Phoneutria nigriventer Venom and Toxins: A Review

Maria Elena de Lima[a]*, Suely Gomes Figueiredo[b], Alessandra Matavel[c], Kenia Pedrosa Nunes[d], Carolina Nunes da Silva[a], Flávia de Marco Almeida[a], Marcelo Ribeiro Vasconcelos Diniz[c], Marta Nascimento do Cordeiro[c], Maria Stankiewicz[e] and Paulo Sérgio Lacerda Beirão[f]

[a]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

[b]Departamento de Ciências Fisiológicas, Centro Biomédico, Universidade Federal do Espírito Santo, Vitória, ES, Brazil

[c]Centro de Pesquisa e Desenvolvimento Prof.Carlos Diniz, Fundação Ezequiel Dias, Belo Horizonte, MG, Brazil

[d]Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, WI, USA

[e]Department of Biophysics, Faculty of Biology and Environment Protection, Nicolaus Copernicus University, Torun, Poland

[f]Laboratório de Membranas Excitáveis, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

Abstract

The venom of *Phoneutria nigriventer* spider has been extensively studied. It is highly neurotoxic, with clinical manifestations occurring a few minutes after the bite, mainly in children. Among the intoxication signs that may lead to neurogenic shock are agitation, somnolence, sweating, nausea, profuse vomiting, lacrimation, excessive salivation, hypertension, tachycardia, tachypnea, tremors, muscle spasms, and priapism. Many toxins from this venom have been purified and characterized for their structure and/or function. The most studied *Phoneutria nigriventer* toxins are PnTx1, PnTx2-5, and PnTx2-6, which show complex effects on sodium channel kinetics. PnTx2-5 and PnTx2-6 are both described as responsible for priapism; PnTx4(6-1) and PnTx4(5-5) show insecticidal activities and act on insect sodium channels; PnTkPs are muscle-active peptides; and nigriventrine is a piperidine derivative that has neuroactive properties and causes convulsive spells. PnTx3-3 and PnTx3-4 toxins were demonstrated to be effective on preventing cell death after ischemia injury. On the other hand, PnTx3-6 was shown to be efficient in the treatment of persistent pathological pain. The present chapter compiles biochemical, physiological, and pharmacological studies of fractions and purified toxins from *Phoneutria nigriventer* venom, showing their great potential as new tools for pharmacological studies and drug development.

Keywords

Phoneutria nigriventer; Spider venom; Spider toxin and nociception; Spider toxin and erectile function; Spider toxin as insectides; Spider toxins and ionic channels

Introduction

The spiders of the genus *Phoneutria* are members of the family Ctenidae, suborder Labdognata, and order Araneidae. They inhabit forests of the neotropical region from Southern Central America (Costa Rica) throughout South America, from the East of the Andes to the North of Argentina. The genus comprises the

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*Email: melenalima@icb.ufmg.br

*Email: lima.mariaelena@gmail.com
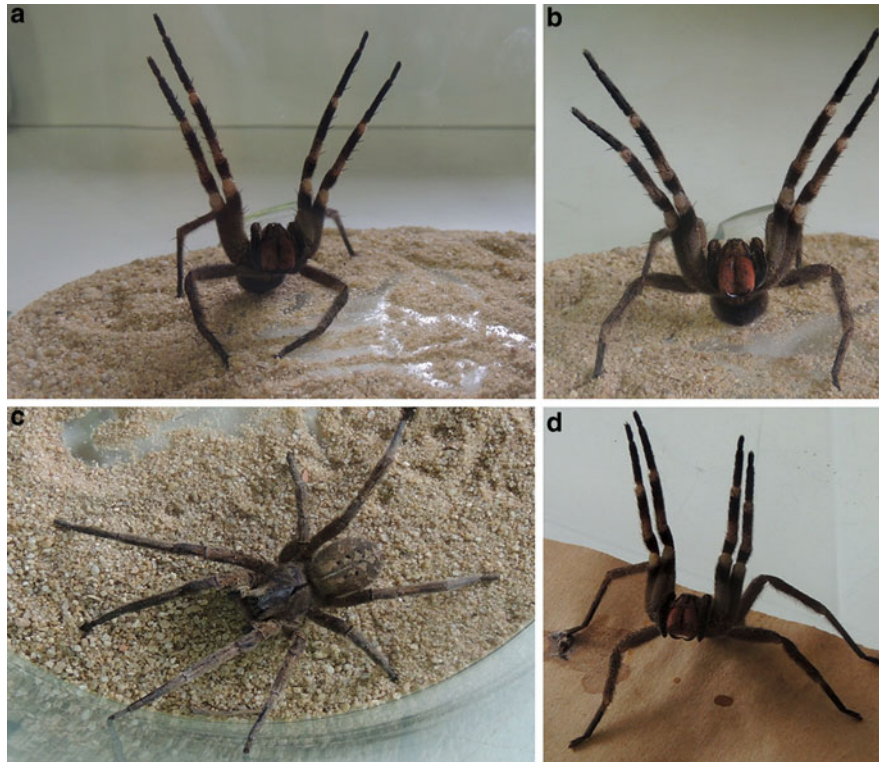
Fig. 1 *Phoneutria nigriventer*, the "armed spider" (Photographed by Alcides Sousa, at Fundação Ezequiel Dias, Belo Horizonte, Minas Gerais, Brazil)

largest known true spiders, considering their size and weight. In Brazil they are also known as "armed spiders," because they display an "armed" position when threatened. The other common name, "banana spider," comes from their tendency to hide in banana bunches. They are wandering spiders with nocturnal habits and fast-moving hunters that make little use of silk, which is employed mainly for the manufacture of egg sacs and nursery webs. *Phoneutria* genus belongs to RTA clade, whose adaptive and evolutionary process is associated with the loss of cribellate silk and prey-capture webs.

The species *Phoneutria nigriventer* (Fig. 1) is the most important species of the genus *Phoneutria*, considering its clinical significance. The venom of this aggressive spider is highly toxic and it is the most studied among the venoms of *Phoneutria* species. *Phoneutria nigriventer* is a synanthropic species, what may explain the large number of human accidents with this spider. Accidents involving *Phoneutria* spiders occur mainly in Brazil, but there are reports of sporadic cases in Central America and in neighboring countries, as well as in other nations of Europe, as a result of banana imports from Brazil. Most accidents involving humans are mild, being 0.5–1.0 % of severe cases. Despite the venom being highly neurotoxic, the amount inoculated through the bite is too small to induce lethal effects, and the poisoning rarely leads to death. The clinical manifestations of severe systemic intoxication are usually seen in children. In such cases, the penile erection (priapism) is one of the most common sign of phoneutrism. Systemic manifestations can occur in children after a few minutes. Agitation, somnolence, sweating, nausea, profuse vomiting, lacrimation, excessive salivation, hypertension, tachycardia, tachypnea, tremors, muscle spasms, and priapism are the most significant symptoms that can lead to neurogenic shock. Cases of systemic poisoning in adults are uncommon but may happen. The effects

observed in experimental animals after venom injection are very similar to those observed in humans after the accidents with this spider.

Bucaretchi et al. (2008) described the case of a 52-year-old man bitten on the neck by a female specimen of *Phoneutria nigriventer*. At the medical ambulatory, immediately after the bite, they observed that the man felt intense pain at the bite site, blurred vision, excessive sweating, tremors, and vomiting episodes. Two hours after the bite, the patient became agitated with elevated blood pressure (200/130 mmHg) and then was treated with captopril and meperidine. Four hours after the bite and under treatment, the patient presented stabilized blood pressure (130/80 mmHg) and heartbeat (150/min), tachypnea, gentle shaking, cold extremities, profuse sweating, generalized tremors, and priapism. One hour after the treatment with an antivenom, the systemic manifestations disappeared.

Studies concerning *P. nigriventer* venom have been reported since the 1950s and have shown that this venom consists of molecules with diverse biological activities. The venom contains a wide variety of proteins and peptides, including neurotoxins, which act on ion channels and chemical receptors of the neuromuscular systems of insects and mammals. Spider venoms have been described as a treasure chest for the discovery and development of new biologically active molecules, with potential application in medicine and agriculture (Richardson et al. 2006; Borges et al. 2009). This chapter describes the biochemical and pharmacological characteristics of *Phoneutria nigriventer* spider venom and its toxins.

In the earliest reports about *P. nigriventer* venom, in addition to the biologically active protein constituents (peptides, proteases, and hyaluronidase), other active compounds such as biogenic amines (histamine and serotonin) and some free amino acids were identified. These early studies revealed that *P. nigriventer* venom displays a pronounced neurotoxic activity, which causes a complex scenario of excitatory symptoms following venom injection in experimental animals and/or in humans by accidental stings (Diniz 1963; Schenberg and Pereira Lima 1978).

However, it was only after 1990 that the biochemical and the pharmacological characterization of the "armed spider" toxins was greatly accelerated, with the valuable contributions of the research group headed by Dr. Carlos Ribeiro Diniz at Ezequiel Dias Foundation and of the research groups at Universidade Federal de Minas Gerais, in Belo Horizonte, Minas Gerais state, Brazil. Since then, *P. nigriventer* venom has been extensively studied, resulting in a large amount of information on this venom and its components. A large number of bioactive molecules, mainly polypeptides and some non-proteic low molecular mass toxins, have been isolated from the crude venom and characterized. The biochemical characterization of these molecules has been carried out by a combination of different methods, including liquid chromatography, molecular biology, automated Edman degradation, mass spectrometry (MS), and nuclear magnetic resonance (NMR).

This chapter presents a general overview of the biochemical and pharmacological properties of the components/toxins from *P. nigriventer* venom.

## Structure and Function of *Phoneutria nigriventer* Toxins

A summary of the best-characterized toxins with identified targets is given in Table 1. The use of synonyms represents a great problem in the nomenclature of *Phoneutria nigriventer* venom toxins. Table 1 includes the original nomenclature described for the toxins, which was usually given after a code derived from the purification procedure (for a review see Richardson et al. 2006; Borges et al. 2009; De Lima et al. 2007), and other synonyms that appeared later in literature.

An initial fractionation procedure, using gel filtration and reversed-phase chromatography, monitored by the assessment of lethal activity and toxic effects, yielded five toxic fractions from *Phoneutria nigriventer* crude venom, with different targets in mammals and/or insects (Rezende et al. 1991;

Table 1 Alignments of the amino acid sequences of precursors and mature toxins from the venom of the spider *Phoneutria nigriventer*

The beginning of the signal peptide, propeptide, and the mature toxin chain sequences is indicated by ↓. Amino acid residues in structural motifs: *PQM* (processing quadruplet motif), *PSM* (principal structural motif), *ESM* (extra structural motif) are shown in green, red, and blue, respectively. Propeptides at the end of the chain are shown in gray. (L) length of the mature toxin; (MM) molecular mass; (ND) not determined; (———) gaps were introduced to facilitate the alignment of the Cys (C) residues; (. . . .) sequences not yet determined; accession number of sequences deposited at SWISS-PROT/TREMBL database; some MM* were obtained from Richardson et al. (2006)
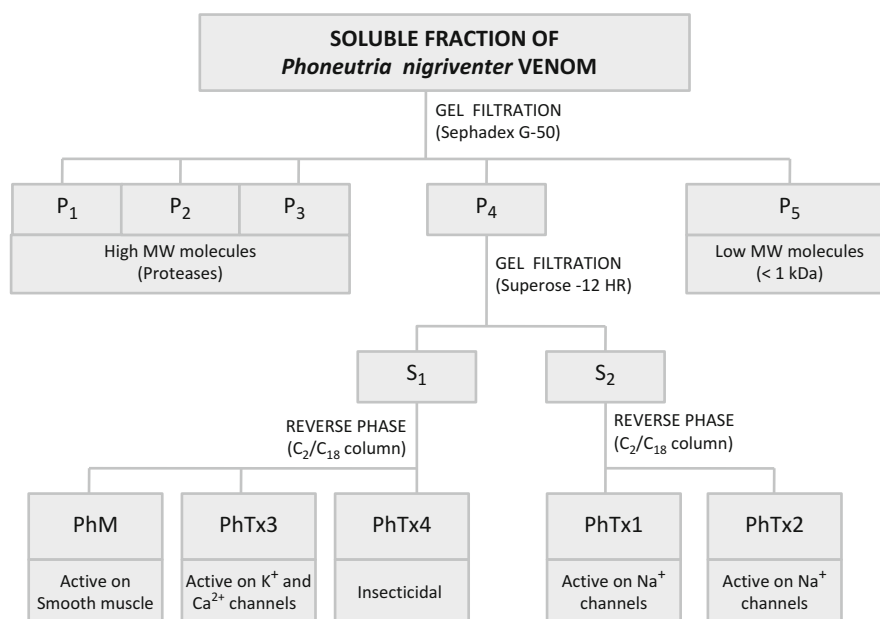
Fig. 2 Flowchart showing the purification procedure of *Phoneutria nigriventer* venom fractions (PhTx groups) (For more details see the classic paper Rezende et al. 1991. *MW* molecular weight)

Figueiredo et al. 1995). At that time, toxicity was evaluated in vivo by intracerebral (i.c.) or intrathoracic injections in mice and insects, respectively, and in vitro by smooth muscle assays using guinea pig ileum.

Using these approaches, four distinct families of neurotoxic polypeptides were demonstrated, named PhTx1, PhTx2, PhTx3, and PhTx4. PhTx1, PhTx2, and PhTx3 are active on mammals and differ in their lethality and effects in mice (Rezende et al. 1991). PhTx4 produces marked stimulatory effects in insects and is more toxic to insects than to mammals (Figueiredo et al. 1995). Another fraction (PhM), apparently not toxic to mammals, is active on smooth muscle, causing contraction (Rezende et al. 1991). The average LD_{50} by i.c. injection in mice for the whole venom, PhTx1, PhTx2, PhTx3, and PhTx4, was 47, 45, 1.7, 137, and 480 µg/kg, respectively (Rezende et al. 1991; Figueiredo et al. 1995). PhTx2 is the most toxic group and PhM (15 mg/kg) has no lethal effect in mice (Rezende et al. 1991). Figure 2 shows a flow diagram describing the purification of *Phoneutria nigriventer* venom fractions.

PhTx1 proved to be a homogeneous fraction (Diniz et al. 1990), whereas the other active fractions (PhTx2, PhTx3, PhTx4, and PhM) were found to be collections of isotoxins with some particular aspects (Cordeiro et al. 1992, 1993; Figueiredo et al. 1995; Pimenta et al. 2005). The neurotoxic fractions were shown to be composed of polypeptides, typically with 30–80 amino acid residues in length (MW from 3.5 to 9 kDa), comprising 3–7 disulfide bridges. The amino acid sequences of some of these neurotoxins were determined chemically or by the analysis of clones from cDNA libraries constructed using the venom gland of the spider (Table 1).

Using biochemical and pharmacological approaches, the mechanisms of action of many of these molecules have also been investigated by several authors. Based on these studies, it was demonstrated that most of these peptides interact with neuronal ion channels, altering their activity, and/or with chemical receptors of the neuromuscular systems of mammals and insects, resulting in alterations in neurotransmitter release (Table 1). The next paragraphs describe the pharmacological and electrophysiological studies that have been done with the fractions and the purified toxins from *Phoneutria nigriventer* venom.
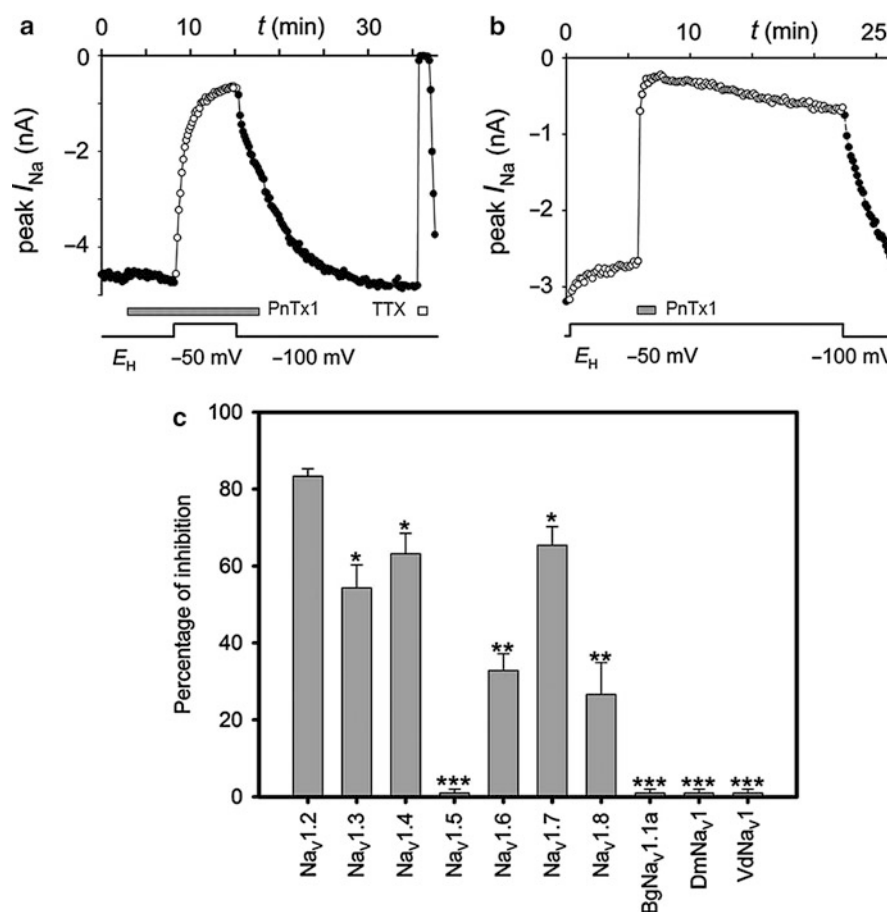
Fig. 3 Fraction PhTx1 inhibits sodium currents. The only toxin present in PhTx1 fraction, named PnTx1, inhibits $Na_v1.2$ sodium channels expressed in CHO cells in a state-dependent mode. The holding potential was shifted from -100 to -50 mV after (**a**) or before (**b**) the addition of PnTx1. Test pulses were preceded by a deinactivating prepulse (200 ms to -100 mV) at a holding potential of -50 mV. (**c**) Average percentage of Na_v current inhibition by rPnTx1 (1 mM) of sodium channel subtypes expressed in oocytes. No effect was observed on cardiac $Na_v1.5$ or on the arthropod isoforms (*Drosophila melanogaster*, DmNaV1; *Blattella germanica*, BgNaV1.1a; and *Varroa destructor*, VdNaV1). The symbols (*), (**), and (***) denote the isoforms on which the toxin effects were not statistically different among them (Credit: (**a**–**b**) Martin-Moutot et al. 2006 © 2006 American Society for Pharmacology and Experimental Therapeutics. (**c**) Silva et al. 2012 © 2012 Elsevier Masson SAS)

The Fraction PhTx1

Fraction PhTx1 induces excitation, tail elevation, and spastic paralysis in mice upon i.c. injection (Rezende et al. 1991). This fraction contains only one toxin, initially called "Tx1" (later PnTx1), which represents 0.45 % of the total protein in the venom. PnTx1 was the first purified and sequenced neurotoxin from *P. nigriventer* venom (Diniz et al. 1990). The molecular target of this toxin remained obscure for many years. It was first suggested that PnTx1 acted on calcium channels, although the authors did not show any significant competition with toxins that act on calcium (ω-conotoxin GVIA) or sodium (PnTx2-6) channels (Santos et al. 1999). Instead, they showed a partial competition with fractions PhTx2 and PhTx3, predominantly active on sodium and calcium channels, respectively. Later, it was shown that PnTx1 competes with μ-conotoxin GIIIA, but not with tetrodotoxin, for the site 1 of voltage-gated sodium channels, and inhibits the neuronal sodium channel $Na_v1.2$ in a state-dependent manner, binding preferentially to the depolarized membrane (Fig. 3a, b) (Martin-Moutot et al. 2006). In the same work, the sample of PnTx1 was analyzed by MS and a contamination of nearly 5 % with PnTx3-3 (originally named Tx3-3) was demonstrated. PnTx3-3 is a well-characterized toxin that blocks high-voltage-activated (HVA) calcium

channels, but not low-voltage-activated (LVA) calcium channels (Leão et al. 2000). This contamination can explain the previous results that showed PnTx1 as a calcium channel inhibitor, partially competing with ω-conotoxin GVIA. However, subsequent experiments using a highly purified PnTx1 showed that this toxin (50 nM) partially displaced the calcium-antagonist dihydropyridine derivative ^{3}H-PN200-110 in GH3 cell membranes and at 1 μM the toxin inhibited 50 % of the calcium influx in GH3 cells (Santos et al. 2006). On the other hand, experiments with the recombinant PnTx1 (rPnTx1) showed no modification in the calcium currents of dorsal root ganglia (DRG) neurons at the concentration of 1 μM (Silva et al. 2012). The recombinant PnTx1 was expressed in a bacterial heterologous system (Diniz et al. 2006) and inhibited a variety of sodium channel isoforms expressed in *Xenopus laevis* oocytes (Fig 3c) and native sodium channels of DRG neurons (Silva et al. 2012). It also had neurotoxic effects in mice, similarly to the native toxin, when intracerebrally injected (Diniz et al. 2006). In addition, it showed a higher affinity to the target than the wild-type toxin (IC_{50} = 33.7 nM for rPnTx1 and 105 nM for PnTx1), probably due to the presence of three additional amino acids: alanine and methionine at the N-terminus and a glycine at the C-terminus. The recombinant toxin, rPnTx1, inhibited mammalian voltage-gated sodium channels, but not the insect isoforms, with the following order of potency: rNav1.2 > rNav1.7 ≈ rNav1.4 ≥ rNav1.3 > mNav1.6 ≥ hNav1.8 (Silva et al. 2012). No effect was observed with the cardiac isoform Nav1.5. Similarly, rPnTx1 was less effective on TTX-resistant sodium channels of DRG neurons (Silva et al. 2012). Curiously, both native and recombinant toxins were not able to block 100 % of the $Na_v1.2$ currents, reaching the maximal inhibition at 85 % and 83 %, respectively, at saturating conditions and a depolarized holding potential (-50 mV) (Silva et al. 2012). As the experiment was carried out with a homogeneous population of sodium channels, this observation suggests that rPnTx1 incompletely inhibits the channel conductance, as demonstrated for μ-conotoxins GIIIA and KIIIA.

There are three basic amino acids conserved in μ-conotoxin GIIIA that are putative binding residues to voltage-gated sodium channels: R13, K16, and K19. PnTx1 has basic residues in two correspondent positions, R35 (instead K16) and K39 (corresponding to K19). However, PnTx1 lacks the first arginine (R13) and has a glycine (G32) in the corresponding position. Arginine-13 was postulated to be a general residue for peptide toxins to interact with the receptor site of sodium channels. This residue is particularly critical, since it is postulated to compete with the guanidinium group of TTX or STX for the binding site 1. The toxin binding sites of sodium channels were classified based on their ability to compete with other toxins in binding experiments. Site 1 is the binding site of TTX and STX and toxins that can displace them, such as μ-conotoxin GIIIA. Since PnTx1 competes with μ-conotoxin GIIIA but not with TTX, it would be more appropriate to consider it as a macrosite 1, instead.

Based on all information to date, it was suggested that PnTx1 binds to the outer mouth of the channel pore and constrains the passage of sodium ions without its complete occlusion, allowing the passage of TTX, which can inhibit the residual current. Therefore, the likely mechanism of action of PnTx1 would be the reduction of the unitary conductance of the channel, similarly to what is seen for the mutated toxin μ-conotoxin GIIIA (R13Q) (Becker et al. 1992). Single-channel experiments in the presence of PnTx1 are necessary to confirm this hypothesis.

The Fraction PhTx2

The fraction PhTx2 is responsible for the prevailing excitatory symptoms of the venom, including salivation, lacrimation, priapism, convulsions, and spastic paralysis of the anterior and posterior extremities, besides being the most lethal fraction of all, showing an LD_{50} of 1.7 μg/kg mice when intracerebrally injected (Rezende et al. 1991). In addition to the effects in mice, PhTx2 was also shown to be highly toxic to insects (Figueiredo et al. 1995). Direct experiments with frog skeletal muscle using a modified loose-patch-clamp technique showed that fraction PhTx2 markedly altered voltage-gated sodium channel kinetics, inhibiting channel inactivation, partially blocking the channel, and shifting the steady-state

inactivation and the conductance activation toward hyperpolarized potentials. It did not affect the potassium current (Araújo et al. 1993).

PhTx2 increased the entry of Na^+ in cortical synaptosomes by inducing membrane depolarization and thus increasing neurotransmitter release, as observed for glutamate and acetylcholine, in a TTX-sensitive manner (Romano-Silva et al. 1993; Moura et al. 1998). This fraction also caused damage and myonecrosis of skeletal muscles and produced alterations in myelinated axons (Mattiello-Sverzut and Cruz-Hofling 2000). It was suggested that these alterations could be related to the osmotic imbalance that results from the opening of sodium channels induced by these toxins. Some works showing a possible mechanism that enables *Phoneutria* venom to cross the blood-brain barrier were published by this group and may be presented in another chapter of this book.

Four pure toxins were studied from PhTx2 fraction: PnTx2-1, PnTx2-5, PnTx2-6, and PnTx2-9 (Cordeiro et al. 1992). PnTx2-1, PnTx2-5, and PnTx2-6 exhibited high identity (77 %), but had only limited similarities with PnTx2-9, which was less toxic to mice (Cordeiro et al. 1992). Among these toxins, the first three reproduced the neurotoxic effects induced by PhTx2 upon i.c. injection and were toxic to mice. All three toxins caused pruritus, lacrimation, hypersalivation, sweating, and agitation followed by spastic paralysis of the anterior and posterior extremities, with LD_{50} at dose levels of 1.62 μg/mouse for PnTx2-1, 0.24 μg/mouse for PnTx2-5, and 0.79 μg/mouse for PnTx2-6. PnTx2-9, at the dose of 1.40 μg/mouse, was much less toxic to mice, causing only tail erection, pruritus, and a reduction in motility. Other toxins (PnTx2-2, PnTx2-3, PnTx2-4, PnTx2-7, and PnTx2-8) were also identified in this fraction. However, they presented low toxicity to mice (Cordeiro et al. 1992).

Among the polypeptides purified from PhTx2 fraction, PnTx2-5 and PnTx2-6 have high sequence homology, differing in five amino acid residues (89 % similarity) (Cordeiro et al. 1992). At the cellular level, these two toxins were shown to have complex effects on sodium channel kinetics, inhibiting its inactivation and shifting the activation voltage dependence toward negative potentials. However, they have significant differences in their interaction with neuronal Na_v channels. PnTx2-6 has an affinity six times higher than that of PnTx2-5 (Matavel et al. 2009). Both were identified as responsible for priapism, a painful and persistent penile erection, which can be observed following this spider bite in severe accidents. The mechanism involved in this effect is described below.

PnTx2-6 toxin exerted complex effects on frog skeletal muscle Na^+ currents, which were similar to the effects of the whole PhTx2 fraction: (1) slowed the time constant for fast inactivation, (2) shifted the sodium conductance voltage dependence and the steady-state inactivation to the hyperpolarized direction, and (3) decreased the peak of the current at almost all potentials (Matavel et al. 2002). Its calculated $K_{0.5}$ was 0.81 μM. These effects are similar to those of scorpion toxins, which bind to sites 3 and 4 of sodium channels. α-Scorpion toxins slow down sodium channel inactivation, and β-scorpion toxins shift the activation potential toward a hyperpolarized potential, respectively. However, binding experiments in brain synaptosomes showed that PnTx2-6 partially competes (59 %) with the typical α-scorpion toxin AaHII (from *Androctonus australis hector*) but not with β-scorpion toxin CssIV (from *Centruroides suffusus suffusus*). The decrease in the inactivation rate leads to a hyperexcitability and can account for the symptoms of *Phoneutria* poisoning (Matavel et al. 2009).

The cDNA sequence of PnTx2-6 was elucidated, showing that it is expressed as a prepropeptide. After this, it became of great interest to express this toxin in heterologous system, since its most prominent effect is a spontaneous penile erection (priapism). In a preliminary study, recombinant toxins expressed in prokaryotes were not able to modify sodium channel currents (Matavel et al. 2002). More recently, PnTx2-6 was expressed in *E. coli* using another approach, and the recombinant toxin was able to produce erection, as was the native toxin (Torres et al. 2010) (see below). This result suggests that it is also active in sodium channels, but this remains to be confirmed.
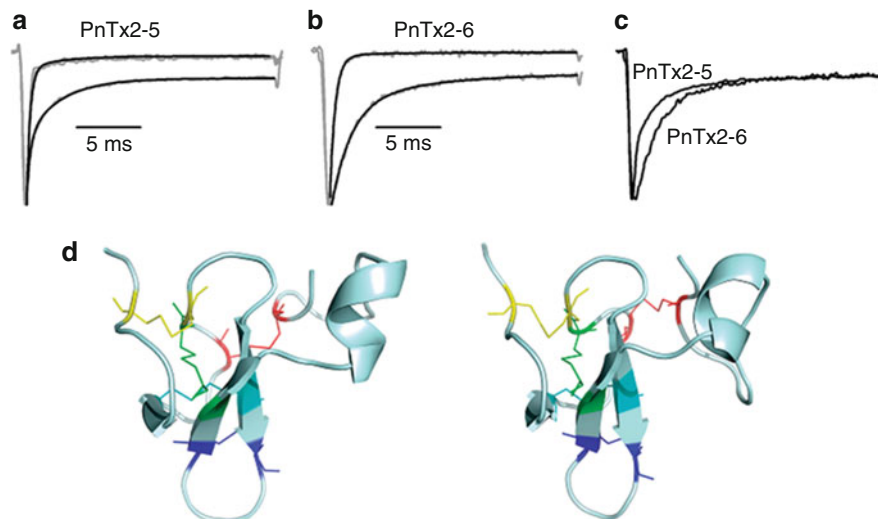
Fig. 4 Electrophysiological effects and molecular modeling of PnTx2-5 and PnTx2-6, both from PhTx2 fraction. Superimposed normalized paired records obtained at 0 mV in the absence or presence of 400 nM PnTx2-5 (**a**) or 100 nM PnTx2-6 (**b**). The records in the presence of PnTx2-5 and PnTx2-6 were superimposed to emphasize the difference in their effects (**c**). (**d**) Cartoon representation of the three-dimensional theoretical models of PnTx2-5 (*left*) and PnTx2-6 (*right*). Disulfide bridges Cys3-Cys17, Cys10-Cys23, Cys14-Cys46, Cys16-Cys31, and Cys25-Cys29 are colored in *yellow*, *turquoise*, *red*, *green*, and *dark blue*, respectively. The amino and carboxy termini are located on the *left* and *right* of the molecule, respectively (Credit: Matavel et al. 2009 © 2009 Biochemistry)

The activity of PnTx2-6 on neuronal sodium channels was compared with that of PnTx2-5, using whole-cell patch clamp (Fig. 4a–c) (Matavel et al. 2009). Both toxins had similar effects on sodium channels, markedly delaying the fast inactivation kinetics and shifting the steady-state inactivation and the conductance activation toward hyperpolarized potentials, as seen in muscle cells. However, PnTx2-6 had more pronounced effects than PnTx2-5, since it shifted the steady-state inactivation potential more potently than PnTx2-5 (−20 mV for PnTx2-6 and −14 mV for PnTx2-5). In addition, PnTx2-6 showed higher affinity and was more effective in slowing the inactivation kinetics than PnTx2-5 ($K_{0.5}$ equal to 140 and 23 nM and maximal effects of 58 and 71 %, for PnTx2-5 and PnTx2-6, respectively) (Matavel et al. 2009).

Phoneutria nigriventer spider toxins do not show similarity with the primary sequence of other known toxins. Moreover, they have a high proportion of disulfide bridges, which hampers their structural analysis: around 20 % of PhTx2 toxins are cysteine, making the structure-function studies very difficult. Fortunately, the structural model of PnTx2-5 and PnTx2-6, which have five disulfide bridges, was achieved (Fig. 4d) (Matavel et al. 2009). Four cysteine bridges were deduced by analogy with the ICK motif of spider cysteine-rich toxins, and the fifth pair was inferred. By setting the disulfide bridges, the conformation of the peptide loops was predicted by Molecular Dynamics software. The three-dimensional model suggested the formation of a short triple-strand antiparallel β-sheet between Gln8-Cys10, Gly21-Cys25, and Cys29-Gln33. In addition, a short alpha-helix, formed from residues 35 (Asn in PnTx2-5 and Tyr in PnTx2-6) to Ala39, was predicted. The model also predicted that the tryptophan residues were exposed on the surface of the toxin, as predicted by fluorescent experiments. The small number of α-helix in these structures contrasts with α-scorpion toxins but was confirmed by circular dichroism analyses. A hydrophobic core surrounded by positive residues was identified, and it was similar to those seen in α-scorpion toxins, consistent with the hypothesis that the active surface of gating modifier toxins is an arrangement of hydrophobic and basic residues (Matavel et al. 2009).

PnTx2-5 and PnTx2-6 Potentiate Erectile Function

As described above, priapism, a painful and persistent involuntary penile erection, is one of the symptoms that may be observed following severe accidents caused by *P. nigriventer*. PnTx2-5 and PnTx2-6 were identified as being directly responsible for priapism (Nunes et al. 2008; Yonamine et al. 2004), and, since then, many studies have been published trying to elucidate the mode of action of these toxins in the erection mechanism.

The mechanism of action through which PnTx2-5 and PnTx2-6 promote cavernosal relaxation and enhance erectile function is not completely clarified (Yonamine et al. 2004; Nunes et al. 2010, 2012a). However, the results strongly suggest that it is *via* nitric oxide (NO)/cyclic GMP pathway. Upon sexual stimulation, NO release evokes penile smooth muscle relaxation, increasing blood flow and intracavernosal pressure, leading to penile erection. NO, released from penile endothelial cells or nitrergic nerves, is the main mediator involved in erectile function, and PnTx2-6 injection was shown to increase NO release in *corpus cavernosum* tissue (Nunes et al. 2008). In addition, PnTx2-5, when intraperitoneally injected in male mice, caused penile erection, hypersalivation, severe respiratory distress, and death. These effects are partially prevented by L-NAME and completely abolished by the nNOS-selective inhibitor 7-nitroindazole, suggesting that nNOS is the major player in this effect (Yonamine et al. 2004).

Functional experiments using cavernosal tissue from knockout mice to neuronal nitric oxide synthase or endothelial nitric oxide synthase (nNOS−/− and eNOS−/−) demonstrated that the relaxation promoted by PnTx2-6 depends on nNOS but not on eNOS (Nunes et al. 2012b). In addition, φ-conotoxin GVIA, an inhibitor of N-type calcium channels ($Ca_v2.2$), blocks the relaxation induced by PnTx2-6. Thus, considering that PnTx2-6 slows down the inactivation of sodium channels, the toxin was suggested to increase the amount of intracellular Ca^{+2}, which activates nNOS and consequently increases NO availability.

Additionally, it was shown that the cavernosal relaxation provoked by PnTx2-6 is not dependent on phosphodiesterase-5 (PDE5) inhibition (Nunes et al. 2012b). Strengthening this idea, a gene expression study in mice erectile tissue showed two overexpressed genes that might be involved in the priapism induced by PnTx2-6 (Villanova et al. 2009). One of these genes directly activates the NO/cGMP pathway. PnTx2-5 has been less investigated, compared to PnTx2-6, but the results suggest that this toxin could also be involved in penile neuronal depolarization (Yonamine et al. 2004). Both toxins, PhTx2-5 and PhTx2-6, represent interesting pharmacological tools to study erectile dysfunction (ED).

The use of these toxins in the treatment of erectile dysfunction shows obvious difficulties: their side effects. Therefore, experiments have been performed aiming at minimizing the toxic symptoms while preserving the pro-erectile effect. PnTx2-6 was cloned and functionally expressed, providing effects on erectile function that were similar to the native toxin (Torres et al. 2010). Ongoing experiments introducing point mutations in the sequence of this toxin have been performed in order to investigate the specific amino acid residues that have an essential role in this action. Preliminary results seem to confirm the role predicted by bioinformatics. In addition, a smaller peptide, designed after the study of the structure of PnTx2-6, was synthesized and seems to be a promising tool to study and treat erectile dysfunction (Nunes et al. 2013). Studies are in progress to investigate the mechanism of action, efficacy, and toxicity of this peptide, as well as its potential use as a pro-erectile drug model.

At present, PDE5 inhibitors are the most common and successful drugs used to treat ED. The problem is that a broad range of patients (30–35 %) failed to respond to these drugs, clearly indicating the need of alternative treatments. It is estimated that 70 % of ED cases are due to vasculogenic problems. Interestingly, PnTx2-6 was able to reverse, at least in part, the erectile dysfunction in diabetic, elderly, and hypertensive animals (rat or mice). This toxin seems to be a promising model for the design of new drugs for the treatment of erectile dysfunction.

The Fraction PhTx3

PhTx3 fraction induces a progressive flaccid paralysis of all legs in experimental animals and acts on calcium and potassium channels (Rezende et al. 1991; Prado et al. 1996; Kushmerick et al. 1999). From the fraction PhTx3, six toxins, named PnTx3-1 to PnTx3-6 (formerly Tx3-1 to Tx3-6), were isolated and presented diverse pharmacological properties and neurological effects. Their primary sequences were total or partially determined (see Table 1). Later, these toxins were cloned and their sequences were confirmed or corrected (Kalapothakis et al. 1998b; Carneiro et al. 2003; Cardoso et al. 2003). All toxins identified by molecular biology techniques are encoded as a precursor peptide composed of a signal peptide, an intervening propeptide, and the mature toxin. Additionally, a C-terminal peptide may be present or not (Table 1). Several isoforms identified by molecular cloning have never been found in the crude venom. Some toxins were functionally expressed in heterologous systems, as described below (Carneiro et al. 2003; Souza et al. 2008). For the sake of standardization, in the present text, the toxin nomenclature proposed by the group of Dr. Beirão is used (Matavel et al. 2002), and synonymous names can be found in Table 1.

In the first experiments, it was observed that a nondialyzable component in *P. nigriventer* venom (PNV) was able to contract vascular smooth muscle, suggesting a peptide component. This contraction was not antagonized by either TTX or phenoxybenzamine, excluding the participation of voltage-gated sodium channels or catecholamine released from autonomic nerve endings, respectively (Antunes et al. 1993). PNV caused a biphasic response on blood pressure, characterized by a transient hypotension followed by a long-lasting hypertension when injected intravenously (Costa et al. 1996). Voltage-gated L-type calcium channel antagonists or extracellular calcium chelators reduced the ability of PNV to induce vascular contraction (Costa et al. 1996; Teixeira et al. 2004). In addition, it was observed that the calcium released from the sarcoplasmic reticulum did not play a role in PNV-mediated contractile effects (Teixeira et al. 2004). Therefore, it was concluded that the effect on vascular contraction could be indirect. The molecular mechanism involved in this effect still remains to be elucidated. When the fraction PhTx3 was isolated, a great effort was made to identify its molecular target. PhTx3 decreased the release of $[H^3]$-ACh (tritiated acetylcholine) in the brain and more effectively in the myenteric plexus, suggesting that its target is linked to the process of ACh release in the brain and in the autonomic nervous system and could probably be a calcium channel (Gomez et al. 1995). In support of this idea, PhTx3 was shown to abolish calcium-dependent glutamate release in rat brain cortical synaptosomes, but not calcium-independent exocytosis. PhTx3 blocked 50 % of glutamate release, similarly to the extracellular calcium chelator EGTA, without affecting the glutamate release triggered by intracellular calcium stocks, thus suggesting that these toxins interfere in the calcium influx in synaptosomes (Prado et al. 1996).

PhTx3 presents the most heterogeneous toxins, with low sequence identity, what explains the different sets of activities of this fraction. For example, PnTx3-1, PnTx3-5, and PnTx3-6 induce paralysis of the posterior limbs. PnTx3-2 induces immediate clockwise gyration and flaccid paralysis. PnTx3-3 and PnTx3-4 are the most toxic: at 5 μg/mouse they reproduce the fast flaccid paralysis followed by death observed for the whole PhTx3 fraction (Cordeiro et al. 1993). Besides the paralytic effect, PnTx3-6 has an analgesic action in rodent models of pain (Souza et al. 2008). It was shown that PnTx3-1 reduces calcium oscillation and pacemaker activity in GH3 cells by blocking potassium currents (Kushmerick et al. 1999).

Whole-cell patch clamp experiments showed that PnTx3-1 reversibly inhibits type-A potassium current (IA) (Fig. 5a) but not other potassium currents (i.e., delayed rectifying, inward rectifying, and large conductance calcium sensitive) or calcium channels (T and L type) in neuroendocrine GH3cells. The inhibition of IA favors cell depolarization and calcium channel activation, increasing the frequency of calcium oscillation (Kushmerick et al. 1999). In the heart, PnTx3-1 had an antiarrhythmogenic effect, decreasing the ACh-mediated heart rate by doubling the frequency of spontaneous miniature end plate potential (Almeida et al. 2011) protecting ischemia/reperfusion heart against arrhythmia. It is important to
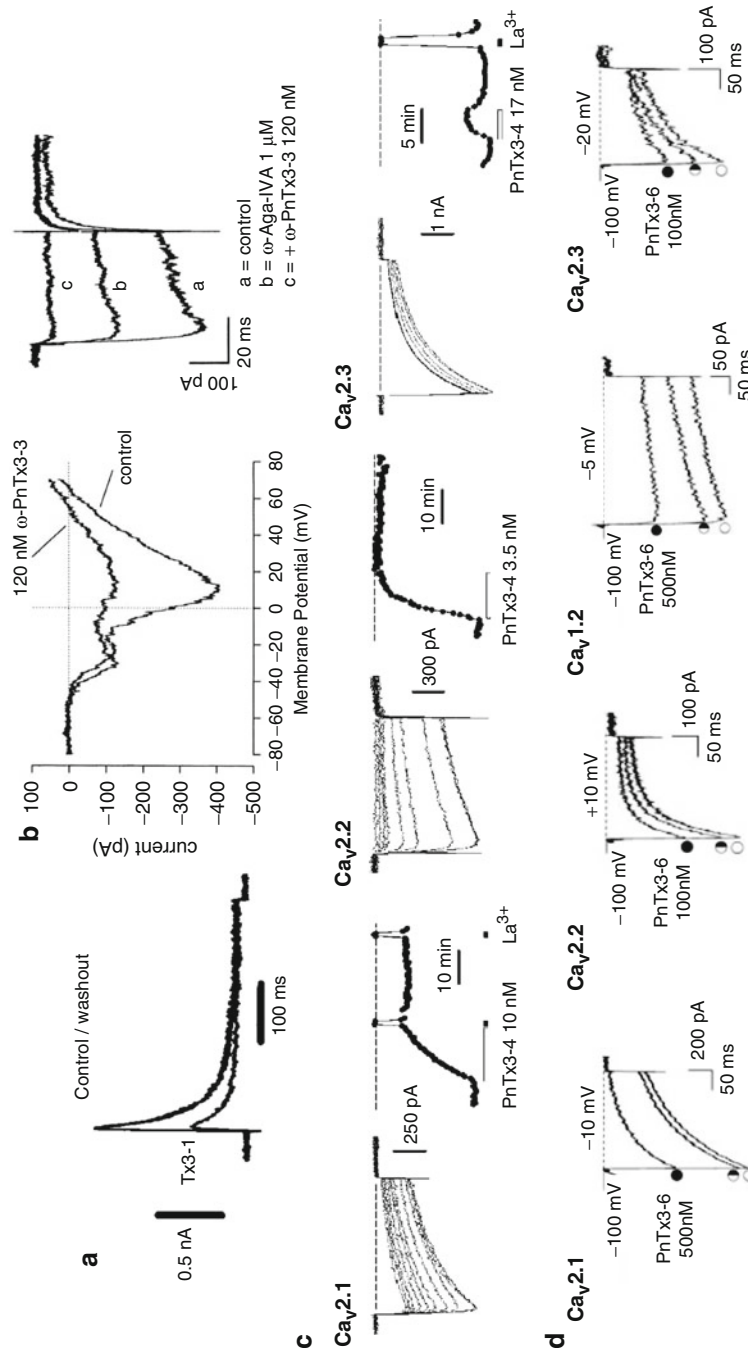
Fig. 5 PhTx3 fraction blocks potassium and calcium channels. (**a**) Toxin PnTx3-1 (4.8 μM) blocks I_A potassium current, in a whole-cell voltage-clamp experiment. GH3 cells were kept at a holding potential of −80 mV and depolarized to +30 mV after a −100 mV prepulse for 200 ms to remove inactivation (Kushmerick et al. 1999). (**b**) PnTx3-3 blocked calcium channels in cerebellar granule neurons. *Left panel:* PnTx3-3 (120 nM) blocked 74 % of HVA but no LVA calcium channels. The cells were kept at a holding potential of −80 mV and the currents were acquired by 170 ms ramps of voltage from −80 to +70 mV. *Right panel:* representative current showing the inhibition of R-type calcium current before (*a*) and after (*b*) the perfusion of 1 μM ω-Aga-IVA (to block P/Q component) or ω-Aga-IVA plus PnTx3-3 120 nM (*c*) (Leao et al. 2000). (**c**) *Left panel:* superimposed currents before and during application of PnTx3-4 on BHK cells transfected with calcium channels as marked. Currents were incited by pulses of +10 mV from a holding potential of −90 mV. *Right panel:* calcium channel peaks before, during, and after toxin perfusion (dos Santos et al. 2002). (**d**) Trace currents of HVA calcium channels transfected into HEK cells, before (*open circle*) and after perfusion of PnTx3-6 (*filled circles*) and after washout (*semi-filled circles*). Cells were kept at −100 mV and stimulated with depolarization steps to −10 mV ($Ca_v2.1$), +10 mV ($Ca_v2.2$), −5 mV ($Ca_v1.2$), or −20 mV ($Ca_v2.3$) (Vieira et al. 2005). (Credit: (**a**) Kushmerick et al. 1999 © 1999 John Wiley and Sons. (**b**) Leao et al. 2000 © 2000 Elsevier. (**c**) Santos et al. 2002 © 2002 American Society for Biochemistry and Molecular Biology. (**d**) Vieira et al. 2005 © 2005 American Society for Pharmacology and Experimental Therapeutics)

highlight that the recombinant toxin heterologously expressed in bacteria had similar effects to the native PnTx3-1 (Carneiro et al. 2003; Almeida et al. 2011).

Electrophysiology experiments showed that PnTx3-2 (280nM) blocked 50 % of the L-type calcium current in 63 s, and this effect was not reversible after 2 min washout (Kalapothakis et al. 1998b). Since PnTx3-2 did not modify KCl-evoked glutamate release or the rise of intracellular calcium in synaptosomes and both effects were sensitive to 1 μM ω-CTX-MVIIC (Prado et al. 1996), a significant blockage of N- or P/Q-type calcium channels was excluded. PnTx3-3 was identified as the most effective *Phoneutria* toxin to inhibit calcium-dependent glutamate release, and it reproduced the major effects of the whole PhTx3 fraction (Prado et al. 1996; Guatimosim et al. 1997). PnTx3-3 blocked high-voltage-activated (HVA) (Fig. 5b), but not low-voltage-activated (LVA) calcium channels, with the following order of efficacy: P/Q ≥ R > L > N type (Leão et al. 2000). Another potent *P. nigriventer* toxin that affects neurotransmission is PnTx3-4. It was postulated that PnTx3-4 affects the neurotransmission by blocking presynaptic calcium channels associated with exocytosis in mammals, as well as in lower vertebrates and arthropods (Troncone et al. 2003). Previous reports showed complete blockage of HVA calcium channels in the sensory neurons of dorsal root ganglia by PnTx3-4, but not of LVA channels, highlighting the importance of L- and N-type calcium channels in pancreatic β-cells and the cell body of cultured neurons, using electrophysiology (Cassola et al. 1998). Moreover, the toxin impaired the influx of ^{45}Ca in the nerve ending, suggesting an effect on P/Q calcium channels (Miranda et al. 2001). PnTx3-4 produced a potent and almost irreversible inhibition of Ca^{2+} currents of HEK293 cells heterologously expressing $Ca_v2.1$ (P/Q type) or $Ca_v2.2$ (N type), whereas it partially and reversibly inhibited $Ca_v2.3$ (R type) currents (Fig. 5c) (Santos et al. 2002). Furthermore, this toxin blocked potassium-induced (Reis et al. 1999) and capsaicin-induced (Gonçalves et al. 2011) glutamate release from rat brain synaptosomes. Incubation of synaptosomes with 8 nM of PnTx3-4 for 30 min in the presence of the calcium chelator EGTA blocked calcium-independent glutamate release, contrasting with the observation that the fraction PhTx3 did not inhibit calcium-independent components of glutamate release (Prado et al. 1996). It also allosterically blocked over 60 % of L-[^{3}H] glutamate uptake (Reis et al. 1999), in addition to calcium-dependent glutamate release (Gonçalves et al. 2011). This apparently conflicting result is probably due to the low proportion of PnTx3-4 in the fraction. In summary, PnTx3-4 inhibited neurotransmission by blocking presynaptic calcium channels. It is noteworthy that, in the excitatory glutamatergic transmission, PnTx3-4 also blocks calcium-independent neurotransmission and neurotransmitter uptake, which can be a valuable tool to avoid neuronal intoxication by glutamate and neuronal death in ischemic episodes.

When compared with other calcium channel blockers, such as ω-conotoxin GVIA and ω-conotoxin MVIIC, the fraction PhTx3 displayed a greater neuroprotection from ischemic injury induced by oxygen deprivation and low glucose in rat hippocampus or retina. Only 18 % of the cells in hippocampal CA1 region died compared to 68 % and 54 % for each conotoxin, respectively (Pinheiro et al. 2006).

In the retina, the fraction PhTx3 protected 79.5 % of the cells from the injury (Agostini et al. 2011). The search for toxins in this fraction that was responsible for the protective effect led to the identification of PnTx3-3 and PnTx3-4 as the main candidates. Both toxins were effective in preventing cell death after ischemic injury, with even better protective effects than the whole fraction. Furthermore, PnTx3-4 showed superior protection when compared to PnTx3-3, the fraction PhTx3, or the calcium channel blockers ω-conotoxin GVIA and ω-conotoxin MVIIC (Agostini et al. 2011; Pinheiro et al. 2009). To date, no pharmacological data has been published concerning the toxin PnTx3-5.

Electrophysiology experiments indicated that the toxin PnTx3-6 (also called Phα1b) reversibly inhibited a broad range of HVA calcium channels heterologously expressed in either HEK293 mammal cell or N18 neuroblastoma cell lines, with the following order of potency: N-(α_{1B}/$Ca_v2.2$) > R-(α_{1E}/$Ca_v2.3$) > P/Q-(α_{1A}/$Ca_v2.1$) > L-(α_{1C}/$Ca_v1.2$) (Fig. 5d), with IC_{50} values of 122, 136, 263, and 607 nM, respectively. However, the toxin was ineffective to LVA T-type calcium channels (α_{1G}/$Ca_v3.1$) (Vieira et al. 2005).

PnTx3-6 inhibited potassium-induced calcium-dependent glutamate release by blocking voltage-gated calcium channels, but it was not able to modify the calcium-independent process. The calculated IC_{50} for the blockage of neurotransmission release (74.4 nM) was significantly higher than that for the inhibition of intracellular calcium concentration (9.5 nM) and seems to involve different types of calcium channels (Vieira et al. 2005). Because glutamate is a pro-nociceptive neurotransmitter and PnTx3-6 was more effective and potent to block calcium channels than the refractory pain relief ω-conotoxin MVIIA (commercially called ziconotide), the analgesic proprieties of both, native and recombinant PnTx3-6, were tested. They were shown to be efficient for the treatment of persistent pathological pain (i.e., neuropathic and inflammatory pain) mediated by either glutamate release or capsaicin-induced calcium influx, but not involving capsaicin receptor inhibition (Souza et al. 2008; Castro-Junior et al. 2013).

The Fraction PhTx4

PhTx4 is referred to as the insecticidal fraction, due to its high toxicity/lethality toward insects and minor toxicity when injected in mice. This fraction causes hyperactivity (cramps, quivering, jerking of the limbs, and violent trembling of the body and the legs), leading to muscle fatigue and therefore causing paralysis in insects of the orders Diptera and Dictyoptera (Figueiredo et al. 1995). It is suggested that PhTx4 acts on the glutamatergic system of both insects and mammals. Three excitatory insecticidal toxins PnTx4(6-1), PnTx4(5-5), and PnTx4-3 (Table 1) were purified to homogeneity from PhTx4 (Figueiredo et al. 1995, 2001; Oliveira et al. 2003). These insecticidal toxins have 64–97 % sequence identity. PnTx4(6-1) and PnTx4(5-5), formerly called Tx4(6-1) and Tx4(5-5), respectively, act on insect sodium channels (De Lima et al. 2002, 2007; Stankiewicz et al. 2005). Despite their apparent lack of toxicity to mammals, they have been shown to inhibit glutamate uptake in the mammalian central nervous system (Mafra et al. 1999; Oliveira et al. 2003). It was shown that PnTx4(5-5) inhibits NMDA ionotropic glutamate receptor in rat brain neurons (Figueiredo et al. 2001). A noteworthy feature of the sequence of these toxins is the presence of Cys as the amino- and carboxyl-terminal residue (Table 1). The most active toxin of this fraction, studied so far, is the anti-insect neurotoxin PnTx4(6-1). This basic, 48-amino acid polypeptide with 5 disulfide bridges is toxic to houseflies (*Musca domestica*), cockroaches (*Periplaneta americana*), and crickets (*Acheta domesticus*) (Figueiredo et al. 1995). A detailed mode of action of PnTx4(6-1) and PnTx4(5-5) has been tested on cockroach (*Periplaneta americana*), axonal preparations using the double-oil-gap method (De Lima et al. 2002), and on neurosecretory dorsal unpaired median (DUM) neurons in terminal ganglion of nerve cord using the microelectrode technique. Both toxins (10^{-6} M) induced evoked action potential prolongation in axonal preparations. This effect was stronger after PnTx4(6-1) administration than after PnTx4(5-5) (Fig. 6a). When the axonal membrane was artificially hyperpolarized from −60 to −70 mV, low-level *plateau* action potentials appeared with repetitive activity at the end (Fig. 6b). With a higher PnTx4(6-1) concentration (3×10^{-6} M), the *plateau* action potentials (20–30 ms) were recorded also at the potential of −60 mV. In the presence of PnTx4(5-5), the *plateau* action potentials were observed only after co-application of a potassium channel blocker. This confirms that both toxins are active on sodium channels and indicates that their effect was balanced by strong axonal potassium conductance. Post-application of an anti-insect scorpion toxin, LqhαIT (10^{-6} M), after the administration of both *Phoneutria* toxins transformed the slightly prolonged action potentials into *plateau* ones or increased their duration in more than ten times, in experiments where a potassium channel blocker was applied together with the toxins. Tests performed in a voltage-clamp configuration showed that PnTx4(6-1) and PnTx4(5-5) prolonged the axonal sodium current in a manner similar to toxins binding to site 3 of sodium channels. In the presence of PnTx4(6-1), a late current at the end of a 5 ms depolarizing pulse (from −60 to −10 mV) was evident, but it never exceeded 30 % of the sodium current peak. After PnTx4(5-5) (at the same concentration), a late current was much smaller at the corresponding time (Fig. 6c). This indicates that PnTx4(5-5) is less efficient in inhibiting sodium channel inactivation
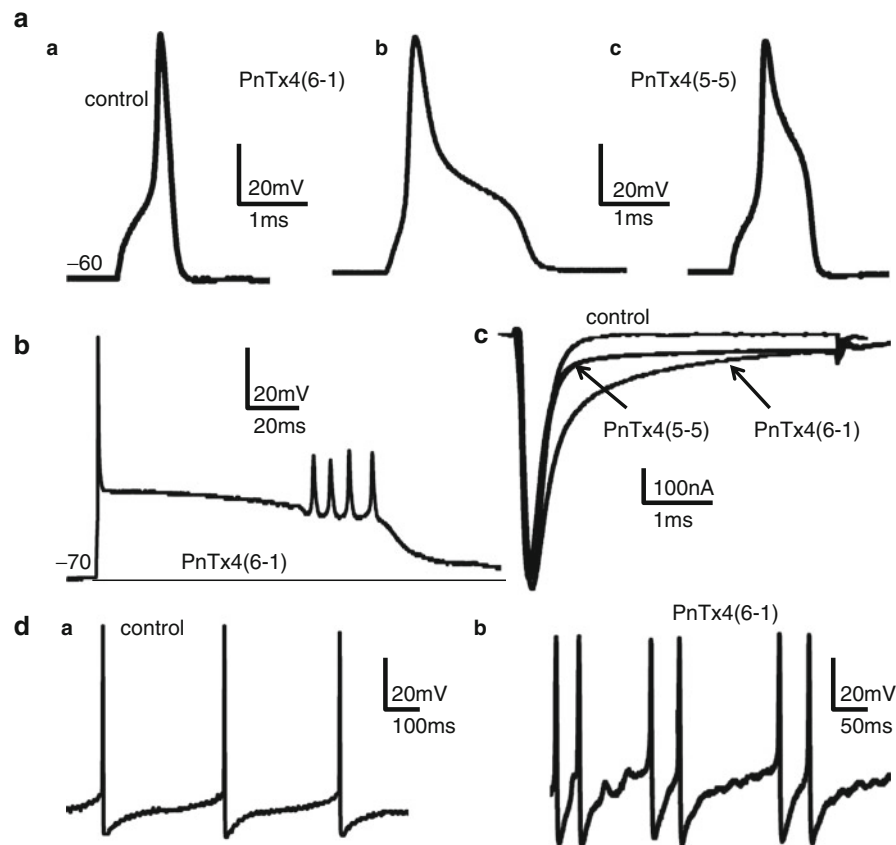
Fig. 6 Effects of toxins PnTx4(6-1) and PnTx4(5-5) on cockroach isolated giant axons and DUM neurons (studied using the double-oil-gap method on axons and the microelectrode technique on DUM neurons in situ, in terminal abdominal ganglion of the nerve cord). (**a**) Evoked action potential (from resting potential -60 mV) in control conditions and in 15 min of PnTx4(6-1) and PnTx4(5-5) (10^{-6} M) action. Note that the prolongation of the action potential is larger after PnTx4(6-1) than after PnTx4(5-5) administration. (**b**) *Plateau* action potential with short repetitive activity at the end, recorded in the presence of PnTx4(6-1) -3×10^{-6} M when the axon was artificially hyperpolarized to -70 mV. (**c**) Control- and toxin (10^{-6} M)-modified axonal sodium current induced by membrane depolarization from -60 to -10 mV. Note that the presence of late currents is larger after PnTx4(6-1) than after PnTx4(5-5). (**d**) Effect of PnTx4(6-1), 10^{-5} M, on the spontaneous activity of DUM neurosecretory neuron. Note the transformation of spontaneous beating firing activity into irregular, higher frequency discharges

than PnTx4(6-1), what has been shown in experiments on action potentials. Post-application of LqhαIT (10^{-6} M) increased the late sodium current up to 90 % (not shown).

Corresponding tests in voltage clamp were performed in mammalian (rat brain rBIIA and rat skeletal muscle rSkM1) sodium channels reconstructed in *Xenopus* oocytes. No effect was observed even at 10^{-5} M PnTx4(6-1) concentration (De Lima et al. 2002).

On DUM cells, PnTx4(6-1) (3×10^{-6} M) changed the regular spontaneous firing pattern of action potential generation into an irregular activity. A group of 2-3 action potentials, generated with high frequency, was separated by a period of neuron silence of several milliseconds (Fig. 6d). A similar but much more expressed modification was observed after LqhαIT (10^{-6} M) administration (Stankiewicz et al. 2012). The results obtained with electrophysiological experiments suggested that PnTx4(6-1) is active on site 3 of sodium channels. This was confirmed with binding assays (De Lima et al. 2002). Bom IV, an alpha-like toxin that binds to receptor site 3 on insect sodium channels, was displaced by PnTx4(6-1). The affinity of PnTx4(6-1) to sodium channels is much lower than that of LqhαIT
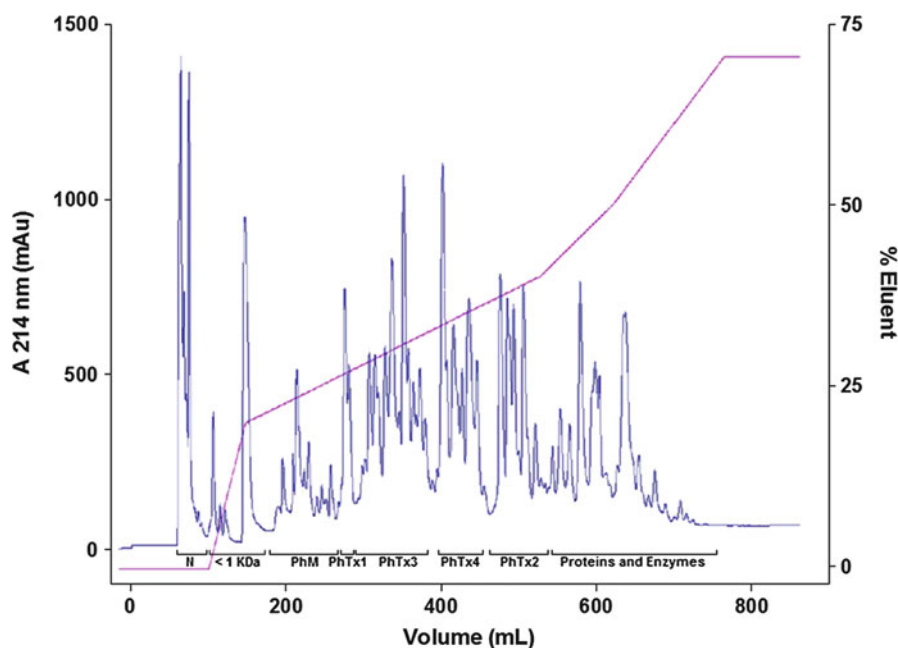
Fig. 7 Elution profile of reversed-phase (RP-HPLC) fractionation of *Phoneutria nigriventer* venom. Venom sample was loaded on a preparative Vydac C4 column (2.2 × 25 cm). Column was eluted at a flow rate of 5 mL/min, monitored at 214 nm, under a gradient of acetonitrile (Richardson et al. 2006). The solid bars indicate the eluted fractions and (N) nigriventrine

($K_{0.5} = 25$ nM and $K_{0.5} = 0.09$ nM, respectively). The mode of action of PnTx4(6-1) and PnTx4(5-5) is highly similar to other spider toxins.

New Bioactive Molecules

Richardson et al. (2006) improved the purification method of *P. nigriventer* venom components: instead of the conventional gel filtration fractionation used in the previous studies (Rezende et al. 1991; Cordeiro et al. 1992, 1993; Figueiredo et al. 1995), a preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) was employed as the initial step. This improved approach resolved the venom in 55 proteic peaks. Figure 7 shows details of *P. nigriventer* venom RP-HPLC (for a review, see Richardson et al. 2006). This new method is time saving, since it reduces the time and chromatography steps to get to the previously isolated toxins (from PhTx1 to PhTx4 fractions).

This new purification procedure also contributed for the characterization of the smooth muscle-active fraction PhM (Pimenta et al. 2005). Like the other toxic fractions, it was demonstrated that PhM consists of a pool of similar isoforms of smaller (<2 kDa) peptides. The amino acid sequences of 15 of these isoforms (Table 2) were determined by mass spectrometry (Pimenta et al. 2005). These muscle-active peptides contain 7–14 amino acid residues and have a common scaffold composed of basic and acid amino acids (PyrKKDKKDx), where x can be either K or R. Since all of these molecules are structurally related to the tachykinin family of neurohormone peptides, which possess N-terminal pyroglutamate residues, they were named *Phoneutria nigriventer* tachykinin peptides (PnTkP), as shown in Table 2. A diversity of posttranslational modifications such as proteolysis, C-terminal amidation, and cyclization are other features of PnTkP group that, combined, generate additional isoforms (Pimenta et al. 2005).

The new procedure also led to the discovery of other two new structural families of small 4 kDa and 3.5 kDa peptides (Richardson et al. 2006) in the armed spider venom. The family of 4 kDa toxins targets Ca^{2+} channels (Lúcio et al. 2008). Two different isoforms (PnTx27C4 and PnTx26AN0C3) were isolated and characterized (Table 1) and showed 92 % sequence identity. These toxins, like the PhTx3 fraction,

Table 2 Alignment of the amino acid sequences of tachykinin family from *P. nigriventer* venom

Name	Sequence	Length	MM	Accession number[a]
PnTkP-I	QKKDKKD————————	7	871.70	P86298
PnTkP-II	QKKDKKDK———————	8	999.88	P86299
PnTkP-IIi	QKKDKKDR———————	8	1,027.95	P86300
PnTkP-IV	QKKDKKDKF—————	9	1,146.86	P86301
PnTkP-IV	QKKDKKDRF—————	9	1,174.80	P86302
PnTkP-VI	—QKKDRFLGLM-[b]	10	1,217.00	P86303
PnTkP-VII	—QKKDRFLGLF-[b]	10	1,232.79	P86304
PnTkP-VIII	QKKDKKDRFY—————	10	1,337.89	P86305
PnTkP-IX	-QKKDKDRFYGLM-[b]	12	1,509.87	P86306
PnTkP-X	QKKDKKDKFYGLM-[b]	13	1,610.12	P86307
PnTkP-XI	QKNDKKDRFYGLM-[b]	13	1,623.92	P86308
PnTkP-XII	QKKDKKDKFYGLF-[b]	13	1,626.36	P86309
PnTkP-XIII	QKKDKKDRFYGLM-[b]	13	1,637.93	P86310
PnTkP-XIV	QKKDKKDRFYGLF-[b]	13	1,653.83	P86311
PnTkP-XV	QKKDKKDRFPNGLV[b]	14	1,653.94	P86297

N-terminal = pyroglutamic acid from the glutamine shown as Q
[a]SWISS-PROT
[b]C-terminally amidated peptides

```
          10         20         30         40         50         60
       IVYGTVTTPG KYPWMVSIHE RVKDVMKQAC GGAILNENWI VTAAHCFDQP IILKDYEVYV

          70         80         90        100        110        120
       GIVSWLHKNA PTVQKFQLSK IIIHDKYVKD GFANDIALIK TATPIDIKGS KYGVNGICFP

         130        140        150        160        170        180
       SGATDPSGEA TVIGWGMIRG GGPISAELRQ VTLPLVPWQK CKQIYGHPDS EFEYIQVVPS

         190        200        210        220        230        240
       MLCAGGNGKD ACQFDSGGPL FQYDKKGVAT LIGTVANGAD CAYAHYPGMY MKVSAFRSWM

       DKVMT
```

Fig. 8 Amino acid sequence of PN47 serine protease from *P. nigriventer* venom (accession number of the sequence deposited at SWISS-PROT: P84033)

produce spastic paralysis and death in mice when intracerebrally injected. Concerning the smaller 3.5 kDa peptides, three very similar peptides (PnTx13C3, PnTx24An0C3, and PnTx24An0C4) were obtained in pure state; they show 79 % amino acid sequence identity (Table 1). Similar to PhTx4 toxins, 3.5 kDa toxins are very toxic to houseflies and induce no observable toxic effects in mice by i.c. injection (Richardson et al. 2006).

Two proteases were purified from the later eluted peaks of RP-HPLC (Fig. 7): PN44 (proteinase PN44) and PN47 (proteinase PN47). The complete amino acid sequence of the PN47 (Fig. 8) and the N-terminal sequence of PN44 demonstrated that both are serine proteases belonging to the peptidase S1 family. It has been suggested that the endogenous proteolytic enzymes in the venom may be responsible for the posttranslational modification observed in some of the venom components.
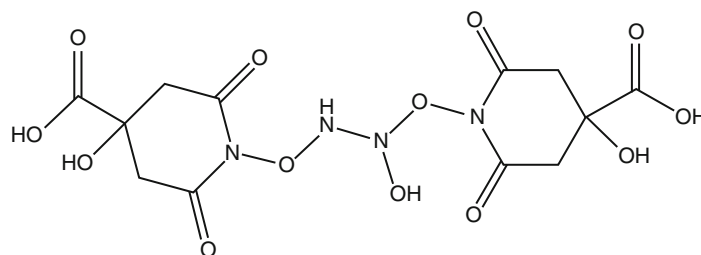
Fig. 9 Proposed structure of nigriventrine (Gomes et al. 2011)

A Nonprotein Neurotoxin: Nigriventrine

In addition, a novel non-proteic low molecular mass neurotoxin named nigriventrine (Gomes et al. 2011) was isolated from the hydrophilic fractions obtained from *P. nigriventer* venom by RP-HPLC (Fig. 7). Nigriventrine, a piperidine derivative, has neuroactive properties and causes convulsion when injected in mice, intracerebrally or peripherally (i.v.). Its structural elucidation was carried out with ESI-MS, HRESI-MS/MS, ^{1}H-NMR, and ^{13}C-NMR spectroscopy, and it was characterized as hydroxyl-hydrazyl-dioxopiperidine (Fig. 9) with a molecular mass of 422.0631 Da (Gomes et al. 2011).

General Characteristics of the Sequence and Structure of Toxins from *P. nigriventer*

Employing molecular biology strategies, several investigators isolated genes encoding *Phoneutria nigriventer* toxins (Diniz et al. 1993; Kalapothakis et al. 1998a,b; Kushmerick et al. 1999; Matavel et al. 2002). Some *P. nigriventer* toxins, such as PnTx1 and PnTx2-6, were cloned and expressed to produce recombinant toxins (Diniz et al. 2006; Torres et al. 2010). Both biochemical characterization and cDNA analyses showed that (see Table 1) (i) *P. nigriventer* venom contains several families of homologous polypeptides that differ in their effects and/or targets; (ii) *P. nigriventer* venom is constituted by single-chain toxins with variable sequence length and predominantly cationic (basic) character (pI > 7); (iii) with exception of the tachykinin-like peptides (Table 2), its toxins exhibit high content of Cys residues, all cross-linked by disulfide bonds, thus conferring great stability to these molecules; (iv) cysteine positions are highly conserved among toxins belonging to the same family; and (v) its toxins have a similar structural mRNA organization, encoded as prepropeptides that undergo posttranslational modifications to yield the mature toxin.

It is noteworthy that the amino acid sequence of *P. nigriventer* toxins from the same family, with the exception of PhTx3, exhibited sequence similarity (Tables 1 and 2). They also showed similarity with neurotoxins from the venoms of other spiders. The similarity between spider toxins that act on ion channels is more evident in the position of the Cys residues and in the pattern of disulfide bridges.

To date, no tertiary structure of any *Phoneutria* toxin has been elucidated experimentally. However, it has been suggested that the majority of *Phoneutria nigriventer* neurotoxins share a common structural motif known as cystine knot. This knot motif comprises an embedded ring formed by two disulfide bonds and their connecting backbone segments which is threaded by a third disulfide bond. It has been defined after comparisons with the three-dimensional structures of known polypeptides from snails of *Conus* genus and spider venoms.

This widely distributed disulfide-rich framework commonly found in spider venom toxins that act on ion channels was predicted for *Phoneutria* neurotoxins by similarity.

In addition, as can be seen in Table 1, *Phoneutria* toxins exhibit all three structural motifs suggested by Kozlov et al. (2005) for the identification of toxin-like structures in spider venoms based on analyses of the primary structure: (i) the PSM, principal structural motif, with 6 amino acid residues between the first and second cysteine residue and the Cys-Cys sequence at a distance of 5–10 amino acid residues from the

second cysteine; (ii) the ESM, extra structural motif, characterized by the presence of a pair of CXC fragments in the C-region; and (iii) the PQM, processing quadruplet motif, which specifies the Arg residue at position -1 and Glu residues at positions $-2, -3$, and -4 in the precursor sequences just before the post-processing site. These characteristics are typical for ion channel-blocking toxins. As shown in Table 1, PnTx1 and toxins from PhTx2, PhTx3, and PhTx4 families carry all three structural motifs. On the other hand, the new families (4.0 and 3.5 kDa) have only the PSM in their chain.

Conclusion and Future Directions

It is estimated that *P. nigriventer* venom contains more than 150 peptides/protein components (Richardson et al. 2006). Considering that only about 50 toxins were pharmacologically and/or chemically characterized and deposited in "protein knowledge base" so far, a wide diversity of new molecules, with possible different biological targets and activities, remains to be discovered in this venom. The studies conducted with *P. nigriventer* venom revealed several toxins acting on sodium, calcium, and potassium channels, among other receptors. In addition, a wide range of activities may result from the action of these molecules. Some of them have shown biotechnological and therapeutic potential, for example, by enhancing erectile function and by acting as analgesics or as insecticides. Although much of the richness and diversity of active peptides of *Phoneutria nigriventer* venom has been revealed, it has not yet been fully explored and other activities still remain to be discovered.

Cross-References

▶ *Phoneutria nigriventer* Venom and Toxins: A Review
▶ Spider Venom and Drug Discovery: A Review
▶ Spider Venom Peptides as Potential Therapeutics

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