Journal of Biological Research

Bollettino della Società Italiana di Biologia Sperimentale

eISSN 2284-0230 - pISSN 1826-883
https://www.pagepressjournals.org/index.php/jbr/index

J Biol Res 2026 [Online ahead of print]

To cite this Article:
Castro H, Karina Bermeo K, Isabel Arenas I, et al. **Comparative effect of cnidarian venoms on the action potential firing of superior cervical ganglion neurons.** *J Biol Res* doi: 10.4081/jbr.2026.14831

Submitted: 9 January 2026
Accepted: 30 March 2026
Early access: 28 May 2026

Comparative effect of cnidarian venoms on the action potential firing of superior cervical ganglion neurons

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Key words: Cnidaria, anthozoans, box jellyfish, action potential firing, superior cervical ganglion neurons.

Abstract

Cnidarians are considered the most ancient venomous phylum on Earth. Their venom is a complex mixture of bioactive compounds that produce a myriad of signs and symptoms in humans. Additionally, studies have revealed the presence of molecules with remarkably

pharmacological potential as modulators of immune responses and ion channels. Herein, we have used Superior Cervical Ganglion (SCG) neurons to assess the effects of five cnidarian species on the action potential firing. Although all venoms tested significantly slowed down the firing rate, differential underlying mechanisms were observed between anthozoan and cubozoan species. These findings show the presence of neurotoxins in all five cnidarian species as modulators of neuronal excitability and reveal differential mechanisms between anthozoans and cubozoans. The underlying mechanisms, likely involving changes in receptors and ion channel kinetics, are key pieces in advanced drug design with clinical purposes.

Introduction

Cnidarian venoms are complex mixtures of peptides, proteins, and low-molecular-weight compounds injected into prey to cause paralysis and likely initiate digestion.[1] These toxic compounds are stored and delivered in special structures called nematocysts, although sea anemones also deliver toxins through ectodermal and endodermal glands along the body.[2] Anthozoans, such as sea anemones and scleractinian corals, are not considered harmful or risky to humans, although some severe injuries resulting from direct or indirect contact have been reported.[3,4] This might be mainly because these sessile animals are rarely in contact with divers or tourists. Zoanthids are a notable exception, as several reports of intoxication exist for people who handle aquariums.[5,6] Hydrozoans are also generally harmless, except for fire corals, which cause severe pain upon contact,[7] and *Physalia physalis*, the “Portuguese man-o’-war”, which has been reported as fatal in some cases.[8] In

contrast, many pelagic species, mainly cubozoans, have been associated with moderate to severe envenomation and even fatalities in humans.[9,10]

Several studies have indicated that cnidarian stings mainly cause cardiotoxic, neurotoxic, myotoxic, hemolytic, and dermonecrotic lesions, as well as anaphylactic reactions.[11–13] The mechanism by which all the venom components act together to produce this variety of signs and symptoms when injected into the victim remains poorly understood. Besides, the presence of these disorders depends on issues such as the species involved in the sting and the method of venom entry into the victim, which might be subcutaneous, intramuscular, intralymphatic, etc.[14]

Among all the bioactive components of cnidarian venom, the proteinaceous compounds have been the most extensively studied, and they are classified into enzymes, pore-forming toxins, and neurotoxins.[15–17] Enzymes and pore-forming toxins have been linked to cytolytic effects and, in some cases, to the generation of reactive oxygen species during inflammatory responses, as reported for the crude venom of *Pelagia noctiluca*.[18] On the other hand, neurotoxins mainly exert their effects by altering membrane conductance through modifications in ion channel activity[15] and by forming pores.[19] In particular, cnidarian venoms are a rich source of ion channel modulators with therapeutic potential.[15] Several ion channels have been identified as targets of cnidarian venom, including Acid-Sensing Ion Channels (ASICs), Transient Receptor Potential (TRP) channels, and, most notably, voltage-gated sodium and potassium channels;[15–17] however, how this is reflected in membrane excitability remains poorly understood. Therefore, the purpose of this work was to investigate and compare the acute effects of a variety of venom extracts on the action potential firing in a native model neuron. In this study, we examined the effect of extracts from five species of cnidarians on the action potential firing of Superior Cervical

Ganglion (SCG) neurons: four anthozoans, including two sea anemones (*Lebrunia neglecta* and *Anemonia sargassensis*), a scleractinian coral (*Pseudodiploria strigosa*), and a zoanthid (*Palythoa caribaeorum*); and one cubozoan, a box jellyfish (*Carybdea marsupialis*).

Materials and Methods

Specimen collection and venom extraction

L. neglecta and *A. sargassensis* specimens were collected at Puerto Morelos reef lagoon by SCUBA diving at a depth of 4 m during late spring and summer, respectively. The organisms were separated from rocks using chisel and hammer, deposited in plastic bags, and frozen at -60 ºC until use. Samples were thawed and homogenized with a TEN BROECK 7727-15 PYREX® homogenizer (Corning, NY, USA). The extract was centrifuged at 4,000 rpm and 4ºC, then the supernatant was separated, lyophilized, and stored at -60ºC. Specimen collection was conducted according to the approval of the National Commission of Aquaculture and Fishing (permit number PPF/DGOPA-096/16). Fragments of *P. strigosa* were collected by scuba diving at depths of 4 to 10 m from coral reefs along the coast of Puerto Morelos, Quintana Roo, Mexico in summer season. Fragments were kept wet with seawater for transport to the laboratory and then frozen and stored at -70°C. Specimen collection was conducted according to the approval of the National Commission of Aquaculture and Fishing, of the Secretary of Agriculture, Livestock, Rural Development, Fishing, and Feeding of the Mexican Federal Government (permit number PPF/DGOPA-193/13). Coral fragments were stirred in high-performance liquid chromatography (HPLC)-grade water at 4°C for 24 h. The extract obtained was centrifuged at 3,000 rpm (2,060 × g) for 15 min at 4°C, and the supernatant was separated;

this procedure was repeated twice. Supernatants were freeze-dried, dissolved in HPLC-grade water at concentration of 150 mg/mL, and centrifuged at 3,000 rpm for 15 min at 4°C.

P. caribaeorum specimens were collected from the Puerto Morelos Reef Lagoon at approximately 1 m depth during summer. Organisms were separated from their rocky substrate with chisel and deposited in plastic bags containing seawater. Extraction was performed as described by Lazcano-Pérez *et al*.[1]

C. marsupialis samples were manually captured by free diving at the Puerto Morelos, Quintana Roo seashore in summer season and deposited in 20 L vessels. Immediately after capture, the box jellyfish tentacles were excised and homogenized in deionized water to discharge nematocysts at 4°C. A protease inhibitor (Complete Mini Roche®, Manheim, Germany) was added, and the extract was stirred and centrifuged for 30 min at 4,000 rpm. Supernatant was then lyophilized and stored until use.

The protein content of all samples was determined by the Bradford assay,[20] using a standard curve prepared with lyophilized bovine serum albumin.

Cell culture

SCG neurons were isolated as previously described.[21] Cells were obtained from 5-week-old male Wistar rats. Rats were anesthetized with CO_2 and then decapitated by guillotine. After dissection, ganglia were sliced into eight pieces, and the tissue was incubated in modified Hank's solution supplemented with 20 U/mL papain for 20 min at 37°C. Sample was then transferred into a solution containing 1 mg/mL collagenase type I and 10 mg/mL dispase and incubated for an additional 20 min before mechanical disaggregation of the tissue. Cell suspension was centrifuged at 180 g for 3 min and washed twice in Leibovitz's L-15

medium and once in Dulbecco's Modified Eagle's Medium (DMEM), both supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% penicillin-streptomycin solution. Cells were then plated on polystyrene culture dishes coated with poly-L-lysine and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were used between 18 and 24 h after plating. L-15 and DMEM culture media were obtained from Invitrogen Corp. (Carlsbad, CA, USA), and all other reagents were obtained from Sigma (St. Louis, MO, USA).

Ethical statement and animal care

Male Wistar rats were obtained from the Unidad Academica Bioterio of the Faculty of Medicine at Universidad Nacional Autónoma de México (UNAM). Rats were transported in plexiglass cages and euthanized afterward. All animals were handled according to the guidelines and requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition) and the Mexican Official Norm for Use, Care, and Reproduction of Laboratory Animals (NOM-062-ZOO-1999). Experimental protocols were reviewed and approved (identification code: FM/DI/059/08-07-2018) by the Committee of Research and Ethics of the Faculty of Medicine, UNAM.

Electrophysiology

Recordings were performed in the whole-cell configuration of the patch-clamp technique[22] using an EPC-9 amplifier and PatchMaster software (HEKA Instruments, Holliston, MA, USA) at room temperature. Patch micropipettes were pulled on a P-97 puller (Sutter Instrument, Novato, CA, USA) with a resistance of 1.6-2.2 MΩ. Signals were filtered at 2.9 kHz and sampled at 20 kHz. Repetitive firing was evoked by injection current steps of 50

pA from 0 to 400 pA before, during, and after the extract application. Cells were continuously bathed with either the control or the test solution at a flow rate of 2 mL/min. Control bath solution contained 160 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 5 mM $CaCl_2$, 1 mM $MgCl_2$, and 8 mM D-Glucose (pH adjusted to 7.4 with NaOH). Each venom was diluted into the bath solution to a final concentration of 200 g protein/mL. Micropipette solution contained 140 mM KCl, 15 mM NaCl, 2 mM $MgCl_2$, 1 mM ethyleneglycol- bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 10 mM HEPES, 5 mM Na2-ATP, 0.3 mM Tris-GTP, and 0.1 mM Leupeptin (pH adjusted to 7.2 with KOH). The effect of the cnidarian venom was evaluated after 1 min of application.

Data analysis

Neurons included in the analysis had a Resting Membrane Potential (RMP) more negative than -45 mV and overshooting, which agrees with other works.[23] Repetitive firing was evoked by the current injection of 50 pA steps from 0 to 400 pA before, during, and after the extract application. The duration (width at half of the maximal spike amplitude), height (the amplitude from threshold to positive peak), and trough voltage (the most negative voltage in between two spikes) of the first action potential were measured at the highest level of repetitive firing.[24] Phase-plane plots were performed to characterize and compare the first action potential.[25]

Results are presented as mean ± Standard Error of the Mean (SEM). The Shapiro-Wilk test was used to evaluate the normal distribution of the groups, while the F-test was employed to test for equal variances. No outliers were identified by the Robust Regression and Outlier Removal (ROUT) analysis method. Statistically significant differences in the number of spikes between the control, venom, and wash conditions were analyzed using a two-way

Analysis of Variance (ANOVA) by repeated measures (during 600 ms) at different current injections (from 50 to 400 pA), followed by Dunn's *post hoc* test. The paired t-test was used to compare the features of the first action potential in control and venom conditions. Statistical significance was taken to $p \leq 0.05$. Graphics and statistical analyses were constructed using Igor Pro (version 6.3, WaveMetrics, Portland, OR, USA, 2009), NeuroMatic software,[26] and GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA).

Results

Sea anemones: A. sargassensis *and* L. neglecta

Firstly, the RMP was measured, since this sets the stage for action potential firing. As shown in Fig.1A, the RMP was depolarized in the presence of *A. sargassensis* extract, from -81.2 ± 2.2 mV to -61.6 ± 2.4 mV ($t_{(5)} = 7.69$, n = 6). This effect was accompanied by a significant reduction in the number of action potentials elicited by current injections from 150 to 400 pA; e.g., for a 250 pA current injection, the number reduced from 8.8 ± 1.5 to 4.5 ± 0.9 ($F_{(2,10)} = 8.98$, n = 6) with recovery during washout by using control solution (Figure 1A and 1B). Out of the different features analyzed for the first spike, the height was decreased from 117 ± 1.8 mV to 109 ± 3.3 mV ($t_{(5)} = 5.26$; n = 6), and the trough voltage was depolarized from -65.06 ± 1.02 mV to -59.44 ± 1.9 mV ($t_{(5)} = 4.78$; n = 6) in the presence of *A. sargassensis* extract (Figure 1C). In addition, to measure the action potential dynamics the plotting of voltage against its rate of change in a phase-plane plot was made. As shown in Figure 1D, the maximum downstroke velocity of the falling phase decreased in the presence of *A. sargassensis* extract from 52.29 ± 2.81 V/s to 45.83 ± 4.18 V/s ($t_{(5)} = 2.83$; n = 6).

Similarly, in the presence of *L. neglecta* extract, the RMP was depolarized from -82.5 ± 1.2 mV to -69.4 ± 2.1 mV ($t_{(5)}$ = 6.34; n = 6) (Figura 2A). Also, the mean number of spikes was decreased by application of *L. neglecta* extract; e.g., for 200 pA current injection, the mean was decreased from 10 ± 1.1 to 5.5 ± 0.9 ($F_{(2,10)}$ = 8.09, n = 6) with reversion in washout condition (Figure 2A and 2B). In addition, height was reduced from 117 ± 2.5 mV to 108 ± 1.9 mV ($t_{(5)}$ = 8.96; n = 6), and the trough voltage was depolarized from -64.4 ± 1.4 mV to -59.4 ± 1.7 mV ($t_{(5)}$ = 6.34; n = 6) in the presence of *L. neglecta* extract, as shown in Figure 2C. No significant changes were observed in maximum upstroke and downstroke velocities (Figure 2D).

A scleractinian coral: **P. strigosa**

In the presence of *P. strigosa* extract, RMP was depolarized from -86.1 ± 2.04 mV to -79.2 ± 1.6 mV ($t_{(4)}$ = 3.15; n = 5) (Figure 3A). Regarding repetitive firing, it was significantly decreased over current injection from 200 to 400 pA (Figure 3A); e.g., for a 250 pA current injection, the rate decreased from 10.6 ± 0.9 to 7.2 ± 0.8 during the application of *P. strigosa* extract ($F_{(2,8)}$ = 17.09, n = 5). Interestingly, no recovery was observed after washout as shown in Figure 3B. Figure 3C shows the effect of *P. strigosa* extract on the features of the first spike. Height decreased from 114 ± 1.1 mV to 108 ± 0.9 mV ($t_{(4)}$ = 8.28; n = 5), while the trough voltage was depolarized from -66.1 ± 0.2 mV to -64.4 ± 0.3 mV ($t_{(4)}$ = 3.11; n = 5) in the presence of *P. strigosa* extract. Additionally, maximum upstroke and downstroke velocities decreased due to the application of *P. strigosa* extract, from 121.2 ± 4.42 V/s to 109.8 ± 4.59 V/s ($t_{(4)}$ = 3.21; n = 5) and from 37.6 ± 1.27 V/s to 35.3 ± 0.99 V/s ($t_{(4)}$ = 3.49; n = 5), respectively (Figure 3D).

***A zoanthid:* P. caribaeorum**

In the experiment shown in Figure 4A, RMP was depolarized from -82.9 ± 3.7 mV to -66.8 ± 3.05 mV ($t_{(5)} = 2.95$; n = 6) in the presence of *P. caribaeorum* extract. Along with this effect, the mean number of spikes evoked by current injection from 300 to 400 pA was significantly reduced; e.g., for a 300 pA current injection, it decreased from 11.8 ± 2.4 to 8.1 ± 2.5 ($F_{(7,35)} = 16.82$, n = 6) with reversion in washout condition (Figure 4A and 4B). In collecting results for the first action potential, the height was reduced from 112 ± 3.2 mV to 106 ± 3.4 mV ($t_{(5)} = 2.85$; n = 5), while the trough voltage was depolarized from -65.5 ± 2.5 mV to -61.4 ± 2.1 mV ($t_{(5)} = 3.65$; n = 5) (Figure 4C) in the presence of *P. caribaeorum* extract. No effects on maximum upstroke and downstroke velocities were observed.

***A box jellyfish*: C. marsupialis**

As shown in Figure 5A and 5B, the number of spikes decreased in the presence of *C. marsupialis* extract; e.g., for a 250 pA current injection, it decreased from 9.1 ± 1.1 to 4.4 ± 0.5 ($F_{(2,12)} = 9.49$, n = 7). This effect was observed for firing evoked by current injections from 200 to 400 pA. The recovery of excitability in the washout condition was absent and mainly observed at 200 and 250 pA of current injection (Figure 5B). In contrast with the other cnidarian venoms, no changes in the RMP were observed, and the features of the first spike remained unchanged (Figure 5C and 5D).

Discussion

The systemic effect of cnidarian venoms, whether injected into a prey or used to deter potential predators, results from the simultaneous action of multiple compounds targeting various physiological organs. The overall activity of these compounds, including potassium

channel inhibitors, sodium channel modulators, porins, phospholipases, and others, is manifested as the signs and symptoms observed after contact. Depending on the species, these can cause paralysis, mild pain, or burning sensations that may progress to severe pain or even lead to cardiac and respiratory failure. Given the nature of these responses, it is important to examine how cnidarian venoms affect neuronal activity.

To characterize and compare the effects of different cnidarians on action potential, we performed electrophysiological assays in SCG neurons. These cells can be classified based on their firing patterns as i) Phasic-1, which fires few spikes during stimulation even when current injection is increasing; ii) Phasic-2 (the most common type), where a weak stimulus elicits a small number of spikes, but the spike count gradually increases with the current injection; and iii) Tonic, neurons that exhibit sustained firing of action potentials even with minimal stimulus.[27–29] In this work, neuronal firing was classified as Phasic-2 type.

It has been suggested that cells become hyperactive in response to cnidarian neurotoxins.[16] However, this study shows a decrease in neural firing rate after acute application of five different cnidarian venoms, indicating reduced excitability. Changes in the RMP and in the features of the first action potential can be used to propose possible molecular mechanisms for the different poisons. However, more detailed pharmacological research is necessary to examine the toxins in these venoms as well as the molecular targets underlying changes in neuronal excitability.

In SCG neurons, the RPM was determined by currents carried by the TWIK-related K+ channel 2 (TREK-2), voltage-gated potassium (KV) channels (especially KV7.2/7.3), Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) channels, Na+/K+-pump, and voltage-gated sodium (NaV) channels (persistent current).[30,31] All of these are potential targets for tested venoms that depolarized the RMP, such as those from *A. sargassensis*, *P.*

strigosa, *P. caribaeorum*, and *L. neglecta*. Additionally, another possible mechanism involved is the influx of Na+ through pores formed by toxins, as reported for the crude venom of *P. noctiluca*.[19] In this study, only *P. strigosa* shows an irreversible effect upon washout, possibly due to a pore-forming mechanism.

On the other hand, changes after venom application in the waveform of the first action potential may indicate which molecular targets are involved. In our preparation, the depolarizing phase is mediated by voltage-gated sodium and calcium channels,[32–34] while the repolarizing phase depends on voltage-gated and calcium-activated potassium channels.[24,27,35,36] Except for *C. marsupialis*, cnidarian venoms decreased the height and increased the trough voltage of the first action potential, with consistent changes in the maximum upstroke and downstroke velocities observed. Accordingly, multiple targets have been identified for *P. caribaeorum* venom, including voltage-gated potassium, calcium, and sodium channels,[1,37] while in the case of sea anemones used in this work, only for *L. neglecta* it has been reported to inhibit voltage-gated calcium channels in chromaffin cells.[38] Finally, there are no reports of selective effects on ion channels caused by *P. strigosa* and *A. sargassensis* venoms.

The case of *C. marsupialis* extract is also worth noting, as a decrease in neuronal excitability was observed without significant changes in RMP or in the features of the first action potential. We suggest that the compounds in the *C. marsupialis* extract primarily target the open state of ion channels involved in the depolarization and repolarization phases, leading to widespread changes after the first action potential. According to these findings, the potential neurotoxic activity of *C. marsupialis* venom was explored in a heterologous expression system. The effect of this venom is partially mediated by the

blockade of inwardly rectifying K+ channels and by the activation of a G protein-coupled receptor signaling.[39,40]

Conclusions

This work highlights the effects of venoms from different cnidarian species acutely modulating the firing rate in native neurons, mostly by reducing excitability. Interestingly, these findings shed light on mechanisms preserved in cnidarian species, driven by ancient molecules influencing neuronal excitability throughout evolution. Understanding these mechanisms is crucial in managing the clinical signs and symptoms they may cause and, more importantly, for identifying molecules with pharmacological potential, including traits to antibiotic resistance, pancreatic β-cell exhaustion, pain, epilepsy, cancer or neurodegenerative diseases.

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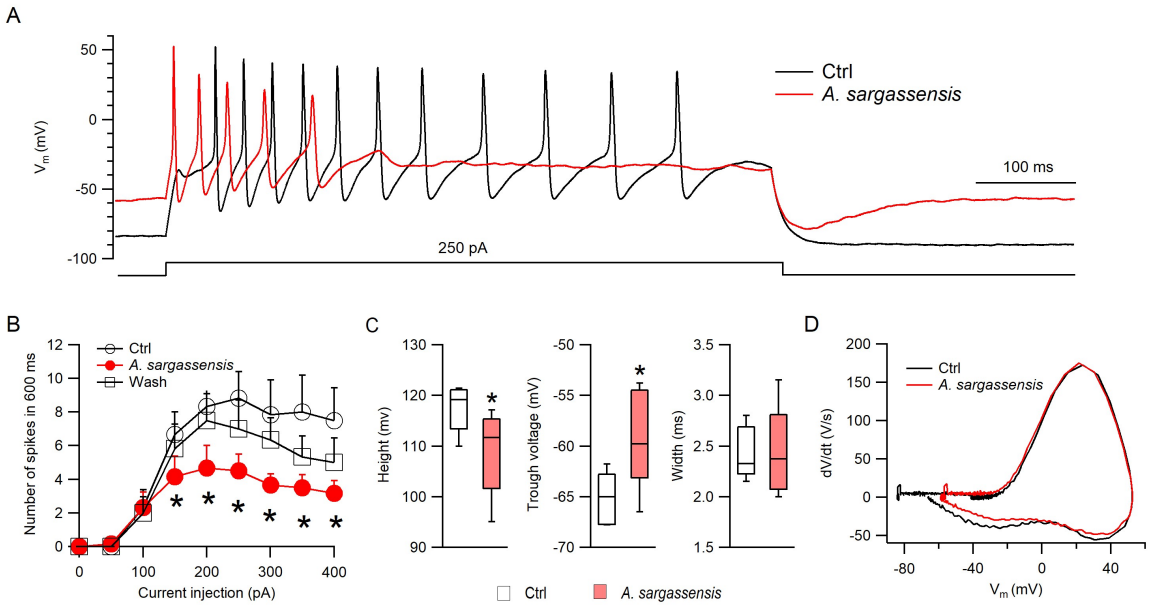

Figure 1. Effect of *A. sargassensis* extract on firing properties in SCG neurons. A) Representative traces of neuron firing patterns in the control (Ctrl) condition and in the presence of *A. sargassensis* extract for a 250 pA current injection. B) Number of spikes elicited by 50 pA current steps in control, in the presence of *A. sargassensis* extract, and after washout (Wash). The wash condition was established after three minutes of perfusion with control solution. Data are presented as the mean ± Standard Error of the Mean (SEM) and were analyzed by two-way Analysis of Variance (ANOVA); $F_{(2,10)} = 8.98$, n = 6. C). Height, trough voltage, and width of the first action potential elicited for a 250 pA current injection in control and in the presence of *A. sargassensis* extract. Data are presented in box and whisker plots and were analyzed by t-test: $t_{(5)} = 5.26$, n = 6, and $t_{(5)} = 4.78$, n = 6 for height and trough voltage, respectively. D) Representative phase-plane plot of the first action potential in control and in the presence of *A. sargassensis* extract; $t_{(5)} = 2.83$; n = 6. * Represents significant differences between control and *A. sargassensis* ($p \leq 0.05$).

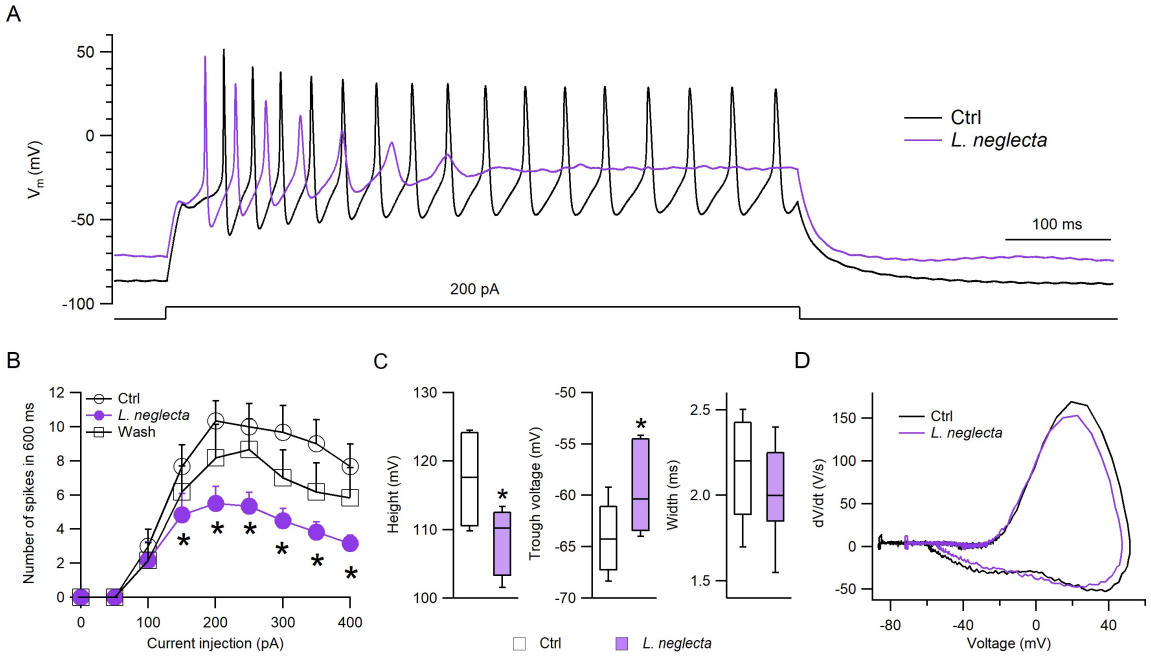

Figure 2. Effect of *L. neglecta* extract on firing properties in SCG neurons. A) Representative traces recorded in the control (Ctrl) condition and in the presence of *L. neglecta* extract for a 200 pA current injection. B) Number of spikes elicited by 50 pA current steps in control, in the presence of *L. neglecta* extract, and after washout (Wash). Data are presented as the mean ± Standard Error of the Mean (SEM) and were analyzed by two-way Analysis of Variance (ANOVA); $F_{(2,10)} = 8.09$, n = 6. C). Height, trough voltage, and width of the first action potential elicited for a 200 pA current injection in control and in the presence of *L. neglecta* extract. Data are presented in box and whisker plots and were analyzed using t-tests: $t_{(5)} = 8.96$, n = 6, and $t_{(5)} = 6.34$, n = 6 for height and trough voltage, respectively. D) Representative phase-plane plot of the first action potential in control and

in the presence of *L. neglecta* extract. * Represents significant differences between control and *L. neglecta* ($p \leq 0.05$).

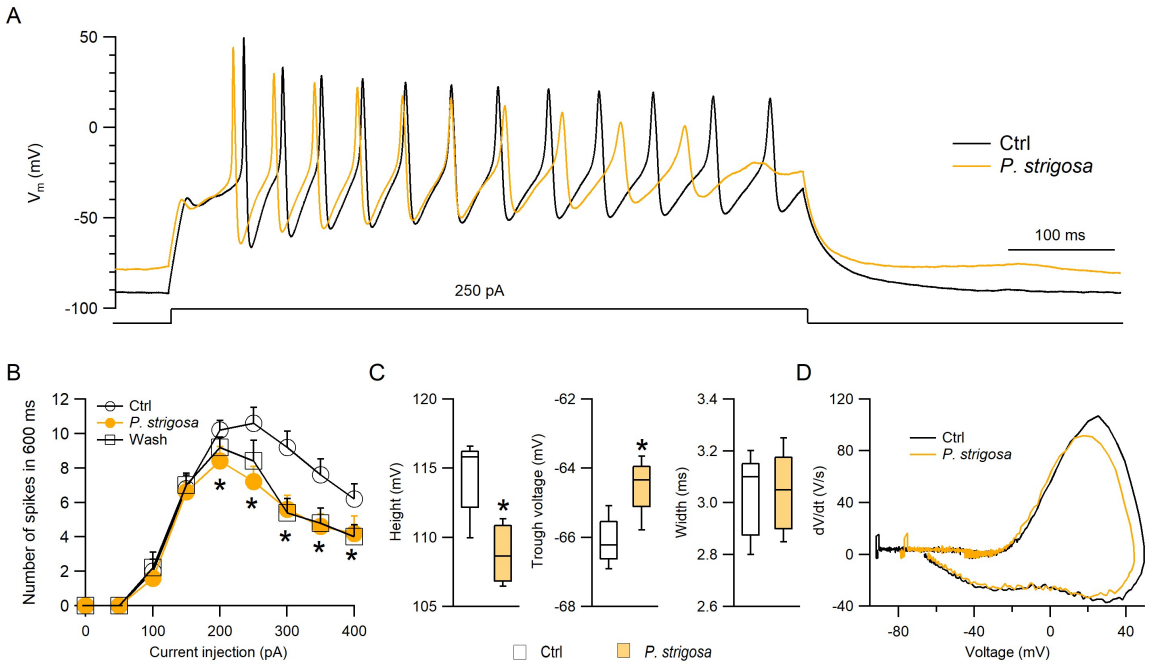

Figure 3. Effect of *P. strigosa* extract on firing properties in SCG neurons. A) Representative traces recorded in control (Ctrl) condition and in the presence of *P. strigosa* extract for a 250 pA current injection. B) Number of spikes elicited by 50 pA current steps in control, in the presence of *P. strigosa* extract, and after washout (Wash). Data are presented as the mean ± Standard Error of the Mean (SEM) and were analyzed by two-way Analysis of Variance (ANOVA); $F_{(2,8)} = 17.09$, n = 5. C). Height, trough voltage, and width of the first action potential elicited for a 250 pA current injection in control and in the presence of *P. strigosa* extract. Data are presented in box and whisker plots and were analyzed by t-test: $t_{(4)} = 8.28$, n = 5, and $t_{(4)} = 3.11$, n = 5 for height and trough voltage,

respectively. D) Representative phase-plane plot of the first action potential in control and in the presence of *P. strigosa* extract. * Represents significant differences between control and *P. strigosa* ($p \leq 0.05$).

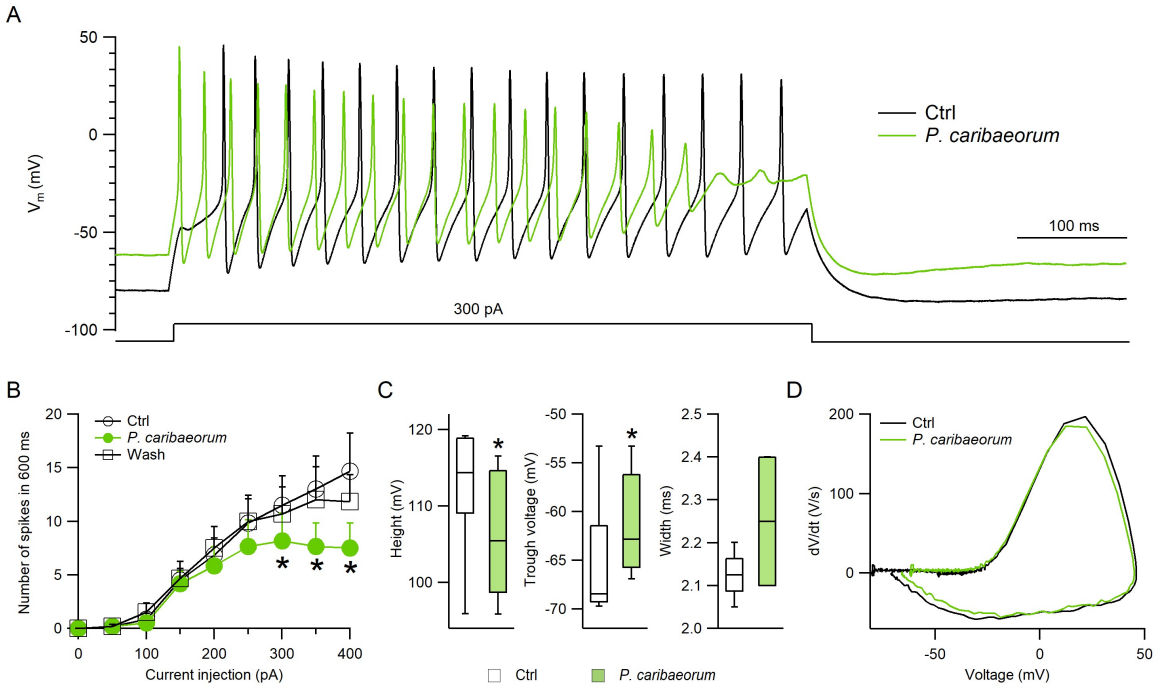

Figure 4. Effect of *P. caribaeorum* extract on firing properties in SCG neurons. A) Representative traces recorded in the control (Ctrl) condition and in the presence of *P. caribaeorum* extract for a 300 pA current injection. B) Number of spikes elicited by 50 pA current steps in control, in the presence of *P. caribaeorum* extract, and after washout (Wash). Data are presented as the mean ± Standard Error of the Mean (SEM) and were analyzed by two-way Analysis of Variance (ANOVA); $F_{(2,10)} = 1.84$, n = 6. C). Height, trough voltage, and width of the first action potential elicited for a 300 pA current injection in control and in the presence of *P. caribaeorum* extract. Data are presented in box and

whisker plots and were analyzed using a t-test: $t_{(5)} = 2.85$, n = 5, and $t_{(5)} = 3.65$, n = 5 for height and trough voltage, respectively. D) Representative phase-plane plot of the first action potential in control and in the presence of *P. caribaeorum* extract. * Represents significant differences between control and *P. caribaeorum* ($p \leq 0.05$).

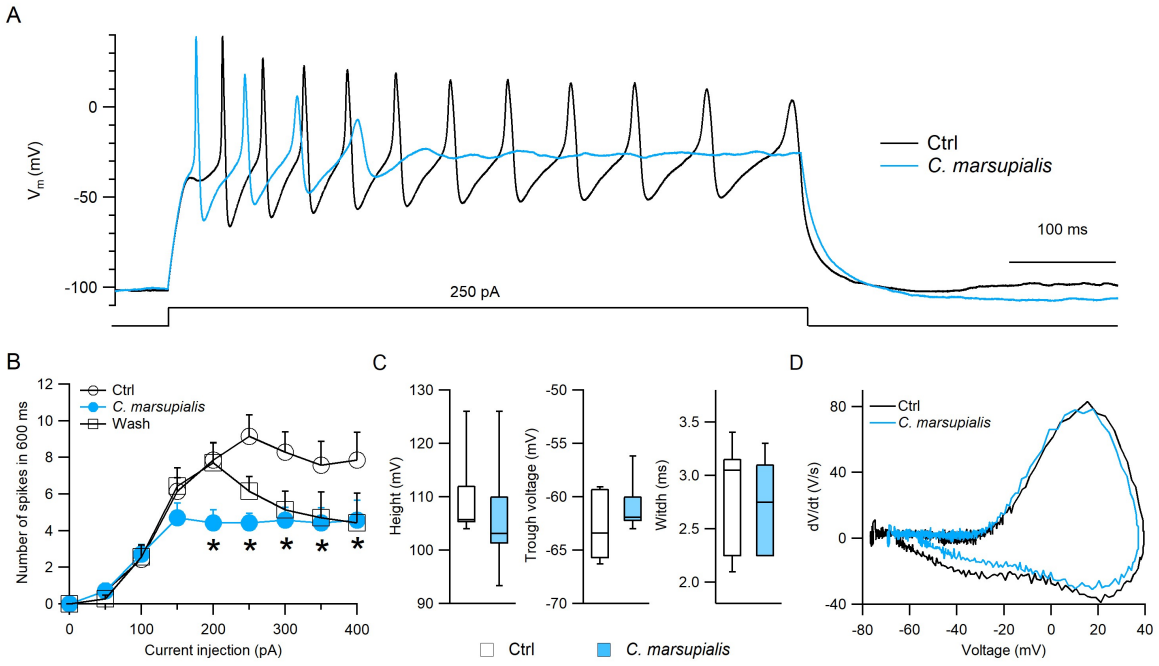

Figure 5. Effect of *C. marsupialis* extract on firing properties in SCG neurons. A) Representative traces of neuronal firing in the control (Ctrl) condition and in the presence of *C. marsupialis* extract for a 250 pA current injection. B) Number of spikes elicited by 50 pA current steps in control, in the presence of *C. marsupialis* extract, and after washout (Wash). Data are presented as the mean ± Standard Error of the Mean (SEM) and were analyzed by two-way Analysis of Variance (ANOVA); $F_{(2,12)} = 9.49$, n = 7. C). Height,

trough voltage, and width of the first action potential elicited for a 250 pA current injection in control and in the presence of *C. marsupialis* extract. Data are presented in box and whisker plots and were analyzed by t-test. D) Representative phase-plane plot of the first action potential in control and in the presence of *C. marsupialis* extract. * Represents significant differences between control and *C. marsupialis* ($p \leq 0.05$).

Contributions: Hector Castro, David E. Garcia, Karina Bermeo, and Fernando Lazcano-Pérez conceived and designed research; Fernando Lazcano-Pérez Karina Bermeo, H.C., and Isabel Arenas performed experiments; Karina Bermeo and Hector Castroanalyzed data and prepared figures; David E. Garcia, Fernando Lazcano-Pérez, Karina Bermeo, and H.C. interpreted results of experiments; Karina Bermeo, Hector Castro, Fernando Lazcano-Pérez, Isabel Arenas, Fernando Lazcano-Pérez, Judith Sánchez-Rodríguez, and David E. Garcia drafted the manuscript. All authors revise the manuscript and approve the final version.

Conflict of interest: the authors declare that they have no competing interests.

Ethics approval: all animals were handled according to the guidelines and requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th edition) and the Mexican Official Norm for Use, Care, and Reproduction of Laboratory Animals (NOM-062-ZOO-1999). Experimental protocols were reviewed and approved (identification code: FM/DI/059/08-07-2018) by the Committee of Research and Ethics of the Faculty of Medicine, UNAM.

Availability of data and material: the datasets used and/or analyzed during the current study are available upon reasonable request from the corresponding author.

Fundings: this work was supported by Universidad Nacional Autónoma de México-Dirección General de Asuntos del Personal Académico-Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica (UNAM-DGAPA-PAPIIT), Grant IN210325.

Acknowledgements: D.E.G. is grateful to the Alexander von Humboldt Stiftung, Germany for the equipment provided.