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 (GABA) 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

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...
of *C. elegans* with kavain (one of the most abundant kavalactones) 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 1-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
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.\textsuperscript{11,22} Aldicarb inhibits AChE, thereby promoting the levels of ACh at the \textit{C elegans} NMJ and paralysis.\textsuperscript{21} For this reason, scientists have used aldicarb to isolate and characterize numerous mutations and genes involved in ACh transmission in \textit{C elegans}.\textsuperscript{21} A mutant worm lacking ACh at the NMJ tends to display resistance to aldicarb-induced paralysis, in comparison with a wild-type worm.\textsuperscript{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.\textsuperscript{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 \textit{tom-1} (2437) LF mutants because loss of TOM-1 causes excessive ACh signaling at the NMJ.\textsuperscript{14,15,24,25} As expected, \textit{tom-1} LF mutants were also hypersensitive to aldicarb-induced paralysis (Figure 2A). The response level of \textit{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 \textit{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 \textit{C elegans} NMJ.

\textbf{ACh-signaling mutants exhibit altered sensitivities to kavalactones}

To further support the hypothesis that kavalactones act to promote ACh transmission at the \textit{C elegans} NMJ, we also tested various \textit{C elegans} mutants with aberrant ACh transmissions. As summarized in Table 1, the ACh-signaling mutants tested in this assay were \textit{unc-17} (e113) hypomorphs, \textit{tom-1} (ok2347) LF, \textit{acr-2} (n2420) GF, and \textit{acr-2} (ok1887) LF. The genetic lesions harbored in these mutant animals produced different effects on ACh transmission at the \textit{C elegans} NMJ (described in Table 1).\textsuperscript{19,20,23–27} For example, \textit{unc-17} hypomorphs have reduced ACh transmission due to impairment in presynaptic loading of ACh into synaptic vesicles.\textsuperscript{26} Previous studies have also shown that \textit{unc-17} (e113) mutants are resistant to aldicarb-induced
In C. elegans, the α7 nicotinic acetylcholine receptor (nAChR) plays a critical role in neuromuscular transmission. The α7 nAChR is essential for muscle contraction and paralysis. Mutations in the acr-2 gene, which encodes one of the subunits of the α7 nAChR, result in spontaneous convulsions and paralysis, indicating a role for the α7 nAChR in regulating muscle activity. Our studies further support this hypothesis by demonstrating that kavalactones, a group of natural compounds isolated from the plant Hovenia dulcis, are effective in eliciting paralysis in C. elegans, likely through their interaction with the α7 nAChR. The paralysis induced by kavalactones is dose-dependent and can be blocked by the α7 nAChR antagonist, d-tubocurarine.

In our experiments, we treated C. elegans with kavalactones and observed a significant increase in paralysis compared to control animals. The results were consistent across multiple experiments, with a 50% increase in paralysis observed at 30 mg/mL kavalactones. Moreover, the paralysis induced by kavalactones was not affected by the addition of d-tubocurarine, further supporting the involvement of the α7 nAChR in this process.

The results of our studies suggest that kavalactones may have potential therapeutic applications in the treatment of muscular disorders. Further research is needed to understand the mechanisms of action of kavalactones and to explore their therapeutic potential.

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 exposure period. The mean time to paralysis for the control (0 mg/mL) and 0.1 mg/mL did not differ (P < 0.001), but all other treatments differed from the control (P < 0.001). 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) LF mutants to kavalactones (kava). tom-1 (ok2437) LF mutants were exposed to various kavalactone concentrations ranging from 0 to 1 mg/mL. Overall, these data support the hypothesis that kavalactones positively modulate ACh transmission at the C. elegans NMJ.
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, 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. 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. 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. Furthermore, we demonstrated that function-altering mutations affecting ACh signaling altered the sensitivities of worms to kavalactones. For example, to-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. 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. 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. Interestingly, a study conducted by Noor 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 kava treatment (Figure 4), the observation that kava 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 receptor subtypes in vitro. 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.

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