Scorpion venom increases acetylcholine release by prolonging the duration of somatic nerve action potentials.

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Highlights

- Low concentrations of *T. bahiensis* venom enhanced spontaneous and evoked ACh release.
- At high concentrations, the venom blocked nerve conduction;
- Partial membrane depolarization could account for the enhanced spontaneous release;
- A conduction delay increased the quantal content by causing longer nerve depolarization;
- Tetrodotoxin-sensitive voltage-gated sodium channels as a major target of the venom.

Abstract

Scorpionism is frequently accompanied by a massive release of catecholamines and acetylcholine from peripheral nerves caused by neurotoxic peptides present in these venoms, which have high specificity and affinity for ion channels. *Tityus bahiensis* (*T. bahiensis*) is the second most medically important scorpion species in Brazil but, despite this, its venom remains scarcely studied, especially with regard to its pharmacology on peripheral (somatic and autonomic) nervous system. Here, we evaluated the activity of *T. bahiensis* venom on somatic neurotransmission using myographic (chick and mouse neuromuscular preparations), electrophysiological (MEPP, EPP, resting membrane potentials, perineural waveforms, compound action potentials) and calcium imaging (on DRG neurons and muscle fibres) techniques. Our results show that the major toxic effects of *T. bahiensis* venom on neuromuscular function are presynaptically driven by the increase in evoked and spontaneous neurotransmitter release. Low venom concentrations prolong the axonal action potential leading to a longer depolarization of the nerve terminals that enhances neurotransmitter release and facilitates nerve-evoked muscle contraction. The venom also stimulates the spontaneous release of neurotransmitters, probably through partial neuronal depolarization that allows the calcium influx. Higher venom concentrations block the generation of action potentials and resulting muscle twitches. The venom pharmacology was reversed by low concentrations of TTX, indicating voltage-gated sodium channels as the primary target of the venom toxins. These results suggest that the major neuromuscular toxicity promoted by *T. bahiensis* venom is probably mediated mainly by α- and β-toxins interacting with presynaptic TTX-sensitive ion channels on both axons and nerve terminals.

Key-words: *Tityus bahiensis*; somatic nervous system; electrophysiology; ion channels; nerve conduction.

Abbreviations

BC: chick *biventer cervicis* preparation;

CAP: compound action potential;

EDL: *extensor digitorum longus* preparation;

PND: phrenic nerve-diaphragm preparation;

RPM: resting membrane potential.

1. Introduction

Scorpion envenomation is a major health problem in tropical and subtropical regions worldwide with >1.2 million cases reported every year (Chippaux and Goyffon, 2008). In Brazil, scorpionism has assumed epidemic proportions, with data from the Brazilian Ministry of Health showing that the number of registered cases in this country has increased by 332 % over the last 10 years, with >124,000 cases reported in 2017; scorpion stings are now the primary cause of human envenomation countrywide (Brasil, 2018). Almost all cases of scorpionism in Brazil are caused by the genus *Tityus*, with the two most commonly implicated species being *T. bahiensis* (brown scorpion) and *T. serrulatus* (yellow scorpion), the latter being clinically the most important (Bucaretchi et al., 2014), particularly in the states of Minas Gerais and São Paulo in southeastern Brazil (von Eickstedt et al., 1996). Other species involved include *T. obscurus* (Pardal et al., 2014; Torrez et al., 2015) and *T. silvestris* in the eastern Brazilian Amazon (Monteiro et al., 2016) and *T. stigmurus* in northeastern Brazil (Albuquerque et al., 2013). To date, the most studied and best characterized venom of the various *Tityus* spp. is that of *T. serrulatus* (Bordon et al., 2015; Pucca et al., 2015; Nencioni et al., 2018).

The clinical manifestations of envenomation by *Tityus* spp. include local and systemic effects. The local effects consist of immediate severe pain (described as an excruciating and burning sensation along the affected area) and varying degrees of erythema, swelling (edema) and paraesthesia; the intense pain can trigger transient bradycardia and hypertension can be present (Chippaux and Goyffon, 2008). The systemic effects, in turn, are mediated predominantly by the massive release of sympathetic and parasympathetic neurotransmitters that contribute to central and peripheral neurotoxicity, as well as cardiac and hemodynamic alterations (arrhythmias, bradycardia, tachycardia, hypertension and hypotension) that can lead to cardiogenic shock and pulmonary edema, the primary causes of death (Bucaretchi et al., 2014, 2016; Pucca et al., 2015; Housley et al., 2017). Additional systemic effects such as acute kidney injury and pancreatitis have also been observed (Pucca et al., 2015).

Scorpionism by *Tityus* spp. can be life-threatening, with children being more susceptible to fatalities than adults (Bucaretchi et al., 1995, 2014; Chippaux and Goyffon, 2008; Albuquerque et al., 2013).

Transcriptomic and proteomic analyses have shown that *Tityus* venoms contain a variety of toxins, most of which have molecular masses <10 kDa (Barona et al., 2006; Batista et al., 2006, 2007; Dias et al., 2018); toxin classes identified include metalloproteinases (the predominant toxin class), Na^+ and K^+ ion channel toxins, hyaluronidase, hypotensin and a variety of peptides (Pucca et al., 2015; Oliveira et al., 2015, 2018). The modulation of ion

channel activity by scorpion toxins is believed to contribute to most of the sympathetic and parasympathetic actions of these venoms (Lourenço et al., 2002; Pucca et al., 2015). Whereas the sympathetic and parasympathetic effects of *Tityus* venoms have been extensively investigated in laboratory animals (Pucca et al., 2015; Nencioni et al., 2018) and clinically (Bucaretchi et al., 2014; Torrez et al., 2015), much less is known about the effects of these venoms on neurotransmission in skeletal muscle, although the uncoordinated neuromuscular activity may contribute to respiratory failure (Bucaretchi et al., 1995; Torrez, et al., 2015).

Envenomation by the Amazonian species T. *obscurus* in the western Amazon (northern Brazilian state of Pará) is characterized by electric shock-like sensations throughout the body associated with myoclonus, ataxia, dysarthria, dysmetria, dyslalia and muscle fasciculations that are not observed in individuals stung by this species in the eastern part of this state (Pardal et al., 2014; Torrez et al., 2015) or in patients stung by *T. bahiensis* or *T. serrulatus* (Bucaretchi et al., 2014). These alterations have been associated with acute cerebellar dysfunction rather than with direct interference in peripheral neurotransmission by venom toxins (Pardal et al., 2014; Torrez et al., 2015). Monteiro et al. (2016) described a case of envenomation by *T. silvestris* in the Brazilian Amazon that involved recurrent generalized severe muscle spasms for three days post-envenomation. As with *T. obscurus*, the spasms were of central rather than peripheral origin as they were progressively attenuated by diazepam, phenytoin and promethazine; antivenom was ineffective in treating these spasms.

Few experimental studies have examined the effects of *Tityus* venoms on neurotransmission in skeletal muscle, and those that have, have generally used *T. serrulatus* venom. Vital Brazil et al. (1973) reported that *T. serrulatus* venom evoked spontaneous muscle twitches in innervated rat diaphragm muscle without electrical stimulation and caused sustained muscle contraction in low Ca^{2+} solution. The venom also enhanced the twitch responses to supramaximal stimulation and delayed subsequent muscle relaxation. These effects were attributed to the presynaptic release of acetylcholine (ACh) by venom since they were abolished by *d*-tubocurarine (*d*-Tc), muscle denervation, the absence of Ca^{2+} or the presence of Mg^{2+}, neomycin (aminoglycoside antibiotic) or lidocaine (local anaesthetic) in the physiological solution. The venom contained no antiacetylcholinesterase activity that could have contributed to these effects. The venom also had no effect on muscle contractility in directly stimulated curarized preparations although a slight initial transitory increase in baseline tension was observed. Most of these effects were subsequently shown to be reproduced by 'tityustoxin' [probably a mixture of toxins containing true tityustoxin, now referred to as Ts3; see Bordon et al. (2015)] (Warnick et al., 1976).

Oliveira et al. (1989) later showed that *T. serrulatus* venom enhanced the contractile responses to exogenous ACh in rat phrenic nerve-diaphragm (PND) preparations, an effect also seen with toxin γ (currently, Ts1) and fraction T_2III_1 (currently, Ts3) from this venom. Both toxins caused a transitory increase in miniature endplate potentials (MEPPs) and a transitory depolarization of diaphragm muscle that was maximal after 15 min and could be prevented by tetrodotoxin (TTX) and *d*-Tc. Borja-Oliveira et al. (2009) subsequently reported that *T. serrulatus* venom (0.5 μg/ml) caused a marked increase (~230%) in the twitch tension of indirectly stimulated mouse PND preparations compared to that caused by *T. cambridgei* (a junior synonym for *T. obscurus*) venom (~75%; 10 μg/ml); the latter venom caused ~44% muscle facilitation in curarized, directly stimulated preparations whereas *T. serrulatus* venom had no such effect. These increases in tension in indirectly stimulated preparations were virtually abolished by TTX (both venoms) and attenuated (*T. serrulatus*) or reversed (*T. cambridgei*) by the K^+ channel opener, cromakalim. In directly stimulated preparations, TTX abolished the facilitation caused by *T. cambridgei* venom, whereas cromakalim had no significant effect on the response. These findings indicate that *Tityus* venoms can affect skeletal muscle contractility, although they vary in their profiles of activity and potency in indirectly and directly stimulated preparations.

Tityus bahiensis (brown scorpion), endemic to Sao Paulo State of Brazil, has a wide distribution in southeastern and southern Brazil, particularly in the states of São Paulo and Minas Gerais, where most envenomations occur. This specie also occurs in northern Argentina (provinces of Misiones, Corrientes, Entre Ríos, Santa Fé; Chaco and Formosa; de Roodt et al., 2014; Martinez et al., 2018) and eastern Paraguay (in provinces such as Cordillera, Guairá, Alto Paraná and Itapuá; Maury, 1984; Lourenço, 1994). *T. bahiensis* is the second major species responsible for scorpion stings countrywide (von Eickstedt et al., 1996; Brasil 2018) but is not a major cause of clinical envenomation in Argentina or Paraguay, with no severe envenoming or deaths reported (Ojanguren Affilastro et al., 2005; de Roodt et al., 2014). Most of its venom gland DNA transcripts have been determined to encode for potassium and sodium channel toxins, metalloproteases and cysteine-rich secretory peptides, but transcripts for hypotensin-like substances, antimicrobial and anionic peptides, protease inhibitor and serine proteinase are also present (Oliveira et al., 2015). Clinical studies have shown that, among other life-threatening peripheral effects, *T. bahiensis* envenomation promotes neuromuscular toxicity such as tremors (Bucaretchi et al., 1995). Despites *T. bahiensis* has great medical importance,

its venom remains poorly studied, especially regarding to its actions on the peripheral nervous system.

Since few reports have investigated the effects of *Tityus* venoms in skeletal muscle and since the venom of *T. bahiensis* has been studied less than that of *T. serrulatus* (Lourenço et al., 2002; Oliveira et al., 2015; Miyamoto et al., 2018; Nencioni et al., 2018) (despite the clinical importance of *T. bahiensis*), in this work we examined the activity of *T. bahiensis* venom on somatic neurotransmission by using chick and mouse neuromuscular preparations, as well as mouse sciatic nerve and dorsal root ganglia (DRG).

2. Materials and methods

2.1. Reagents and *T. bahiensis* venom

Acetylcholine, carbachol, dimethyl sulfoxide (DMSO) and *d*-tubocurarine were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Fluo-4 AM was from Thermo Fisher Scientific Inc. (Waltham, MA, USA), RPMI 1640 culture medium and Hank's balanced salt solution (HBSS) were from Life Technologies Ltd. (Paisley, Scotland), and isofluorane was from Cristália (Itapira, SP, Brazil). Dispase (type II) was from Roche Life Sciences (Burgess Hill, UK) and collagenase (type P) was from Worthington Biochemical Co. (Lakewood, NJ, USA). The reagents (analytical grade) for physiological solutions were purchased from J.T. Baker Chemicals/Mallinckrodt (Mexico City, DF, Mexico), Merck (Rio de Janeiro, RJ, Brazil) or local suppliers.

Lyophilized *T. bahiensis* venom was provided by the Arthropods Laboratory of the Butantan Institute (São Paulo, Brazil) and was obtained from scorpions of both sexes.

2.2. Animals

Male Balb/c mice (20 - 35g, 8 weeks-old) and Wistar rat pups of either sex (aged 3-8 days old) were obtained from the Multidisciplinary Centre for Biological Investigation (CEMIB, the central animal house at UNICAMP) or by the Strathclyde Institute of Pharmacy and Biomedical Sciences, and male HY-LINE W36 chicks (4-8 days old) were supplied by Granja Globo Aves Agrícola Ltda. (Campinas, SP, Brazil). The animals were housed in plastic cages (mice and rats; 5/cage with a wood shaving substrate) or metal cages (chicks; 10/cage with a sawdust substrate) at 22 ± 3 ºC on a 12 h light/dark cycle with free access to standard diet and water. The experiments were approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol nos. 4068-1 and 4189-1) and were performed

according the general ethical guidelines of the Brazilian Society for Laboratory Animal Science (SBCAL), the Brazilian National Council for Animal Experimentation (CONCEA), Brazilian legislation (Federal Law no. 11,794, of October 8, 2008), EU Directive 2010/63/EEC for the Protection of Animals Used for Scientific Purposes and the UK Government Animals (Scientific Procedures) Act (ASPA) 1986 and associated guidelines.

2.3. Myographical studies

2.3.1. Mouse extensor digitorum longus (EDL) preparation

Mice were euthanized with an overdose of isoflurane (via inhalation) and the *extensor digitorum longus* muscles were carefully removed along with the tendons. The preparations were mounted under a resting tension of 1 g in a 5 mL organ baths containing Tyrode solution (37 °C) of the following composition (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 0.49, NaH_2PO_4 0.42, $NaHCO_3$ 11.9 and glucose 11.1, gassed with 95% O_2 - 5% CO_2. A bipolar platinum ring electrode was placed around the muscle for field stimulation with a Grass S48 stimulator (0.1 Hz, 0.2 ms, supramaximal voltage; Grass Instruments, Quincy, MA, USA). Muscle contractions were measured isometrically via a four-channel PowerLab ML866 physiograph coupled to a Quad Bridge ML224 amplifier and a computer containing LabChart7 software (all from ADInstruments, Newcastle, NSW, Australia). The preparations were allowed to stabilize for 20 min before the addition of venom (1-10 µg/mL). Control preparations were incubated with Tyrode solution alone.

2.3.2. Mouse phrenic nerve diaphragm (PND) preparation

Mice were euthanized with an overdose of isoflurane (via inhalation) followed by exsanguination. The hemidiaphragms and corresponding phrenic nerves were carefully removed and mounted under a tension of 5 g in a 5 mL organ baths containing Tyrode solution (composition in section 2.3.1) gassed with 95% O_2 and 5% CO_2 at 37 ºC. The muscles were stimulated either indirectly (nerve-evoked contractions; 0.1 Hz, 0.2 ms, supramaximal voltage; Grass S48 stimulator) or directly (70 V, 0.1 Hz, 20 ms), the latter after incubation with *d*-Tubocurarine (5µg/mL) to block possible nerve-mediated responses. Muscle contractions were recorded isometrically as indicated in the previous section. The preparations were allowed to stabilize for 20 min before the addition of venom (1-30 µg/mL). Control preparations were incubated with Tyrode solution alone.

2.3.3. Chicken biventer cervicis (BC) preparation

Male chicks were euthanized with an overdose of isoflurane (via inhalation) and the *biventer cervicis* muscles were carefully removed. The preparations were mounted under a tension of 1 g in a 5 mL PanLab organ baths coupled to a PanLab LE13206 thermostat containing Krebs solution (composition, in mM: NaCl 118.7, KCl 4.7, $CaCl_2$ 1.88, KH_2PO_4 1.17, $MgSO_4$ 1.17, $NaHCO_3$ 25.0 and glucose 11.65, pH 7.5, 37 ºC) gassed with 95% O_2 and 5% CO_2 at 37 °C (temperature maintained with a PanLab LE13206 thermostatted water bath). The muscles were stimulated indirectly with field stimulation (0.1 Hz, 0.2 ms, supramaximal voltage) or directly (70 V, 0.1 Hz, 20 ms) after neuromuscular inhibition with *d*-Tubocurarine (5µg/mL). Some experiments were also done in Krebs solution without calcium. Muscle contractions were recorded isometrically as indicated in section 2.3.1. After allowing the preparations to stabilize for 20 min, contractures to exogenous acetylcholine (ACh, 110 µM), potassium chloride (KCl, 20 mM) and carbachol (CCh, 8 µM) were recorded in the absence of stimulation before and 120 min after the addition of venom (1-30 µg/mL). Control preparations were incubated with Krebs solution alone.

2.4. Electrophysiological study

2.4.1. Muscle resting membrane potential (RMP)

Mouse diaphragm muscle was isolated, and carefully pinned to a Sylgard-based (Dow-Corning, Allesley, Coventry, UK) perfusion chamber connected to a temperature controller (Hugo Sachs Elektronic, Munich, Germany) filled with aerated (95%O_2 and 5% CO_2) Tyrode solution (composition in section 2.3.1) at 37 ºC. The resting membrane potentials were measured with glass microelectrodes filled with 3M KCl (resistance of 15-25 MΩ, made in a vertical puller model P-30, Sutter Instruments, Novato, CA, USA) inserted superficially into muscle cells using a Leica Letz mechanical manipulator. The potential difference between the reference electrode and the recording microelectrode in the cell was measured with a unitary gain high input impedance electrometer (model Electro 705, World Precision Instruments, Hertfordshire, UK). The electric potential from the microelectrode was amplified using a CED 1902 (Cambridge Electronic Design) and digitized with a NIQAQ-MX 16bit A/D convertor connected via BCN-2110 connector block (National Instruments, Newbury, UK); the signs were then recorded and analysed using WinEDR software (John Dempster, University of Strathclyde, UK). The resting membrane potential was measured before and after incubation with venom (0.3 µg/mL) for 5, 10, 15, 30, 60, 90 and 120 min.

2.4.2. Miniature end-plate potentials (MEPPs)

Miniature end-plate potentials (MEPPs) were measured in mouse phrenic nerve – diaphragm preparations using conventional microelectrode techniques. The preparations were isolated and carefully pinned to a Sylgard-based (Dow-Corning, Allesley, Coventry, UK) perfusion chamber connect to a temperature controller (Hugo Sachs Elektronic, Munich, Germany) filled with Tyrode solution (composition in section 2.3.1) gassed with carbogen (95% O_2 and 5% CO_2), as described in preceding section at room temperature. To record MEPPs, glass microelectrodes (filled with 3M KCl, resistance of 15-25 MΩ) were inserted into superficial muscle fibres close to end-plates; these regions were identified by following the nerve branches under a compound microscope (x 200) and were chosen for the MEPPs to have rise-times faster than 1ms. The potential differences between the reference electrode and the recording microelectrode were measured as described above. The potentials were measured as described in the preceding section using the same time intervals.

2.4.3. End-plate potentials (EPPs)

End-plate potentials (EPPs) were measured in mouse phrenic nerve – diaphragm preparations using conventional microelectrode techniques. The preparations were isolated and carefully pinned to a Sylgard-based perfusion chamber connected to a temperature controller (Hugo Sachs Elektronic, Munich, Germany) filled with Tyrode solution (composition as in section 2.3.1, except for a lower calcium concentration of 0.4 mM) gassed with carbogen (95% O_2 and 5% CO_2) at room temperature. The EPPs were obtained in response to indirect nerve stimulation with a Grass S48 stimulation (1 Hz, 0.2 ms, twice the threshold voltage). The 'cut muscle' technique, which consists of cutting muscle fibres connected to the central tendon and to the ribs, was employed to avoid the generation of muscle action potentials and the accompanying contractions; this approach depolarizes the muscle cells, inactivating muscle sodium channels which prevents the generation of action potentials. To ensure that EPPs were not affected by non-linear summation, the calcium concentration was reduced to ~0.4mM to ensure that the EPP amplitude was <10% of the resting membrane potential. To record EPPs, glass microelectrodes (filled with 3M KCl, resistance of 15-25 MΩ) were insert into superficial muscle fibres close to end-plates; these regions were identified by following the nerve branches under a compound microscope (x 200) and were chosen for EPPs to have rise-times faster than 1ms. The potential differences between the reference electrode and the recording

299 microelectrode were measured as described above. The EPPS was recorded before and after 5,
300 10, 15, 30, 60, 90 and 120 min of exposure to *T. bahiensis* venom (0.3 μg/mL).
301
302 *2.4.4. Perineural waveforms*
303 Mice were euthanized with an overdose of isoflurane (via inhalation) and the left
304 *triangularis sterni* nerve-muscle preparations were dissected immediately before the
305 experiments. The rib cage was removed and transferred to a constant-flow Petri dish superfused
306 with carbogen-gassed (95% O_2 and 5% CO_2) Tyrode solution (composition as in section 2.3.1)
307 at a rate of 20–25 mL/min. The connective tissue, layers of intercostal muscle and ribs were
308 removed; after the dissection, the muscles connected to the sternum were cut to prevent muscle
309 contractions during the experiment. The preparation was then pinned thoracic side downwards
310 to Sylgard-coated chamber containing carbogen-gassed Tyrode solution. The intercostal nerve
311 was stimulated via a suction electrode (1 Hz, 0.1 ms) coupled to a Grass stimulus isolation unit
312 and Grass 88 stimulator (Grass Instrument Co.) to produce a maximal twitch. Postsynaptic
313 potentials were abolished by adding *d*-tubocurarine (14 μM) to the Tyrode solution in addition
314 to using the "cut muscle" technique. To measure the perineural waveforms, the glass
315 microelectrodes (filled with 2M NaCl, resistance: 15-20 MΩ) made in a vertical puller (model
316 P-30, Sutter Instruments, Novato, CA, U.S.A) were positioned on one of the branches of the
317 intercostal nerve. The potentials were measured as described in the preceding section in section
318 2.4.1 before and after 5, 10, 15, 30, 60, 90 and 120 min of exposure to venom (0.3 μg/mL).
319
320 *2.4.5. Axonal compound action potentials (CAP)*
321 Axonal compound action potentials were obtained from mouse sciatic nerves arranged
322 horizontally in a recording chamber composed by three compartments electrically isolated from
323 each other by the addiction of petroleum jelly (Vaseline®), so that the proximal, medial and
324 distal regions of the axon were each located in one of three isolated compartments filled with
325 gassed (95% O_2 and 5% CO_2) Tyrode solution (composition described in section 2.3.1). The
326 proximal end of the axon was submersed in the first compartment and field stimulated (at twice
327 the threshold voltage, 1 Hz, 0.05 ms; Grass S48 stimulator) while the middle portion was placed
328 in the central compartment with the reference electrode and the distal extremity was placed in
329 the last compartment with the recording electrode. The CAP waveforms were recorded as
330 described in section 2.4.1 before and for 90 min following the incubation with venom (0.3
331 μg/mL).
332

2.5. Calcium imaging

Intracellular Ca^{2+} transients were monitored using Ca^{2+} sensitive fluorescent indicators (Fluo-4 AM; Thermo Fisher Scientific Inc., Waltham, MA, USA) in *triangularis sterni* nerve-muscle preparations and neurons isolated from dorsal root ganglia (DRG). The fluorescent dye was prepared as a 1 mM stock in dry dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at - 20ºC until used. The images were recorded using a Grasshopper3 USB3 camera model GS3-U3-15S5M-C (FLIR Integrated Imaging Solutions Inc.) attached to a Zeiss Axioskop 50 upright epifluorescence microscope (Carl Zeiss, Germany). Excitation light was provided by a 50 W mercury short arc lamp (Osram, Germany) and filter set 9 (Carl Zeiss), that consisted of an excitation filter (BP 450 – 490 nm), beam splitter (FT 510 nm) and emission filter (LP 520 nm). Image acquisition was controlled with the software WinFluor software (John Dempster, University of Strathclyde, UK) and obtained at rates of 2 frames/s and exposure times of 100 – 500 ms. All Ca^{2+} measurements were done at room temperature.

2.5.1. Triangularis sterni preparation

Mouse *triangularis sterni* preparations were isolated as already described (section 2.4.4), denervated and incubated with 10 μM Fluo-4AM in Tyrode solution (composition as in section 2.3.1) gassed with carbogen (95% O_2 and 5% CO_2) for at least 60 min in the dark at room temperature. Following incubation, the muscles were washed to remove extracellular indicator and pinned to a Perspex Sylgard-coated recording chamber containing Tyrode solution. The preparations were allowed to equilibrate for 20 min before the addition of venom. Intracellular Ca^{2+} fluorescence was recorded before (control) and for 15 min after the addition of venom (30 μg/mL).

2.5.2. Primary culture neurons

Rat pups were killed by cervical dislocation and their dorsal root ganglia (DRG) from all the spinal levels were removed aseptically and immediately transferred to a Gibco® RPMI 1640 culture medium (Life Technologies Ltd., Paisley, UK), after which the remaining connective tissue and attached nerves were removed. The cleaned ganglia were centrifuged (1000 rpm for 4 min) and resuspended to 5 mL of Gibco® Hank's Balanced Salt Solution (HBSS; Life Technologies Ltd., Paisley, Scotland) containing dispase (type II; 1.5 mg/mL; Roche Life Sciences, Burgess Hill, UK) and collagenase (type P; 1.5 mg/mL; Worthington Biochemical Corporation, Lakewood, NJ, USA) for 1 h at 37 ºC for enzymatic dissociation. After incubation,

the DRGs were gently mechanically disrupted using micropipettes and the cell suspension was centrifuged (1000 rpm, 4 min) and resuspended in RPMI 1640 medium. Aliquots were then placed on coverslips precoated with HBSS containing poly-D-lysine (0.1 mg/mL) and laminin (0.01 mg/mL) and left to adhere for 20-30 min at 37 ºC. Once seeded, the cells were kept in RPMI 1640 medium supplemented with fetal bovine serum (10%), streptomycin (1%), penicillin (1%), L-glutamine (1%), sodium pyruvate (1%) and non-essential amino acids (1%) at 37 ºC in a humidified atmosphere containing 5% CO_2. Cytosine β D arabinofuraside (10 µM) was included in the culture medium to inhibit the growth of non-neural cells. The cells were used within five days of isolation. For use, the cells were incubated with of Fluo-4 AM for 1 h in a tissue culture incubator at 37 ºC and the cover slips then washed with Tyrode solution to remove the extracellular indicator and left to equilibrate for 20 min prior to the addition of venom (30 µg/mL). Intracellular Ca^{2+} fluorescence was recorded before (control) and for 15 min after venom addition.

2.6. Statistical analysis

All results were expressed as the mean ± SEM, as indicated in the legends. Statistical comparisons were done using either Student´s unpaired *t*-test or two-way analysis of variance (ANOVA) followed by the Tukey-Kramer test, depending on the analysis, with $p<0.05$ indicating significance. All data analyses were done using Prism v.6 (GraphPad Inc., La Jolla, CA, USA).

3. Results

3.1. Electromyography

3.1.1. Mouse extensor digitorum longus (EDL) preparation

Tityus bahiensis venom was initially tested on mouse EDL, a fast-twitch muscle preparation (Riquelme et al., 2013). At the lowest concentration tested (1 µg/mL), the venom caused a significant facilitation on nerve-evoked muscle contraction (Fig. 1A, B). As the venom concentration increased (3 µg/mL), this facilitatory effect gave way to a concentration-dependent blockade of muscle contraction, as evidenced at 90 min; the concentration of 10 µg/mL promoted an immediate and total blockade (Fig. 1A, C).

400 *3.1.2. Mouse phrenic nerve – diaphragm (PND) preparation*

401 As with mouse EDL preparations, the incubation of indirectly stimulated PND
402 preparations with the lowest venom concentration (1 μg/mL) caused marked, prolonged
403 facilitation of the contractile responses that gradually gave way to neuromuscular blockade with
404 increasing venom concentrations; at the highest concentration (30 μg/mL), a rapid initial
405 facilitation was replaced by irreversible blockade that was virtually complete after 50 ± 12 min
406 (n=5) (Fig. 2A,C). There was no muscle facilitation in curarized, directly stimulated
407 preparations at any of the venom concentrations tested (Fig. 2B). Instead, the venom produced
408 time- and concentration-dependent partial neuromuscular blockade that was maximal (14 ± 7%)
409 at the highest venom concentration (Fig. 2B,D). In contrast to indirectly stimulated
410 preparations, there was no significant difference in the blockade produced by the two highest
411 concentrations during direct stimulation; the blockade caused by the two lowest concentrations
412 was also similar (not significantly different).

413 The velocity of muscle contraction (time required to develop peak tension) and
414 relaxation (time required for return from peak to basal tension) in the absence and presence of
415 venom was calculated from the myographic recordings. Figure 2E shows that incubation with
416 venom (10 μg/mL) resulted in slower muscle contraction and relaxation in indirectly stimulated
417 preparations, whereas no such effect was seen in directly stimulated muscle (Fig. 2F); this
418 finding suggested a presynaptic action of the venom. In addition, a high venom concentration
419 (>10 μg/mL) caused fasciculations and small spontaneous twitches, even after complete
420 neuromuscular blockade; this effect was absent or greatly reduced in curarized preparations
421 (dada not shown).

422

423 *3.1.3. Chick biventer cervicis (BC) preparation*

424 In indirectly stimulated BC preparations the venom produced the same pattern of
425 responses as seen in EDL and NFD preparations, namely, lower venom concentrations (1 and
426 3 μg/ml) caused muscle facilitation, an intermediate concentration (10 μg/ml) had minimal
427 effect and a high concentration (30 μg/ml) caused fast, total block of nerve-evoked muscle
428 contractions (Fig. 3A). Muscle contractures to exogenous ACh, KCl and carbachol (CCh) were
429 not significantly altered by incubation with venom when compared to control preparations or
430 basal contractures (Fig. 3B).

431 In directly stimulated BC preparations, the venom produced a similar pattern of
432 responses to that seen with indirect stimulation involving muscle facilitation (1-10 μg/ml) and
433 partial blockade at 30 μg/ml (Fig. 3C). However, the facilitation was more intense and

prolonged than in indirectly-stimulated preparations, whereas the blockade was only about half of that seen in the latter preparations. In nominally Ca^{2+}-free medium there was no facilitation and muscle blockade was evident at venom concentrations ≥3 µg/ml, with little concentration-dependence over the range of 3-30 µg/ml (maximal blockade of 41.5 ± 9 %, 33.8 ± 9 % and 40.4 ± 4 % for 3, 10 and 30 µg/mL, respectively, after 120 min) (Fig. 3D); these results also indicated that the facilitatory effects were likely to be presynaptic in origin.

The twitch velocity was assessed after incubation with the highest venom concentration (30 µg/mL). Venom slowed the twitch waveform after a 10 min incubation in both curarized and Ca^{2+}-free experiments and this effect was completely reversed by a low concentration of tetrodotoxin (TTX, 5 nM; data not shown). This effect was not observed upon indirect stimulations because after 10 min the indirect contractions were almost totally blocked. At 30 µg/mL, venom also caused sustained muscle contracture (elevation of baseline) that was reduced in Ca^{2+}-free medium (Fig. 4AB) and absent in curarized preparations (Fig. 4C). The venom also caused contraction in the absence of electrical stimulation (Fig. 4D), indicating that this effect was independent of a presynaptic action potential that evoked ACh release; further, the addition of d-tubocurarine completely reversed this contraction (Fig. 4D). Incubation with botulinum toxin (BoNT-A, 10 µg/mL) did not prevent the contracture but shortened its duration (Fig. 4E).

3.2. Electrophysiological study

Next, we used mouse PND preparation to record the resting membrane potential (RMP) and post-junctional potentials (MEPPs and EPPs). Mouse *triangularis sterni* preparation was adopted further for more detailed evaluation of pre-junctional potentials (perineural waveforms) and the intracellular movement of Ca^{2+} in the muscle fibres; mouse sciatic nerve was used for extracellular recording of nerve conduction (action potentials) and, isolated GRD cells were used to investigate the mobilizarion of intracellular Ca^{2+} in the neuron.

3.2.1. RMP, MEPPs and EPPs in PND preparations

In control experiments, the RMP of mouse diaphragm muscle ranged from -71 mV to -64 mV during a 120 min incubation; basal values (time 0; before venom incubation) were -67.4 ± 2 mV for the control group and -69.8 ± 5 mV for the treated group. The addition of *T. bahiensis* venom caused a slight but significant depolarization during the first 30 min of incubation (-67.0 ± 2, -70.7 ± 4 and -71 ± 4 mV in control versus -60.7 ± 6*, -59.6 ± 3* and -

61 ± 5* mV in venom-incubated muscles after 5, 15 and 30 min, respectively; *$p<0.05$ compared to control).

Concomitantly with RMP depolarization, the venom significantly ($p<0.05$) increased the MEPP frequency from 0.4 ± 0.04 Hz (control) to 0.7 ± 0.1 Hz (venom) after 5 min, and from 0.4 ± 0.1 Hz (control) to 0.6 ± 0.08 Hz (venom) after 30 min (Fig. 5A). In contrast, the average rise and decay time constants and MEPPs amplitude were not significantly altered. However, there was a leftward-displacement in the amplitude-frequency distribution curve was seen in the first 5 min of incubation (Fig. 5B).

The venom significantly increased the EPP amplitude after 30 min without altering rise and decay time constants (Fig. 6A). This increase in EPPs amplitude was concomitant with the nerve-evoked facilitation of twitch-tension. After a prolonged incubation (90 min), the venom triggered multiple EPPs per stimulus (Fig. 6B). A high venom concentration (30 µg/mL) completely blocked the EPPs (data not shown).

3.2.2. Perineural waveforms

The perineural waveform obtained from the perineural nerve trunk in *triangularis sterni* nerve-muscle preparations typically consists of two negative deflections, the first of which is related to Na^+ influx at the recording site while the second is related primarily to K^+ efflux at distal nerve terminal during the action potential in the nerve ending. Incubation with *T. bahiensis* venom significantly reduced the amplitude of both waveforms when compared to control, with the K^+- related compound being firstly reduced followed by the Na^+-related deflection (Fig. 7A, B). The waveform rise times, rates of rise (mV/ms) and latency (time between stimulus and response) were not affected by the venom (data not shown). However, the exposure to venom resulted in multiple waveforms per stimulus (repetitive firing) that merged with each other resulting in a marked prolongation of the 2nd negative deflection and a largeincrease in the area under the curve (AUC) from 60 min onwards (Fig. 8A). The addition of a low-concentration TTX (20 nM) abolished the repetitive firing and reversed the venom-induced prolongation of the second negative deflection (Fig. 8B,C) and the increase in AUC (Fig. 8A).

3.2.3. Compound action potential (CAP) of sciatic nerve

Exposure of the sciatic nerve to venom (0.3 µg/mL) slowed the rise phase and decay time constants of the CAP and reduced its amplitude (Fig. 9). These marked delays in repolarization was reversed by the addition of a low-concentration of TTX (20 nM; data not shown). Whereas

high concentration of venom (30 µg/mL) reduced and abolished the ability of the axon to generate an action potential (data not shown).

3.3. Calcium imaging

The DRGs neurons and *triangularis sterni* muscle cells were loaded with the Ca^{2+} sensitive fluorescent dye (Fluo-4-AM) and the changes in fluorescence were recorded before and after a single concentration of *T. bahiensis* venom (10 µg/mL, 15 min). Venom increased the fluorescence levels in DRG neurons, especially in the first 5 min of incubation (Figure 10); this time-scale coincided with the presynaptic neuromuscular facilitation and the increase in MEPP frequency. In contrast, there were no marked changes in the intracellular calcium content of *triangularis sterni* muscle cells (Fig. 10).

4. Discussion

Tityus scorpion venoms are well-known for their actions on sympathetic and parasympathetic pathways that account for most of the clinical manifestations of envenomation. In contrast, the action of these venoms on somatic neurotransmission is less well understood.

In human envenoming by *T. bahiensis* and *T. serrulatus*, the effects on motorsomatic nerve system are not as common as observed for other species (*T. obscurus, T. silvestris*) which skeletal muscle effects (myoclonus/fasciculations, spasms) have been clearly observed and described in clinic (Pardal et al., 2014; Torrez et al., 2015; Monteiro et al., 2016); these muscular effects are believed to be mediated by acute cerebellar dysfunction and possibly by a direct effect of the venoms on skeletal muscle fibres. This could be explained by the finding that most stings by positively identified *T. bahiensis* (~88.6%) and *T. serrulatus* (~72%) are 'dry stings' (no venom injection) or class I stings characterized by local manifestations such as pain, edema, hyperemia or paresthesia (Bucaretchi et al., 2014); the low frequency of class II and class III stings would tend to make it more difficult to detect possible somatic effects.

In choosing to investigate cholinergic nicotinic neurotransmission, we opted to specifically study somatic neuromuscular transmission once studies that examined such effects of *Tityus* venoms in these systems are very poor (Vital Brazil et al., 1973; Warnick et al., 1976; Oliveira et al., 1989; Borja-Oliveira et al., 2009) and all with *T. serrulatus* venom. The pharmacological approaches used included a combination of twitch-tension, electrophysiological and calcium imaging techniques In the three different neuromuscular preparations studied (PND, EDL and BC), a low concentration of venom generally caused

facilitation of the nerve-evoked muscle twitches, while a high concentration of venom blocked these contractions. Low concentrations of venom also increased the spontaneous release of ACh and quantal content of evoked release, prolonged the axonal action potential and perineural waveform, and increased the intracellular calcium concentration in neurons. All of these effects could be attributed to the presence of toxins that delay the closing of voltage-gated sodium channels. Nerve-evoked twitches in mammalian preparations have shown different sensitivities to *T. bahiensis* venom: while EDL was more sensitive to the blocking activity than PND, the PND was more sensitive to the facilitatory effect than EDL. The sensitivity of EDL muscle to blockade could be explained by the higher density of sarcolemmal Na^+ channels since i) fast-twitch muscles such as EDL have a higher density of voltage-gated sodium channels in the junctional and extra-junctional regions when compared to slow-twitch muscles such as PND (Ruff and Whittlesey, 1993; Milton and Behforouz, 1995), ii) *Tityus* venoms contain α- and β-toxins with high affinity and specificity for voltage-gated sodium channels (Bordon et al., 2015; Pucca et al., 2015; Kozlov, 2018), and iii) the neuromuscular blockade observed here also had a postsynaptic origin.

In contrast, the PND was more sensitive to the presynaptic facilitation compared with EDL, a divergence that could be explained by differences in the quantal content (number of ACh quanta released upon a single nerve impulse) of nerve terminals - the quantal content in PND is 3.5 times higher than in EDL (Urbano et al., 2002; Herrera and Zeng, 2003; Nguyen-Huu et al., 2009), and by the density of post-synaptic nicotine receptors in these two preparations - PND muscle has a higher density and greater availability of nicotinic receptors than EDL muscle (Nguyen-Huu et al., 2009). Presynaptic facilitation, muscle fasciculations, and a delay in muscle relaxation have been also observed in PND incubated with *Tityus serrulatus* and *Odontobuthus doriae* venoms (Vital Brazil et al., 1973; Oliveira et al., 1989; Jalali et al., 2007; Borja-Oliveira et al., 2009). *Tityus bahiensis* venom did not prolong the EPPs, but repetitive and spontaneous ACh release that could prolong and facilitate the muscle contractions were observed, which is consistent with a previous study using 'tityustoxin' from *T. serrulatus* venom (Warnick et al., 1976).

Different than the focal innervation of mammal neuromuscular junctions in which nicotinic acetylcholine receptors (nAChR) are restricted to the terminal plate region, avian muscles such as BC have multifocal innervation comprehending on nicotine receptors disposed not only in the terminal plate but also distributed along the entire fibre length (Fernandes et al., 2014). This peculiar arrangement makes it possible to examine the contractile effects of exogenous cholinergic agonists (such as acetylcholine – ACh and carbachol – CCh) and KCl

on tissue depolarization and, consequently, on sarcoplasmic release of Ca^{2+} and muscle contraction. As shown here, *T. bahiensis* venom did not significantly affect the muscle response to ACh, CCh and KCl, thus excluding the possibility that the venom interfere with the binding of ACh to nAChR, contained antiacetylcholinesterase activity or adversely affected the integrity of the postsynaptic membrane and the release of Ca^{2+} from the sarcoplasmic reticulum. These findings and conclusion are consistent with those for venoms from other scorpions such as *Buthus tamulus* (Rowan et al., 1992).

A high concentration of venom induced an immediate tonic contracture of BC muscle (an effect not seen in PND preparations) that was independent of the nerve action potential and the release of ACh evoked by electrical stimulation. This effect was prevented or reversed by *d*-Tubocurarine, reduced in the absence of Ca^{2+} in the bath solution, and unaffected by botulinum toxin (BoNT-A) that blocks quantal ACh release without modifying the nerve conduction, affecting the nicotine receptor sensibility or abolishing the spontaneous release of ACh (Thyagarajan et al., 2010, Wheeler et al, 2013). These results suggest that the *T. bahiensis* venom-induced contracture involved the spontaneous release of ACh from BC terminals, independently of the nerve action potential, a conclusion supported by the finding that the absence of Ca^{2+} in the physiological solution reduced the MEPP frequency (Onur et al., 1995) and the venom-induced muscle contracture.

Since recording ACh from BC preparations is technically demanding, we used PND to gain a clearer insight into the mechanism of action of the venom. In PND preparations, *T. bahiensis* venom increased the MEPP frequency without changing the shape (rise and decay times) of the potentials; despite their average amplitude was unaffected, the MEPPs tended to be smaller as the frequency of release increased. A similar effect was observed with *O. doriae* scorpion venom that displaced the Gaussian distribution curve to the left, indicating a larger number of smaller amplitude potentials as the MEPPs frequency increased (Jalali et al., 2007). This increase may reflect a delay in Na^{+} channels inactivation as well as alterations in the channel opening threshold, leading to a partially depolarized nerve terminal that triggers the spontaneous ACh release (Jalali et al., 2007). This notion agrees with a previous study showing that *T. bahiensis* venom dislocated the Na^{+} channel activation curve to more negative potentials and markedly reduced the rate of channel inactivation (Moraes et al., 2011).

A presynaptic toxicity produced by venoms can also be observed by measuring the movement of intracellular Ca^{2+} (Vetter and Lewis, 2010; Grienberger and Konneth, 2012). As shown here, *T. bahiensis* venom markedly increased the intracellular Ca^{2+} content of DRG neurons, but not of skeletal muscle fibres (*triangularis sterni* muscle cells). The increase in

603 neuronal intracellular Ca^{2+} possibly reflected the partially depolarized neuron membrane that
604 allowed the influx of Ca^{2+}. However, it is unlikely that the increased Ca^{2+} movement was
605 secondary to nAChR activation because electrophysiological and myographic experiments
606 excluded this possibility.

607 Since the neuromuscular facilitation, muscle fasciculation, spontaneous contracture and
608 the effects on electrical potentials (RPM, MEPPs and EPPs) were apparently presynaptic in
609 origin, we next evaluated the effects of venom on nerve terminals and axonal conduction.
610 Perineural waveforms, characterized by two negative deflections related to the influx of Na^+
611 and (principally) efflux of K^+ in nerve terminals, are commonly used to monitor ion channels
612 activity in nerve terminals and nearby regions (Braga et al., 1995; Hodgson et al., 2007). *Tityus*
613 *bahiensis* venom reduced both of these negative peaks and triggered repetitive waveforms in
614 response to electrical stimulation; these repetitive firing became increasingly frequent until they
615 merged with each other to produce a massive delay in repolarization of the nerve. The venom
616 initially reduced the amplitude of the second peak, suggesting an action on K^+ channels. The
617 blockade of these channels in nerve endings delays repolarization, prolongs nerve
618 depolarization and increases neurotransmitter release (Jiménez-Vargas et al., 2017).

619 The delayed repolarization and repetitive firings were reversed by ow concentrations of
620 TTX, indicating that sodium channels are involved in such effect. Similar TTX-dependent
621 change in perineural waveforms have also been reported for ATX-II, an *Anemonia sulcata* toxin
622 that disrupts the sodium channels inactivation mechanisms (Vatanpour et al., 1993). Venoms
623 from *B. tamulus* (Rowan et al., 1992) and *O. doriae* (Jalali et al., 2007) produced similar
624 alterations leading to a reduction in both waveforms, repetitive firing upon stimulation and a
625 marked delay in nerve terminal repolarization. The *T. bahiensis* venom also markedly delayed
626 the axonal repolarization of the sciatic nerve (two times slower than normal) as shown by
627 extracellular recordings of action potentials in this tissue. This phenomenon was also reversed
628 by TTX indicating that, similarly to perineural waveforms, the sodium channels play an
629 important role in the pharmacological mechanism of action of the venom.

630 The primary function of scorpion venom, including that of *T. bahiensis*, is to capture
631 small prey (cockroaches, crickets and other insects) and protect the scorpions from predation
632 (Gwee et al., 2002). In the course of evolution, the neurotoxins of scorpion venom conferred
633 them the ability of attack and immobilizing prey (Gwee et al., 2002), however, as many of the
634 pharmacological targets are shared between insects and mammals (Evans and Maqueira, 2005;
635 Gordon et al, 2005), these venoms can have a profound "off prey" effect and produce severe
636 symptoms of envenomation of larger mammals, including man (Lourenço et al., 1996). The

amount of venom injected in one sting (approximately 0.4 mg, obtained by electrical stimulation; Candido and Lucas, 2004) would be roughly equivalent to the high concentration of venom used in our experiments, particularly in view of the small size of the scorpion's more common prey. This dose is enough to paralyze insects (Duzzi et al., 2016) and is a similar outcome (paralysis) to that reported here for high concentrations of venom. Since the mouse LD_{50} of *T. bahiensis* venom is 1.62 mg/kg, one sting would be enough to rapidly kill an animal weighing <250 g.

In larger vertebrates such as humans, the venom is used as a defensive mechanism (Bordon et al., 2015). However, in view of the larger size of the victim, the amount of venom injected in one sting is insufficient to paralyze skeletal muscle neurotransmission, but produces a massive increase in neurotransmitter release leading to an "autonomic storm", as observed clinically; this latter profile reflects the enhanced neurotransmitter release seen here with the low concentrations of venom and probably explains why major blockade of skeletal neuromuscular transmission is not observed in experimentally envenomed animals (Chávez-Olórtegui et al., 1994; Revelo et al., 1996; Santana et al., 1996) and clinically in humans (Barral-Netto et al., 1991; de Rezende et al., 1995a,b, 1996; D´Suze et al., 2003). Differences in patient size (and hence variations in the blood volume available for venom dilution) could also explain the greater severity of envenomation by Tityus spp. in young children compared to adults (Bucaretchi et al., 2014).

Antivenom therapy is still the recommended treatment for envenoming by *Tityus* spp., although ancillary measures such as mechanical ventilation (to alleviate respiratory problems associated with pulmonary edema) and ionotropic drugs such as dobutamine (for cardiac failure/circulatory shock) may also be used in severe envenomation (Bucaretchi et al., 2014; Cupo, 2015). The cholinergic manifestations of envenomation by *Tityus* spp. are generally not a focus of clinical pharmacological interventions and such cholinergic-mediated effects on somatic neurotransmission as have been observed for some species (e.g., *T. obscurus*) the efficacy of diazepam in treating these manifestations provides evidence in support of the central origin of these muscular effects. Since few somatic effects are observed clinically with *T. bahiensis*, for the moment, there is no need to alter the therapeutic approaches currently used to treat envenomation by this species.

Although we studied the somatic effects of the venom, we believe our findings, particularly with regard to the divergent effects of low and high venom concentrations, and the presynaptic activities which are also likely to be applicable to the autonomic nervous system. In this regard, the divergent responses of neurotransmission to low and high venom

concentrations could reflect the fluctuations (excitation and depression) seen in the relative involvement of the cholinergic branch of the autonomic system during envenomation.

5. Conclusion

The results of this investigation show that low concentrations of *T. bahiensis* venom prolong the axonal and nerve terminal repolarization mainly by modulating neuronal Na^+ channels. This persistent nerve depolarisation leads to longer depolarization of the nerve terminal that enhances neurotransmitter release and facilitates nerve-evoked muscle contraction. The venom also stimulates the spontaneous release of neurotransmitters probably through partial neuronal depolarization that allows the influx of calcium.

Even though the crude venom pharmacology is very complex considering the biological activities of other components, the TTX-dependence of the major neurotoxic effects revels the TTX-sensitive sodium channels as a main target of the venom, probably through its interaction of α- and β-toxins present with these channels.

This work mains to contribute with the understanding of neurotoxicity on somatic neurotransmission following the scorpionism, especially regarding to *Tityus bahiensis* envenomation's.

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Conflict of interest statement

The authors have no conflict of interest regarding to the publication of this study.

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Legends

Fig. 1. Neuromuscular activity of *T. bahiensis* venom in mouse *extensor digitorum longus* preparations. The preparations were field stimulated (0.1 Hz, 0.2 ms, supramaximal voltage) in the absence (control) and presence of venom (1-10 μg/mL) for 120 min at 37 °C. Panel A shows the mean contractile responses and panels B and C show representative recordings for venom concentrations of 1 μg/mL and 10 μg/mL, respectively. Note that the lowest venom concentration caused muscle facilitation while the highest concentration (10 μg/mL) caused complete neuromuscular blockade within 19.8 ± 9 min. Venom was added at time zero (0). The points in A represent the mean ± SEM of four experiments. *$p<0.05$ compared to the corresponding control responses.

Fig. 2. Neuromuscular activity of *T. bahiensis* venom in mouse phrenic nerve-diaphragm (PND) preparations. The muscles were subjected to indirect (nerve-evoked twitches) or direct (curarized preparations) electrical stimulation in the absence (control) and presence of venom (1-30 μg/mL) for 120 min at 37 °C. Panels A, C and E correspond to indirectly stimulated preparations, whereas panels B, D and F are for directly stimulated preparations. In indirectly stimulated muscle, the lowest venom concentration caused twitch facilitation while the highest concentration caused complete neuromuscular blockade preceded by a brief, intense facilitation (A). Muscle facilitation and total blockade occurred only in indirectly stimulated preparations (A-D). Venom significantly slowed the contraction and relaxation of nerve-evoked twitches but did not alter the responses to direct muscle stimulation (E,F). Venom was added at time zero (0). The points in panels A, B, E and F represent the mean ± SEM of 5-6 experiments. *$p<0.05$ compared to the corresponding control or basal value.

Fig. 3. Neuromuscular activity of *T. bahiensis* venom in chick *biventer cervicis* preparations (BC). The muscles were stimulated indirectly (A; nerve-evoked twitches) or directly (C, curarized preparations; D, non-curarized preparations in nominal 0 mM Ca^{2+} Krebs solution) in the absence (control) and presence of venom (1-30 μg/ml) for 120 min at 37 °C. B) Responses to exogenous KCl (110 μM), ACh (20 mM) and carbachol (CCh; 8 μM) were obtained before (basal) and after (at the end of the experiment) incubation with venom. The responses obtained in the presence of venom were expressed as a percentage of the basal contractions. Note in A and C that low concentrations caused facilitation while higher concentrations caused neuromuscular blockade; the lack of Ca^{2+} in the bath solution prevented

facilitation (D). The points and columns represent the mean ± SEM of 4-6 experiments. *p<0.05 compared to control responses.

Fig. 4. Representative recordings of the activity of *T. bahiensis* venom (V) on chick *biventer cervicis* preparations. The muscles were stimulated indirectly (A) or directly (B, nominally 0 mM Ca^{2+} solution; C, curarized preparations) and venom (30 μg/ml) was added at time 0. In C, the addition of d-tubocurarine (↑) prior to venom prevented the usual venom-induced contractures (increase in baseline tension). D. Indirectly stimulated preparations in which the stimulation was turned off and venom (V) was added at time zero (0). Note the muscle contracture caused by venom alone; the subsequent addition of d-tubocurarine (↑) abolished this contracture. E. Incubation with BoNT-A blocked the nerve-evoked contractions but did not prevent the venom-induced muscle contracture, although the duration of contracture was shortened. These recordings are representative of 5-6 experiments for each protocol.

Fig. 5. Activity of *T. bahiensis* venom on miniature end-plate potentials (MEPP) amplitude and frequency. The potentials were recorded from mouse diaphragm muscle using conventional microelectrodes. A. The venom (0.3 μg/ml) increased the MEPP frequency without affecting the amplitude or the depolarization/repolarization times. B. Gaussian curves of the amplitude-frequency relationship before (Basal) and at various times after incubation with venom. The points in A represent the mean ± SEM (n=8-11) and are expressed as a percentage of the basal (control) values. The curves in B were plotted using the Gaussian equation and are representative of 11 experiments. *p<0.05 compared to the basal (pre-venom) values of the corresponding parameter.

Fig. 6. The activity of *T. bahiensis* venom on end-plate potentials (EPPs). The EPPs were measured in mouse diaphragm muscle using the cut-muscle technique and conventional microelectrodes. A. Venom (0.3 μg/ml) increased the EPP amplitude without affecting the depolarization and repolarization times. B. Representative recordings of EPPs after incubation with venom for 60 min and 90 min. Multiple EPPs per stimulus were observed after 90 min of incubation. Black – basal recording, light grey – recording after 60 min and blue – recording after 90 min. The points in A represent the mean ± SEM (n=6-11) expressed as a percentage of the basal values. *p<0.05 compared to basal (pre-venom) values.

Fig. 7. *Tityus bahiensis* venom attenuates the perineural waveform of *triangular sterni* nerve-muscle preparations. A. Incubation with venom (0.3 µg/ml) resulted in a significant blockade of the first and second components of the waveform related to K^+ and Na^+ currents, respectively. The second peak (K^+ currents) was affected before the first (Na^+ currents). B. Representative recordings of perineural waveforms before (basal) and after incubation with venom for 45 min and 90 min. The stimulus artifact was digitally removed to improve the clarity of the figure. The points in A represent the mean ± SEM (n=5) and the recordings in B are representative of 5 experiments. *$p<0.05$ compared to basal (pre-venom) values.

Fig. 8. Tetrodotoxin (TTX) abolishes the delay in repolarization caused by *T. bahiensis* venom. A. Area under the curve of the second component (Na^+ current) of the perineural waveform after exposure to venom (0.3 µg/ml). Note the significant increase after 60 min and 90 min (indicative of a delay in repolarization) that was reversed by TTX (20 nM, at 90 min). B and C. Representative recordings before and after incubation with venom for 45 min and 60 min (both in panel B) and 90 min (panel C). Note the multiple waveforms per stimulus (↑) that increased in frequency with time until they merged into each other; this delay was reversed by TTX (C). The columns in A represent the mean ± SEM (n=5) while the recordings in B and C are representative of 5 experiments. *$p<0.05$ compared to basal (pre-venom) values; $^{\#}p<0.05$ compared with the 90 min incubation.

Fig. 9. *Tityus bahiensis* venom adversely affects the compound action potential (CAP) of mouse sciatic nerve. A. Incubation with venom (0.3 µg/ml) resulted in a time-dependent decrease in the action potential amplitude, with a concomitant decrease in the rate of rise and a delay in the decay time constants. Note that the changes in the latter two parameters are shown graphically as increases above the basal values. B. Changes in CAP amplitude during a 90 min incubation with venom**.** There was a time-dependent decrease of amplitude and delay on decay time constant towards basal parameters. The points in A represent the mean ± SEM (n=5) and the recordings in B are representative of 5 experiments. *$p<0.05$ compared to basal values.

Fig. 10. Representative images of the effect of *T. bahiensis* venom on intracellular calcium movement in the soma of DRG neurons and in *triangular sterni* muscle cells. Changes in fluorescence after venom addition reflected a corresponding increase in intracellular calcium content. The changes were particularly marked in neurons but not very apparent in muscle cells

(indicated by ↑ in both cases). Fluorescence scale (from low to high): light blue < dark blue < green < yellow < red. Magnification: neurons 100x; muscle cells: 400x.

Figure 10

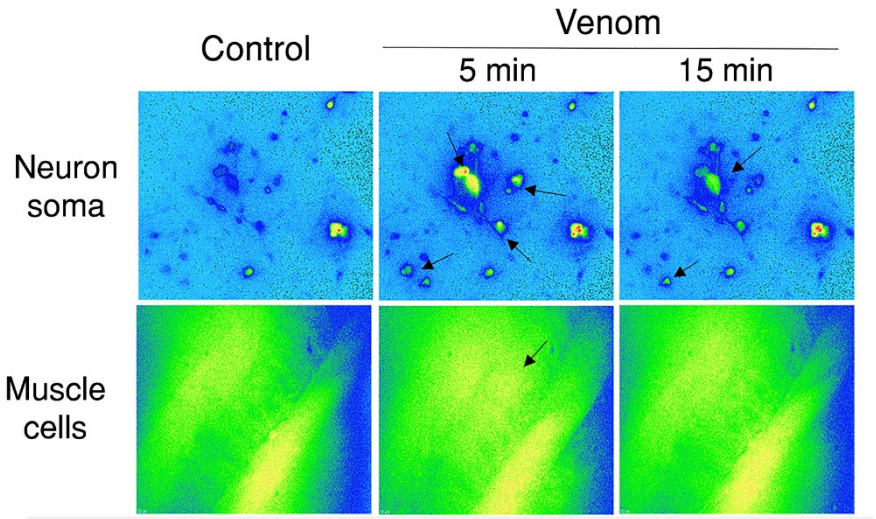

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Figura 9

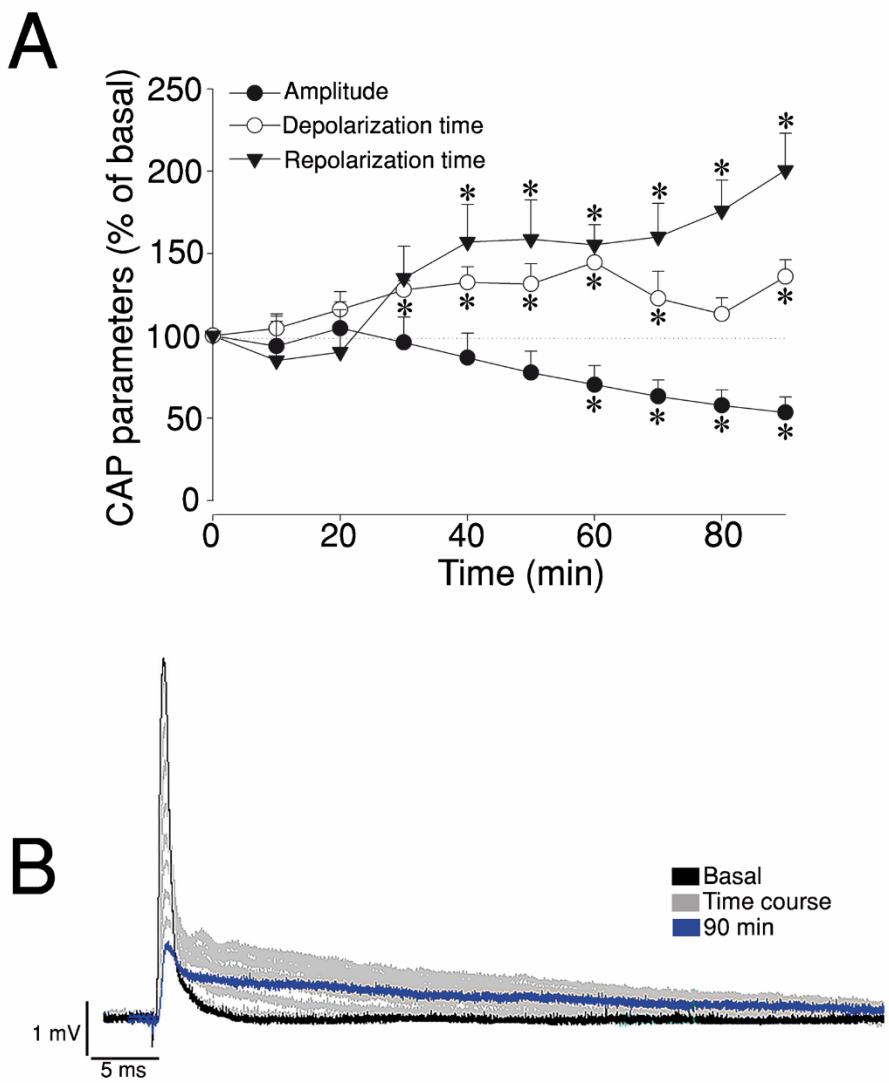

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Figure 8

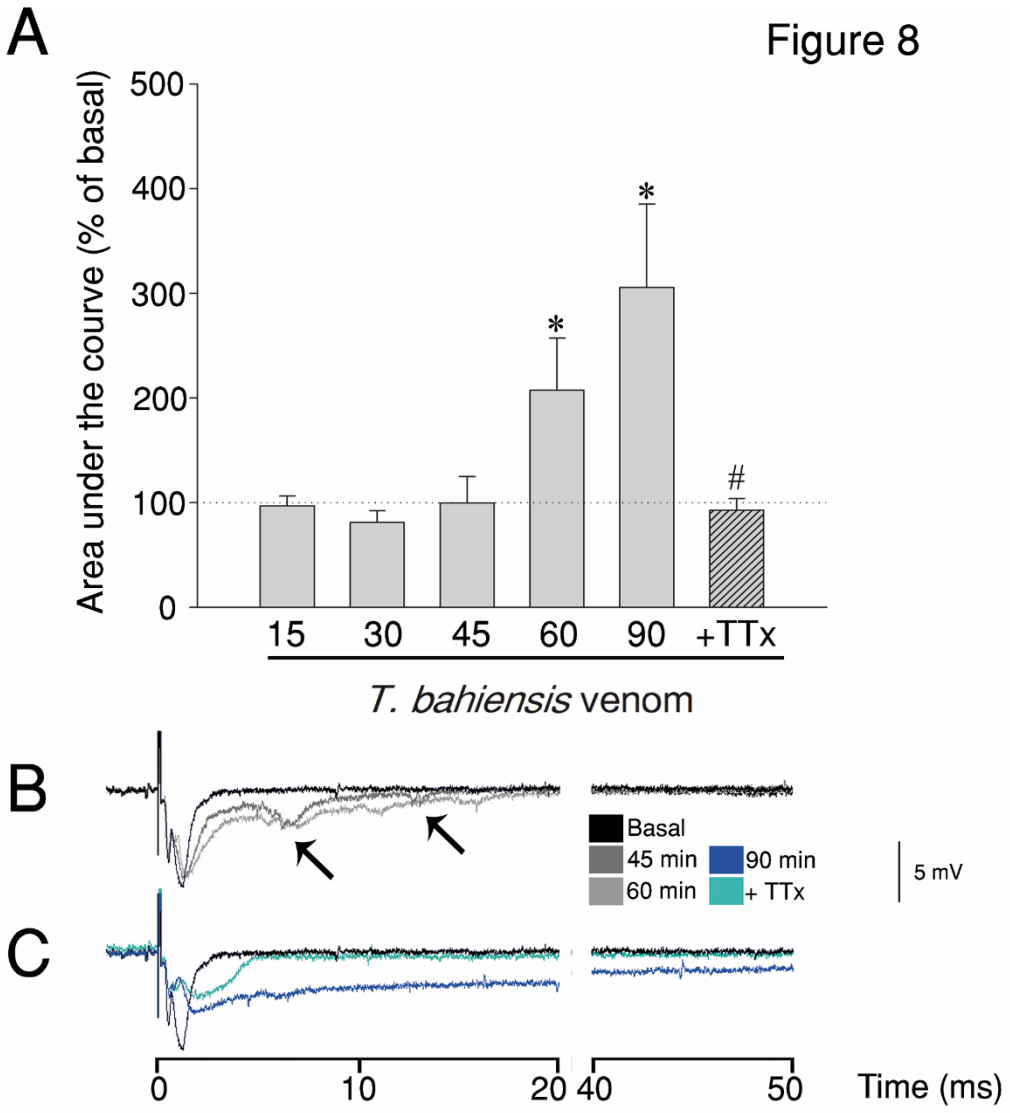

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Figure 7

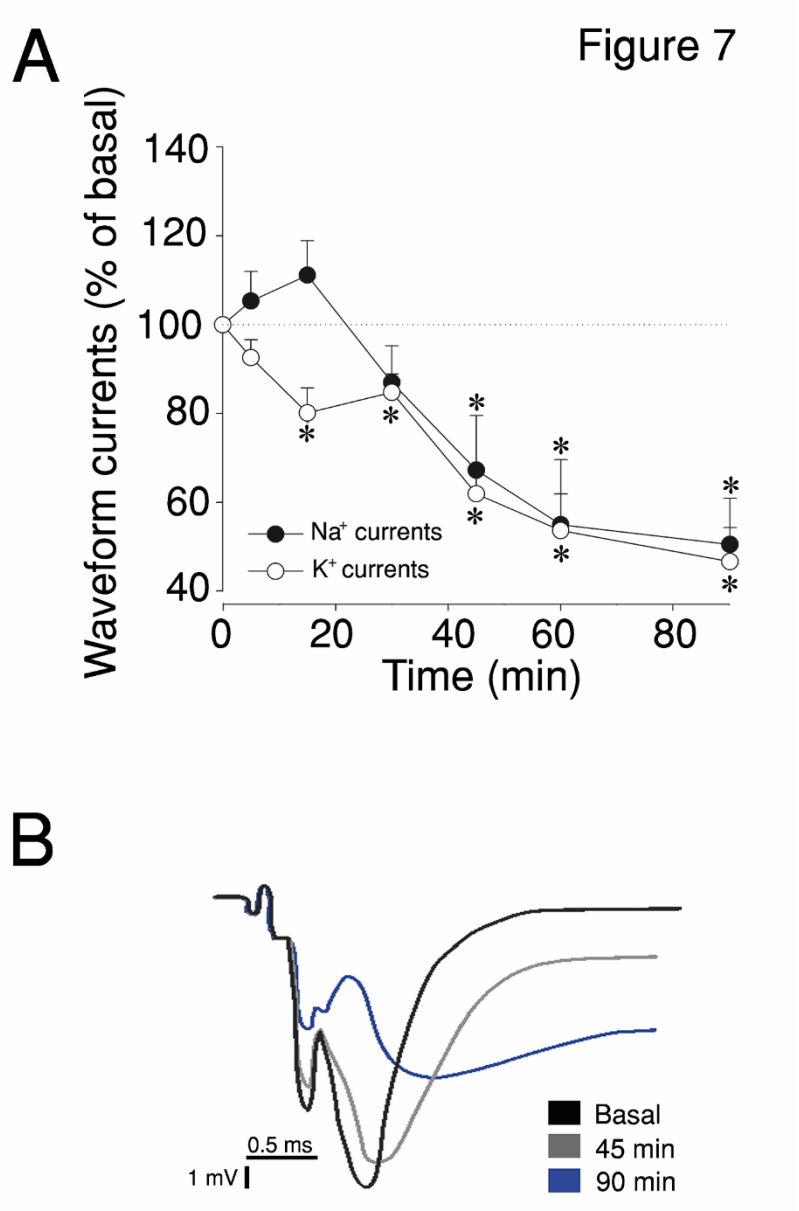

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Figure 6

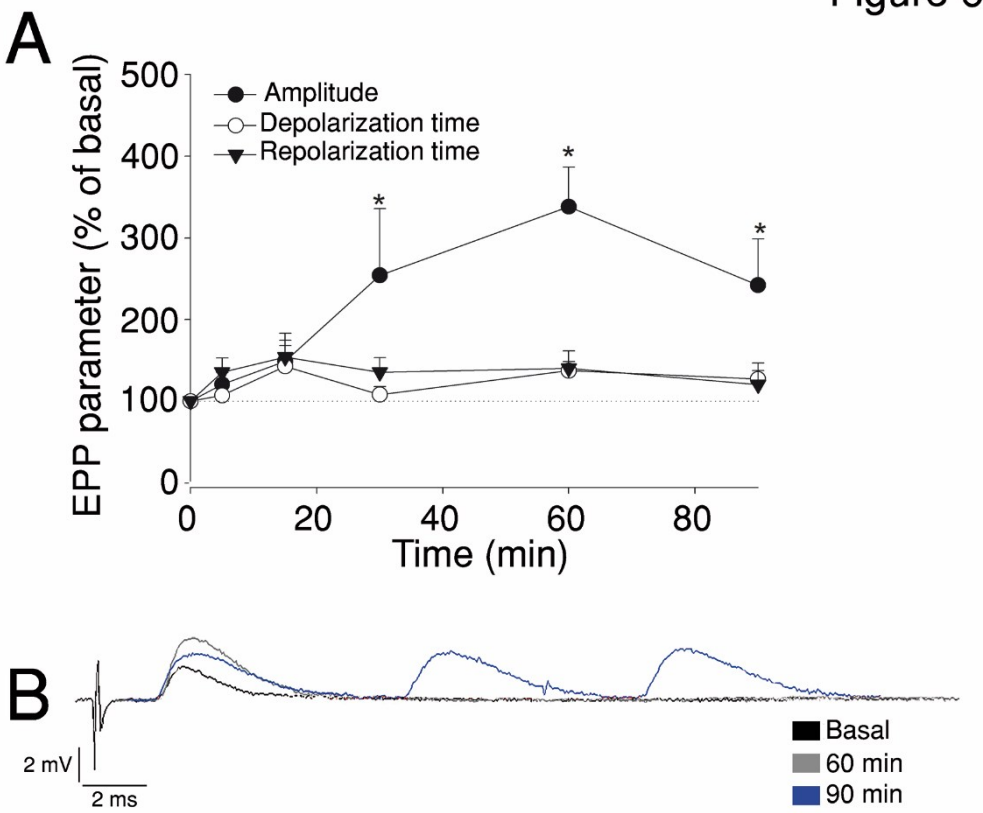

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Figure 5

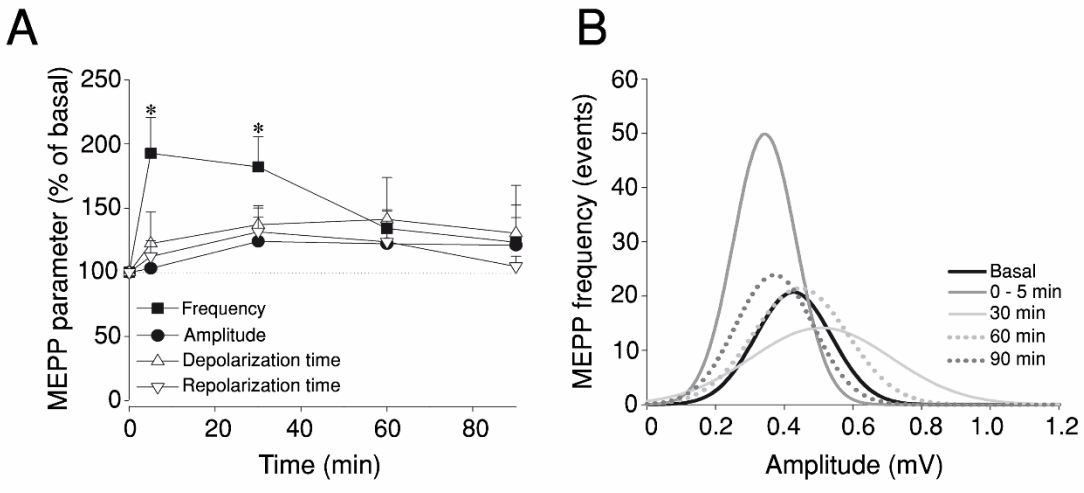

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Figure 4

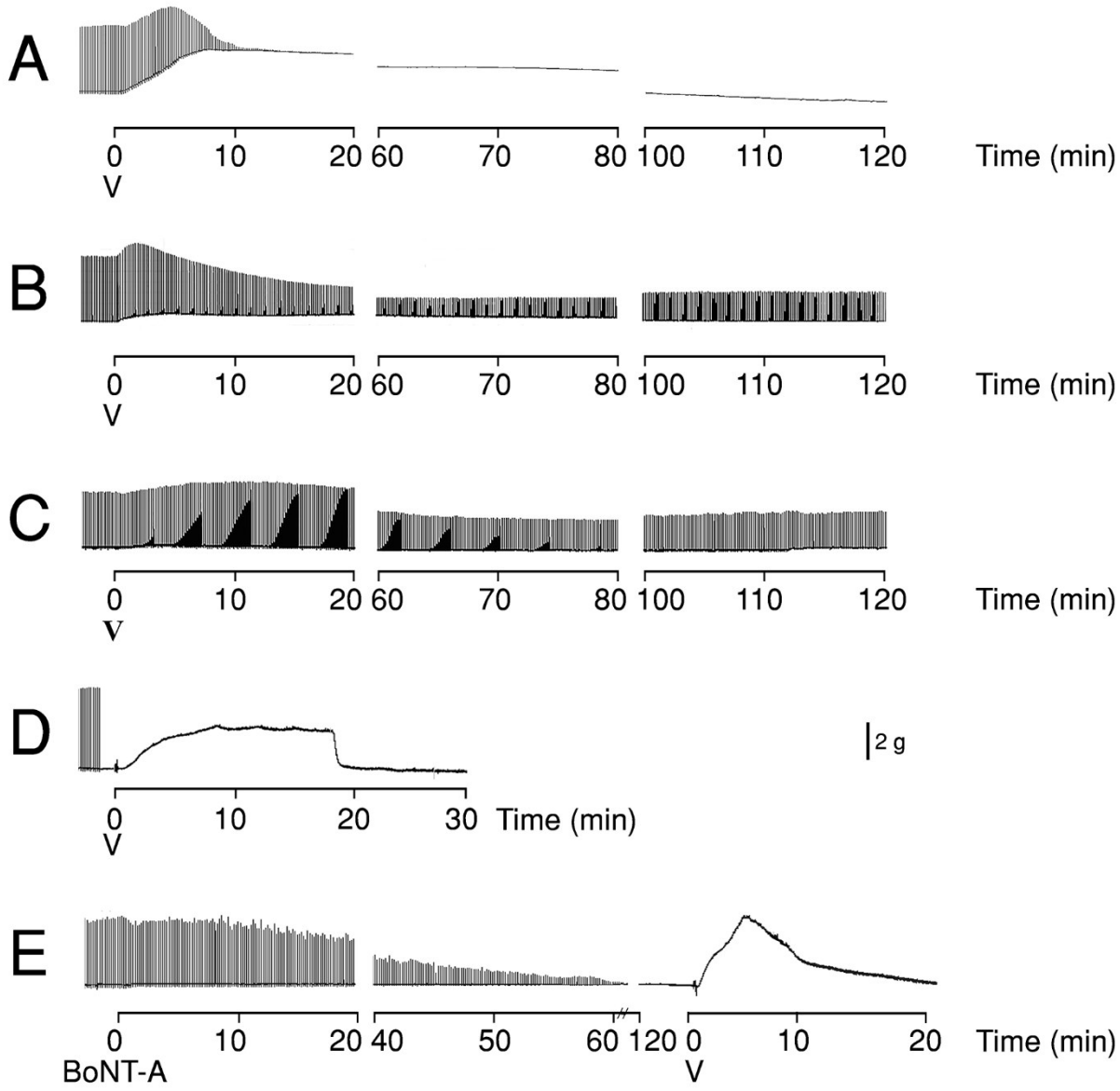

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Figure 3

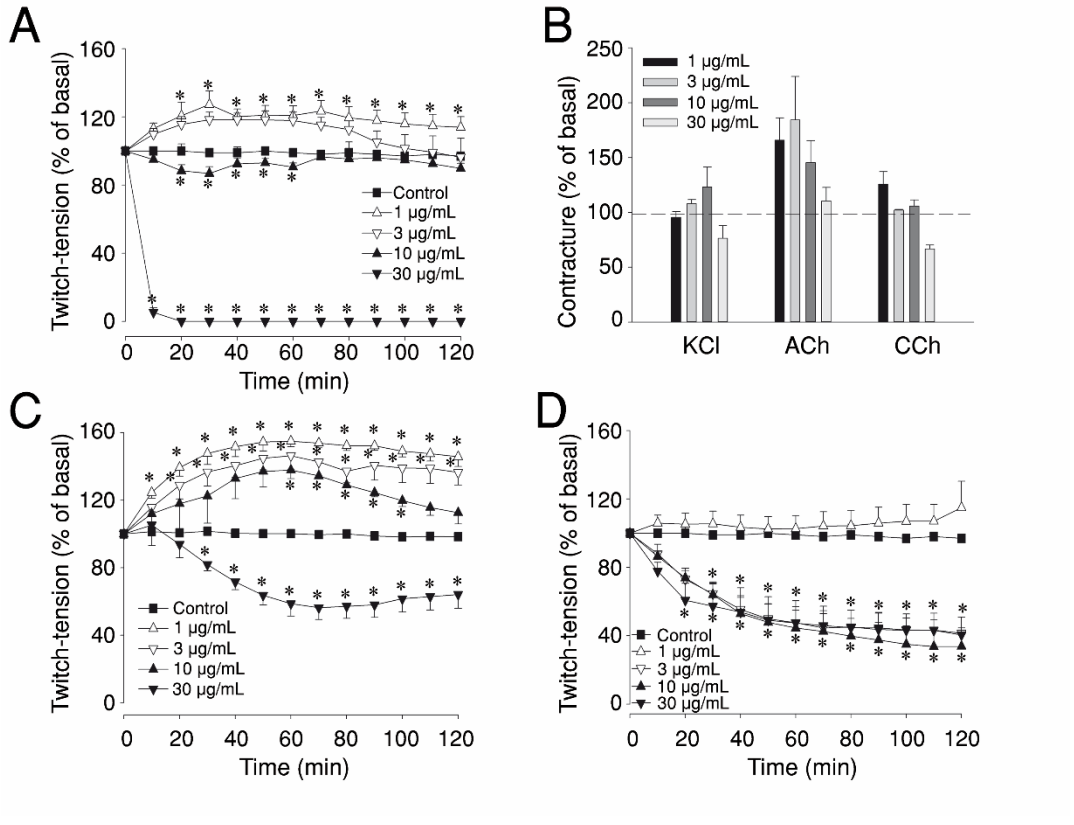

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Figure 2

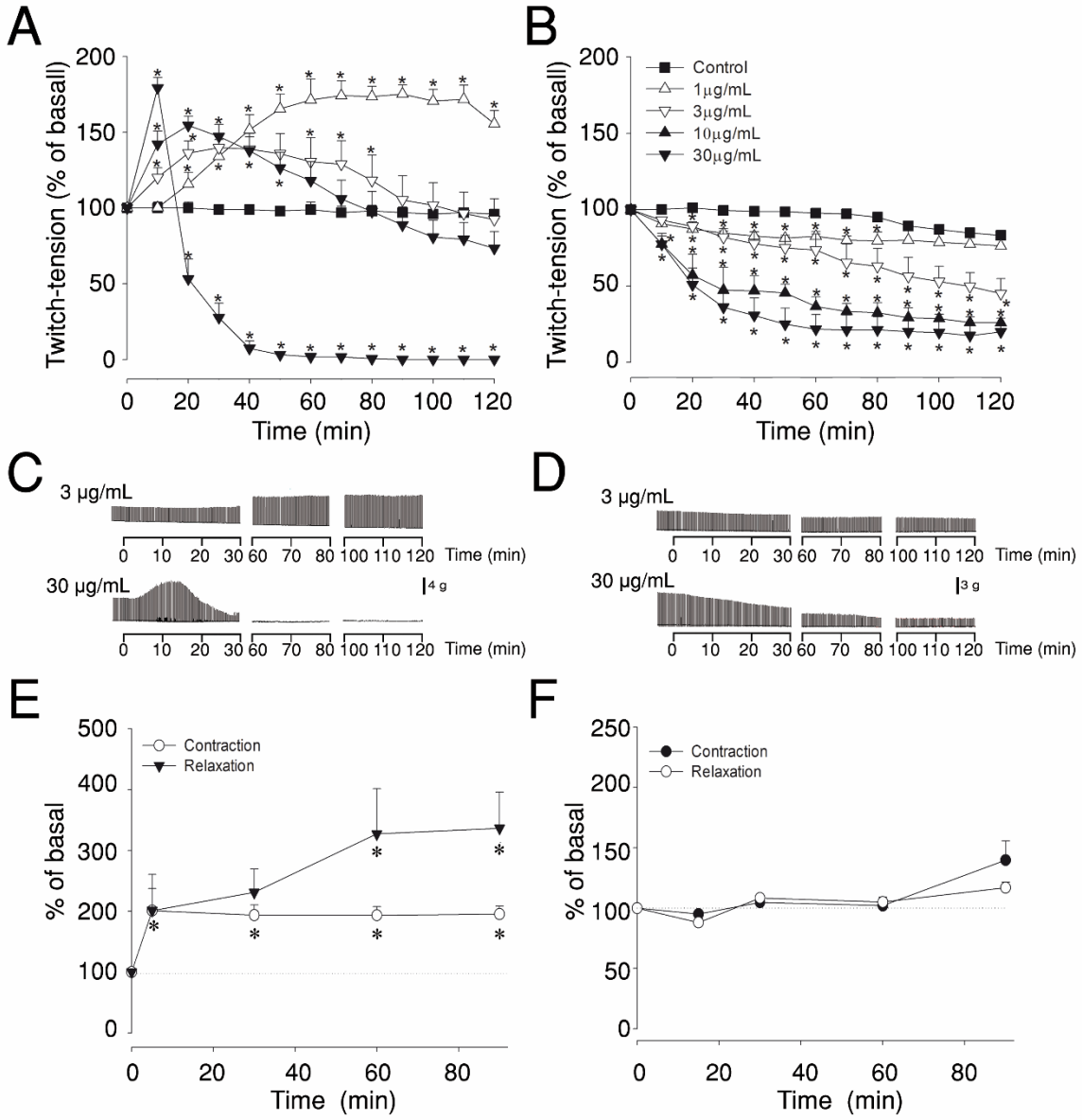

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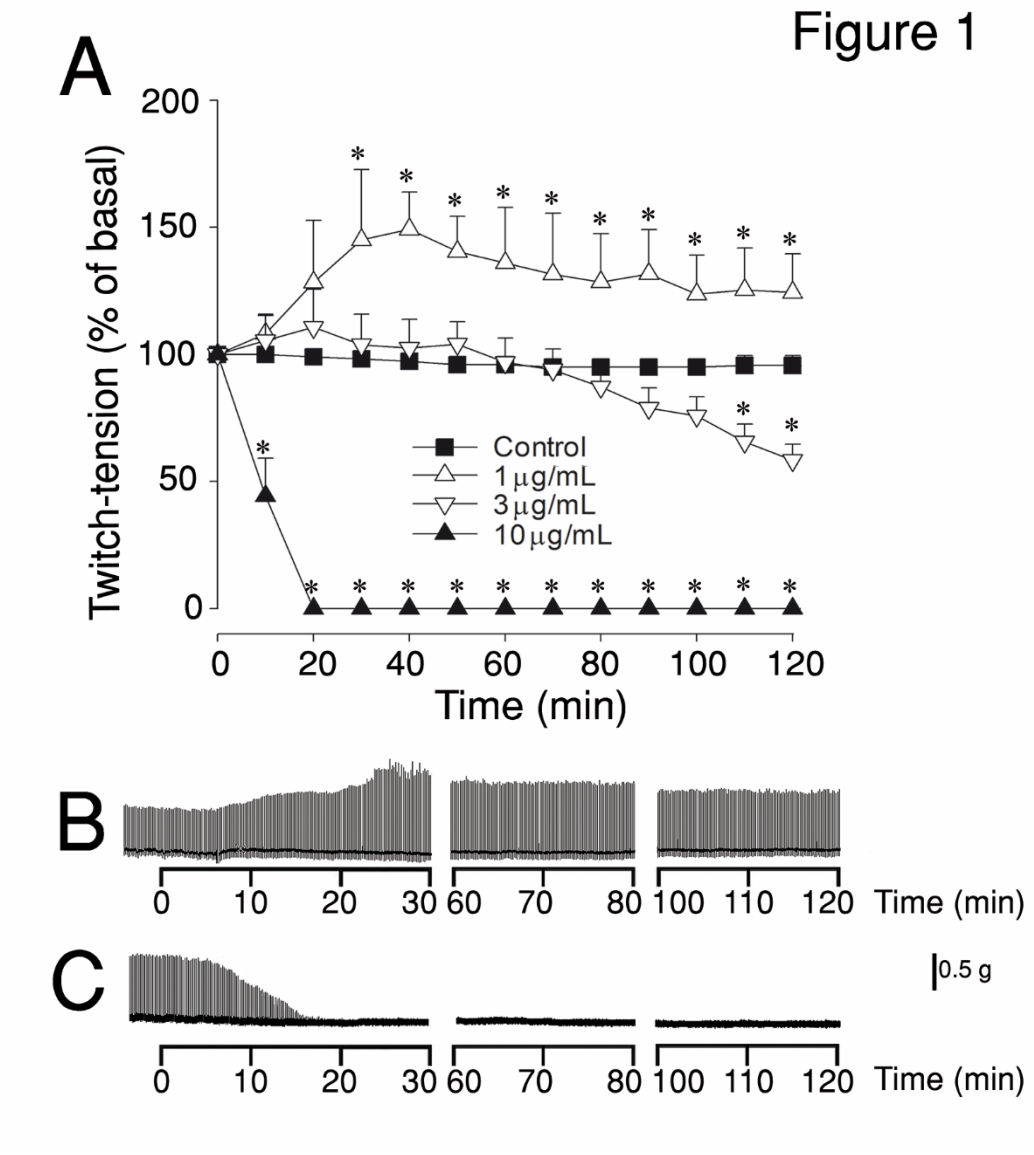
Figure 1



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