Peripheral neurotoxicity of *Micrurus lemniscatus lemniscatus* (South American coral snake) venom assessed *in vitro* and neutralization by antivenom

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Abstract

We investigated the effect of South American coral snake (*Micrurus lemniscatus lemniscatus*) venom on neurotransmission in vertebrate nerve-muscle preparations *in vitro*. The venom showed calcium-dependent PLA_2 activity and caused irreversible neuromuscular blockade (0.1-30 μg/ml) in chick biventer cervicis (BC) and mouse phrenic nerve-diaphragm (PND) preparations. In BC preparations, contractures to exogenous acetylcholine and carbachol (CCh), but not KCl, were abolished by venom concentrations ≥0.3 μg/ml; in PND preparations, the amplitude of the tetanic response was progressively attenuated, but with little tetanic fade. In low Ca^{2+} physiological solution, venom (10 μg/ml) caused neuromuscular blockade in PND preparations within ~10 min that was reversible by washing; the addition of Ca^{2+} immediately after the blockade temporarily restored the twitch responses, but did not prevent the progression to irreversible blockade. Venom (10 μg/ml) did not depolarize diaphragm muscle, prevent depolarization by CCh, or cause muscle contracture or histological damage. Venom (3 μg/ml) had a biphasic effect on the frequency of miniature end-plate potentials, but did not affect their amplitude; there was a progressive decrease in the amplitude of evoked end-plate potentials. The amplitude of compound action potentials in mouse sciatic nerve was unaffected by venom (10 μg/ml). Pre-incubation of venom with coral snake antivenom (Instituto Butantan) at the recommended antivenom:venom ratio did not neutralize the neuromuscular blockade in PND preparations, but total neutralization was achieved with a 10-fold greater volume of antivenom. The addition of antivenom after 50% and 80% blockade restored the twitch responses. These results show that *M. l. lemniscatus* venom causes potent, irreversible neuromuscular blockade, without myonecrosis. This blockade is apparently mediated by pre- and postsynaptic neurotoxins and can be reversed by coralsnake antivenom.

Keywords: coral snake venom, facilitation, neuromuscular blockade, α-neurotoxin, phospholipase A_2 (β-neurotoxin), three-finger toxins

Introduction

Coral snakes of the genus *Micrurus* are the major representatives of the family Elapidae in the Americas and are widely distributed from the southeastern United States to central-southern Argentina (Roze, 1996; Silva Jr. et al., 2016a). Despite the large diversity of *Micrurus* in Brazil (~34 spp.) (Silva Jr. et al., 2016b), bites by this genus account for ≤1% of venomous snakebites reported annually in this country (Bucaretchi et al., 2016a,b). This low incidence of bites probably reflects the low population density of these snakes, their unaggressive behavior and the difficulty in inoculating venom because of their small mouth and short fangs.

Systemic envenomation by coral snakes is characterized by peripheral neurotoxicity that is mediated by two major groups of toxins: (1) α-neurotoxins that block postsynaptic nicotinic (cholinergic) receptors and are part of the three-finger toxin (3FTx) family, and (2) β-neurotoxins that are phospholipases A_2 (PLA_2) and cause potent presynaptic blockade (Gutiérrez et al., 2016; Lomonte et al., 2016; Aird et al., 2017). Clinically, neurotoxicity is the most important activity of coral snake venoms because of the risk of respiratory failure and death as a consequence of peripheral neuromuscular blockade (Warrell, 2004; Bucaretchi et al., 2016a,b; Risk et al., 2016).

Despite the large number of *Micrurus* in Brazil, only a few of them (*M. altirostris, M. averyi, M. corallinus*, *M. decoratus*, *M. filiformis*, *M. frontalis*, *M. hemprichii*, *M. ibiboboca*, *M. lemniscatus*, *M. spixii* and *M. surinamensis*) have been conclusively implicated in human envenomation (Bucaretchi et al., 2016a,b; Melgarejo et al., 2016; Risk et al., 2016; Rodrigo et al., 2016; Souza et al., 2016; da Silva et al., 2018; Strauch et al., 2018), with *M. lemniscatus* being the third most frequent species involved (Bucaretchi et al., 2016b). Currently *Micrurus lemniscatus* is a species composed of three subspecies (*M. l. carvalhoi*, *M. l. helleri* and *M. l. lemniscatus*). Figure 1 shows the geographic distribution of these three subspecies in Brazil

and neighbouring countries: (1) *M. l. carvalhoi* is distributed along the Brazilian east coast from the northeast to southeast of the country and in parts of central, central-western, southeastern and southern Brazil, as well as eastern Paraguay and northeastern Argentina, (2) *M. l. helleri* occurs in the western Brazilian Amazon and in Colombia, Ecuador, Peru and Bolivia east of the Andes, and (3) *M. l. lemniscatus* is found in the central Brazilian Amazon, northern Brazil (states of Amapá, Maranhão e Pará) and in Guyana, Suriname and French Guiana.

The precise taxonomic relationship among these three subspecies is unclear, with *M. l. helleri* probably being synonymous with *M. l. lemniscatus*, and *M. l. carvalhoi* possibly being a separate species (Silva Jr. et al., 2016b). As shown in Fig. 1, there is considerable overlap in the geographic distributions of *M. diutius* and *M. l. lemniscatus*, and the distribution of *M. l. lemniscatus* may well include the indicated range of *M. l. helleri*, although the true extent of overlap between the latter two subspecies is unclear (Roze, 1996; Campbell and Lamar, 2004; Silva Jr. et al., 2016b). There is also uncertainty regarding the eastern limits of *M. l. lemniscatus* and western limits of *M. l. carvalhoi* and the potential areas of overlap between these two subspecies throughout most of their ranges, primarily because of the low number of specimens for these subspecies in herpetological collections of these regions. Human envenomation by these three subspecies has been reported, e.g., *M. l. carvalhoi* (Rosenfeld, 1971; Ribeiro and Jorge, 1986; Nishioka et al., 1993; Bucaretchi et al., 2006; Risk et al., 2016), *M. l. helleri* (Warrell, 2004; Manock et al., 2008) and *M. l. lemniscatus* (Brazil and Brazil Filho, 1933).

Appreciation of the taxonomic status of this species is important in order to avoid confusion in the toxinological literature relating to the subspecies currently included in this species. Thus, although various studies have reported the characterization of venom (Brazil and Brazil Filho, 1933; Vital Brazil, 1965; Cecchini et al., 2005; Ciscotto et al., 2011; Santos

et al., 2012; Tanaka et al., 2010, 2016; Ramos et al., 2017) and toxins (Oliveira et al., 2008; Silva et al., 2011; Carvalho et al., 2014; Casais-e-Silva et al., 2016) from '*M. lemniscatus*', examination of the source of the venoms used (geographic origin and/or supplier) indicates that these studies relate to *M. l. carvalhoi* and not to *M. l. lemniscatus* or *M. l. helleri*. Therefore, these reports cannot be considered to be representative of the species *M. lemniscatus*. As with other *Micrurus* venoms, proteomic analyses have shown that 3FTx and PLA_2 are the main constituents of *M. l. carvalhoi* and *M. l. lemniscatus* venoms (Ciscotto et al., 2011; Aird et al., 2017), although there is considerable variation in the proportion of these toxin groups in each venom: 2.3% 3FTx and 48.6% PLA_2 for *M. l. carvalhoi* from the state of Goiás, compared to 59% 3FTx and 9% PLA_2 for this subspecies from 'southeastern Brazil' (probably the state of Minas Gerais), and 34.3% 3FTx and 19.4% PLA_2 for *M. l. lemniscatus* from Altamira, in the state of Pará.

Whereas the peripheral (Brazil and Brazil Filho, 1933; Vital Brazil, 1965; Cecchini et al., 2005) and central (Oliveira et al., 2008; Carvalho et al., 2014) neurotoxicity of *M. l. carvalhoi* venom has been studied, nothing is known of the neurotoxicity of other *M. lemniscatus* subspecies (*M. l. lemniscatus* and *M. l. helleri*), although a clinical report suggests that neuromuscular blockade by the latter subspecies may be insensitive to reversal by neostigmine (Manock et al., 2008), a potentially useful ancillary measure for treating envenomation by coral snake venoms with a predominantly postsynaptic site of action (Coelho et al., 1992; Vital Brazil and Vieira, 1996; Bucaretchi et al., 2016b). In this work, we undertook a detailed investigation of the neurotoxicity of *M. l. lemniscatus* venom on neurotransmission in vertebrate (avian and mammalian) nerve-muscle preparations *in vitro* using a combination of myographic and electrophysiological approaches to determine the principal sites of action. We also examined the ability of coral snake antivenom produced by the Instituto Butantan (São Paulo, SP, Brazil) to neutralize the neurotoxicity of this venom *in*

vitro. The findings reported here will be of interest to those investigating the pharmacology of *Micrurus* venoms and to clinicians who may have to treat envenomation by *M. l. lemniscatus* with antivenom produced by the Instituto Butantan.

Materials and methods

Reagents and venom

Acetylcholine chloride, carbachol, *p*-bromophenacyl bromide, neutral red, 4-nitro-3-octanoyloxy-benzoic acid and tetrodotoxin (TTX) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) and *d*-tubocurarine chloride was from Abbott Laboratórios do Brasil Ltda. (São Paulo, SP, Brazil). All salts for the physiological solutions were of analytical grade. *Micrurus l. lemniscatus* venom was obtained from twelve adult snakes (7 males; 5 females) from Altamira in the northern Brazilian state of Pará in the Brazilian Amazon (3°25'55"S and 51°56'6"W); the snakes were identified by one of the co-authors (NJS), a professional herpetologist with extensive experience in coral snake taxonomy. The venom was lyophilized and stored at -20 °C until used.

Animals

Male BALB/c mice (25-30 g; 2-3 months old) obtained from the Multidisciplinary Center for Biological Investigation (CEMIB/UNICAMP) were housed in plastic cages (5-10/cage) with a wood-shaving substrate, at 23 ± 1 °C in ventilated stands (Alesco®) on a 12 h light/dark cycle with lights on at 6 a.m. Male HY-line chicks (4-8 days old) were provided by Globo Aves Agricola Ltda. (Campinas, SP, Brazil) and housed in metal cages with a sawdust substrate at 23-25 °C. The rodents and chicks had free access to food and water. When required, the animals were killed with isoflurane immediately prior to the experiments. The animal experiments were approved by an institutional Committee for Ethics in Animal Use

(CEUA/UNICAMP, protocol no. 3477-1) and were done according to the general ethical guidelines for animal use established by the Brazilian Society of Laboratory Animal Science (SBCAL) and Brazilian legislation (Federal Law no. 11,794, of October 8, 2008), in conjunction with the guidelines for animal experiments established by the Brazilian National Council for Animal Experimentation (CONCEA) and EU Directive 2010/63/EU for the Protection of Animals Used for Scientific Purposes.

PLA_2 activity and inhibition by *p*-bromophenacyl bromide (*p*-BPB)

PLA_2 activity was assayed essentially as described by Carregari et al. (2013). The standard assay mixture contained 200 µl of buffer (10 mM Tris-HCl, 10 mM $CaCl_2$ and 100 mM NaCl, pH 8.0), 20 µl of substrate (3 mM 4-nitro-3-octanoyloxy-benzoic acid) and 20 µl of venom (0.1 mg/ml dissolved in saline) in a final volume of 240 µl. Some assays were done in low Ca^{2+} (0.36 mM) buffer solution. After adding venom, the mixture was incubated for 30 min at 37 °C or room temperature. Enzymatic activity was expressed as the initial velocity of reaction and was calculated based on the increase in absorbance (425 nm) after 20 min. All assays were done in triplicate with readings at 30 s intervals using a SpectraMax 340 multiwell plate reader (Molecular Devices). PLA_2 activity was inhibited by incubating venom with *p*-BPB essentially as described elsewhere (Díaz-Oreiro and Gutiérrez, 1997); aliquots of these *p*-BPB incubated mixtures were then tested for biological activity.

Twitch-tension experiments

Chick biventer cervicis (BC) preparations

Chick biventer cervicis (BC) nerve-muscle preparations were mounted under a resting tension of 1 g in 5 ml organ baths (Panlab, Barcelona, Spain) containing aerated (5% CO_2 and 95% O_2) Krebs solution (composition, in mM: NaCl 119, KCl 4.7, $CaCl_2$ 1.9, KH_2PO_4 1.2,

$MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.7, pH 7.5) at 37 °C and allowed to stabilize for 10 min prior to use, as described elsewhere (Floriano et al., 2013; Fernandes et al., 2014). Field stimulation (0.1 Hz, 0.2 ms) was delivered from an LE 12406 TC stimulator (Panlab) and the muscle twitches were recorded using a TRI201AD force displacement transducer coupled to a Quad Bridge Amp and LabChart 6.0 software (all from ADInstruments, Bella Vista, Australia). Muscle responses to exogenous acetylcholine (ACh, 1 mM), carbachol (CCh, 20 μM) and KCl (40 mM) were obtained before and after incubation with venom (0.1-30 μg/ml) to screen for postsynaptic neurotoxicity and myotoxicity (Harvey et al., 1994).

Mouse phrenic nerve-diaphragm (PND) preparations

Mouse phrenic nerve-diaphragm (PND) preparations were mounted under a resting tension of 1 g in 5 ml organ baths containing aerated (5% CO_2 and 95% O_2) Tyrode solution (composition, in 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, pH 7.0) at 37 °C and allowed to stabilize for 10 min prior to use, as described elsewhere (Floriano et al., 2013; Herrera et al., 2016). Supramaximal stimuli (0.1 Hz, 0.2 ms) were delivered to the nerve from a Grass S88 stimulator (Grass Instrument Co., Quincy, MA, USA) and the muscle twitches were recorded using the same system as described above. After stabilization, the preparations were incubated with venom (0.1-30 μg/ml) for 60 min or until complete neuromuscular blockade. In some experiments, PND preparations were maintained in physiological salt solution containing low Ca^{2+} (0.36 mM) for at least 15 min to ensure that the twitch amplitude was stable prior to the addition of venom (10 μg/ml). To ensure that direct stimulation of the nerve-muscle preparations did not contribute to the overall tension recorded, $MgCl_2$ (40 mM) was added to the bath and in all cases the indirectly evoked twitches were completely blocked. The $MgCl_2$ was subsequently removed by successive washes until the twitch amplitude had returned to control level.

The reversibility of the neuromuscular blockade in normal and low Ca^{2+} solution was assessed by adding neostigmine (29 μM, an acetylcholinesterase inhibitor) or 3,4-diaminopyridine (3,4-DAP, a potassium channel blocker) to the preparations after >90% blockade and assessing the recovery of twitch tension. In the low Ca^{2+} protocols, after incubation with NEO or 3,4-DAP Ca^{2+} was added to the organ bath to restore the concentration to normal (1.8 mM Ca^{2+}) and the effect of this restoration on muscle twitch tension was assessed. To examine the effects of venom (10 μg/ml) on direct muscle stimulation, some preparations in normal Ca^{2+} solution were incubated with *d*-tubocurarine (*d*-Tc, 14.7 μM); direct muscle stimulation was achieved with supramaximal pulses (0.1 Hz, 2 ms). Some preparations were indirectly stimulated with high frequency stimuli (70 Hz, 0.2 ms, ~4V) to produce tetanic responses; the preparations were stimulated at 10 min intervals, with tetanic responses being recorded for 10 s.

Neutralization of neurotoxicity by coral snake antivenom

The ability of coral snake antivenom produced by the Instituto Butantan (São Paulo, SP, Brazil) to neutralize the venom-induced neuromuscular blockade in mouse PND was assessed by incubating venom with varying amounts of antivenom at antivenom:venom ratios of 1:1.5, 3:1.5 and 10:1.5 (v/w). These ratios were based on the manufacturer´s stated neutralizing capacity for the antivenom (1 ml of antivenom neutralizes 1.5 mg of *M. frontalis* venom). The venom was either pre-incubated with the desired volume of antivenom at 37 ºC for 30 min before assaying for residual neuromuscular activity (Gutiérrez et al., 2017), or the required volume of antivenom was added directly to the organ bath after 50% or 80% blockade had been reached so as to assess the reversal of blockade. The venom of *M. frontalis*, which is included in the venom pool used to raise coral snake antiserum in horses (Cardoso et al., 2009), served as a positive control.

Extracellular recordings

Mouse sciatic nerve compound action potentials (CAPs) were recorded from a 3-6 cm length of sciatic nerve comprising the proximal and distal regions. The nerve was dissected and mounted in a Perspex recording chamber containing physiological salt solution (composition, in mM: NaCl 150, KCl 5.4, HEPES 10, $NaHCO_3$ 12, KH_2PO_4 0.4, $MgCl_2$ 1.2, $CaCl_2$ 1.8 and glucose 10, pH 7.3-7.4) previously gassed with 100 % O_2 (Dal Belo et al., 2005; Floriano et al., 2015). Standard extracellular recording techniques were used to record CAPs via pellet-type Ag/AgCl electrodes dipped into two of the chambers. CAPs were evoked by placing two platinum wires into the end chamber and attaching them to a Grass S48 stimulator via a stimulus isolation unit (SIU 5A) to supply supramaximal voltages (0.4 Hz, 0.05 ms duration) (Grass Instruments). The signals were amplified by an Electro 705 electrometer (World Precision Instruments, Sarasota, FL, USA) and Tektronix 5A22N transducer (Tektronix, Beaverton, OR, USA) and digitized through an A/D BCN-2110 card (National Instruments, UK). CAPs were recorded and analyzed using custom-built software (WinWCP v.4.5.7) (Dempster, 1988). In each experiment, the amplitude, rise time and latency of the CAPs were measured. Prior to adding venom, the CAP amplitude was monitored for 15 min (pre-treatment control period); if the CAP amplitude decreased by more than 10% of the initial value during this period the nerve was remounted and a new equilibration period was initiated. To verify whether the nerve was desheathed, tetrodotoxin (TTX, 1 μM) was added at the end of the experiments (in the absence of complete blockade by venom).

Intracellular recordings

The effects of venom on the frequency and amplitude of miniature end-plate potentials (MEPPs), quantal content (QC) from evoked end-plate potentials (EPPs) and membrane

resting potential (RP) were recorded using mouse PND preparations mounted in a Perspex Sylgard-coated chamber containing Tyrode solution (composition in section 2.4.2), essentially as described elsewhere (Floriano et al., 2013). To measure the MEPPs, EPPs and RP, a microelectrode (15-20 MΩ) filled with KCl (3 M) was positioned on one muscle fiber near the endplate region and the signals were amplified, digitized and analyzed as described in section 2.5. The QC was estimated as the quotient between the squared average and the variance of the EPPs (indirect method) (McLachlan and Martin, 1981) using the software WinWCP v.4.5.7 (Dempster, 1988). The MEPP frequency was monitored in multiple neuromuscular junctions at t_0 (zero time, pre-venom basal value) and at various times after venom addition (t_5-t_{60}). RP measurements were obtained from different regions of the muscle at the same intervals as the MEPP frequency determinations. To assess the functionality of postsynaptic nicotinic receptors (absence or presence of blockade by venom neurotoxins), a single concentration of carbachol (CCh, 68.5 μM) was added prior to the venom and 15 min later the level of membrane depolarization was recorded followed by washing of the preparation; the same procedure was repeated at the end of the incubation with venom. All of the electrophysiological protocols were done at room temperature.

Morphological analysis

Samples of mouse hemidiaphragm were fixed in 10% formaldehyde, dehydrated in an increasing ethanol series, cleared in xylol and embedded in paraffin. Serial sections 5 μm thick were obtained from the mid-region of the muscle using a Leica RM2245 microtome. The sections were stained with hematoxylin-eosin (HE) and examined with a Leica DFC 300FX CCD light microscope coupled to a computer with Q Win Plus v.3.2.0 software.

Statistical analysis

All results (myographic and electrophysiological) were expressed as the mean ± SEM of the number of independent experiments (preparations) indicated in the text and figure legends. Changes in the twitch-tension responses of BC and PND preparations were expressed as a percentage relative to baseline (time zero) values. Statistical comparisons were done using Student's *t*-test or ANOVA followed by the Tukey-Kramer test, with $p < 0.05$ indicating significance. All data analyses were done using Microcal Origin 8 SR4 v.8.0951 (Microcal Software Inc., Northampton, MA, USA) or GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) software.

Results

Venom PLA_2 activity and inhibition by *p*-BPB

Micrurus l. lemniscatus venom exhibited PLA_2 activity (4.56 ± 0.02 nmol/min/mg at 37 ºC, n = 3) that was attenuated by 33 ± 0.2% when the assay was done at 25 °C (3.07 ± 0.1 nmol/min/mg, $p < 0.05$, n = 3) (Fig. 2A). In low (0.36 mM) and nominal zero Ca^{2+} buffer, the activity at 37 ºC was attenuated by 60 ± 0.1% and 73 ± 0.2%, respectively; a similar profile of attenuation was seen when the assay was done at 25 ºC. However, proportionally, the reduction in enzyme activity in low and nominal zero Ca^{2+} conditions was greater for activity measured at 37 ºC than at 25 ºC, although in absolute values there was no significant difference in the activities observed in these two Ca^{2+} conditions at both temperatures. Pre-incubation with *p*-BPB significantly reduced the PLA_2 activity of the venom [from 4.59 ± 0.01 to 0.62 ± 0.01 nmol/min/mg (86.5 ± 0.1% reduction) at 37 ºC ($p < 0.05$, n = 3)] (Fig. 2B).

Neuromuscular effects of venom in BC preparations

In BC preparations, *M. l. lemniscatus* venom (0.1-30 µg/ml) caused concentration-dependent neuromuscular blockade that was maximal at 10 µg/ml, with complete blockade occurring at concentrations ≥1 µg/ml between 20-40 min after venom addition. However, all venom concentrations caused a significant decrease in twitch amplitude at the end of the 60 min incubation (Fig. 3A). This concentration-dependent blockade was evident in the times required for 50% and 90% blockade, which decreased progressively with increasing venom concentration, except for the two highest concentrations for which the times were similar at each level of blockade (Table 1). Figure 3B provides a representative recording of the neuromuscular blockade induced by the venom (10 µg/ml) in an indirectly stimulated preparation at 37 °C and shows that the blockade was not reversible by repeated washing of the preparation; there was also no increase in baseline tension normally associated with muscle contracture. Muscle contractions to exogenous ACh and CCh were completely abolished at venom concentrations ≥0.1 µg/ml onwards, indicating interaction of venom neurotoxins with extrajunctional postsynaptic nicotinic receptors. However, the venom did not significantly alter the contractures to KCl (101 ± 4.9%, 88.5 ± 8.8%, 90.9 ± 3.1%, 102.2 ± 4.8%, 103.8 ± 9.8% and 109.3 ± 0.2% for 0.1, 0.3, 1, 3, 10 and 30 µg of venom/ml, respectively, expressed as a percentage of the contracture to KCl prior to venom addition, considered as 100%; n = 4 each), thus indicating the lack of a direct effect on muscle contractility.

Neuromuscular effects of venom in PND preparations

In PND preparations, *M. l. lemniscatus* venom caused irreversible time- and concentration-dependent neuromuscular blockade from 3 µg of venom/ml onwards, although only 10 and 30 µg/ml caused complete blockade in <60 min (52 ± 2.3 min and 17 ± 1.3 min,

respectively, with the higher concentration causing significantly faster blockade; $p < 0.05$, n = 4-5) (Fig. 4A). This time- and concentration-dependence was evident in the times required for 50% and 90% blockade, which decreased progressively with increasing venom concentration (Table 1). Figure 4B shows representative recordings of the complete neuromuscular blockade induced by 10 µg/ml (B_1) and 30 µg/ml (B_2) in ~55 and ~15 min, respectively, that was not reversed by washing the preparations. As with BC preparations, the venom did not cause an increase in the baseline tension. The venom (10 and 30 µg/ml) also did not cause a decrease in muscle twitch tension in directly-stimulated PND preparations pre-treated with d-Tc (14.7 µM), indicating the lack of a direct effect on muscle contractility [twitch responses after a 60 min incubation: 87.8 ± 3.4%, 88.3 ± 1.7% and 87.2 ± 1.2% of basal values (considered as 100%) for control (Tyrode solution alone), 10 and 30 µg of venom/ml, respectively, n = 4 each]. Slight, transient facilitation (9.6 ± 1.4% above basal twitch response; n = 4) was seen within 10-20 min after the addition of venom in directly stimulated preparations incubated with 30 µg of venom/ml. Figure $4B_3$ shows the initial neuromuscular facilitation and the lack of blockade by the venom (30 µg/ml) in a curarized, directly stimulated PND preparation. Based on the results described above, a venom concentration of 10 µg/ml was chosen for subsequent experiments with PND preparations as this was the lowest concentration that caused complete blockade in <60 min. The neuromuscular blockade caused by venom (10 µg/ml) was accompanied by an equally rapid inhibition of the tetanic response to electrical stimulation, but without significant tetanic fade (Supplementary Fig. 1); this inhibition was considerably faster than that observed with the venom of *M. frontalis*, used for comparison (t_{50} for blockade of PND muscle twitches by 10 µg of *M. frontalis* venom/ml: 32 ± 3.8 min (n = 4), which was greater ($p < 0.05$) than the corresponding time for blockade by *M. l. lemniscatus* (Table 1); t_{50} for blockade of tetanic twitches by *M. l. lemniscatus* and *M.*

frontalis venoms was 50 ± 2.5 min (n = 4) and 20 ± 2.2 min (n = 4), respectively; $p < 0.05$) (Supplementary Fig. 1).

The *M. l. lemniscatus* venom (10 μg/ml)-induced neuromuscular blockade was not prevented by pre-treatment with *p*-BPB but there was a significant delay in the times required for 90% blockade [28 ± 1.1 min (positive control - ethanol) *vs*. 37 ± 1.4 min (*p*-BPB-treated venom), $p < 0.05$, n = 3]; the change in the time for 50% blockade was not significant [14 ± 1.2 min (positive control - ethanol) vs. 18 ± 1.8 min (*p*-BPB-treated venom), n = 3] (Fig. 4C).

Histological analysis of diaphragm muscle at the end of the experiments revealed little muscle damage, in agreement with the lack of increase in baseline tension and the unaltered contractile responses in directly stimulated PND preparations (Supplementary Fig. 2).

Neuromuscular effects of venom in PND preparations maintained in low Ca^{2+} physiological solution

To examine whether *M. l. lemniscatus* venom exhibited PLA_2-independent actions, as commonly seen in other Elapidae venoms that contain β-neurotoxins, some experiments were done in low Ca^{2+} physiological solution to reduce the PLA_2 activity of the venom. Under these conditions, and before adding venom, there was a decrease in twitch amplitude as a result of a reduction in quantal content and the consequent failure of motor units.

When PND preparations were maintained in low Ca^{2+} physiological solution (0.36 mM) under indirect stimulation, the venom (10 μg/ml) caused rapid neuromuscular blockade that was complete in 20 ± 2.5 min (n = 5); this concentration of venom caused complete blockade in 52 ± 2.3 min (n = 5) in preparations maintained in normal Ca^{2+} conditions (1.8 mM) (Fig. 5A). When the preparations were washed with physiological solution containing normal Ca^{2+} (1.8 mM) immediately after total neuromuscular blockade in low Ca^{2+} conditions there was immediate and complete reversal of the blockade to the level of contractility seen

before venom addition; recovery of contractility to the level seen before incubation in low Ca^{2+} required several washes with normal Ca^{2+} solution (Fig. $5B_1$). There was no reversal of the blockade when the preparations were initially washed with low Ca^{2+} (0.36 mM) physiological solution, but subsequent washing with normal Ca^{2+} solution restored the neurotransmission (Fig. $5B_2$). The addition of Ca^{2+} (1.8 mM) immediately after venom-induced blockade (with no prior washing of the preparation to remove the venom, in contrast to Fig. $5B_1$ in which there was washing with normal Ca^{2+} solution to remove venom) caused a transient increase in twitch amplitude that was followed by complete, irreversible blockade (Fig. $5B_3$); adding a high concentration of Ca^{2+} (10 mM), the twitch responses were temporary recovered similarly to that seen with 1.8 mM Ca^{2+} (Fig. $5B_4$).

Influence of neostigmine and 3,4-diaminopyridine on the venom-induced blockade in PND preparations

In PND preparations maintained under normal Ca^{2+} conditions (1.8 mM), neostigmine (NEO, 29 µM) and 3,4-diaminopyridine (3,4-DAP, 230 µM) did not restore muscle contractility after complete venom (10 µg/ml)-induced neuromuscular blockade (Fig. $6A_1$-A_2). However, in preparations incubated in low Ca^{2+} conditions, NEO caused a small, transient reversal of the venom (10 µg/ml)-induced neuromuscular blockade [maximum reversal of 28 ± 3.5% relative to the basal contractile responses in low Ca^{2+} basal values (considered as 100%); this reversal was maximal in 2.2 ± 0.08 min; n = 4]; the addition of Ca^{2+} (1.8 mM) after incubation with NEO for 10 min resulted in partial temporary restoration of the twitch responses (maximum reversal of 102 ± 21% that was reached in 4.1 ± 0.3 min; n = 4) followed by irreversible blockade 14 ± 1.1 min after the maximum response (Fig. $6B_1$). 3,4-DAP (230 µM) produced greater reversal of the venom (10 µg/ml)-induced neuromuscular blockade compared to that seen with NEO (maximum reversal of 300 ± 37%

that was reached in 5.5 ± 0.4 min; n = 4); however, as with NEO, this reversal was transient, with complete blockade occurring 22 ± 0.8 min after reaching the maximum response. The addition of Ca^{2+} (1.8 mM) shortly after complete blockade in the presence of 3,4-DAP did not restore the muscle twitches, nor did repeated washing of the preparation (Fig. $6B_2$).

For comparison, similar protocols run with the venom of *M. frontalis*, considered to be predominantly postsynaptic in action, yielded essentially the results as seen with *M. l. lemniscatus*, i.e., minimal reversal by NEO and 3,4-DAP in normal Ca^{2+}, little reversal by NEO but a marked response to 3,4-DAP (~180%) in low Ca^{2+} solution, and temporary/transitory enhancement of muscle twitches with the addition of normal Ca^{2+} (~33% of twitch responses in low normal Ca^{2+}; Supplementary Figs. 3 and 4).

Effect of venom on the membrane resting potential, miniature end-plate potentials and evoked end-plate potentials in mouse PND preparations

Micrurus l. lemniscatus venom (10 μg/ml) did not affect the membrane resting potential (RP) of diaphragm muscle as there was no depolarization of the muscle membrane during a 60 min incubation [RP: -79.4 ± 1.7 mV (t_0) *vs.* -82.3 ± 0.5 mV (t_{60}), n = 5] and did not affect carbachol (CCh)-induced depolarization (responses to CCh before and after venom: -58 ± 6 mV and -59.5 ± 8.7 mV, respectively; n = 5) (Fig. 7), indicating a lack of myotoxicity (unaltered RP) and no interference with postsynaptic receptors (unaltered response to CCh).

Venom (3 μg/ml) caused a biphasic change in MEPP frequency that involved an initial increase in neurotransmitter release 5-15 min after venom addition followed by a progressive decrease from 45 min onwards (Table 2); this biphasic effect was particularly marked in MEPPs with amplitudes of 0.6-2.0 mV (Fig. 8). There was also a significant decrease in MEPP amplitude during both the excitatory and inhibitory phases (t_5 and t_{45}, respectively) (Table 2). In these experiments and in the EPP measurements described below, a venom

concentration of 3 μg/ml was used as this gave a clearer response than a concentration of 10 μg/ml.

Fig. 9A_1 and A_2 show representative recordings of the changes in EPP amplitude in control (Tyrode solution) and venom-treated (3 μg/ml) preparations, respectively; note that of six preparations studied, only the one indicated in Fig. 9A_2 showed an excitatory phase of neurotransmitter release (at 5 min after venom addition). Fig. 9B shows that incubation with venom (3 μg/ml) resulted in a significant decrease in EPP amplitude from 30 min onwards.

Effect of venom on the compound action potential recorded in mouse sciatic nerve preparations

Incubation with venom (3, 10 and 30 μg/ml) did not affect neuronal conduction assessed through compound action potential (CAP) measurements in mouse sciatic nerve preparations (Fig. 10); there was a slight, non-significant decrease in CAP amplitude at the two highest venom concentrations (10 and 30 μg/ml), with no important alterations in the latency and rise time of the potentials (Table 3). Tetrodotoxin (TTX, 1 μM) added at the end of the experiments abolished the CAP, indicating that sodium channels were not affected by the venom.

Neutralization of neuromuscular blockade by coral snake antivenom in PND preparations

The neuromuscular blockade induced by *M. l. lemniscatus* venom (10 μg/ml) was not significantly attenuated by pre-incubating venom with the manufacturer´s recommended antivenom:venom ratio of 1:1.5 [the t_{50} (in min) for blockade in the absence and presence of antivenom was 20 ± 4.4 and 24 ± 4.1, and complete blockade occurred in 53 ± 3.9 min and 62 ± 4.3 min, respectively; n = 4]. Pre-incubation with a higher antivenom:venom ratio (3:1.5) produced partial neutralization, with neuromuscular blockade reaching a maximum of

91 ± 4.2% after a 120 min incubation; complete neutralization occurred at an antivenom:venom ratio of 10:1.5 (Fig. 11A). In contrast to these findings, similar experiments with *M. frontalis* venom (positive control) resulted in complete neutralization of the neuromuscular activity at all of the antivenom:venom ratios tested, including the recommended ratio of 1:1.5 (Fig. 11B).

The addition of antivenom after venom-induced blockade had reached 50% and 80% was also effective in reversing the neuromuscular blockade and restoring the muscle contractility. At the recommended antivenom:venom ratio of 1:1.5 the reversal of 50% and 80% blockade by *M. l. lemniscatus* venom was 53.2 ± 2.7% and 39.2 ± 2.1% (n = 4) after the first addition of antivenom. Two further additions of antivenom (at the same antivenom:venom ratio) were required to achieve complete reversal of the blockade in preparations with 50% blockade, while in preparations with 80% blockade the recovery was still <100% after the third addition (Fig. 12). With *M. frontalis* venom, the first application of antivenom resulted in 69.4 ± 2.3% reversal after 50% blockade and 52.4 ± 2.3% reversal after 80% blockade; the reversal in both of these cases was greater that the corresponding reversal with *M. l. lemniscatus* venom. A second application of antivenom resulted in full reversal after 50% blockade, whereas a third application was required to achieve full recovery after 80% blockade. Overall, reversal of the neuromuscular blockade was easier with *M. frontalis* than with *M. l. lemniscatus* venom. These findings indicate that while antivenom was able to restore neurotransmission after the onset of blockade, more antivenom was required than suggested by the pre-incubation protocols or by the manufacturer´s recommended antivenom:venom ratio. Furthermore, the reversal experiments indicated that the greater the neuromuscular blockade prior to antivenom administration, the more difficult it was to restore neurotransmission. This finding agrees with clinical observations regarding the difficulty in

reversing neuromuscular blockade with antivenom in patients with manifestations of severe neurotoxicity (Bucaretchi et al., 2016a).

The addition of antivenom corresponding to an intermediate antivenom:venom ratio of 3:1.5 (without prior addition of the lower antivenom:venom ratio of 1:1.5) produced 70 ± 3.4% and 61 ± 4.1% of reversal after 50% and 80% blockade, respectively (n = 4), with two further additions of this ratio resulting in a final reversal of 120 ± 4.7% and 109±3.1% after 50% and 80% blockade, respectively. The highest antivenom:venom (10:1.5) produced complete reversal of the twitches responses (105 ± 3.8% and 99 ± 4.2% for 50% and 80% blockade, respectively) with just a single addition. Against *M. frontalis* venom, the addition of antivenom at the intermediate antivenom:venom ratio (3:1.5) completely restored the twitch tension responses after 50% and 80% blockade (recordings not shown). In view of the complete reversal obtained with the latter ratio, the highest antivenom:venom ratio (10:1.5) was not tested with this venom.

Discussion

Systemic envenomation by coral snakes (*Micrurus* spp.) in humans is characterized by neurotoxicity (Bucaretchi et al., 2016a,b; Risk et al., 2016; Anwar and Bernstein, 2017; Corbett and Clark, 2017) and various studies have confirmed this neurotoxicity *in vivo* (Vital Brazil, 1965; Weis and McIssac, 1971; Vital Brazil et al., 1976/77; Vital Brazil and Fontana, 1983/84) and *in vitro* (Vital Brazil et al., 1976/77; Vital Brazil and Fontana, 1983/84; Goularte et al., 1995; Vital Brazil et al., 1995; Abreu et al., 2008, Camargo et al., 2011; Renjifo et al., 2012; Carbajal-Saucedo et al., 2014; Yang et al., 2017). The results described here show that, in common with other *Micrurus* spp., *M. l. lemniscatus* venom caused irreversible blockade of neurotransmission in mammalian and avian neuromuscular preparations. Mouse PND preparations were less sensitive to neuromuscular blockade than

chick BC preparations, as reflected in the times required for 50% and 90% blockade (Figs. 3 and 4, Table 1): in the former, venom caused complete blockade at concentrations ≥3 μg/ml, while in the latter complete neuromuscular blockade occurred at ≥1 μg/ml. This interspecific variation in sensitivity to neuromuscular blockade has been noted for other coral snake venoms (Cecchini et al., 2005; Abreu et al., 2008; Camargo et al., 2011; Carbajal-Saucedo et al., 2014) and probably reflects differences in the mode of innervation of these two preparations, *i.e.,* monofocal in mammalian and multifocal in avian muscle, in addition to the presence of extra-junctional nicotinic receptors (nAChR) in the avian preparation (Chang and Tang, 1974; Chang and Su, 1975; Silva et al., 2017).

Mechanism of blockade

The neurotoxicity of *Micrurus* venoms is mediated by a combination of postsynaptic α-neurotoxins and presynaptic β-neurotoxins (Gutiérrez et al., 2016; Lomonte et al., 2016; Aird et al., 2017), and proteomic and transcriptomic studies have shown that the venoms of *M. l. carvalhoi* and *M. l. lemniscatus* contain a variety of α- and β-neurotoxins (Ciscotto et al., 2011; Aird et al., 2017). We therefore sought to assess the relative contribution of these two groups of toxins to the blockade caused by *M. l. lemniscatus* venom.

In BC preparations, the contractures induced by exogenous ACh and CCh were abolished by venom concentrations ≥0.1 μg/ml, indicating the presence of α-neurotoxins in the venom. The sensitivity of the post-junctional receptors to blockade by α-neurotoxins was quite high since venom concentrations of 0.1 and 0.3 μg/ml abolished the responses to exogenous ACh and CCh while causing only partial neuromuscular blockade in indirectly stimulated preparations after a 60 min incubation. Thus, although venom blocked the extra-junctional postsynaptic receptors at low concentrations, the junctional postsynaptic receptors of the end-plate region remained responsive.

In PND preparations, the blockade at a venom concentration of 10 μg/ml was accompanied by a rapid progressive decrease in the tetanic muscle contractures but without tetanic fade. A decrease in tetanic contractures has also been reported for *M. pyrrhocryptus* venom at the same concentration (10 μg/ml) as used here for *M. frontalis* and *M. l. lemniscatus*. In his seminal study of the neuropharmacology of *Micrurus* venoms, Vital Brazil (1965) found that the neuromuscular blockade caused by '*M. lemniscatus*' (=*M. l. carvalhoi*) venom in rat PND preparations was partially reversed by neostigmine, a finding suggestive of a predominantly postsynaptic action. However, as shown here for mouse PND preparations, the *M. l. lemniscatus* venom-induced blockade was not reversed by neostigmine or 3,4-DAP in normal Ca^{2+} solution. We also observed similar minimal reversal of the blockade caused by the venom of *M. frontalis* that has a predominantly postsynaptic action (Vital Brazil et al., 1976/77). This lack of reversal could reflect (a) very high affinity ('irreversible') binding of α-neurotoxins to nAChR, (b) the fact that these compounds were tested only after complete blockade, when reversal can be very limited (Goularte et al., 1995), and (c) a general refractoriness of rodent preparations to the reversal of coral snake venom-induced blockade. Indeed, the reversal of *Micrurus* venom-induced blockade in rodent PND preparations rarely exceeds 30-50% and is transient, with subsequent progression to complete neuromuscular blockade (Goularte et al., 1995; Vital Brazil et al., 1995; Serafim et al., 2002; Abreu et al., 2008; Camargo et al., 2011; Renjifo et al., 2012). This finding contrasts with the complete reversal by neostigmine of the blockade caused by *M. frontalis* venom *in vivo* in pigeons, dogs and monkeys (Vital Brazil, 1987) and humans (Vital Brazil and Vieira, 1996).

A further possibility for the limited reversal by neostigmine and 3,4-DAP could be that the blockade was mediated by a predominantly presynaptic rather than postsynaptic action (Vital Brazil, 1987). Indeed, the finding that CCh-induced depolarization of diaphragm muscle was not prevented by prior incubation with the venom for 60 min (enough time for

venom-induced neuromuscular blockade at the concentration tested – 10 μg/ml) indicated that postsynaptic nAChR were not blocked by the venom. Clinically, the inability of neostigmine to reverse coral snake venom-induced neuromuscular blockade has also been considered indicative of a predominantly presynaptic action (Vital-Brazil and Vieira, 1996; Manock et al., 2008; Bucaretchi et al., 2016a).

This conclusion regarding a presynaptic action was supported by changes in the MEPP frequency that showed a biphasic response consisting of an initial excitatory phase at ~15 min (corresponding to ACh release) followed by an inhibitory phase, leading to blockade. A similar response has been reported for *M. laticorallis* venom (Carbajal-Saucedo et al., 2014) and this phenomenon is characteristic of elapid presynaptic neurotoxins (Chang et al., 1977; Chang and Su, 1982; Pungerčar and Križaj, 2007; Rossetto and Montecucco, 2008; Šribar et al., 2014). This biphasic ACh release was not seen in evoked EPPs (although the venom caused a significant reduction in neurotransmitter release) or in twitch-tension experiments under low Ca^{2+} conditions. The lack of effect on compound action potentials from mouse sciatic nerve preparations confirmed that the neuromuscular effect of *M. l. lemniscatus* venom was restricted to the motor end-plate where it affected the presynaptic mechanisms of ACh release. The irreversible neuromuscular blockade was Ca^{2+}-dependent, possibly involving PLA_2 activity.

PLA_2 activity

Micrurus venoms, including those of *M. l. carvalhoi* and *M. l. lemniscatus* contain numerous PLA_2 (Ciscotto et al., 2011; Lomonte et al., 2016; Aird et al., 2017) that can potentially contribute to neuromuscular blockade in experimental animals and humans (Vergara et al., 2014). However, relatively few of these enzymes have been purified and their neurotoxicity assessed pharmacologically (Dal Belo et al., 2005). Oliveira et al. (2008)

reported that the intracerebroventricular injection of several PLA_2 purified from *M. l. carvalhoi* venom in rats resulted in a range of behavioral, electroencephalographic, and morphological alterations as well as lethality (at doses of ~4.4-17 μg/kg), whereas these enzymes were not lethal when administered intravenously at up to ~1.6 mg/kg in mice. Carvalho et al. (2014) examined the cytotoxicity of some of these same PLA_2 in rat cultured hippocampal neurons, and more recently, Casais-e-Silva et al. (2016) described the myotoxicity and pro-inflammatory activity of lemnitoxin, a PLA_2 from *M. l. carvalhoi* venom.

As shown here, *M. l. lemniscatus* venom had PLA_2 activity, in agreement with this activity previously reported for *M. lemniscatus* ssp. venoms (Aird and Silva Jr., 1991). The PLA_2 activity was significantly reduced when assayed in low Ca^{2+} and at room temperature (25 ^oC) and after preincubation with *p*-bromophenacyl bromide (*p*-BPB), a widely used inhibitor of snake venom PLA_2 activity (Lomonte et al., 2003; Soares and Giglio, 2003). Although these interventions attenuated the PLA_2 activity, they caused only a slight rightward shift in the time curves for venom (10 μg/ml)-induced blockade in PND preparations; there was a slight delay (~10 min) in the onset of blockade and a small increase (also ~10 min) in the time for complete blockade. The exception to this trend was low Ca^{2+} solution that considerably potentiated the onset and time for total blockade. These findings indicate that PLA_2 activity, a temperature of 37 ^oC and the presence of Ca^{2+} are not essential for the venom-induced blockade. Overall, these results suggest that PLA_2 may not have a central role in this venom-induced blockade or possibly that non-catalytic PLA_2 (not detected in the enzymatic assay) may be more important in this response. Catalytic and non-catalytic PLA_2 have been detected in transcriptomic analysis of *M. l. lemniscatus* venom (Aird et al., 2017). Neuromuscular blockade that is independent of Ca^{2+} and unaffected by a reduction in temperature (and, by inference, PLA_2 activity) has also been reported for the venoms of *M.*

altirostris (Abreu et al., 2008) and *M. pyrrhocryptus* (Camargo et al., 2011). In contrast, the neuromuscular blockade caused by venoms that are myotoxic, such as that of *M. nigrocinctus* (Goularte et al., 1995), is markedly attenuated by a reduction in temperature.

Role of calcium

In indirectly-stimulated PND preparations maintained in low (0.36 mM) Ca^{2+} Tyrode solution, *M. l. lemniscatus* venom produced potent neuromuscular blockade in <20 min compared to ~50 min in normal Ca^{2+} (1.8 mM) solution. Incubation in low Ca^{2+} physiological solution reduced twitch amplitude by decreasing the release of ACh, with consequent deactivation of motor units. In these conditions, the few remaining responsive motor units were rapidly blocked by the venom neurotoxins. When the preparations were washed with Tyrode solution containing normal Ca^{2+} (1.8 mM) immediately after complete neuromuscular blockade, there was total recovery of the twitch responses; no such recovery was seen when the preparations were washed with low Ca^{2+} Tyrode solution. However, the addition of 1.8 mM Ca^{2+} to the bath once blockade had been achieved resulted in temporary recovery of the twitch responses followed by complete, irreversible blockade. This observation suggested the possibility that Ca^{2+} was involved in modulating the blockade. To examine the latter possibility, the effect of adding 10 mM Ca^{2+} to the organ bath once blockade had been achieved was examined; this concentration of Ca^{2+} produced a temporary recovery of the twitch responses similar to that seen with 1.8 mM Ca^{2+} (data not shown).

These results suggest that by re-establishing the ideal Ca^{2+} concentration for normal presynaptic release of ACh, the enhanced presence of neurotransmitter in the synaptic cleft could displace postsynaptic toxins that were blocking nicotinic receptors, resulting in the restoration of twitch responses. Alternatively, this enhanced response to restored Ca^{2+} could simply represent an increase in the EPP amplitude of motor units that were previously set at

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slightly below threshold but were now contributing to the twitch responses. When the preparations were maintained in low Ca^{2+} and subsequently exposed to *M. l. lemniscatus* venom, there was no damage to the pre- and postsynaptic machinery involved in motor neurotransmission. On the other hand, when Ca^{2+} (1.8 mM) was added directly to the bath physiological solution after complete blockade, there was a transient reversal of the twitch responses that subsequently progressed to complete, irreversible neuromuscular blockade in ~30 min, a time scale similar to that for preparations exposed to venom in normal Ca^{2+} conditions.

As indicated above, neostigmine and 3,4-DAP failed to reverse the venom-induced blockade in PND preparations in normal Ca^{2+} solution. However, in low Ca^{2+} conditions, the neuromuscular blockade was temporarily reverted by 3,4-DAP followed by irreversible blockade, indicating that the machinery involved in ACh release was not affected by the venom. The irreversible blockade seen after the 3,4-DAP-induced facilitation probably resulted from the depletion of synaptic vesicle stores subsequent to the enhanced entry of Ca^{2+} into the motor nerve terminal. The very poor response to neostigmine in low Ca^{2+} conditions largely reflecte[illegible] mode of action of this compound in enhancing the synaptic content of neurotransmitte[illegible]ther than stimulating the presynaptic release of ACh.

Você tem alguma referência para apoiar esta afirmação? Você fez experimentos controles com apenas DAP (ou seja, DAP sozinha em uma preparação, sem incubação prévia com veneno) para ver se a facilitação com DAP era mantida ou se decaía? (Este controle seria importante para afirmar que o bloqueio irreversível visto após DAP era devido à depleção dos estoques de neurotransmissor).

Myotoxicity has been demonstrated experimentally in mice for a variety of *Micrurus* spp. venoms (Gutiérrez et al., 1980, 1983, 1992), but is rarely seen clinically, mainly in *M. fulvius* from southeastern United States (Kitchens and Van Mierop, 1987; Bucaretchi et al., 2016b). Although *M. lemniscatus* spp. venoms contain PLA_2 (Silva Jr. and Aird, 1991; Cecchini et al., 2005; Oliveira et al., 2008; Carvalho et al., 2014; Casais-e-Silva et al., 2016; Aird et al., 2017), several lines of evidence from the present investigation indicated that *M. l.*

lemniscatus venom was not myotoxic and that myotoxicity was not a contributing factor to the neuromuscular blockade observed here. Specifically, (1) the venom had no effect on muscle contractility in curarized, directly stimulated PND preparations (a venom concentration of 30 μg/ml had no effect in this preparation but produced complete blockade in ~20 min in indirectly stimulated preparations), (2) there was no increase in the baseline tension (indicative of muscle contracture) in either BC or PND preparations, (3) the venom did not depolarize the diaphragm muscle membrane, (4) the contractile responses to exogenous K^+ (generally used as an indicator of venom and toxin-induced myotoxicity; Harvey et al., 1994) were unaltered in BC preparations, even when the responses to ACh and CCh had been abolished, and (5) histological analysis revealed no muscle necrosis or general damage. These findings are reminiscent of those reported by Vital Brazil (1965, 1987) for '*M. lemniscatus*' (=*M. l. carvalhoi*), the venom of which also did not affect the contractile responses to direct muscle stimulation or depress the muscle contractures to exogenous K^+ in rat chronically denervated rat hemidiaphragm; a similar lack of myotoxicity has also been observed for other species such as *M. altirostris* (Abreu et al., 2008) and *M. pyrrhocryptus* (Camargo et al., 2011). In contrast, the venoms of *M. dissoleucus* from Colombia (Renjifo et al., 2012), *M. laticorallis* from Mexico (Carbajal-Saucedo et al., 2014) and *M. nigrocinctus* from Costa Rica (Goularte et al., 1995, 1999) are myotoxic and produce neuromuscular blockade and an increase in baseline tension in PND preparations *in vitro*.

Neutralization by antivenom

Antivenom therapy is the mainstay for treating systemic envenomation by coral snakes and its early administration is important in preventing or reversing neuromuscular blockade since antivenom is considerably less effective once full neuromuscular blockade has been established (Bucaretchi et al., 2016a,b). The coral snake antivenom produced in Brazil by the

Instituto Butantan (São Paulo) and Fundação Ezequiel Dias (FUNED, Belo Horizonte, MG) is raised in horses against a combination of *M. corallinus* and *M. frontalis* venoms, with 1 ml of antivenom neutralizing 1.5 mg of *M. frontalis* venom. Although raised against the venom of only two of the numerous coral snake species in Brazil, this antivenom is used to treat envenoming by any of the other *Micrurus* spp. in this country (Bucaretchi et al., 2016). Whilst various studies have examined the immunological cross-reactivity and neutralization of biological activities (including lethality) of coral snake venoms with this antivenom (Higashi et al., 1995; Tanaka et al., 2010, 2016; Ramos et al., 2017), few have assessed its ability to neutralize the neuromuscular blockade caused by these venoms.

Camargo et al. (2011) showed that Instituto Butantan antivenom neutralized the neurotoxicity of *M. pyrrhocryptus* venom from Argentina in BC preparations at the recommended antivenom:venom ratio, even though this venom is not included in the venom pool used in the immunization protocol. In contrast, Abreu et al. (2008) reported that in preincubation protocols or when added simultaneously with the venom this same antivenom failed to protect against the neuromuscular blockade by *M. altirostris* venom in BC preparations at the recommended antivenom:venom ratio; complete protection against blockade and the reduction in contractures to exogenous ACh was obtained only at an antivenom:venom ratio 20-fold greater than the recommended ratio. For other *Micrurus* venoms, Goularte et al. (1995) showed that the neuromuscular blockade by *M. nigrocinctus* venom in PND preparations was prevented by an equine monovalent antivenom to this venom (produced by the Instituto Clodomiro Picado, Costa Rica), whether in preincubation protocols or when added 10-20 min after the venom. More recently, Yang et al. (2017) demonstrated that Coralmyn (Bioclon, Mexico), an equine antivenom raised against *M. nigrocinctus nigrocinctus* venom, effectively neutralized the neuromuscular blockade by *M. fulvius* venom in BC preparations, but was ineffective against the venoms of *M. pyrrhocryptus*, *M. spixii*, *M.*

tener and the Sonoran coral snake *Micruroides euryxanthus*. None of these studies assessed the ability of antivenom to restore neurotransmission after ≥50% blockade.

As shown here, in pre-incubation experiments, complete neutralization of the neuromuscular blockade caused by *M. l. lemniscatus* venom required a 10-fold higher antivenom:venom ratio than that recommended by the manufacturer, whereas complete neutralization of the neuromuscular blockade by *M. frontalis* venom was observed at the recommended antivenom:venom ratio. In agreement with these findings, the reversal of neuromuscular blockade when antivenom was added at the recommended antivenom:venom ratio after 50% or 80% blockade was more efficient against *M. frontalis* venom than against *M. l. lemniscatus* venom although, in both cases, more than one addition of antivenom was required and the extent of reversal was dependent on the initial degree of blockade. For both venoms, the higher the initial antivenom:venom ratio tested, the quicker the reversal of blockade, *i.e.,* complete reversal of *M. frontalis* and *M. l. lemniscatus* blockade was achieved with a single addition of a three-fold and ten-fold higher initial antivenom:venom ratio, respectively.

Although preincubation protocols are the gold standard for assessing antivenom neutralizing capacity in toxinology (Gutiérrez et al., 2017), the addition of antivenom after the onset of blockade such as used here is more representative of the clinical situation in which antivenom is given after envenomation. The results reported here suggest that the efficacy of antivenom in reversing neuromuscular blockade by *M. l. lemniscatus* venom in humans may be less than for *M. frontalis*, *i.e.,* greater amounts of antivenom may be required to treat bites by the former species. In addition, initiating treatment with an antivenom:venom ratio greater than that recommended by the manufacturer may hasten the recovery from neuromuscular blockade, as shown by the reversal obtained with an initial antivenom:venom ratio of 10:1.5.

Intraspecific comparison

Comparison of the neuromuscular effects of *M. l. lemniscatus* venom described here with the findings reported for *M. l. carvalhoi* (Vital Brazil, 1965; Cecchini et al., 2005) indicate that there are similarities (*e.g.,* lack of myotoxicity, as shown by the inability to affect contractile responses to direct stimulation, membrane potential and contractures to exogenous K^+) and potential differences (*e.g.,* in the extent of reversibility of the venom-induced blockade by neostigmine) between the venoms of these two subspecies. However, the lack of detailed neuromuscular and electrophysiological studies for the venoms of *M. l. carvalhoi* and *M. l. helleri* precludes further comparison among the subspecies.

Conclusion

The results of this study indicate that *M. l. lemniscatus* venom causes neuromuscular blockade by a combination of pre- and postsynaptic mechanisms, in agreement with the presence of α- and β-neurotoxins in the venom of this subspecies (Aird et al., 2017). The presence of presynaptic activity and the poor reversibility of blockade by neostigmine are reminiscent of those reported for *M. corallinus* (Vital Brazil and Fontana, 1983/84) and suggest that human envenomation by this species may not respond adequately to treatment with anticholinesterase drugs such as neostigmine (Bucaretchi et al., 2016b). This conclusion agrees with the clinical observation of a case of envenomation by the closely related *M. l. helleri* in which neostigmine was ineffective in reversing the neuromuscular blockade (Manock et al., 2008). In addition, the neutralization experiments indicated that while antivenom is useful in reversing the blockade by this venom, a 10-fold greater antivenom:venom ratio is required compared to that which protects against blockade by *M. frontalis* venom used to raise the antivenom.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conflict of interest

The authors declare that they have no conflict of interest with this work.

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Figure legends

Fig. 1. Distribution of *M. lemniscatus* subspecies in Brazil. Note that all three subspecies also occur outside of Brazil. The range of *M. diutius*, recently separated from the *M. lemniscatus* complex (Starace, 2013), is also shown. Hatched areas – overlap between subspecies. "?" – regions of uncertainty with regard to distribution and potential overlap of subspecies.

Fig. 2. PLA_2 activity of *M. l. lemniscatus* venom. A. Influence of temperature and calcium concentration on enzymatic activity. B. Attenuation of enzymatic activity by *p*-BPB. Buffer (0.1 M ammonium bicarbonate, 0.7 mM EDTA, pH 8.0, used to incubate the venom with *p*-BPB) and ethanol (solvent for *p*-BPB, final concentration 8.3%) were tested alone to examine their effect on enzyme activity. The columns are the mean ± SEM (n = 3). $^{*}p < 0.05$ compared to enzymatic activity at 37 °C and 10 mM Ca^{2+}; $^{\#}p < 0.05$ compared to enzymatic activity at 25 °C and 10 mM Ca^{2+}; $^{\dagger}p < 0.05$ compared to activity without *p*-BPB.

Fig. 3. Neuromuscular activity of *M. l. lemniscatus* venom in chick biventer cervicis preparations. A. Twitch blockade caused by venom (0.1-30 µg/ml). B. Representative recording showing the irreversible blockade caused by venom (10 µg/ml) in a field stimulated preparation at 37 ºC. Note that contractures to exogenous ACh (1 mM, ▲) and CCh (20 µM, ●) were abolished by the venom whereas those to KCl (40 mM, ■) were unaffected. In A, the points are the mean ± SEM (n = 4); $^{*}p < 0.05$ compared to control preparations; w – wash. Scale bar in B: grams (g).

Fig. 4. Neuromuscular activity of *M. l. lemniscatus* venom in mouse phrenic nerve-diaphragm preparations. A. Twitch blockade caused by venom (0.1-30 µg/ml). B. Representative

recordings showing the irreversible blockade caused by the venom (B_1 – 10 μg/ml and B_2 – 30 μg/ml) in an indirectly stimulated preparation at 37 ºC, as well as the slight neuromuscular facilitation and lack of blockade in a curarized, directly stimulated preparation exposed to 30 μg of venom/ml (B_3). C. Neuromuscular blockade caused by venom pre-treated with *p*-BPB. Note that the inhibition of PLA_2 activity had a minimal effect on the blockade. In A and C, the points are the mean ± SEM (n = 4-5). *$p < 0.05$ compared to control preparations; AB – ammonium bicarbonate buffer (see Fig. 2 legend for buffer composition), DS – direct stimulation, IS – indirect stimulation, *d*-Tc – d-tubocurarine, w – wash. Scale bars in B_1-B_3: grams (g).

Fig. 5. Neuromuscular activity of *M. l. lemniscatus* venom in PND preparations maintained in low (0.36 mM) Ca^{2+} physiological solution. A. Comparison of the venom-induced blockade in normal (1.8 mM) and low Ca^{2+} physiological solutions. B. Representative recordings showing the immediate reversal of the venom-induced blockade after washing in normal Ca^{2+} physiological solution (B_1), the lack of reversal during washing in low Ca^{2+} conditions followed by immediate reversal when normal Ca^{2+} solution is used (B_2), and the transient increase in twitch tension followed by irreversible blockade of the twitch response when 1.8 mM Ca^{2+} was added to the bath immediately after neuromuscular blockade in low Ca^{2+} conditions (no removal of venom prior to addition of 1.8 mM Ca^{2+}) (B_3). In (A), the points are the mean ± SEM (n = 4); *$p < 0.05$ compared to control preparations. Scale bars in B_1-B_3: grams (g). w – wash.

Fig. 6. A. The lack of effect of neostigmine (NEO; 29 μM, A_1) and 3,4-diaminopyridine (3,4-DAP; 230 μM, A_2) in reversing the neuromuscular blockade induced by *M. l. lemniscatus* venom (10 μg/ml) in PND preparations in normal Ca^{2+} (1.8 mM) physiological solution. B.

Transitory reversal of venom (10 μg/ml)-induced blockade by NEO (29 μM, B_1) and 3,4-DAP (230 μM, B_2) in low Ca^{2+} (0.36 mM) physiological solution. Note that when 1.8 mM Ca^{2+} was added at the end of both protocols (B_1 and B_2) partial restoration of muscle twitches was seen only after exposure to NEO. All representative recordings correspond to one of four experiments done at 37 °C.

Fig. 7. Membrane resting potential recorded in PND preparations incubated with *M. l. lemniscatus* venom. The venom (10 μg/ml) did not cause membrane depolarization during a 60 min incubation and also did not prevent carbachol (CCh)-induced depolarization at the end of the incubation. The columns are the mean ± SEM (n = 5). *$p < 0.05$ compared to basal values.

Fig. 8. Frequency and amplitude of MEPPs recorded in PND preparations incubated with *M. l. lemniscatus* venom. Note that, with the exception of high amplitude MEPPs (≥2.6 mV), the other amplitude intervals showed an excitatory phase prior to the inhibitory phase in their frequencies. The columns are the mean ± SEM (n = 5) of MEPPs (frequency and amplitude) recorded at each interval. *$p < 0.05$ compared to basal values.

Fig. 9. End-plate potentials (EPPs) in PND preparations incubated with *M. l. lemniscatus* venom (3 μg/ml). A. Superposed recordings showing the decrease in EPP amplitude caused by venom; the times for 0 (basal), 5, 15, 30, 45 and 60 min incubation are indicated close to their respective trace. S – stimulus artefact. A_1 – Control preparation incubated with Tyrode solution alone for 60 min. A_2 – Preparation incubated with venom for 60 min. Note that in this preparation, there was a slight facilitation after 5 min followed by blockade (the only preparation of six in which this was observed). B. Time course of the changes in EPP

amplitude following exposure to venom. All experiments were done at room temperature. In panel B, the columns are the mean ± SEM (n = 6). *$p < 0.05$ compared to t_0 (basal) values; $^{\#}p < 0.05$ compared to corresponding control preparations (Tyrode solution alone).

Fig. 10. Compound action potentials recorded in mouse desheathed sciatic nerve preparations. A. Minimal effect of *M. l. lemniscatus* venom (3, 10 and 30 μg/ml) on compound action potential (CAP) amplitude. B. Representative recordings showing the unaltered CAP amplitude in basal conditions (t_0, before venom addition) (B_1), after a 30 min incubation with venom (B_2) and after the addition of tetrodotoxin (TTX, 1 μM) at the end of the incubation with venom (without venom removal) (B_3). TTX was added at the end of the experiments to confirm that the connective tissue sheath had been removed and sodium channels were responsive. All experiments were done at room temperature. S – stimulus artefact, t_{30} – end of the 30 min incubation with venom. In panel A, the points are the mean ± SEM (n = 5). The traces in B_1–B_3 are representative recordings of the experiments summarized in panel A. There were no significant changes in the amplitude, rise time and latency of the potentials (see Table 3).

Fig. 11. Neutralization by coral snake antivenom of the neuromuscular blockade caused by (A) *M. l. lemniscatus* and (B) *M. frontalis* venom in PND preparations. The preparations were obtained and mounted as described in section 2.4.2 and allowed to stabilize for 15 min prior to the addition of venom or venom preincubated (30 min, 37 °C) with antivenom. Control preparations were incubated with Tyrode solution alone. The antivenom used was raised in horses against a pool of venoms from *M. corallinus* and *M. frontalis*, with 1 ml neutralizing 1.5 mg of *M. frontalis* venom (according to the manufacturer; Instituto Butantan). The venoms were preincubated with varying antivenom:venom ratios prior to testing, starting with

the manufacturer´s recommended ratio and then increasing the amount of antivenom three (3:1.5) and ten (10:1.5) times. Note that the neuromuscular blockade caused by *M. frontalis* venom was effectively neutralized by the manufacturer´s recommended antivenom:venom ratio whereas similar neutralization of the blockade by *M. l. lemniscatus* required a 10 times higher amount of antivenom. The points are the mean ± SEM (n = 4). *$p < 0.05$ compared to venom alone.

Fig. 12. Reversal by coral snake antivenom of the neuromuscular blockade caused by *M. l. lemniscatus* and *M. frontalis* venoms after 50% and 80% blockade by each venom in mouse PND preparations. Three consecutive additions of antivenom added to the organ baths at the manufacturer´s recommended antivenom:venom ratio of 1:1.5 (1 ml of antivenom neutralizes 1.5 mg of *M. frontalis* reference venom) were screened for their ability to reverse venom-induced neuromuscular blockade. The extent of reversal for each addition of antivenom was calculated in relation to the corresponding initial degree of blockade. The columns represent the mean ± SEM (n = 4). *$p < 0.05$ compared to the corresponding first addition, ♦$p < 0.05$ compared to the corresponding second addition, $^{\#, \lozenge}p < 0.05$ compared to the first addition for 50% blockade and 80% blockade, respectively, with *M. l. lemniscatus* venom.


Figure 5.docx


Figure 4.docx


Figure 3.docx


Figure 2.docx


Figure 12.docx


Figure 11.docx


Figure 10.docx


Figure 9.docx


Figure 8.docx


Figure 7.docx


Figure 6.docx


Figure 1.pptx


Table 1.docx


Table 3.docx


Table 2.docx