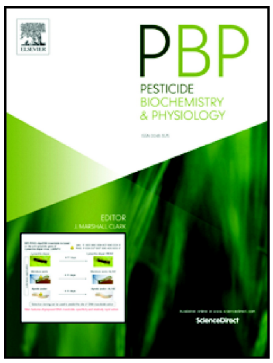

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Toxicity, Mode of Action, and Synergist Potential of Flonicamid Against Mosquitoes

Jennina Taylor-Wells[1,†,a], Aaron D. Gross[1,‡,a], Shiyao Jiang[1], Fabien Demares[1], Joseph S. Clements[2], Paul R. Carlier[2], and Jeffrey R. Bloomquist[1*]

[1]Emerging Pathogens Institute, Department of Entomology and Nematology, University of Florida, Gainesville, FL 32610 USA

[2]Department of Chemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

[†]Current address: Oxitec LTD, 71 Innovation Drive, Milton Park, Abingdon, UK, OX14 4RQ

[‡]Current address: Department of Entomology, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

[a]Co-primary authors

*To whom correspondence should be addressed:

Jeffrey R. Bloomquist
Dept. of Entomology and Nematology
Emerging Pathogens Institute, University of Florida
2055 Mowry Road
Gainesville FL 32610
P.O. Box 100009
(352) 273-9417 (office)
(352) 273-9420 (fax)
Email: jbquist@epi.ufl.edu

Abstract

The present study focused on the toxicity of the aphid anti-feedant flonicamid and its main metabolite, 4-trifluoromethylnicotinamide (TFNA-AM) to *Aedes aegypti* and *Anopheles gambiae* mosquitoes. The compounds were toxic to both species via topical application, resulting in un-coordinated locomotion and leg splaying, with a favorable *An. gambiae* LD_{50} value of 35 ng/mg for TFNA-AM, but no significant lethality to *Ae. aegypti* at 10 μg/female. There was mild cross resistance in the Akron-*kdr* (Akdr) strain of *An. gambiae*. Both compounds were non-toxic to intact larvae (LC_{50} >300 ppm); however, headless *Ae. aegypti* larvae displayed spastic paralysis, with PC_{50} values of 2-4 ppm, indicating that the cuticle is a significant barrier to penetration. TFNA-AM showed low mammalian toxicity, with an LD_{50} of >2000 mg/kg in mice. Electrophysiological experiments showed larval *Aedes* muscle depolarization and Kv2 channel blocking activity that required near mM concentrations, suggesting that this potassium channel is not the main target for flonicamid nor its metabolite. However, TFNA-AM was a potent blocker of evoked body wall sensory discharge in dipteran larvae, suggesting that some component of the chordotonal organ system may be involved in its toxicity. Finally, flonicamid and TFNA-AM showed about 2-fold synergism of permethrin toxicity against *An. gambiae* adult females whose mechanism should become more clear once the mode of action of these compounds is better defined.

Keywords: *Aedes aegypti, Anopheles gambiae,* chordotonal organ, insecticide, mosquito, permethrin

Abbreviations

CNS central nervous system

IC_{50} 50% inhibitory concentration

KD_{50} dose or concentration in which 50% of subjects are knocked down

LC_{50} concentration that killed 50% of treated subjects

LD_{50} dose that killed 50% of subjects

PC_{50} concentration that paralyzed 50% of subjects

PBO piperonyl butoxide

RR resistance ratio

SR synergist ratio

TEA tetraethylammonium

TFNA-AM 4-trifluoromethylnicotinamide

1. Introduction

Effective mosquito control is hampered by a limited spectrum of insecticides, and widespread insecticide resistance [1], with obvious implications for the incidence of vector-borne diseases such as malaria, dengue, and the recent Zika epidemic [2]. The investigation of compounds with novel modes of action and the successful re-evaluation of agricultural insecticides will contribute to more effective mosquito control. The aphid antifeedant flonicamid is a pyridinecarboxamide that is also active on other insects, including whiteflies, thrips and planthoppers [3] and has 4-trifluoromethylnicotinamide (TFNA-AM, Fig. 1) as a major metabolite [4] and active principle [5]. Upon spray treatment, aphids are able to attach to the plant, but salivation and sap feeding are limited or completely blocked [6]; therefore, mortality is presumably caused by starvation. Flonicamid also has a favorable toxicological profile, with low toxicity to birds, fish, bees, and mammals (male rat oral LD_{50} = 884 mg/kg) [3].

Interestingly, the Insecticide Resistance Action Committee (IRAC) originally classified flonicamid with another anti-feedant, pymetrozine, under Group 9 as a chordotonal organ modulator [7]. Evidence for this target includes the ability of pymetrozine to alter posture and cause hind leg extension, as well as block femoral chordotonal organ discharge [8]. In the most recent classification, flonicamid was re-classified to group 29, to which it is the lone member and mode of action is linked to chordotonal organ modulation, but 'the specific target protein(s) responsible for biological activity are distinct from Group 9 and remain undefined' [9]. A recent paper by Kandasamy et al. [10] investigated the mode of action of chordotonal organ modulators on TRP channels, but both flonicamid and TFNA-AM were inactive on this target. Morita *et al.* [6] also described flonicamid as having a mode of action distinct from pymetrozine, since treated insects did not display contractions of the foregut characteristic of pymetrozine [11], but exposed

aphids did display staggered walking, with their legs extended at the femur/tibia and tibia/tarsus joints. [4]. Flonicamid does not act on γ-aminobutyric acid, nicotinic acetylcholine, glutamate or octopamine receptors. Nor does it act upon the L-type calcium channel, sodium channel, ryanodine calcium channels, or as an inhibitor of the acetylcholinesterase enzyme [12]. However, 10 μM flonicamid did show blockage of A-type (Kv1.5) potassium current with similar, but less extensive blockage of K-type, Kv2 current, when evaluated in patch clamped lepidopteran (*Heliothis virescens*) adult neurons [13].

The present study assessed the toxicity of flonicamid and TFNA-AM to *Ae. aegypti* and *An. gambiae*, as well as the oral mouse toxicity of TFNA-AM. Mode of action was investigated using various larval *in situ* electrophysiological techniques, combined with patch clamp studies of engineered HEK-293 cells expressing *An. gambiae* Kv2.1 channels. Finally, because patent filings claim a synergistic action of flonicamid with other insecticides [14,15], flonicamid and TFNA-AM were tested as synergists for the pyrethroid, permethrin.

2. Materials and Methods

2. 1. Chemicals

Flonicamid and technical permethrin were purchased from ChemService Inc. (West Chester, PA, USA). TFNA-AM was prepared in two steps from 4-(trifluoromethyl)nicotinic acid or purchased from Oakwood Chemical (Estill, SC, USA). PBO (> 90%) and TEA (>98%) were purchased from Sigma Aldrich (St., Louis, MO, USA). Propoxur (99%) was obtained from Fluka (St. Louis, MO, USA). DMSO (99.7%) and ethanol (>99%) were obtained from Fisher Bioreagent (Fair Lawn, NJ, USA). Cell culture materials were obtained from ThermoFisher Scientific (Waltham, MA, USA), unless otherwise stated. Buffer reagents were obtained from various commercial suppliers.

2.2. Insects

Third instar *Ae. aegypti* (Orlando insecticide-susceptible strain) larvae were obtained from a colony maintained by the United States Department of Agriculture-Agricultural Research Service, Center for Medical and Veterinary Entomology (Gainesville, FL, USA). Mosquitoes were reared according to Pridgeon *et al.* [16]. Larvae were fed a ground liver and yeast mixture, and pupae placed in emergence cages (BioQuip Products, Inc., Rancho Dominguez, CA, USA). Adults were held at 28 ^{o}C and provided with cotton balls soaked in 10% sugar water for rearing and for all bioassays.

Two strains of *An. gambiae* wild-type (G3 strain; MRA-112) and (Akron-kdr strain; MRA-1280), were obtained from colonies maintained as part of the Malaria Research and Reference Reagent Resource Center, part of the Biodefense and Emerging Infections Research Resources Repository at the Center for Disease Control and Prevention (Atlanta, GA, USA). The G3 (insecticide-susceptible) and Akron-kdr (Akdr; pyrethroid-resistant) strains were reared at the University of Florida, Emerging Pathogens Institute (Gainesville, FL, USA). Briefly, eggs and larvae were incubated at 28 ^{o}C in tap water and fed ground fish food (Tetra, Blacksburg, VA, USA). Pupae were collected and placed in paper containers secured with fine tulle for adult eclosion.

For other species, third-instar *Musca domestica* larvae (FS strain) were kindly provided by Dr. Phil Kaufman at the University of Florida, Department of Entomology and Nematology (Gainesville, FL, USA). A susceptible strain of *Drosophila melanogaster* (Oregon-R), maintained in culture at the University of Florida since 2009, was reared in plastic vials on artificial media purchased from Carolina Biological Supply (Burlington, NC, USA).

2.3. Mouse toxicity experiments

Adult female *Mus musculus* (ICR strain, 20-25 g) were purchased from Envigo (Tampa, FL, USA). A toxicity screen of TFNA-AM was performed using an OECD-approved, modified "up-and-down method" [17]. Mice were dosed orally via gavage using olive oil mixtures ($\leq$ 400 µl). The University of Florida IACUC (Protocol # 201408463) approved all procedures for these experiments. Mice were monitored every 4 hr for 24 hr after the administration of TFNA-AM, and as per the OECD protocol, were sacrificed at any sign of discomfort (vocalization, convulsions, etc.) and counted as dead. Overall lethality was recorded at 24 hr post-exposure.

2.4. Intact and headless Ae. aegypti *larval toxicity assays*

Third instar *Ae. aegypti* larvae (n=10) were placed in 35 mm plastic dishes containing tap water plus ethanol vehicle, final 0.5% (v/v) or with test compound in ethanol. Dishes were held in a 28 ^{o}C incubator and evaluated every 24 hr (up to 72 hr) to determine mortality (LC_{50} in ppm). A headless larval bioassay was used to provide a better estimate of the intrinsic paralytic activity of test compounds under condition of enhanced penetration [18]. Decapitation of fourth instar larvae was performed with forceps, and the larvae (n = 5-10) were placed into 3 ml of chemical/solvent/saline solution and observed for toxic effects for a period of five hr. The mosquito saline was composed of (mM) sodium chloride (154), calcium chloride (1.4), potassium chloride (2.7), and HEPES (1.2) at pH 6.9 [19], and the final solvent (ethanol) concentration was 0.5% (v/v). An unaffected headless larva showed a strong bilateral contractile motion when probed, whereas paralysis was defined by loss of response [18]. At least five concentrations were employed per experiment, and paralysis data expressed as PC_{50} in ppm.

2.5. Mosquito adult toxicity assays

Non-blood fed adult female *Ae. aegypti* and *An. gambiae* (1-5 days post-eclosion) were collected and anesthetized on ice, and methods paralleled those of Francis et al. [20]. Treatments

(0.2 µl) were topically applied using a Hamilton 700 series syringe and a PB600 repeating dispenser (Thermo Fisher Scientific, Hampton, NH, USA). Ethanol (100%) was used as a vehicle, with controls dosed with ethanol alone. For treated females (n = 8-20 per dose) knockdown was assessed at one and four hr, mortality at 24-72 hr. Knockdown was considered loss of postural control and uncontrollable or completely inhibited flight. Mortality was considered as loss of posture (mosquitoes usually lying on their sides or back) with no movement or only slight leg movements. A minimum of five concentrations were tested per compound, and data were expressed as KD_{50} and LD_{50} in ng/mg body weight. For synergism studies, flonicamid or TFNA-AM at the LD_{10} were co-applied topically to *An. gambiae* adults with a range of permethrin doses to assess any effect on the LD_{50}.

Surface contact assays were performed on glass and paper. Glass test tubes (20 x 150 mm) were coated with compound dissolved in acetone (250 µl). The tubes were rotated by hand to ensure complete and even coverage of the inside and left to dry for 30 min. *Ae. aegypti* were anaesthetized on ice and 10-20 insects added to the tube secured with a cotton ball soaked with 1 ml of 10% sugar water. Mortality data were collected at 24 hr, as described for the topical assay, pooled for probit analysis and LC_{50} calculated, expressed as µg/cm^2. Toxicity via paper contact was performed with a slightly modified World Health Organization (WHO) protocol [21]. Briefly, 100% ethanol as control or compound dissolved in ethanol (2 ml) was applied evenly to a 180 cm^2 (12 x 15 cm) sheet of cellulose chromatography paper (Fisher Scientific). The paper was left to dry overnight and placed in a WHO treatment chamber. Twenty adult females were placed in the holding chamber to acclimatize for 1 hr, moved to the treatment chamber for 1 hr to expose them to treated paper, and then moved back to the holding chamber for 24 hr. Mortality was assessed as described above for the glass assay.

A feeding assay [20] was performed by dissolving the compound in 0.5% ethanol as vehicle, then dispersed in 10% sugar water. Mosquitoes were anaesthetized on ice before transferring 10-20 insects to glass tubes and starved for 6 hr. A cotton ball soaked with 1 ml of 10% sugar water containing the compound (or ethanol only as control) was then used to stopper the tubes. Mosquitoes were observed for mortality every 24 hr, for a 7 day (168 hr) period, with the compound/ethanol/sugar solution replaced every day. Mortality was assessed as described above for the surface contact assays.

2.6. Toxicity data analyses

Bioassays were performed as $n \geq 3$ experiments with ≥ 2 different batches of insects, and Abbott's formula was used to correct for control mortality under 20% [22]. Any experiments with control mortality $\geq$ 20% were discarded. Values reported as the mean PC_{50}, KD_{50}, LD_{50} or LC_{50} (n = 3 replicates), with confidence intervals, were all calculated by PROC PROBIT analysis in the statistical analysis package SAS 9.3 (SAS Institute, Cary, NC, USA).

2.7. Ae. aegypti *larval neuromuscular electrophysiology*

Membrane potential was recorded from *in situ* 4th instar *Ae. aegypti* larval muscle (viscera and CNS removed) bathed in saline [19], using a conventional glass microelectrode placed in the large IIa ventrolateral muscle fiber [23]. The signal was amplified, filtered, and digitized as previously described by Larson et al. [24]. The preparation was left for 5 minutes to stabilize the membrane potential before the addition of 0.5 µl DMSO (0.1% v/v final) as a control, followed by various concentrations of test compound in DMSO at 5 min intervals. A minimum of 3 larvae were used for each experimental condition, with 5 test concentrations per preparation.

2.8. M. domestica *larval sensory electrophysiology*

Sensory recordings from the peripheral nerves of *M. domestica* were performed assuming they contain segmental chordotonal organs, similar to *Drosophila melanogaster* larvae, given the striking conservation of these sensilla in immature stages across insect orders [25], and using methods similar to those of Bradfisch and Miller [26]. Third instar larvae were pinned to a dissecting dish and immersed in physiological saline containing (mM) NaCl (140), $CaCl_2$ (0.75), KCl (5), $MgCl_2$ (4), $NaHCO_3$ (5), and HEPES (5), at pH 7.25. After viscera and CNS were removed, peripheral nerves were collected in a glass suction electrode containing physiological saline and spontaneous nerve firing recorded using an AC/DC differential amplifier (Model 3000 A-M Systems, Carlsborg, WA). Noise was eliminated using a HumBug (Quest Scientific, North Vancouver, BC, Canada) and LabChart 7 with Power Lab (ADInstruments, Colorado Springs, CO, USA) was used to record the output in mV, which was then converted into a rate plot, expressed in spikes/sec or Hz. The preparation was left for 5 minutes to stabilize before the addition of 0.5 µl DMSO (0.1%, v/v, final) as a control, followed by various test concentrations in DMSO. Treatments were added at five-minute intervals. A minimum of 3 larvae were used for each experimental condition, with ≥ 4 test concentrations per experiment.

Stimulation of body wall sense organs in this preparation was performed by mechanical deformation of the cuticle using a glass capillary tube, fire polished on the end and glued to a small radio speaker. Mechanical stimuli were delivered as 2-3 V square pulses of 200 msec duration and at a frequency of 0.25 Hz. Sensory action potentials were amplified and noise removed as described above and the signal digitized using Lab Chart Pro 7 Software in Scope® mode. Sensory responses were analyzed before and 5 min post-application of TFNA-AM, where 20 time blocks were signal averaged, a block defined as one 500 msec sweep (100 msec pre-trigger with 400 msec post-trigger recording). DMSO vehicle (0.1%) served as a negative

control. Sensory discharges resulting in potentials greater than the baseline electrical activity (approximately 50 μV) were counted for 50 msec after stimulation, with background noise removed. An unpaired t-test between individual treatments was performed to determine significant difference at discrete time points following treatment (α=0.05). Data were plotted as bar graphs with t-test/ANOVA and IC_{50} determined by nonlinear curve fitting to a four parameter logistic equation, all using GraphPad Prism 7 (La Jolla, CA, USA).

2.9. D. melanogaster *CNS electrophysiology*

Extracellular electrophysiology recordings were performed on dissected CNS from wandering third-instar *D. melanogaster* larvae essentially as described previously [27]. Briefly, the excised, transected CNS was placed in a wax dish, and descending electrical activity from the neve trunks was recorded with a suction pipette. Test solution (1 μL) was added to the bath using DMSO (0.1%) as a vehicle and mixed by pipetting several times. CNS firing frequency was averaged (± SEM) over 3-min intervals, immediately prior to (baseline), and every 3 min after application of the test compound, for 30 min.

2.10. Patch clamp electrophysiology of HEK-293 cells expressing Ag*Kv2.1*

Human embryonic kidney (HEK-293 cells, CRL-1573) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS as described previously [24]. HEK293 cells were engineered by Dualsystems Biotech AG (Schlieren, Switzerland) to express the *An. gambiae* Kv2.1 (*Ag*Kv2.1) channel gene (Accession #XM_315955.4) using a CMV promoter. Tetracycline (1μg/mL) treatment was used to induce *Ag*Kv2.1 (I_K) channel expression, and cells underwent patch clamp using conventional techniques. Currents were recorded, drug treatments applied, and data analyzed exactly as described in Larson et al. [24].

3. Results

3.1. Signs of intoxication in larval and adult mosquitoes

Adult *Ae. aegypti* and *An. gambiae* (G3 and Akdr) mosquitoes intoxicated with flonicamid or TFNA-AM displayed staggered walking and un-coordinated flight, as well as leg tremors and distinctive splaying of all legs, often with curled up tarsi (Fig. 2). Over time, treated mosquitoes lost postural control and often collected on the bottom of the container. These effects were observed within 1 hr of topical treatment, at doses of ≥100 ng flonicamid (less for the Akdr strain) and ≥1 ng TFNA-AM per mosquito, and persisted up to 72 hr post-treatment, at which time observations ceased. Leg splaying was also observed following glass contact, feeding and injection assays, but was absent following paper contact assays. When tested in a headless larvae paralysis assay, both flonicamid and TFNA-AM induced spastic paralysis, with affected larvae showing a bent or twisted posture and hyper-excited movements.

3.2. Mosquito and mouse bioassay results

Flonicamid and TFNA-AM were topically applied to adult *Ae. aegypti* and *An. gambiae* mosquitoes (Table 1). Responses to these compounds were somewhat variable as shown through KD_{50} values at 1 hr and 4 hr, and the 24 hr LD_{50}. Flonicamid was 2.7-fold more effective at knocking down *Ae. aegypti* mosquitoes at 1 h versus 4 h (Table 1), but was not lethal for 24 hr at doses up to 10 µg/mg (Table 1). TFNA-AM also resulted in a lower KD_{50} at 1 hr versus 4 hr, the ratio was much larger (24-fold), and it did not cause lethality at 10 µg/mg (Table 1). At 1 hr, the KD_{50} for TFNA-AM was 11-fold lower than that of flonicamid, but at 4 hr, there was little or no difference between the two compounds (1.3-fold). The slope values for 1 hr knockdown also differed when comparing flonicamid and TFNA-AM in *Ae. aegypti* (Table 1).

For *An gambiae*, flonicamid against the G3 strain (susceptible) had 2.8-fold more potent knockdown (reflected in the KD_{50} value) at 4 hr than at 1 hr, and TFNA-AM 8.8-fold (Table 1),

both opposite to that observed for *Ae aegypti*. TFNA-AM treatment had a 1 hr KD_{50} value that was 6-fold lower than flonicamid, and at 4 hr the difference was 19-fold in the G3 strain (Table 1). In the Akdr strain, flonicamid had a KD_{50} value at 4 hr that was 2.8-fold greater than at 1 hr and TFNA-AM an 18.5-fold difference, similar to the pattern observed for *Ae aegypti*. In Akdr, TFNA-AM treatment had a 1 hr KD_{50} value that was 6.8-fold lower than flonicamid, but at 4 hr the difference was negligible (Table 1). Interestingly, the Akdr strain was significantly more sensitive to flonicamid (5.3-fold), while the TFNA-AM LD_{50} in the *An. gambiae* Akdr strain was not significantly elevated, and the response highly variable.

Several additional bioassays were conducted with *Ae. aegypti* to explore different routes of exposure. Feeding assays with flonicamid and TFNA-AM resulted in a significant effect on mosquito survival. Survival was significantly reduced with both flonicamid and TFNA-AM starting at 4 days post-treatment, and lasting for 7 days (Fig. 3), but did not exceed 30% mortality. Surprisingly, intrathoracic injections of flonicamid and TFNA-AM did not result in mortality >50% at doses up to 500 ng/mg. In contrast, intrathoracic injections with propoxur yielded an LD_{50} value of 0.22 ng/mg, close to the value of 0.24 ng/mg reported in the literature [20]. TFNA-AM showed no lethality to mice at an oral dose of 2,000 mg/kg, and only slight hyperactivity and hyperexcitability were observed. *Ae. aegypti* adult females exposed to flonicamid and TFNA-AM in a glass test tube assay resulted in LC_{50}s of 13 (10 – 15) $\mu g/cm^2$ and 9 (6 – 21) $\mu g/cm^2$, respectively. However, these LC_{50} values are much greater than permethrin, which had an LC_{50} of 0.17 ng/cm^2. The WHO paper contact assay showed that 1 mg/ml of both flonicamid and TFNA-AM (11 $\mu g/cm^2$) did not result in any mortality.

Flonicamid and TFNA-AM were not toxic to intact *Ae. aegypti* larvae (LC_{50} > 300 ppm) at 24 hr. For comparison, propoxur resulted an LC_{50} value of 0.51 (0.38 – 0.83) ppm at 24 hr, in

line with recently published values [19]. However, when headless larvae were exposed to either compound in a 5 hr paralysis assay [13], flonicamid and TFNA-AM resulted in PC_{50}s of 4 (2 – 8) ppm and 2 (0.9 – 11 ppm), respectively. Further, the larvae showed clear signs of neuronal intoxication. When immersed in TFNA-AM at paralytic doses (e.g., 10 ppm) the larvae were contracted, and sometimes showed fine twitching, along with exaggerated and prolonged responses to probing. Those exposed to flonicamid were mostly contracted with less expression of twitching or hyper-excitability.

Because there are claimed synergistic interactions between flonicamid and other insecticides [12,13], flonicamid and TFNA-AM were tested as synergists of the pyrethroid, permethrin against adult female *An. gambiae*. These experiments were performed using a fixed dose of flonicamid or TFNA-AM (calculated LD_{10}), combined with a range of doses of permethrin (Fig. 4). In these studies, permethrin alone had an LD_{50} = 0.212 ng/mg (0.184-0.254), while co-application with flonicamid lowered the LD_{50} to 0.098 ng/mg (0.086-0.112). This latter value gave a synergistic ratio of 2.16 and was significantly different from the expected additive LD_{50} = 0.137 (0.113-0.166) by statistical t-test ($P < 0.04$). Similarly, co-application of permethrin with TFNA-AM reduced the LD_{50} to 0.103 ng/mg (0.080-0.131), giving a synergistic ratio of 2.06 and this LD_{50} was significantly different (t-test, $P < 0.01$) from the expected additive value, 0.155 mg/mg (0.132-0.187).

3.3. Electrophysiological screening of muscle and nerve preparations

The ability of flonicamid and TFNA-AM to depolarize *Ae. aegypti* larval muscle was investigated. Flonicamid showed a weak depolarizing effect on the muscle membrane potential at 1 mM in 2/3 of the preparations (Fig 5A). TFNA-AM was more potent, with some muscles responding at 100 µM (3/5 preparations), and a greater depolarization at 1 mM (Fig 5B). As a

negative control, a preparation with five consecutive applications of DMSO were performed, yielding no effect on the membrane potential (data not shown).

Patch clamp electrophysiological recordings

Because of the depolarizing effect of TFNA-AM on mosquito muscle, comparative studies on *An. gambiae* voltage-sensitive potassium channel (*Ag*Kv2.1) were performed using whole-cell patch clamp of engineered HEK-293 cells (Fig. 6). In this assay, the standard blocker TEA had an IC_{50} = 1.6 (1.1 – 2.3) mM. Flonicamid and TFNA-AM inhibited Kv2.1 current, similar to TEA. About 50% inhibition was observed at 1 mM, and there was no difference in either potency or efficacy between the two compounds (Fig. 6). Because of the low activity in this assay and solubility issues, additional concentrations of flonicamid and TFNA-AM were not attempted.

The sensory nervous system was investigated as a possible target of flonicamid and TFNA-AM using third instar *M. domestica* larvae. While recording spontaneous activity in un-stimulated preparations, flonicamid had no effect on peripheral nerve firing (in 3/3 preparations), while TFNA-AM either increased or decreased discharge rate, depending on concentration. However, the effects were variable, so a stimulation protocol was employed. Repetitive mechanical probing of *M. domestica* larval cuticle resulted in consistent ascending sensory nerve discharges (Fig. 7), presumably via chordotonal organ activation. Spikes occurred at both the onset and offset of the mechanical stimulus, as observed previously [24]. Evoked nerve impulses were not affected by 0.1% DMSO (Fig. 7A) or 100 µM flonicamid (Fig. 7B), whereas TFNA-AM at 100 µM showed complete inhibition of stimulus-evoked potentials (Fig. 7C) without evidence of enhanced discharge. Averaged effects on spiking at 100 µM quantified the clear difference in activity of flonicamid and TFNA-AM (Fig. 7D). Concentration-response studies

showed progressive inhibition of nerve discharge that commenced at 100 nM and resulted in a calculated IC_{50} value of 2.0 (0.5 – 8.8) µM (Fig. 7E). The curve had a shallow slope value of -0.47 and $R^2 = 0.73$ with the top and the bottom of the curve fixed at 100 and 0, respectively.

To ascertain whether effects of TFNA-AM were specific for sensory nerves, the sensitivity of CNS firing was investigated using a third-instar larval *D. melanogaster* preparation (Fig. 8). CNS preparations were treated with 10 µM TFNA-AM, which is 5-fold greater concentration than the sensory IC_{50} and below the threshold concentration for effects on mosquito larval muscle or Kv2 potassium channel blockade. It was observed that 10 µM TFNA-AM did not significantly alter the CNS firing rate for at least 10 min compared to the control (0.1% DMSO).

4. Discussion

Flonicamid and TFNA-AM displayed unique effects when topically applied to *Ae. aegypti* and *An. gambiae*. Treated mosquitoes had un-coordinated locomotion and distinct splaying of the legs, similar to aphids, which displayed uncoordinated walking, and legs extended at the femur/tibia and the tibia/tarsus joints [6]. A similar abnormal posture and extension of the hind legs was also observed in locusts treated with pymetrozine [8], as well as flonicamid and TFNA-AM (Ralf Nauen, personal communication).

Favorable KD_{50} and LD_{50} values were observed for adult *An. gambiae* (Table 1), that were significantly lower than those observed for *Ae. aegypti*. We speculate that the disparity between the lethality of flonicamid and TFNA-AM to the two mosquito strains may relate to differences in cuticular penetration and metabolism. Comparable toxicity was observed with the Akdr strain of *An. gambiae*, a pyrethroid-resistant strain, demonstrating that flonicamid and its metabolite are not affected by the *kdr* mutation in the voltage-sensitive sodium channel, or other

metabolic resistance factors present in this strain [28], an advantage for use in the field. It is interesting to note that feeding flonicamid or TFNA-AM at 1,000 ppm in sugar water did not result in significant mosquito mortality until 4 days of exposure, in contrast to the strong anti-feedant properties associated with flonicamid in aphids [4,6].

In contrast to adult mosquitoes, the larval assays indicate that the cuticle is a barrier to the penetration of both flonicamid and TFNA-AM, as both compounds were poor larvicides (LC_{50} >300 ppm), whereas headless larvae displayed spastic paralysis, with PC_{50} values 2 – 4 ppm for both compounds. The greater sensitivity to headless larvae demonstrates that the site of action is internal and apparently not accessible via aqueous exposure to intact larvae.

Flonicamid has been placed in IRAC group 29 as a modulator of chordotonal organs [7]; however, evidence for this mode of action is poorly documented in the literature. Kandasamy et al. [10] investigated the mode of action of chordotonal organ modulators on TRP channels, but both flonicamid and TFNA-AM were inactive on this target. A number of metabolizing enzymes and neurological receptors have been ruled out as targets of flonicamid [12], and Hayashi et al. [13] reported that flonicamid may act by blockage of potassium channels. We therefore sought to further define its mode of action, investigating the effect of flonicamid and its metabolite on potassium channels in relation to its actions on muscle and nerve preparations. Concentrations of at least 1 mM and 100 µM of flonicamid and TFNA-AM, respectively, were required to depolarize *Ae. aegypti* larval muscle membrane. (Fig. 5). Inhibition of *Ag*Kv2 by flonicamid and TFNA-AM was also observed, but the compounds were equipotent, and required concentrations greater than 100 µM (Fig. 6). Thus, while the low potency for blocking Kv2 is consistent with a weak depolarizing effect on muscle, it indicates that Kv2 potassium channels are at best a secondary target of flonicamid and TFNA-AM.

Compared to muscle, the *M. domestica* sensory nervous system was more sensitive to TFNA-AM, with an IC_{50} = 2 µM for blockage of evoked discharges (Fig. 7), as well as variable changes in spontaneous firing of unstimulated preparations. The shallow Hill slope for this effect (Fig. 7) may reflect impeded drug delivery. These actions were similar to that of pymetrozine in a locust chordotonal organ mechanoreceptive preparation, where it caused a temporary tonic firing response, but whose main effect was complete block of elicited discharges [8]. Flonicamid was inactive on this sensory preparation, consistent with its hypothesized role as a proinsecticide. Further, there was no obvious effect of TFNA-AM on the CNS at 10 µM (Fig.8), indicating that the blocking effect was specific for sensory nerves. Thus, the primary action of TFNA-AM observed in this study is consistent with chordotonal organ modulation, with a less potent blocking action on potassium channels. Further studies are required to confirm the molecular site of action of TFNA-AM, and establish a linkage between effects on chordotonal organs, leg splaying, and in some way, lethality. Whether effects on other potassium channel subtypes might play a role in chordotonal organ disruption remains to be determined.

Finally, the positive responses to flonicamid and TFNA-AM in adult *An. gambiae* led us to test it as a synergist for the pyrethroid, permethrin (Fig. 4). Both flonicamid and its metabolite were effective synergists, significantly increasing the toxicity compared to permethrin alone (RR = 2). As the mode of action of this chemistry is better defined, the physiological basis for this interesting synergistic effect should become more clear.

Acknowledgements

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References

[1] A.F. Read, P.A. Lynch, M.B. Thomas, How to make evolution-proof insecticides for malaria control, PLoS Biol. 7 (2009) e1000058.

[2] N. Liu, Insecticide resistance in mosquitoes: impact, mechanisms, and research directions, Annu. Rev. Entomol. 60 (2015) 537-559.

[3] ISK, Flonicamid new systemic insecticide. https://www.iskweb.co.jp/products/pdf/flonicamid.pdf (accessed 12 December 2017).

[4] APVMA, Public Rlease Summary on the Evaluation of the new active Flonicamid in the product Mainman 500 WG Insecticide, Australian Pesticides and Veterinary Medicines Authority, 2014. https://apvma.gov.au/sites/default/files/publication/13721-prs-flonicamid.pdf (accessed 12 December 2017).

[5] V. L. Salgado, A. Nesterov, R. Kandasamy, C. Spalthoff, M. G. Goepfert, Action of Insecticides on Chordotonal Organs, (2014) p. 61, 13th IUPAC International Congress of Pesticide Chemistry, San Francisco, CA, USA.

[6] M. Morita, T. Ueda, T. Yoneda, T. Koyanagi, T. Haga, Flonicamid, a novel insecticide with a rapid inhibitory effect on aphid feeding, Pest Manag. Sci. 63 (2007) 969-973.

[7] T.C. Sparks, R. Nauen, IRAC: Mode of action classification and insecticide resistance management, Pestic. Biochem. Physiol. 121 (2015) 122-128.

[8] J. Ausborn, H. Wolf, W. Mader, H. Kayser, The insecticide pymetrozine selectively affects chordotonal mechanoreceptors, J. Exp. Biol. 208 (2005) 4451-4466.

[9] IRAC, IRAC Mode of Action Classification Scheme, Version 8.3, July 2017. http://www.irac-online.org/documents/moa-classification/ (accessed 12 December 2017).

[10] R. Kandasamy, D. London, L. Stam,W. von Deyn, X. Zhao, V. L. Salgado, A. Nesterov, Afidopyropen: New and potent modulator of insect transient receptor potential channels, Insect Biochem. Mol. Biol. 84 (2017) 32-39.

[11] L. Kaufmann, F. Schurmann, M. Yiallouros, P. Harrewijn, H. Kayser, The serotonergic system is involved in feeding inhibition by pymetrozine. Comparative studies on a locust (Locusta migratoria) and an aphid (Myzus persicae), Comp. Biochem. Physiol. C Toxicol. Pharmacol. 138 (2004) 469-483.

[12] B. Black, J. Argentine, R. Chaguturu, R. Dargar, L. Dungan, R. Eldridge, L. Kinne, R. Peters, A. Wrzesinski, D. Yuhas, Flonicamid mode of action studies, in: P.B. Goodell, P.C. Ellswort, Second International Lygus Symposium Asilomar, J. Insect Sci. 8 (2007) 4.

[13] J. H. Hayashi, G. Kelly, L. P. Kinne, Flonicamid mechanism-of-action studies using whole-cell patch-clamping of cultured insect neurons, in: P.B. Goodell, P.C. Ellswort (Eds.), Second International Lygus Symposium Asilomar, J. Insect Sci. 8 (2007) 15.

[14] I. Annan, T. Selby, G. Lahm, T. Stevenson, H. Portillo, J. Flexner, Synergistic mixtures for controlling invertebrate pests containing an anthranilamide compound and flonicamid. European patent EP20100009772 (2012) https://www.google.ch/patents/EP2281458B1?cl=en&hl=de (accessed 12 December 2017).

[15] L. Gomez, R. Hunter, M. Shaw, T. Trullinger, M. Kubiszak, Synergistic pesticidal compositions and related methods, U.S. patent US20150111744 A1 (2015) https://www.google.com/patents/US20150111744 (accessed 12 December 2017).

[16] J.W. Pridgeon, R.M. Pereira, J.J. Becnel, S.A. Allan, G.G. Clark, K.J. Linthicum, Susceptibility of *Aedes aegypti, Culex quinquefasciatus* Say, and *Anopheles*

quadrimaculatus Say to 19 pesticides with different modes of action, J. Med. Entomol. 45 (2008) 82-87.

[17] R. Bruce, An up-and-down procedure for acute toxicity testing, Fundam. Appl. Toxicol. 5 (1985) 51-157.

[18] R.M. Islam, J.R. Bloomquist, A method for assessing chemically-induced paralysis in headless mosquito larvae, Methods X 2 (2015) 19-23.

[19] R.O. Hayes, Determination of a physiological saline for *Aedes aegypti* (L.), J. Econ. Entomol. 46 (1953) 624-627.

[20] S. A. M. Francis, J. Taylor-Wells, A. D. Gross, J. R. Bloomquist, Toxicity and physiological actions of carbonic anhydrase inhibitors to *Aedes aegypti* and *Drosophila melanogaster*, Insects 8 (2017) 2, doi:10.3390/insects8010002.

[21] W.H.O., Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets, WHO/CDS/NTD/WHOPES/GCDPP/2006.3 (2006) http://www.who.int/whopes/resources/who_cds_ntd_whopes_gcdpp_2006.3/en/ (accessed 12 December 2017).

[22] W.S. Abbott, A method of computing the effectiveness of an insecticide, J. Econ. Entomol. 18 (1925) 265–267.

[23] B. Samtleben, Anatomie und Histologie der Abdominal- und Thoraxmuskulatur von Stechmückenlarven, Akad. Verlagsges, Leipzig, 1929.

[24] N.R. Larson, P.R. Carlier, A.D. Gross, R.M. Islam, M. Ma, B. Sun, M.M. Totrov, R. Yadav, J.R. Bloomquist, Toxicology of potassium channel-directed compounds in mosquitoes, NeuroToxicol. 60 (2016) 214-223.

[25] L. H. Field, T. Matheson, Chordotonal organs of insects, Int. Adv. Insect Physiol. 27 (1998) 1–230.

[26] G. Bradfisch, T. A. Miller, Behavioural and electrophysiological investigation of 1-(7-ethoxygeranyl)-2-methylbenzimidazole on the house fly *Musca domestica*, Pest Man Sci 17 (1986) 119-127.

[27] J. R. Bloomquist, R. ffrench-Constant, R. Roush, Reduced neuronal sensitivity to dieldrin and picrotoxinin in a cyclodiene-resistant strain of *Drosophila melanogaster* (Meigen), Arch. Insect Biochem. Physiol. 19 (1992) 17-25.

[28] J.M. Mutunga, T.D. Anderson, D.T. Craft, A.D. Gross, D.R. Swale, F. Tong, D.M. Wong, P.R. Carlier, J.R. Bloomquist, Carbamate and pyrethroid resistance in the akron strain of *Anopheles gambiae*, Pestic. Biochem. Physiol. 121 (2015) 116-121.

Table 1. Topical toxicity of flonicamid and TFNA-AM to female *Ae. aegypti* and *An. gambiae* mosquitoes. For 1 and 4 hr time points, lowercase letters after each KD_{50} indicate statistical significance between paired column values within each species/strain. Uppercase letters indicate statistical significance of KD_{50} or LD_{50} values (same row) for the two compounds within each species/strain. Bold letters indicate comparison between G3 and Akdr strain at the LD_{50} level. Statistical significance for ED_{50}s was assessed by non-overlap of the 95% confidence limits.

Mosquito species (strain)	Time (h)	Flonicamid		TFNA-AM	
		ED_{50}[1] (ng/mg)	Slope ± SEM	ED_{50}[1] (ng/mg)	Slope ± SEM
Ae. aegypti (Orlando)	1	251 aA (149 – 391)	1.28 ± 0.20	22 aB (11 - 44)	0.60 ± 0.08*
	4	686 bA (518 – 934)	2.58 ± 0.46	530 bA (143 – 6206)	0.43 ± 0.10
	24	9 ± 4%[2]	---	11 ± 2%[2]	---
An. gambiae (G3)	1	211 aA (143 - 325)	1.41 ± 0.23	35 aB (17 - 99)	0.71 ± 0.12
	4	76 bA (49 – 106)	1.42 ± 0.22	4 bB (1 – 11)	1.05 ± 0.34
	24	484 **a**A (240 – 1462)	0.83 ± 0.20	38 **a**B (19 – 108)	0.62 ± 0.11
An. gambiae (Akdr)	1	54 aA (11 – 153)	0.99 ± 0.14	8 aA (3 – 16)	0.75 ± 0.12
	4	152 aA (85 – 247)	0.99 ± 0.14	178 bA (68 – 636)	0.50 ± 0.80
	24	92 **b**A (37 – 171)	0.87 ± 0.17	126 **b**A (81 – 32950)	0.39 ± 0.11

[1]The effective dose (ED_{50}) represents the pooled data of multiple replicates done on different days with KD_{50} at 1 and 4 hr, and LD_{50} at 24 hr. KD_{50} and LD_{50} values were adjusted for mosquito weight.

[2]Percentage of mortality (± SEM) at 10 µg/mosquito.

*Significantly different slope value ($p < 0.05$, t-test).

Figure 1. Structures of flonicamid and its active metabolite, TFNA-AM

Figure 2. Adult female *An. gambiae* (Akdr) 1hr after topical treatment with 8 µg TFNA-AM in 200 nl ethanol, displaying splayed legs and improper position of wings. This high dose was used to document maximal expression of splayed legs.

Figure 3. Feeding *Ae. aegypti* 10% sugar water that was treated 1,000 ppm flonicamid (open squares), sugar water treated with 1,000 ppm TFNA-AM (closed squares), and a vehicle control (0.1% ethanol; closed circles). All time points from day 4 onward for both flonicamid and TFNA-AM showed a statistically significant difference from control (asterisks), using an unpaired t-test (p-value <0.05).

Figure 4. Synergistic interaction between flonicamid, TFNA-AM, and permethrin against adult female *An. gambiae*. Symbols are mean 24 hr mortality $\pm$ SEM (n = 4). Toxicity curves with data points for each compound were fit to a four-parameter logistic equation. Also shown are theoretical curves without data points for each binary combination for comparison.

Figure 5. Effects of flonicamid (A) and TFNA-AM (B) on the membrane potential of fourth instar *Ae. aegypti* larval muscle. Treatment applications are indicated by the large black vertical lines and labelled with the concentration applied. Initial applications were made with DMSO alone. Each recording was replicated with at least 3 larvae and the recording electrode was withdrawn at the end of the recording (not shown) to assess for drift, which was minimal.

Figure 6. Inhibition of *Ag*Kv2. Inhibition of engineered cells expressing *An. gambiae* Kv2.1 channel by TEA, flonicamid, and TFNA-AM using whole-cell patch clamp. Current was normalized to complete inhibition of potassium current by 30 mM TEA.

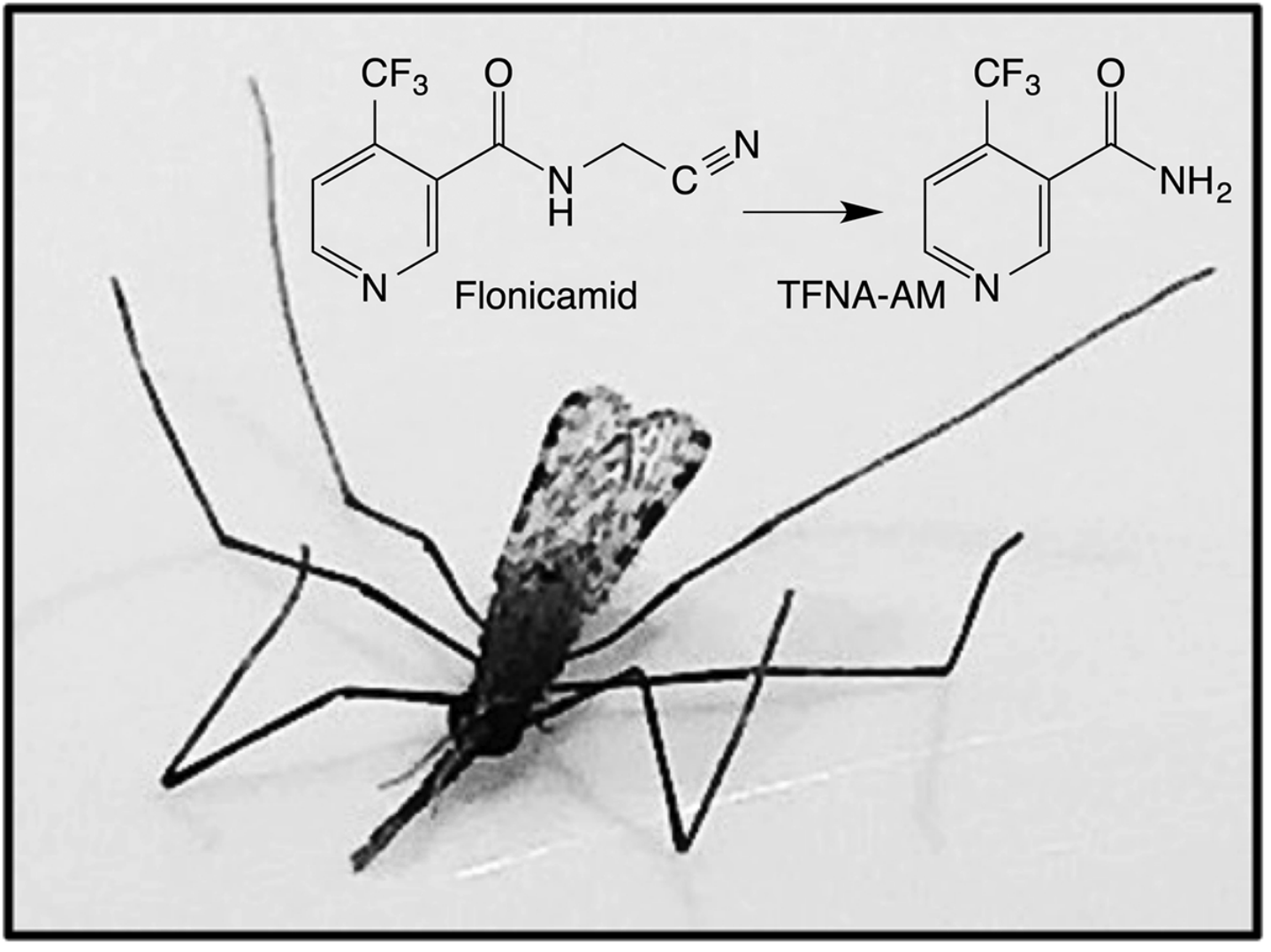
Figure 7. Effects of TFNA-AM on stimulated sensory discharges of *M. domestica*. Spike discharges (A-C) are superimposed on the square pulse used for stimulation. Treatments (5 min exposure) are: A) DMSO (0.1%) negative control; B) 100 μM flonicamid; C) 100 μM TFNA-AM; and D) bar graph (± SEM) comparing control to flonicamid and TFNA-AM; and E) concentration-response curve for TFNA-AM. Oscillations in C are artifacts of the mechanical stimulation observed after complete sensory block. The calibration values in C apply to traces A-B.

Figure 8. The effect of TFNA-AM on *D. melanogaster* CNS firing rate. TFNA-AM was exposed to a transected CNS preparation from wandering third-instar *D. melanogaster* larvae (square) compared to the vehicle control (0.1% DMSO; open circle).

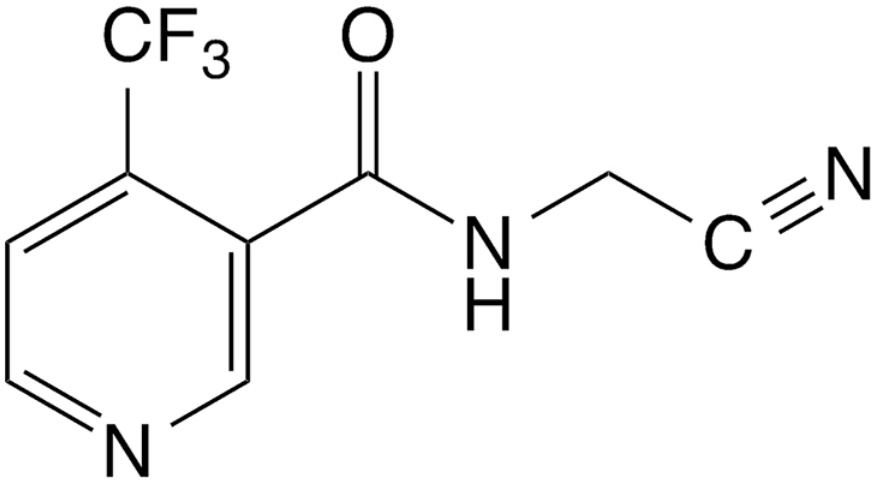
Graphical abstract

Highlights

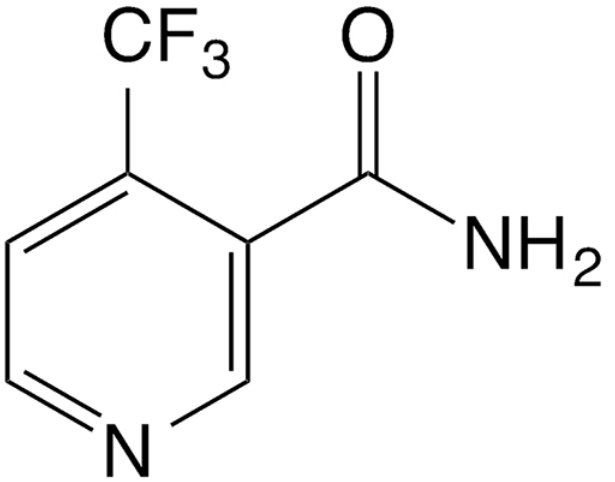
- Flonicamid and its metabolite TFNA-AM had toxic and behavior-modifying properties
- Cross resistance in the Akdr strain of *Anopheles gambiae* was low
- The compounds depolarized *Aedes* muscle and blocked the Kv2 channel at near mM concentrations
- TFNA-AM (2 µM), but not flonicamid, blocked firing of sensory nerves.
- Primary site of action is chordotonal organs, with some effects on potassium channels



Graphics Abstract



Flonicamid



4-(trifluoromethyl) nicotinamide (TFNA-AM)

Figure 1

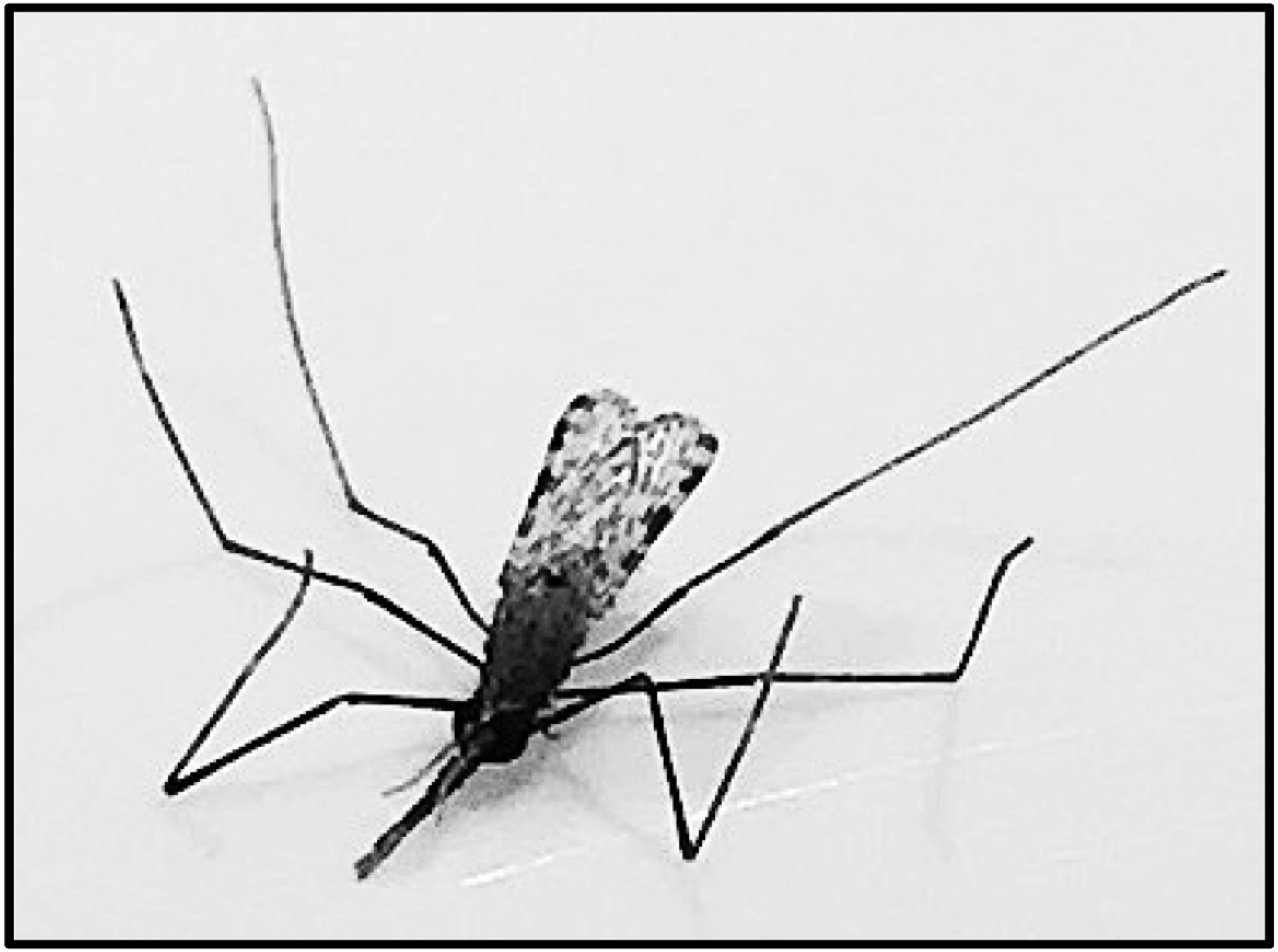


Figure 2

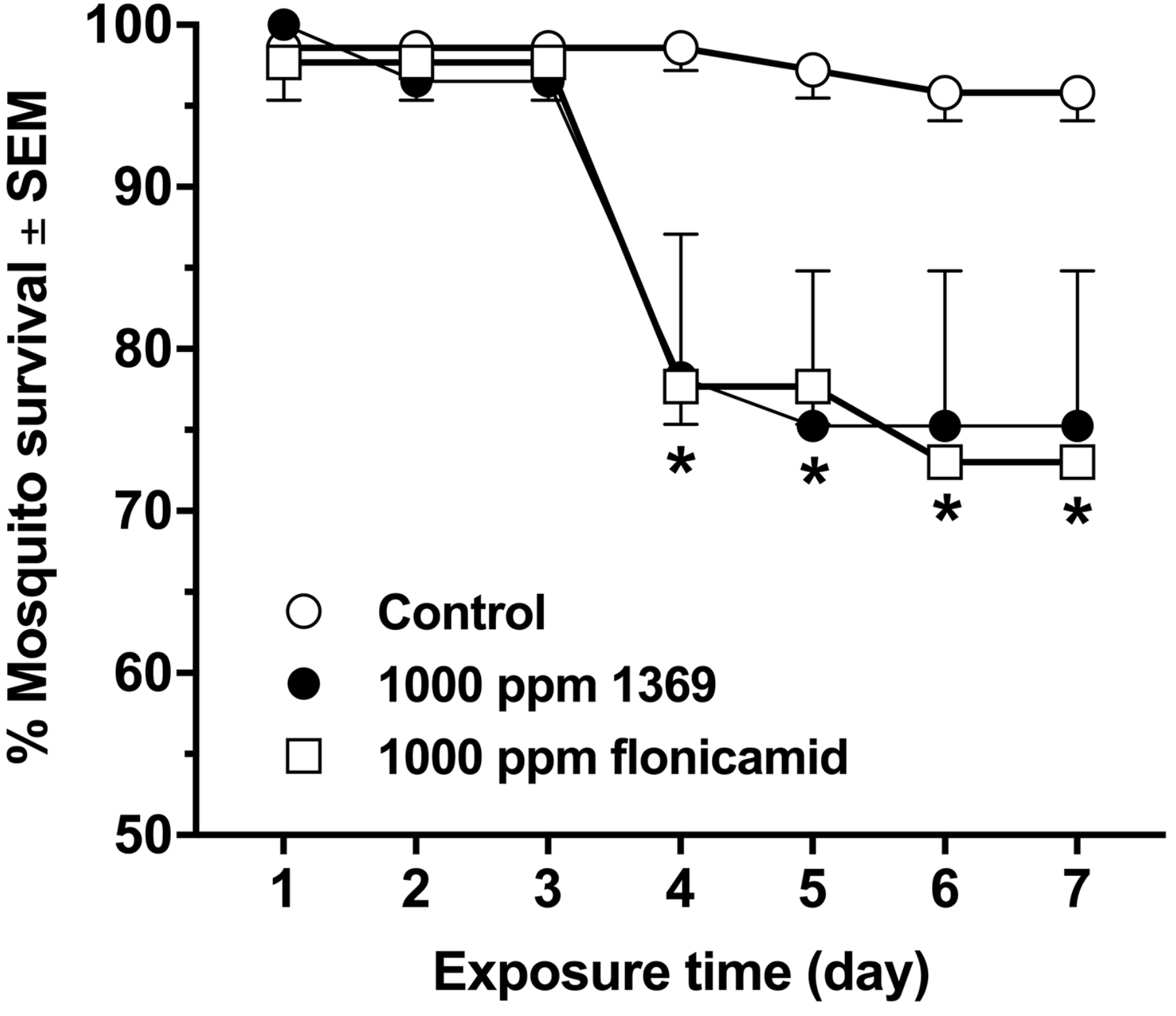

100
90
80
70
60
50
% Mosquito survival ± SEM
*
*
*
*
Control
1000 ppm 1369
1000 ppm flonicamid
1
2
3
4
5
6
7
Exposure time (day)

Figure 3

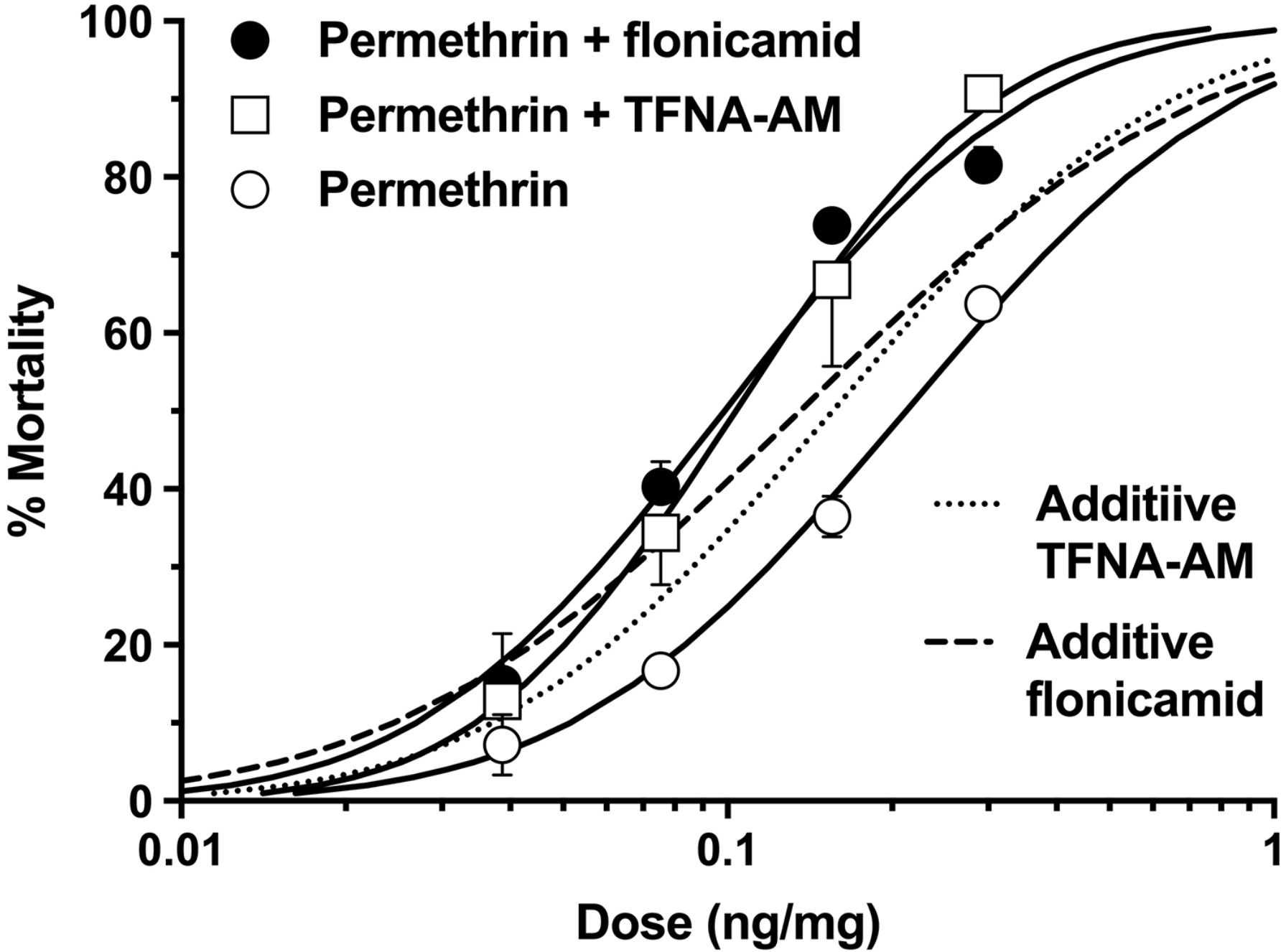

100
80
60
40
20
0
% Mortality
Permethrin + flonicamid
Permethrin + TFNA-AM
Permethrin
Additiive TFNA-AM
Additive flonicamid
0.01
0.1
1
Dose (ng/mg)

Figure 4

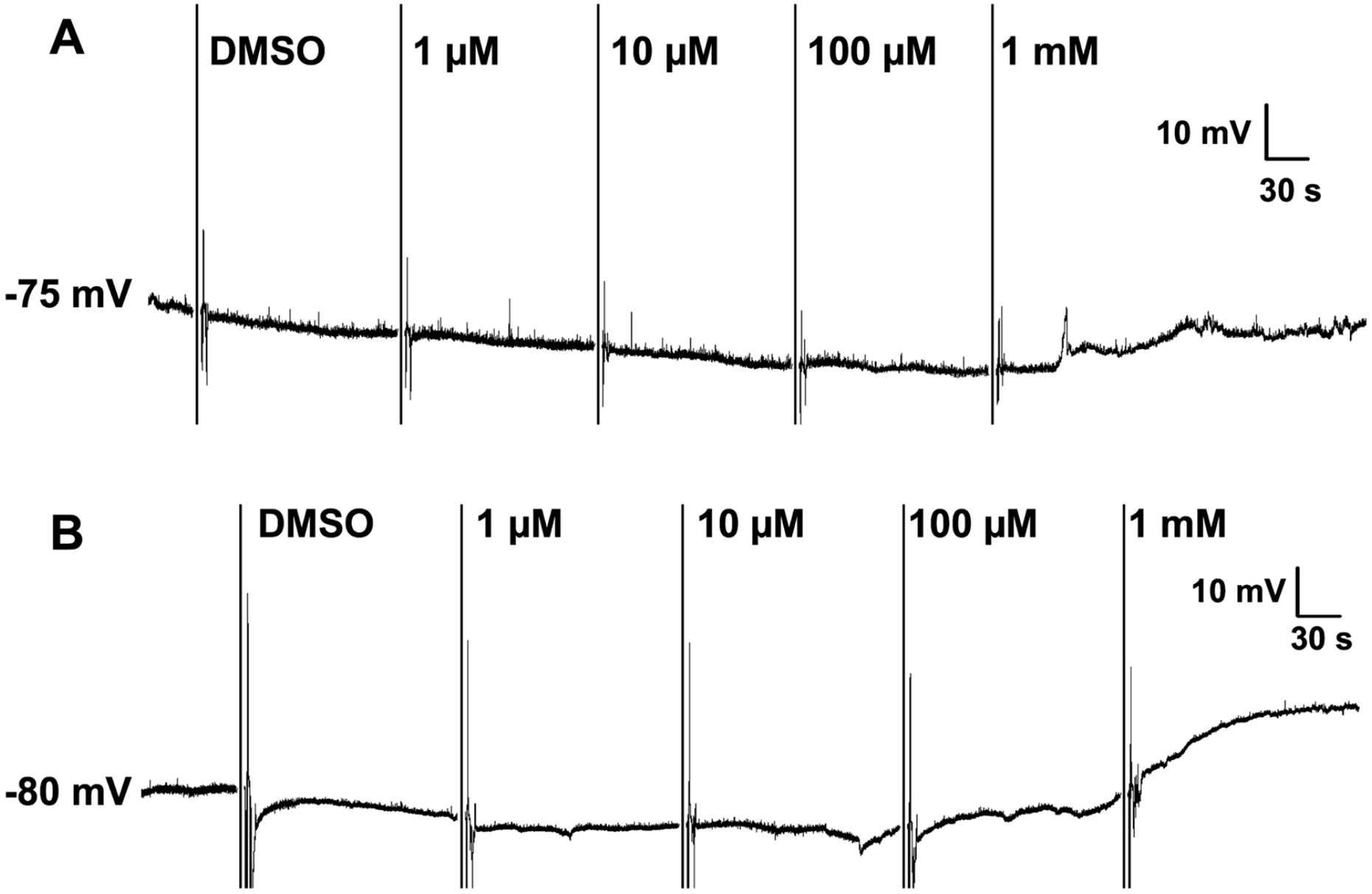

A
DMSO
1 µM
10 µM
100 µM
1 mM
10 mV
30 s
-75 mV
B
DMSO
1 µM
10 µM
100 µM
1 mM
10 mV
30 s
-80 mV

Figure 5

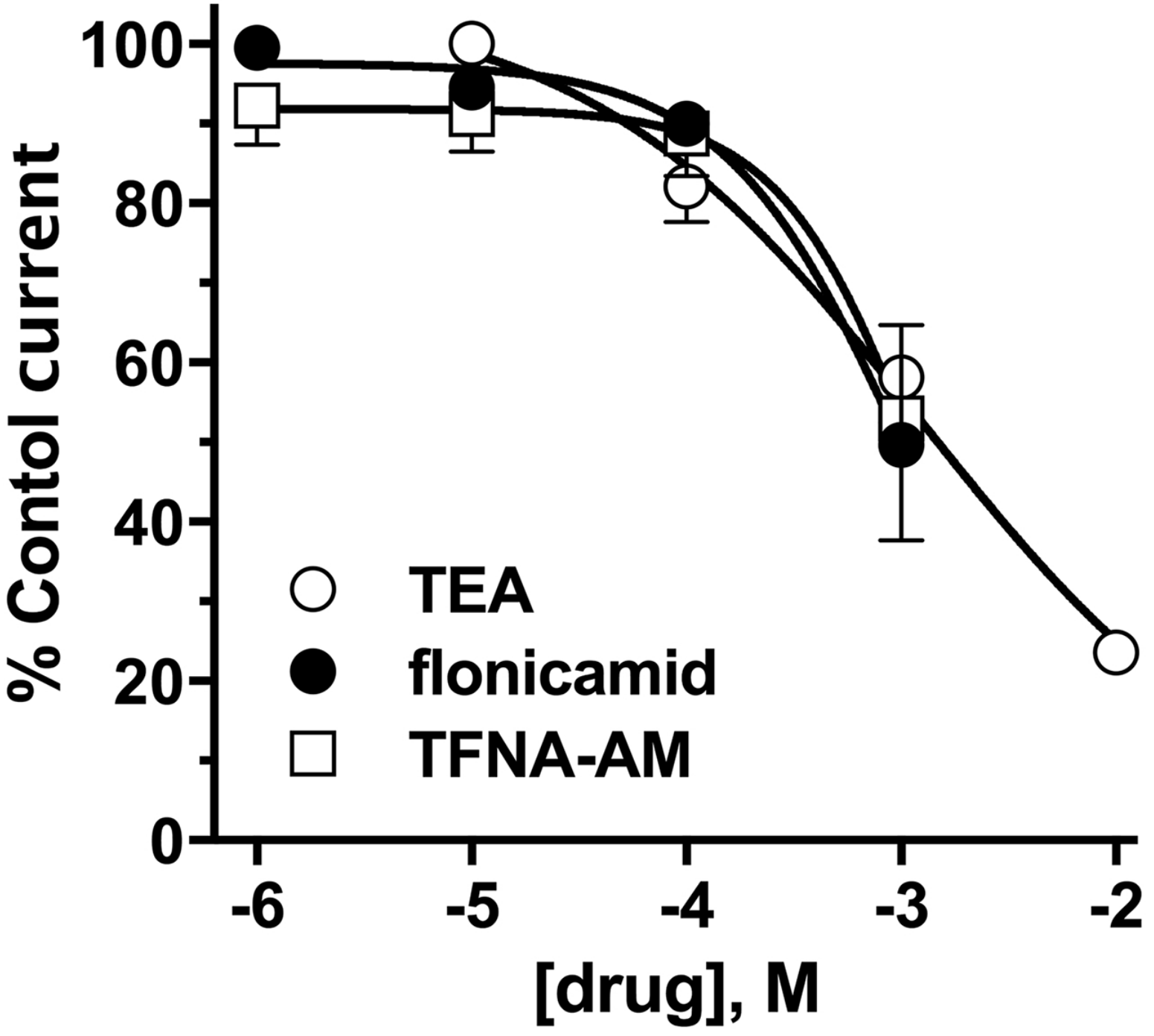

100
80
60
40
20
0
% Contol current
TEA
flonicamid
TFNA-AM
-6
-5
-4
-3
-2
[drug], M

Figure 6

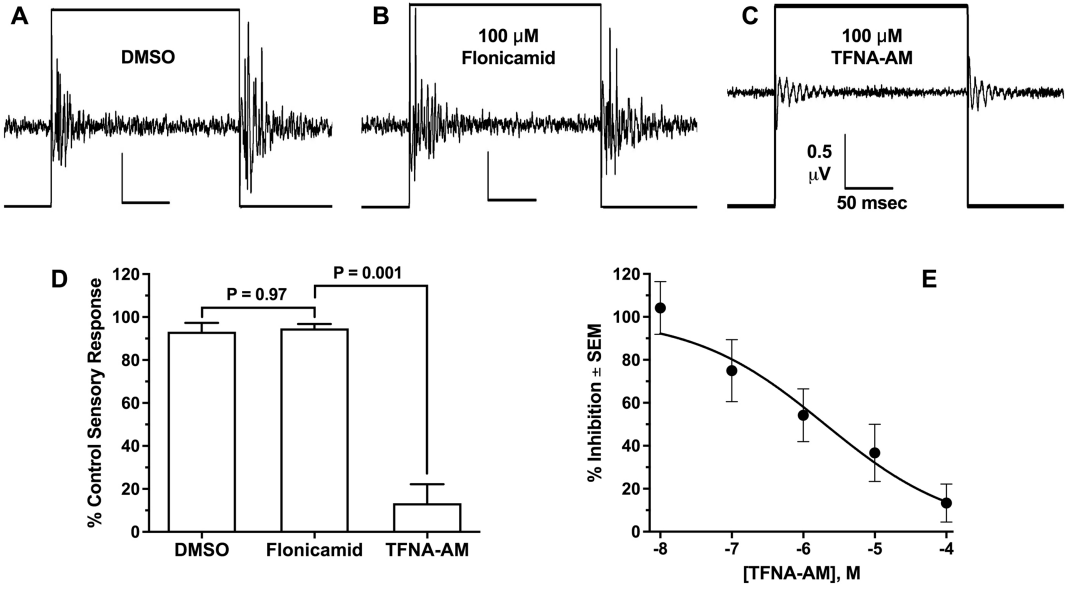

A
DMSO
B
100 μM
Flonicamid
C
100 μM
TFNA-AM
0.5
μV
50 msec
D
% Control Sensory Response
P = 0.97
P = 0.001
DMSO
Flonicamid
TFNA-AM
E
% Inhibition ± SEM
[TFNA-AM], M

Figure 7

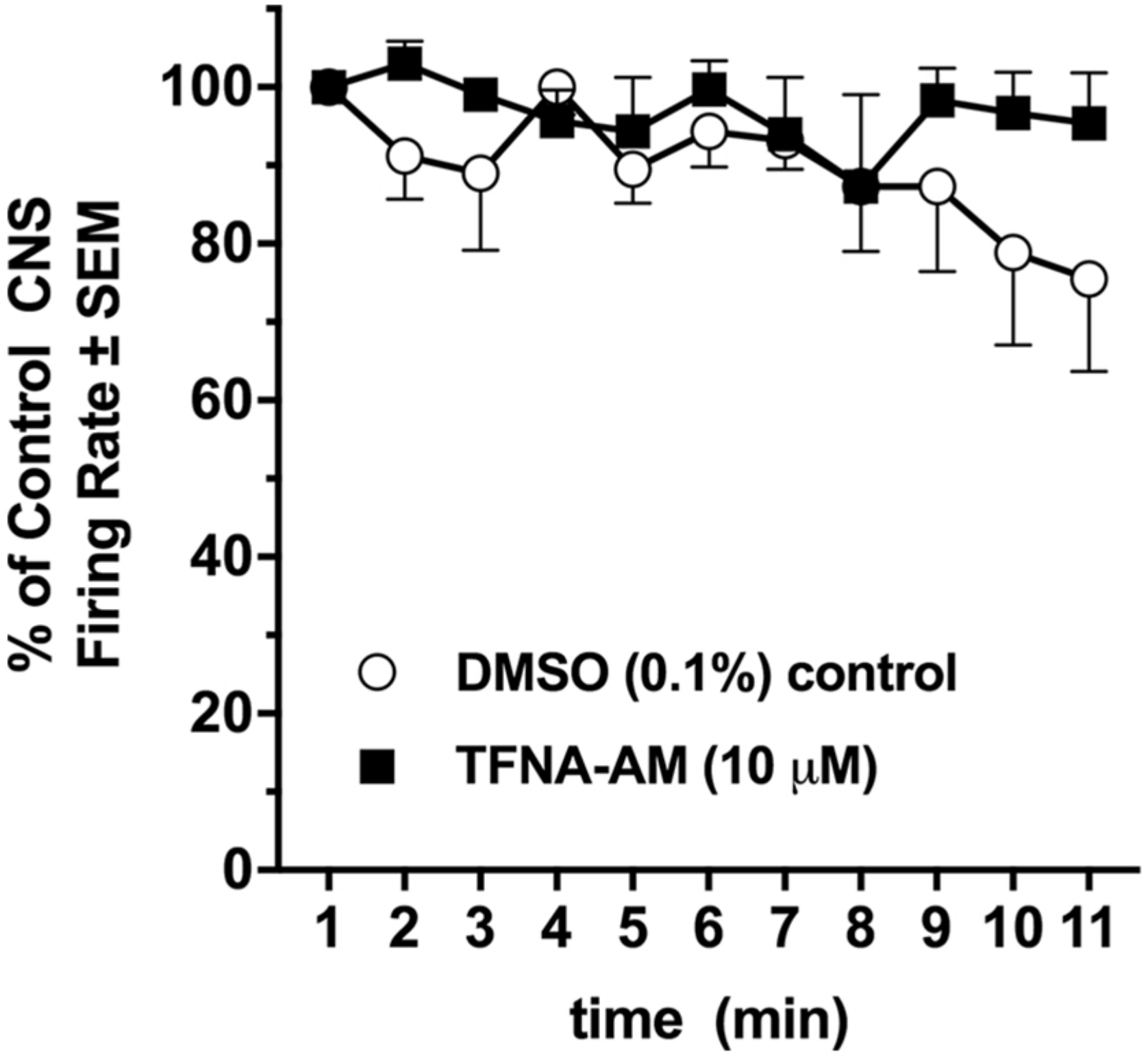

% of Control CNS
Firing Rate ± SEM
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
time (min)
DMSO (0.1%) control
TFNA-AM (10 μM)

Figure 8