Due to poor solubility, we were not able to establish a dose-response curve for kavain in this particular experiment.

Discussion

Pacific Islanders have consumed kava for thousands of years, yet the neurophysiological mechanisms associated with kavalactone metabolism are not fully understood. Although some studies have demonstrated the efficacies of certain kavalactone species on GABA transmission,[3,6] additional studies are needed to uncover the mechanisms underlying the effects of kavalactones on other aspects of the nervous system, including ACh signaling and neuromuscular excitability. Using behavioral and pharmacological assays, we provided in vivo evidence that kavalactones disrupt the inhibitory-excitatory balance at the *C elegans* NMJ. To the best of our knowledge, this is the first evidence showing the cholinergic-enhancing effects of kavalactones at the NMJ of an intact living eukaryote.

The inhibitory-excitatory balance at the *C elegans* NMJ is maintained by the opposing actions of GABA and ACh.[29] When the level of ACh signaling (excitation) is substantially greater than the level of GABA transmission (inhibition) at the *C elegans* NMJ, this results in muscle hypercontraction, which can manifest as a convulsion or paralysis.[19,20,23] In our study, we showed that treatment of *C elegans* with kavalactones

resulted in convulsions and paralysis (Figure 1). We hypothesized that these responses are indicative of elevated or prolonged ACh transmission at the NMJ. This hypothesis was supported by several key observations. First, kavalactone-treated worms are notably hypersensitive to aldicarb-induced paralysis (Figure 2A). In addition, previous studies have shown that mutant worms with elevated levels of ACh are hypersensitive to aldicarb-induced paralysis.[14,15,23] Furthermore, we demonstrated that function-altering mutations affecting ACh signaling altered the sensitivities of worms to kavalactones. For example, *tom-1* LF mutants showed increased hypersensitivity to kavalactones compared with wild-type worms (Figures 2B and 3). These results suggest that kavalactones disrupt the inhibitory-excitatory balance by enhancing ACh signaling.

To corroborate the above results, we also demonstrated the effects of kavalactones on the nicotinic receptor ACR-2. ACR-2 is expressed in the cholinergic motor neurons and plays an important role in maintaining the balance of muscle inhibition and excitation in the locomotory circuit of *C elegans*.[19] It has been shown that the *acr-2* (*n2420*) GF allele causes hyperactivation of the cholinergic motor neurons of *C elegans*, leading to overexcitation of the worm muscles and convulsions. Because ACR-2 is expressed in the cholinergic neurons, it was initially hypothesized that this neuronal nicotinic receptor may function to regulate presynaptic ACh signaling.[19] In our investigation, we demonstrated that *acr-2* (*n2420*) GF allele conferred hypersensitivity to kavalactone-induced convulsions and paralysis. In corroboration of this result, we showed that *acr-2* LF mutants were resistant to kavalactone-induced convulsions/paralysis, in comparison with wild-type worms (Figure 3). These results further support the hypothesis that kavalactones exacerbate ACh signaling. Furthermore, it is possible that ACR-2 partly mediates the effect of kavalactones with respect to neuromuscular activity. Despite that, we do not rule out the possibility that the effect of kavalactones at the NMJ may not be specific to ACR-2, as other ACh receptor mutants were not tested in this study. As such, future studies will need to examine how kavalactones interact with other types of ACh receptors. In addition, it will also be important in the future to examine the biochemical interactions of kavalactones and ACR-2 (or the entire pentameric nicotinic channel).

Although our findings reveal the link between kavalactones and ACh signaling at the NMJ, the extent of our investigation does not offer a mechanism to explain how kavalactones exacerbate ACh signaling. Despite that, with the exception of *unc-17* (*e113*) hypomorphs, one key trend that emerged from this study is that genetic manipulation of genes involved in ACh transmission strongly altered the sensitivities of worms to kavalactones (Figure 3). Hence, future studies will need to examine how kavalactones modify the dynamics of presynaptic and postsynaptic ACh signaling at the cellular and biochemical levels. In addition, it will also be important in the future to investigate whether or not kavalactones modify ACh signaling via inhibition of AChE. This is certainly a possible mode of action for kavalactones because some classes of lactone compounds are known to have inhibitory effects on AChE activity.[30,31] Interestingly, a study conducted by Noor[32] showed that administration of kava affected AChE activity in the brain tissues of male rats. Thus, the cholinergic-enhancing effect of kavalactones in *C elegans* could be a result of AChE inhibition.

It is expected that examination of specific neurotransmitter-modulating kavalactones using *C elegans* would provide new insights into the mechanism(s) of action of kavalactones in the nervous system. For instance, as indicated in our results with kavain treatment (Figure 4), the observation that kavain induced worm paralysis but not convulsions suggests that the modulatory action of kavain may differ from other kavalactone species. Kavain has been shown to potentiate $GABA_A$ receptor subtypes in vitro.[3] Thus, it is possible that the observed kavain-induced paralysis in the worms could be due to altered GABA transmission. Therefore, future studies will need to further examine the specific effects of each kavalactone species (such as kavain) on neuromuscular signaling or in vivo synaptic transmission. Furthermore, electrophysiology and optogenetic approaches will be helpful in characterizing and refining the neurophysiological mechanisms associated with each (or multiple) kavalactone species.

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Author Contributions

BBK, JP, KS, and MSM conceived and designed the experiments. EAN, JP, and BBK analyzed the data. BBK wrote the first draft of the manuscript. JP, EAN, KS, and MSM contributed to the writing of the manuscript. BBK, JP, KS, MSM, and EAN agree with manuscript results and conclusions and jointly developed the structure and arguments for the paper. BBK and EAN made critical revisions and approved final version. All authors reviewed and approved the final manuscript.

REFERENCES

1. Wang J, Qu W, Bittenbender HC, Li QX. Kavalactone content and chemotype of kava beverages prepared from roots and rhizomes of Isa and Mahakea varieties and extraction efficiency of kavalactones using different solvents. *J Food Sci Technol*. 2015;52:1164–1169.
2. Sarris J, Stough C, Bousman CA, et al. Kava in the treatment of generalized anxiety disorder: a double-blind, randomized, placebo-controlled study. *J Clin Psychopharmacol*. 2013;33:643–648.

3. Chua HC, Christensen ET, Hoestgaard-Jensen K, et al. Kavain, the major constituent of the anxiolytic kava extract, potentiates GABAA receptors: functional characteristics and molecular mechanism. *PLoS ONE*. 2016;11:e0157700.
4. Whittaker P, Clarke JJ, San RH, et al. Evaluation of commercial kava extracts and kavalactone standards for mutagenicity and toxicity using the mammalian cell gene mutation assay in L5178Y mouse lymphoma cells. *Food Chem Toxicol*. 2008;46:168–174.
5. Savage KM, Stough CK, Byrne GJ, et al. Kava for the treatment of generalised anxiety disorder (K-GAD): study protocol for a randomised controlled trial. *Trials*. 2015;16:493.
6. Boonen G, Haberlein H. Influence of genuine kavapyrone enantiomers on the GABA-A binding site. *Planta Med*. 1998;64:504–506.
7. Pantano F, Tittarelli R, Mannocchi G, et al. Hepatotoxicity induced by "the 3Ks": kava, kratom and khat. *Int J Mol Sci*. 2016;17:580.
8. White JG, Southgate E, Thomson JN, Brenner S. The structure of the nervous system of *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci*. 1986;314:1–340.
9. Varshney LR, Chen BL, Paniagua E, Hall DH, Chklovskii DB. Structural properties of the *Caenorhabditis elegans* neuronal network. *PLoS Comput Biol*. 2011;7:e1001066.
10. Richmond J. Synaptic function (December 7, 2007), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.69.1, http://www.wormbook.org.
11. Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics*. 1974;77:95–104.
12. Shaik AA, Hermanson DL, Xing C. Identification of methysticin as a potent and non-toxic NF-kappaB inhibitor from kava, potentially responsible for kava's chemopreventive activity. *Bioorg Med Chem Lett*. 2009;19:5732–5736.
13. Leitzman P, Narayanapillai SC, Balbo S, et al. Kava blocks 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in association with reducing O^6-methylguanine DNA adduct in A/J mice. *Cancer Prev Res (Phila)*. 2014;7:86–96.
14. 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.
15. Locke CJ, Kautu BB, Berry KP, Lee SK, Caldwell KA, Caldwell GA. Pharmacogenetic analysis reveals a post-developmental role for Rac GTPases in *Caenorhabditis elegans* GABAergic neurotransmission. *Genetics*. 2009; 183:1357–1372.
16. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2014.
17. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. *Linear and Nonlinear Mixed Effects Models*. R package version 3.1-124. http://CRAN.R-project.org/package=nlme. Published 2015.
18. Hothorn T, Bretz F, Westfall P. Simultaneous inference in general parametric models. *Biom J*. 2008;50:346–363.
19. Jospin M, Qi YB, Stawicki TM, et al. A neuronal acetylcholine receptor regulates the balance of muscle excitation and inhibition in *Caenorhabditis elegans*. *PLoS Biol*. 2009;7:e1000265.
20. Barbagallo B, Prescott HA, Boyle P, Climer J, Francis MM. A dominant mutation in a neuronal acetylcholine receptor subunit leads to motor neuron degeneration in *C. elegans*. *J Neurosci*. 2010;30:13932–13942.
21. Mahoney TR, Luo S, Nonet ML. Analysis of synaptic transmission in *Caenorhabditis elegans* using an aldicarb-sensitivity assay. *Nat Protoc*. 2006;1:1772–1777.
22. Rand JB. Acetylcholine (January 30, 2007), WormBook, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.131.1, http://www.wormbook.org.
23. Vashlishan AB, Madison JM, Dybbs M, et al. An RNAi screen identifies genes that regulate GABA synapses. *Neuron*. 2008;58:346–361.
24. Dybbs M, Ngai J, Kaplan JM. Using microarrays to facilitate positional cloning: identification of tomosyn as an inhibitor of neurosecretion. *PLoS Genet*. 2005;1:6–16.
25. Miller-Fleming TW, Petersen SC, Manning L, et al. The DEG/ENaC cation channel protein UNC-8 drives activity-dependent synapse removal in remodeling GABAergic neurons. *eLife*. 2016;5:e14599.
26. Alfonso A, Grundahl K, Duerr JS, Han HP, Rand JB. The *Caenorhabditis elegans* unc-17 gene: a putative vesicular acetylcholine transporter. *Science*. 1993;261:617–619.
27. Gracheva EO, Burdina AO, Holgado AM, et al. Tomosyn inhibits synaptic vesicle priming in *Caenorhabditis elegans*. *PLoS Biol*. 2006;4:e261.
28. Tang X, Amar S. Kavain inhibition of LPS-induced TNF-α via ERK/LITAF. *Toxicol Res (Camb)*. 2016;5:188–196.
29. Schuske K, Beg AA, Jorgensen EM. The GABA nervous system in *C. elegans*. *Trends Neurosci*. 2004;27:407–414.
30. Ibrahim M, Farooq T, Hussain N, et al. Acetyl and butyryl cholinesterase inhibitory sesquiterpene lactones from *Amberboa ramosa*. *Chem Cent J*. 2013;7:116.
31. Hajimehdipoor H, Mosaddegh M, Naghibi F, Haeri A, Hamzeloo-Moghadam M. Natural sesquiterpen lactones as acetylcholinesterase inhibitors. *An Acad Bras Cienc*. 2014;86:801–805.
32. Noor NA. Anxiolytic action and safety of Kava: effect on rat brain acetylcholinesterase activity and some serum biochemical parameters. *Afr J Pharm Pharmacol*. 2010;4:823–828.